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14. ABSTRACT

Macrophages are a major inflammatory cell in malignant pleural mesothelioma and may help maintain tumor resistance to therapy. In this final year, we have completed the key tasks that confirm the importance of the macrophage in the inflammatory cell environment of mesothelioma. We have confirmed that it is possible to alter the polarization of macrophages to either an M1 (anti-tumor) or M2 (pro-tumor) phenotype and that a shift toward the Th1 phenotype or depletion of macrophages increases mesothelioma cell apoptosis and chemosensitivity. In our orthotopic murine mesothelioma model, the depletion of macrophages increases the mesothelioma response to chemotherapy. We have recently used a non-toxic CSF1 receptor inhibitor already in clinical trials to manipulate macrophages and shown that the tumors become more responsive to chemotherapy regimens used in patients with mesothelioma. At the end of our three year award, we have convincing evidence that manipulation of macrophages can alter the response of mesothelioma to standard therapy. Our major publications on these findings are in preparation and an RO1 application with a clinical trial included is planned for submission this fall.

15. SUBJECT TERMS
None provided.

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I. INTRODUCTION

Inflammation is an early and consistent feature of mesothelioma [1, 2], an incurable tumor with a high level of resistance to chemotherapy [3]. Within this inflammatory milieu, we have discovered that there is a large population of macrophages, in a higher percentage than we have found in other thoracic malignancies. The macrophage is perhaps the earliest cell recruited to the asbestos fibers that are the cause of mesothelioma. After the tumor forms, macrophages are also, as we have found, a constant feature of mesothelioma. The association of macrophages appears thus to be a feature of mesothelioma during its initiation as well as its progression over four decades.

Macrophages, often known as tumor-associated macrophages, are found, albeit to a lesser extent, throughout many solid tumors. Increasingly, they are recognized as a key factor enhancing the malignant features of the tumor [4]. In separate tumors, a greater extent of macrophage infiltration in a tumor correlates with worse prognosis [5, 6]. Macrophages are now understood to have different phenotypes, directed by their cellular and cytokine environment [7]. The classic macrophage phenotype, termed M1 in analogy to the Th1 phenotype of lymphocytes, is directed to phagocytosis and anti-bacterial actions. This classic activation is seen in the pro-inflammatory environment, where LPS or interferons are prominent. More recently, an M2 phenotype was described that is directed by other cytokines, namely IL4, TGF beta. This alternatively activated macrophage is considered to be important at for the resolution of inflammation, dampening the inflammatory response and orchestrating a healing or tissue remodeling phase [8].

We wish to understand the mechanisms by which macrophages influence mesothelioma cells and whether we can manipulate the tumor-associated macrophages within the tumor by deletion or by alteration of the macrophage phenotype to sensitize tumors to apoptosis. To address these questions, we have several in vitro, ex vivo and in vivo approaches to investigate the mechanisms of macrophage-mesothelioma cell interactions. Our studies are designed to test the hypothesis that macrophages influence mesothelioma cell survival and can be manipulated to enhance mesothelioma cell apoptosis and response to chemotherapy.

In this final year of our grant-funded research, we have completed the key aims. We also are poised to continue this research by extending these findings into a new in vivo model, the MexTAG mouse [9, 10]. With substantial progress, we are preparing an RO1 together with a renowned oncologist with significant expertise in mesothelioma, Dr. Hedy Kindler, to propose a clinical trial testing GW2580, an anti-CSF1R, to inhibit the mesothelioma macrophages and enhance the chemoresponsiveness of the tumor.

II. RESEARCH ACCOMPLISHMENTS

Task 1. To determine the functional significance of macrophage phenotype on mesothelioma cell survival.

a. Elucidate the percentage of immune cells (CD45+) in human mesothelioma tumors and correlate immune cell infiltration with histopathologic subtype (months 1-6).

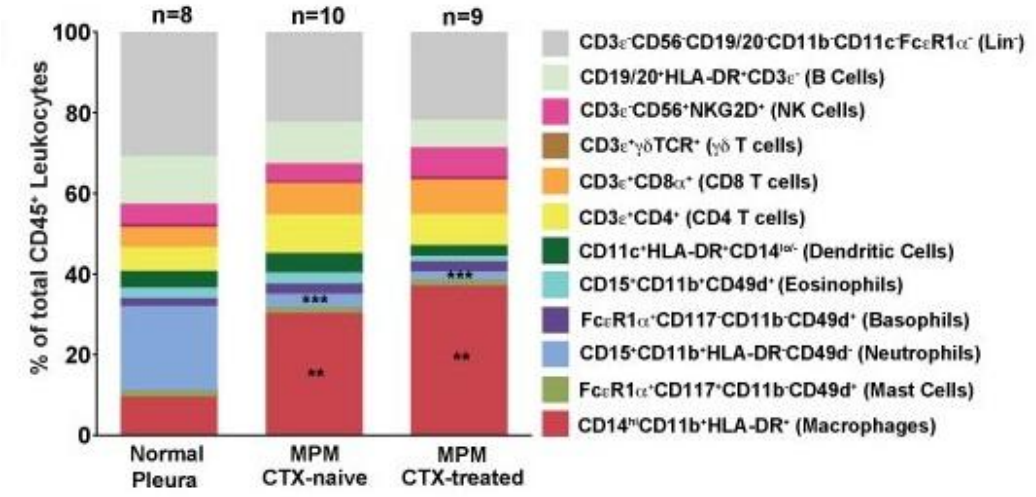
COMPLETE: Results were reported in the 2010 Annual Progress Report, have been presented at several meetings (see Appendix), and are in preparation in manuscript form.

b. Determine the macrophage population (CD14+) as a percentage of the total immune cell population by flow cytometry (months 1-36).

COMPLETE: Results were reported in the 2011 Annual Progress Report, were presented at several meetings this year (see Appendix) and are in preparation in manuscript form.

c. Determine the profile of other immune cells within the microenvironment of the mesothelioma tumor using a panel of cell surface markers (months 1-36).

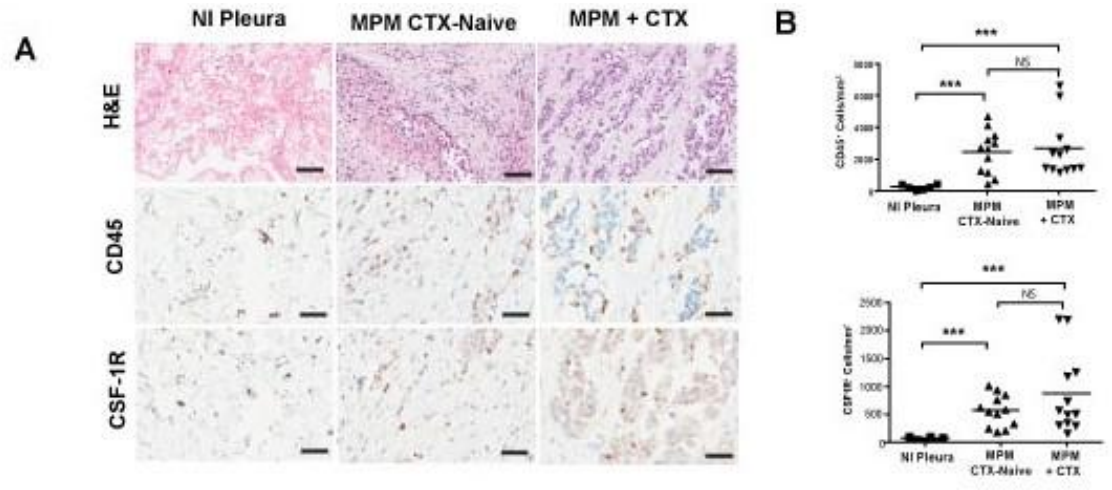
COMPLETE: Mesothelioma tumors (n=19), both chemotherapy naïve and chemotherapy treated, along with normal pleura (n=8) were studied by FACS for the profile of all immune cells. There is a clear difference in the macrophage infiltration in the tumors, whether previously treated or not treated with chemotherapy. There is a trend toward an increase in percentage of the T cells, CD4 and CD8. There is also a decrease in the percentage of neutrophils.



*FACS analysis of leukocytes as a % of total CD45$^+$ cells in normal pleura, CTX-naïve MPM, and CTX-treated MPM. ** p < 0.01 compared to normal pleura, *** p < 0.001 compared to normal pleura by Mann-Whitney test.*

d. Confirm macrophage percentages by immunodetection of the same mesothelioma in fixed tissues (CD68+) (months 1-36).

COMPLETE: Results presented in the 2010 Annual Progress Report have been confirmed using immunohistochemistry of paraffin-embedded mesothelioma. On average, using both immunohistochemistry and flow cytometry, macrophages constitute between 30-70% of the CD45+ (inflammatory) cell population in mesothelioma. We expanded this task to determine whether prior treatment with chemotherapy altered the percentage of macrophages. In the panels below, we can report that there is no difference in these two groups of mesothelioma.



(A) *Representative hematoxylin and eosin (H&E), CD45, and CSF-1R staining of normal pleura and malignant pleural mesotheliomas (MPM) resected from patients who were chemotherapy (CTX) naïve, or who had received neoadjuvant CTX prior to surgery (MPM + CTX).* ***(B)*** *Quantitation of CD45 and CSF-1R staining performed by Aperio software analysis. *** p < 0.001 by Mann-Whitney test.*

e. Isolate macrophages from human mesothelioma disaggregated into single cells by flow cytometry for use in co-culture spheroids with mesothelioma cells (1-12).

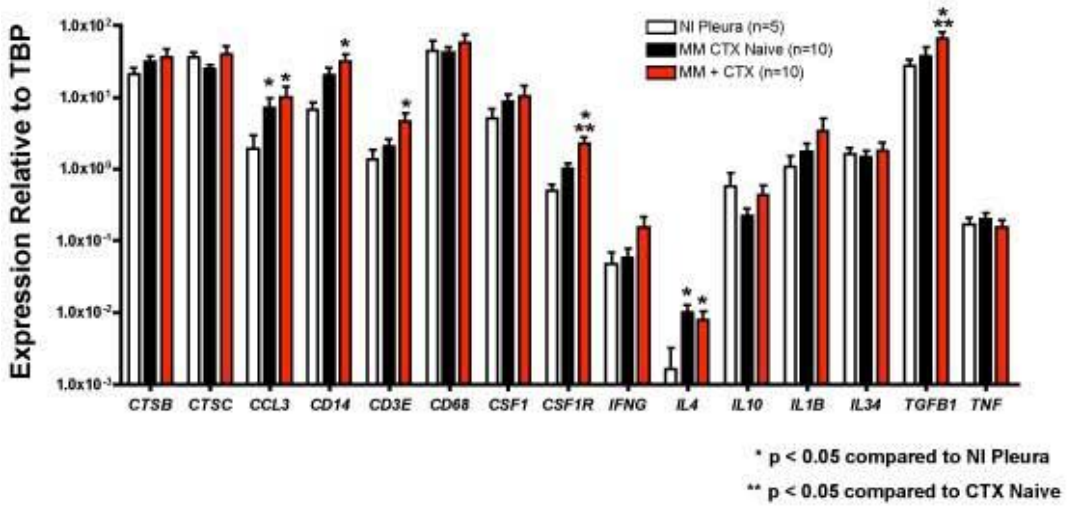
COMPLETE: As presented in preliminary form in the 2010 Annual Progress Report, we have shown that human macrophages from mesothelioma can be harvested from the tumor and co-cultured with mesothelioma cells in culture. In parallel experiments, results with the mesothelioma macrophages and with the macrophages derived from peripheral blood are equivalent.

f. Determine macrophage functional properties in mesothelioma using fixed tissues, by tissue microarray, and by immunohistochemistry for protein expression to define their M1 or M2 microenvironmental status (months 1-12)

COMPLETE: Staining of fixed tissues using immunohistochemistry failed to show consistent patterns. Another limitation was the comparison of staining with normal controls: normal pleura is a thin tissue with significant edge effect, making clearcut immunohistochemical staining difficult to interpret. For these reasons, we moved instead to use PCR to determine the functional status of the macrophages in mesothelioma tissue.

g. Determine the M1 or M2 gene expression signature of macrophages by commercial global chip assay for RNA from tumor tissue or cultures of cells/spheroids for gene profile (months 1-12).

COMPLETE: We have now completed our analysis of 20 tumors with the addition of 5 normal pleural samples, obtained from lungs otherwise normal that were not used for lung transplantation. The recruitment of the normal samples was a slow process, dependent on close coordination with another laboratory and on the availability of normal lungs. With this optimal normal control, we can now show upregulation of message for several inflammatory cytokines, including CCL3, a key macrophage-produced inflammatory chemokine, and CSF-1, a macrophage stimulating and recruitment factor. There is elevation of IL4 indicating an M2/Th2 phenotype.



Human malignant pleural mesotheliomas exhibit increased mRNA expression of macrophage markers compared to normal pleura. Quantiative PCR analysis performed on whole tissues to determine mRNA gene expression of cytokines and immune cell markers in normal pleura (white bar), CTX naïve malignant mesothelioma (black bar), and CTX-treated malignant mesothelioma (red bar). * $p < 0.05$ compared to normal pleura, ** $p < 0.05$ compared to CTX naïve by Mann-Whitney test. Statistically significant changes were found for the macrophage markers CD14 and CSF1R, and the TH2 cytokine IL4.

h. Analyze cytokines produced by mesothelioma tumor fragments by commercial cytokine bead assay from human mesothelioma grown as tumor fragments (months 1-12).

COMPLETE: A variety of inflammatory cytokines have been detected in the supernatant of tumor fragment spheroids, which parallel the RNA message described in g above. In particular, IL-4 is present along with the CSF1 growth factor.

i. Analyze cytokines produced by multicellular spheroids made from either mesothelioma cells alone or mesothelioma cells plus macrophages (THP-differentiated) (months 1-12).

COMPLETE: As presented in the 2010 Annual Progress Report, we have completed studies with hybrid spheroids showing that we are able to polarize the mesothelioma + macrophage hybrid spheroids to an M1 or M2 phenotype. Expression of M1 cytokines (TNF-alpha, IL12 and IL6) were elevated following polarization with interferon gamma plus endotoxin to an M1 phenotype; expression of M2 cytokines (particularly IL-10) was elevated following polarization toward an M2 phenotype using IL-4. The polarization was dependent on the presence of macrophages; no change in cytokine expression was seen in spheroids containing mesothelioma cells alone. Thus, the response of these tumor models to the polarization environment requires the presence of the macrophage population.

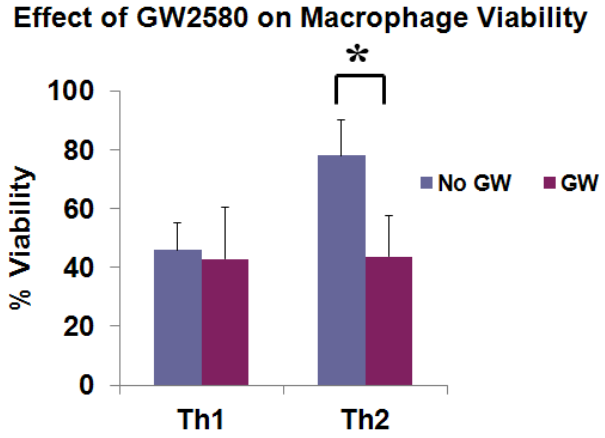
j. Analyze changes in cytokine expression when spheroids (mesothelioma only or mesothelioma plus macrophage) are treated with TRAIL or TRAIL plus gemcitabine (months 1-12).

COMPLETE: We have determined that the polarization state or cytokine profile of the hybrid spheroids is constant after treatment with TRAIL plus gemcitabine (T+G) and after carboplatin plus pemetrexed, the standard chemotherapeutic regimen given to patients with mesothelioma.

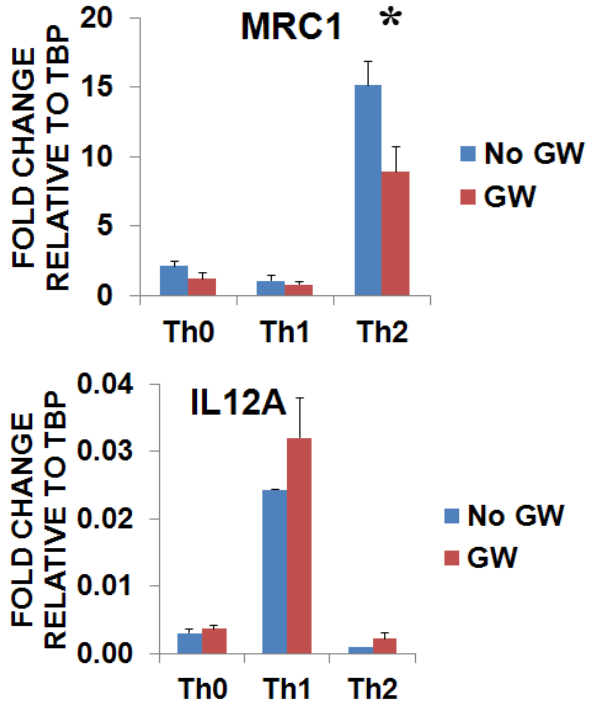
Task 2. To determine the functional significance of macrophages as regulators of mesothelioma apoptosis in vitro.

a. Confirm ability of clodronate-liposomes to deplete macrophages from multicellular spheroids made either with THP-1 differentiated macrophage cells or with primary mesothelioma-derived macrophages and determine optimal dose and timing (months 9-14).

COMPLETE: As described in our previous progress reports, we have moved from toxic clodronate to CSF1R inhibitors, either the Plexxikon compound PLX-3397 or currently GW2580, both of which inhibit the CSF1 receptor. We have determined that the PLX-3397 and the GW2580 compounds both induce loss of macrophage viability, either with macrophages grown in culture and in hybrid spheroids. By qPCR, we can also find that GW2580 alters the polarization by decreasing Th2 markers; there is a trend suggesting an increase in Th1.



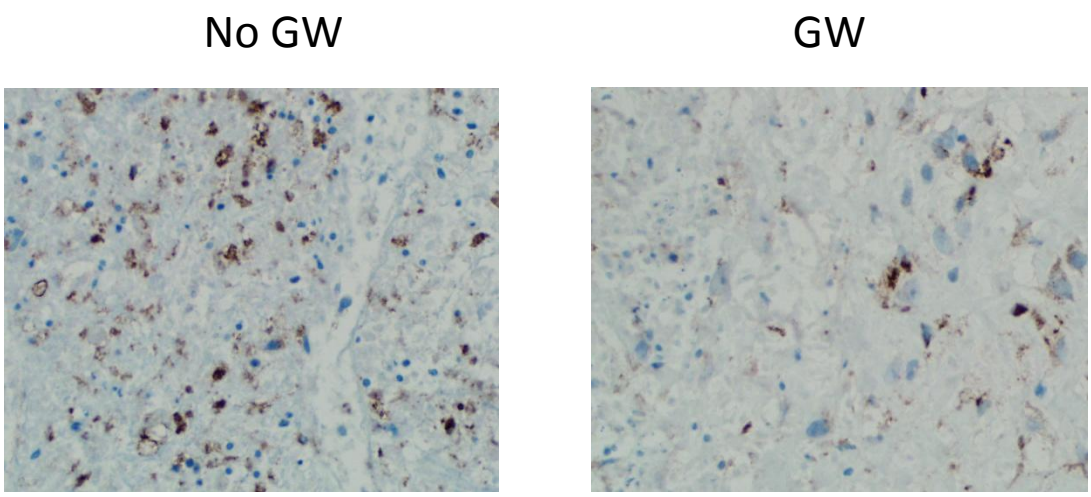
GW2580 decreases the viability of Th2-polarized macrophages: *PBMC derived macrophages were polarized towards Th1 or Th2 with appropriate cytokines, were treated without or with GW2580 (1μM) for 3 days, harvested and stained with trypan blue. GW2580 significantly decreases the macrophages viability when compared to Th1-polarized macrophages. n=3 * p<0.01*



GW2580 also reprograms the macrophages: *PBMC derived macrophages were polarized towards Th1 or Th2 as above and treated without or with GW2580 (1μM) for 3 days, harvested for RNA extraction and analyzed by qpCR. GW2580 significantly increased the expression of Th2 markers (see MRC1). Th1 markers (see IL12A) showed a trend toward an increase. (n=3, p<0.05)*

b. Confirm ability of clodronate-liposomes to deplete macrophages from mesothelioma tumor fragment spheroids with dose and timing established above (months 12-24).

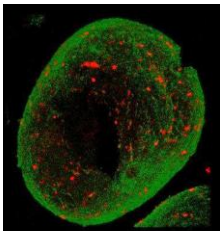
COMPLETE: The two compounds also deplete macrophages from tumor fragment spheroids (*see figure below*).



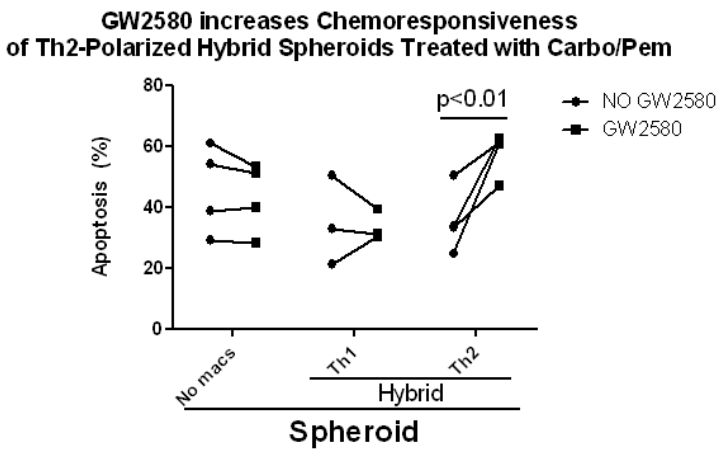
GW2580 decreases the number of macrophages in TFS: *Unpolarized TFS were treated without or with GW2580 (1μM) for 3 days, harvested for paraffin fixation and labeled for CD68, a marker of macrophages by immunohistochemistry (marked brown). GW2580 decreases the number of macrophages in the TFS (representative of 3 different tumors).*

c. Analyze effect of macrophages and of macrophage depletion on apoptosis of mesothelioma cells to treatment with TRAIL or TRAIL plus gemcitabine using multicellular spheroids either with no macrophages, macrophage-depleted or with macrophages (months 12-24).

COMPLETE: We have now shown that the inhibition and relative depletion of macrophages in hybrid spheroids leads to an enhanced chemoresponsiveness of the mesothelioma cells. The increased chemoresponsiveness has been mediated by either of two CSF1R antagonists, PLX3397 or GW2580 (shown below) and can be seen with either TRAIL plus gemcitabine or with carboplatin plus pemetrexed.



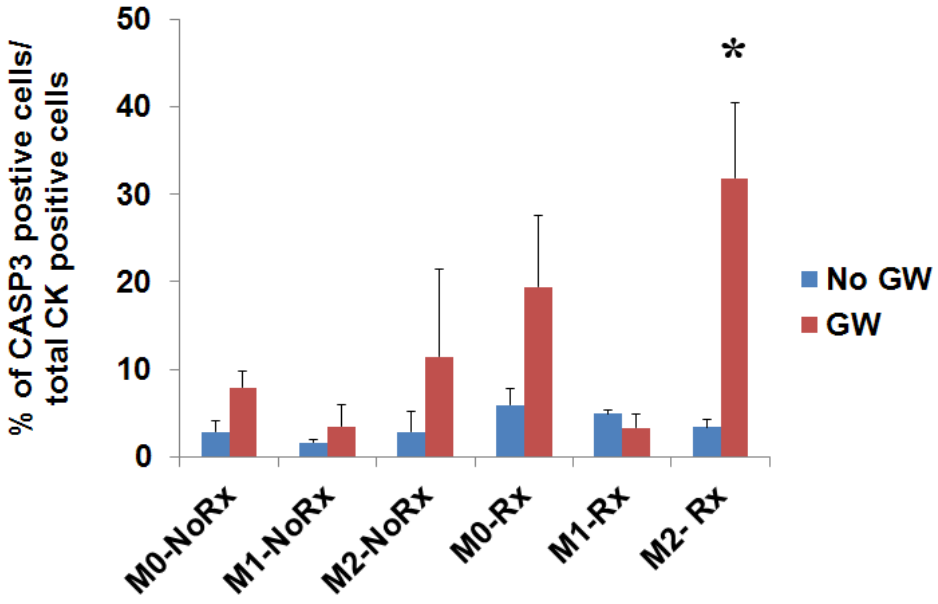
Macrophages and mesothelioma cells form hybrid spheroids*. This spheroid contains 10,000 mesothelioma cells (green, anti-cytokeratin) and 500 (5%) macrophages (red, anti-CD69) (see arrows). By fluorescent microscopy, the macrophages are found to be distributed throughout the spheroid. Spheroid is approximately 0.5 mm in diameter.*



GW2580 enhances the apoptosis in Th2-polarized hybrid spheroids: *M28 and hybrid spheroids were pre-treated without or with GW2580 (1μM) for 3 days, and exposed to carboplatin (250μM) and Pemetrexed (10μM) for 48 hrs. GW2580 has no effect on the mesothelioma cells and in Th1-polarized hybrid spheroids but significantly increased chemo responsiveness in Th2-polarized hybrid spheroids. (n=4, *p<0.01)*

d. Analyze the effect of macrophages and of macrophage depletion on apoptosis of mesothelioma cells in tumor fragment spheroids to treatment with TRAIL or TRAIL plus gemcitabine (months 12-24).

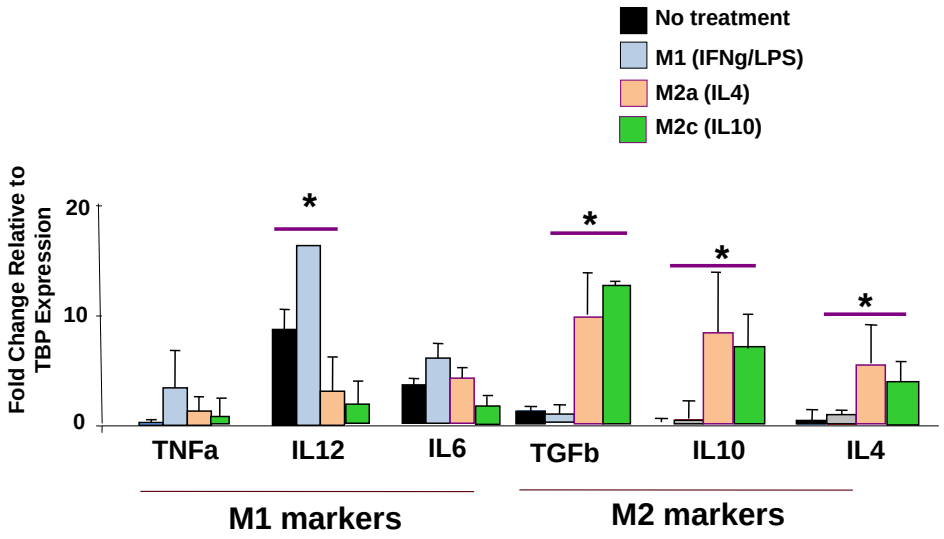
COMPLETE: Similarly to the effect in hybrid spheroids, we have now exposed tumor fragment spheroids from 5 different patients and exposed them to GW2580 prior to treatment with carboplatin plus pemetrexed. This finding confirms the results in cultured macrophages (see Task 2a) and hybrid spheroids (Task 2c) that the Th2 phenotype is most sensitive to blockade of the CSF1R by GW2580.



GW2580 enhances the apoptotic response to chemotherapy in Th2-polarized spheroids: *TFS were polarized in presence of appropriate cytokines, pretreated without or with GW2580, exposed to chemotherapy, harvested for paraffin fixation and double immunostained for cytokeratin (CK), a marker of mesothelioma cells, and cleaved caspase 3 (CASP3), a marker of apoptosis. GW2580 enhances the apoptotic response to chemotherapy in Th2-polarized tumor fragment spheroids (representative of 5 different tumors, p<0.05).*

e. Confirm ability of M1 cytokines to polarize macrophages to a strong M1 phenotype by exposing multicellular spheroids with macrophages (primary mesothelioma or THP-1) to M1 polarizing agents (interferon gamma plus LPS) and confirming with cytokine and gene expression assays for classics M1 or M2 polarization (months 12-24).

COMPLETE: As described in previous progress reports, we have confirmed that polarization is effective in reprogramming macrophages to an M1/Th1 or M2/Th2 phenotype. We continue to see no significant difference in the cytokine expression of the different subsets of M2: M2a and M2c.



Human macrophages derived from peripheral blood monocytes can be polarized to an M1 or M2 phenotype. *Peripheral blood monocytes were matured to macrophages and exposed for 24 h to IFNgamma plus LPS (to stimulate toward a TH1 or M1 phenotype), IL-4 (toward TH2 or M2a) or IL-10 (toward TH2 or M2c). RNA was harvested and analysed by qPCR for expression of message for M1 or M2 marker cytokines.*

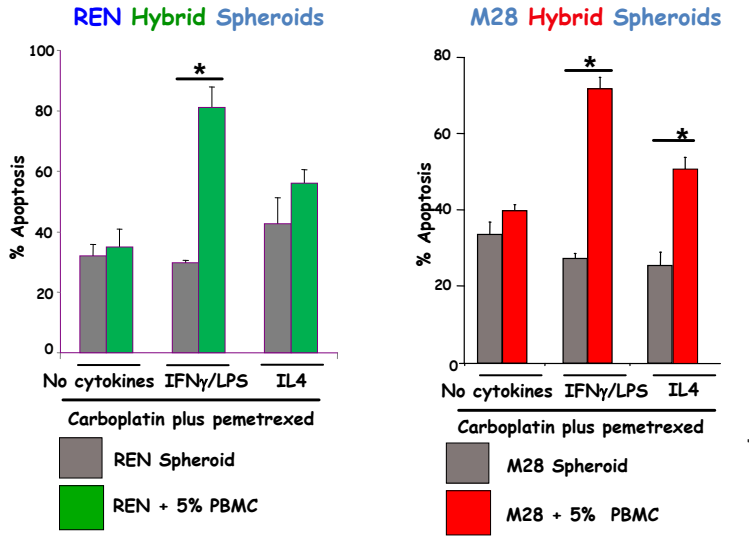
f. Polarize macrophages in tumor fragment spheroids using the approach found best above (months 12-24).

COMPLETE: We were able to confirm polarization of tumor fragment spheroids to Th1 or Th2, with the upregulation of characteristic mRNA for Th1 or Th2 markers. Immunohistochemical staining showed that the expression of iNOS, a marker for Th1, was increased in cultured macrophages and in tumor fragment spheroids polarized toward Th1; expression of the CD206 marker for Th2 was increased when polarized toward Th2.

g. Determine whether repolarization of macrophages enhances apoptosis of mesothelioma cells in multicellular spheroids (grown with primary mesothelioma macrophages or THP-1 macrophages) when treated with TRAIL plus gemcitabine (months 12-24).

COMPLETE: We have extensive data on the effect of repolarization on the apoptotic responses of the mesothelioma cells. As shown in the figure below and reported in the prior progress reports, we have data with multiple different mesothelioma cell lines, a variety of sources of macrophages (from primary mesothelioma, from peripheral blood,

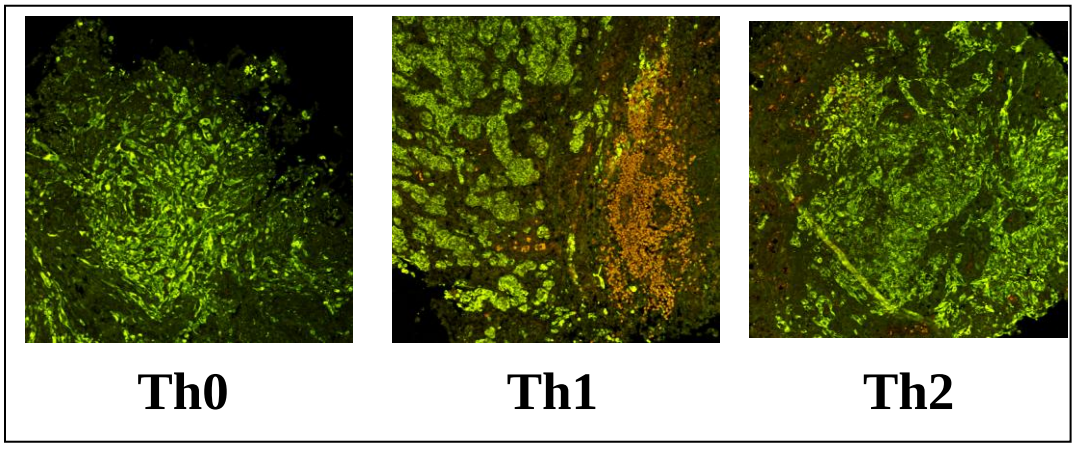
from THP cells) and with different apoptotic regimens (TRAIL containing, or carboplatin plus pemetrexed), that the M1/Th1 phenotype leads to an increased apoptotic response of the mesothelioma cells. The mechanism is still under investigation and may depend on the intracellular cytokine assays planned to show the cytokines responsible. Early studies have implicated TNF for part of the pro-apoptotic response.although other cytokines may be playing an additional role.



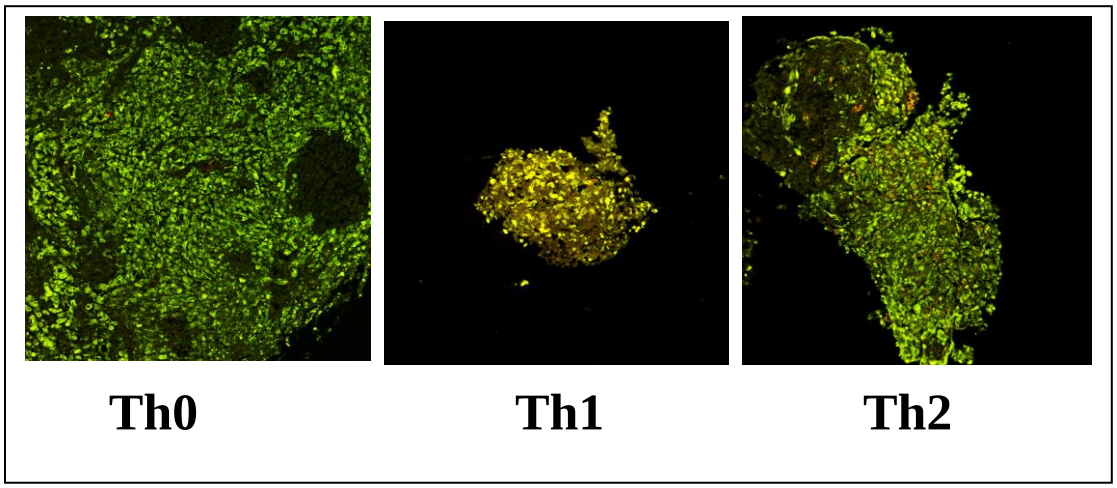
*In 5 experiments in each cell line, polarization increases the apoptotic sensitivity of mesothelioma cells, but only when macrophages are present. This effect is seen for TRAIL plus gemcitabine (not shown) and also for the most clinically relevant therapeutic regimen, carboplatin plus pemetrexed, the treatment considered the standard for patients and the treatment now being used in the mice studies. * p<0.03 n=5*

h. Determine whether repolarization of macrophages enhances apoptosis of mesothelioma cells in tumor fragment spheroids (months 12-24).

COMPLETE: We have completed these studies showing that polarization to a Th1 phenotype leads to significant increases in chemosensitivity to carboplatin plus pemetrexed (**see figure below).** The spheroids are first polarized toward an M1 or M2 phenotype (or a no polarization, or M0 condition) and are then exposed to chemotherapy for 48 h (carboplatin plus pemetrexed). Following fixation and double fluorescent staining for mesothelioma cells (cytokeratin positive) and active apoptosis (cleaved caspase 3), we have found significantly increased apoptosis in spheroids polarized to M1. These findings are similar to those in the hybrid spheroids above and indicate a pro-apoptotic effect of the M1 polarization state of macrophages. However, in the tumor fragment spheroids, the M1 phenotype itself tends to increase apoptosis.

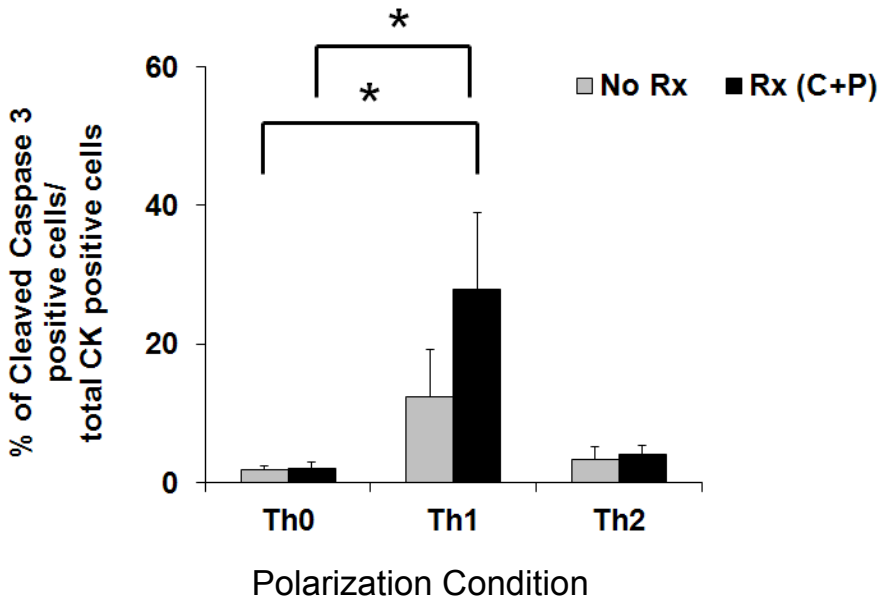


A Untreated



B Treated

*Tumor fragments were exposed to nothing (M0) or to a polarization schedule for 48 h to induce an M1 or an M2 (Th1 or Th2) phenotype and stained for apoptosis **A**) without further treatment or **B**) after exposure to carboplatin plus pemetrexed for another 48 h. The tumor fragments were fixed, embedded, sectioned and stained for cytokeratin (green) and cleaved caspase 3 (red). The merged color (yellow) shows the presence of apoptotic mesothelioma cells. Apoptosis is increased in tumor cells within Th1-polarized TFS, particularly when treated. No difference is seen in untreated TFS (not shown) (Green=cytokeratin indicating mesothelioma cells, red=cleaved caspase 3; yellow=apoptotic mesothelioma cells)*

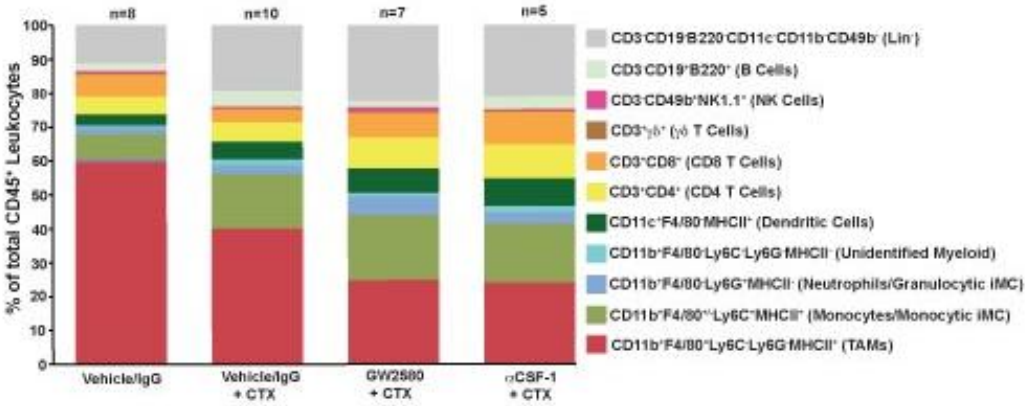


Human Tumor Fragment Spheroids are Sensitized to Chemotherapy by Th1 Polarization
*In tumor fragments derived from 5 different patients, polarization to a Th1 phenotype enhanced apoptosis by itself and also enhanced the apoptotic response to carboplatin plus pemetrexed treatment for 48 h (C+P). (n=5, *p<0.05)*

Task 3. Define functional significance of macrophage depletion or repolarization on mesothelioma survival in vivo.

a. Characterize immune cell profile of the murine mesothelioma model induced by intraperitoneal asbestos injections in the NF2+/- mouse model (months 0-24).

COMPLETE: Full immunophenotyping of the immune cells have been carried out by multicolor FACS in the orthotopic mesothelioma model, in which syngeneic mesothelioma murine cells (40L) are introduced into the peritoneum of the mice and form into mesothelioma tumors that closely resemble human mesothelioma. At baseline, without treatment, the predominant inflammatory cell recruited to the tumor is the macrophage, in a similar pattern to that seen in humans.



Blockade of CSF-1/CSF-1R reprograms the mesothelioma tumor immune microenvironment. *Fluorescence- Activated Cell Sorting (FACS) analysis was performed on single cell suspensions isolated from 40L orthotopic mesothelioma tumors from mice treated with: 1) vehicle or IgG; 2) vehicle or IgG + CTX (carboplatin + pemetrexed); 3) GW2580 + CTX; or 4) anti-CSF-1 + CTX. Colored bars represent the proportion of each cell population identified out of the total CD45+ cells.*

As reported in our last progress report, the asbestos-injected de novo tumor model NF2+/- had significant limitations. The two major limitations were the low and unpredictable number of mice that develop mesothelioma and the time required. After 1 year following multiple injections of asbestos, less than 30% of our mice were shown to have mesothelioma. However, we have used this period to advance several techniques that will help us evaluate mice for early development of mesothelioma and we have arranged, by collaboration with Dr. Richard Lake of Perth Australia, to obtain an improved de novo mouse mesothelioma model, the MexTag model [9, 10]. This mouse has a mesothelin promoter that induces SV40 large T antigen expression in the mesothelial cells; only a few injections of asbestos lead to mesothelioma leading to mesothelioma in all mice, with the development between 20-40 weeks. Such a model has many advantages: almost 100% development of mesothelioma, the 1-2 injections of asbestos and the more rapid development. This model will be much better for the planned treatment studies.

As a result of our difficulties with the NF2+/- mice, we have also developed techniques to screen mice for mesothelioma: 1) cytology of peritoneal lavage, which demonstrated mesothelioma cell clusters (e.g. spheroids) in otherwise normal appearing mice that later were proved to have mesothelioma by autopsy and 2) Doppler ultrasound, which was able to demonstrate pre-morbid mesothelioma in several mice due to the vascular enhancement of the peritoneal wall thickened by tumor. Thus, we have new tools to

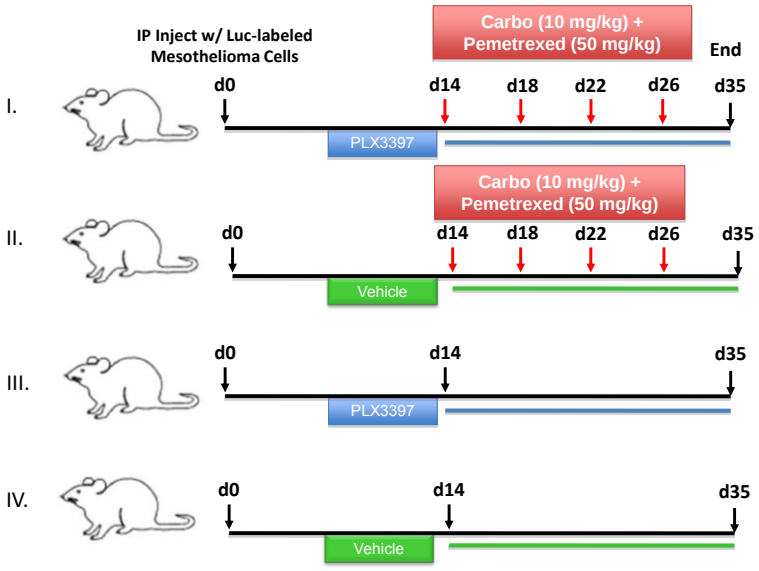
evaluate these new mice to determine the earliest appearance of mesothelioma so that clinical studies can be initiated. The MexTag mice will be used for the studies that continue following this award.

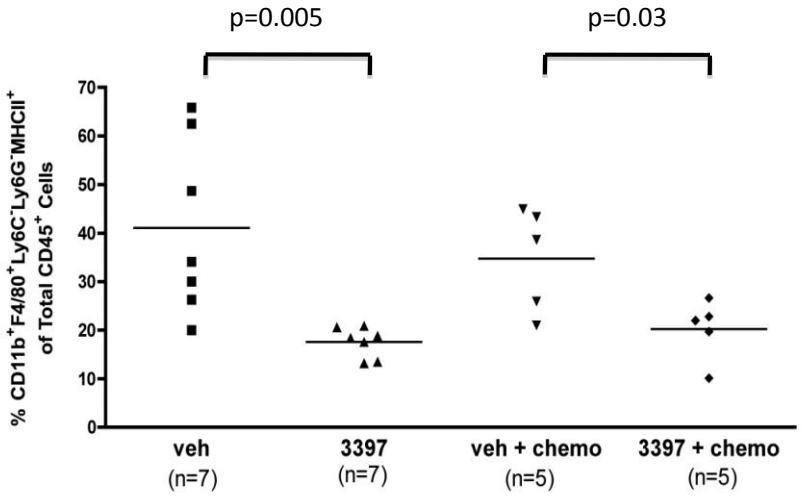
b. Deplete in vivo macrophages with intraperitoneal clodronate-liposomes in mice without mesothelioma to establish protocol (months 4-12).

COMPLETE: We have completed the clodronate studies and followed the in vivo protocol for the use of intraperitoneal clodronate for macrophage depletion. In GFP-macrophage mice, the clodronate was able to eliminate the GFP signal and all cells costaining with two macrophage markers, CD11b+ and F4/80.

However, as mentioned, we have pursued clinically-relevant studies of macrophage inhibition using compounds that are nearing clinical use, non-toxic reagents such as the PLX-3397 or the GW2580 inhibitors of the CSF1 receptor. These studies have begun with the PLX3397 compound and show that PLX3397 does alter the macrophage population in the mouse. These studies have been carried out in mice with orthotopic luciferase-labeled intraperitoneal mesothelioma, not in normal mice, because we wished to go quickly to the relevant model.

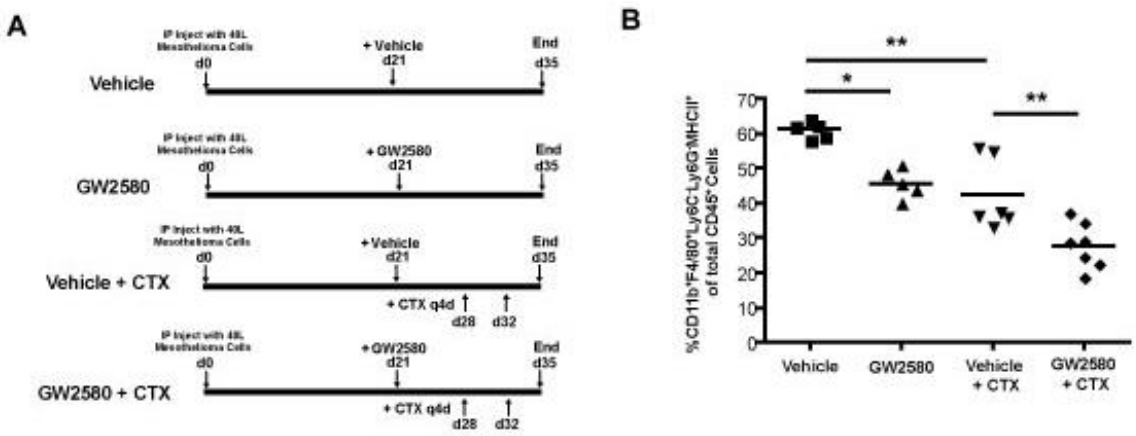
The general treatment schedule is shown:





PLX3397 decreases macrophage number in orthotopic mesothelioma. *In mice with orthotopic 40L luciferase-labeled intraperitoneal mesothelioma, the PLX3397 compound was injected intraperitoneally with or without chemotherapy (carboplatin (10 mg/kg) plus pemetrexed (50 mg/kg) given in 4 doses over 2 weeks). By flow cytometry, the presence of macrophage population in the tumors was significantly reduced. The reduction in macrophage number was seen whether or not the chemotherapy was given.*

As described above, we have moved to the orally available and clinically relevant CSF1R antagonist, GW2580. The studies were repeated with GW2580 in the mouse chow. Both PLX3397 (above) and GW2580 (below) were able to deplete macrophage number in the orthotopic mesothelioma tumors.

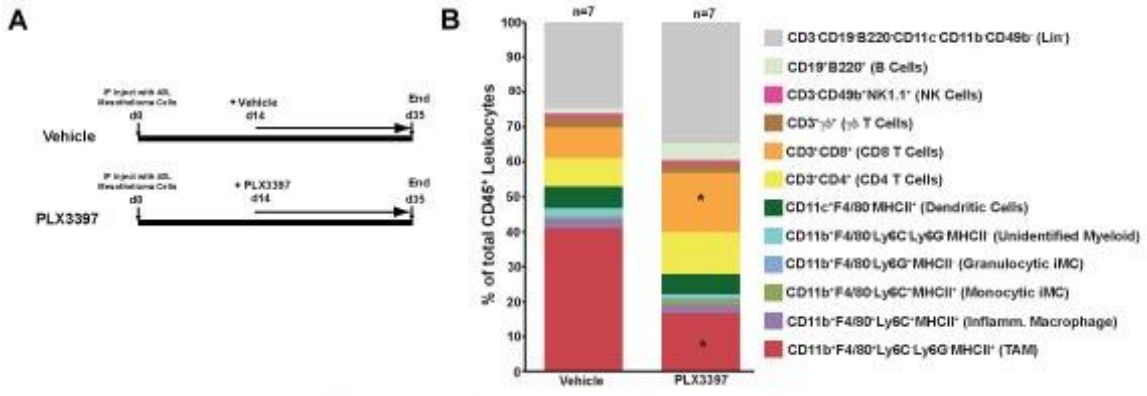


The CSF-1R tyrosine kinase inhibitor GW2580 enhances the antitumor effects of cytotoxic chemotherapy in a syngeneic orthortopic murine model of mesothelioma. (A) *Schematic of experimental design. Wild-type C57BL/6 mice were i.p. injected with syngeneic 40L mesothelioma tumor cells at day 0. Tumor-bearing mice were randomized into one of four experimental groups at day 21. Mice were sacrificed at day 35. Cytotoxic chemotherapy (CTX) consisted of carboplatin (50 mg/kg) and pemetrexed (100 mg/kg) administered i.p. GW2580 was incorporated into chow at 800 mg/kg which mice consumed ad libitum. Experiment was performed twice with a minimum of 5 mice per treatment group in each experiment.* ***(B)*** *Effect of CTX and GW2580 on tumor macrophage infiltration. Quantitation of $CD11b^+F4/80^+Ly6C^-Ly6G^-MHCII^+$ cells (macrophages) as a percentage of total $CD45^+$ cells by FACS analysis of whole mesenteric tumors. * $p < 0.05$, ** $p < 0.01$ by Mann-Whitney test.*

Thus, in this orthotopic model, both of the two different CSF1 receptor antagonists are able to decrease macrophage populations in mesothelioma.

c. Deplete in vivo macrophages in mice with mesothelioma to confirm depletion, to establish effect on other immune cell populations, and to confirm lack of toxicity on the mice (months 12-24).

COMPLETE: In the orthotopic 40L mesothelioma mice, the PLX3397 treatment in the schedule as shown above was also evaluated for several different immune populations by flow cytometry. It was found that the reduction of macrophages was accompanied by an increase in CD8 positive T cells (**figure below, see ***). Such a result parallels findings in breast cancer and may indicate that CD8 T cells will be shown to mediate cytotoxicity against the tumor.



The CSF-1R tyrosine kinase inhibitor PLX3397 exhibits anti-tumor activity in an syngeneic orthotopic model of malignant mesothelioma. (A) *Schematic of experimental design. Wild-type C57BL/6 mice were i.p. injected with syngeneic 40L mesothelioma tumor cells at day 0. Tumor-bearing mice were randomized into experimental groups at day 14. Mice were sacrificed at day 35. PLX3397 was incorporated into chow at 290 mg/kg which mice consumed ad libitum. The experiment was performed twice with a minimum of 10 mice per treatment group in each experiment.* ***(B)*** *Effect of PLX3397 on tumor immune cell infiltration by FACS analysis. * $p < 0.05$ by Mann-Whitney test.*

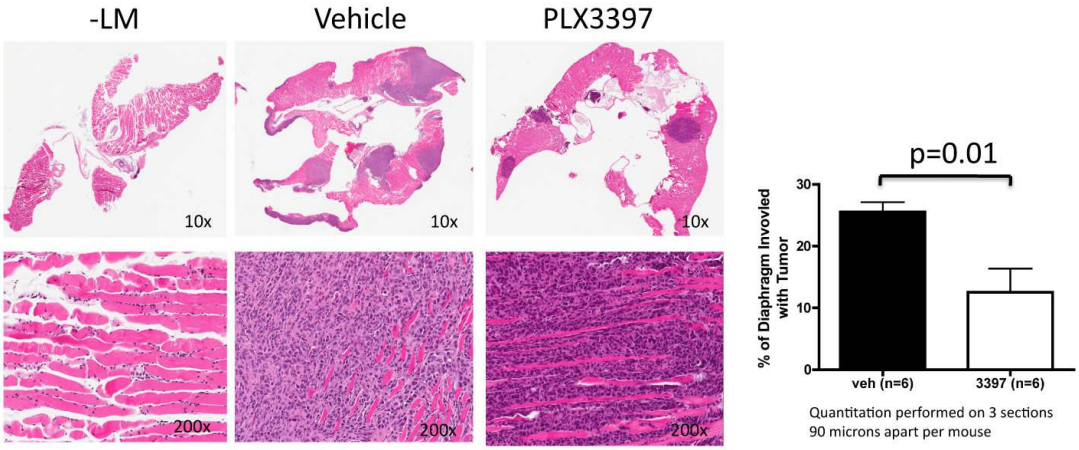
In these studies, the PLX3397 compound can be seen to alter the inflammatory cellular balance within the tumor microenvironment. By reducing macrophages and increasing CD8+ T cells, the PLX3397 compound may be fortifying the anti-tumor forces within the tumor.

d. Determine whether macrophage depletion in mice with mesothelioma enhances apoptotic and therapeutic response of mice with mesothelioma to TRAIL plus gemcitabine over 1-4 weeks after treatment (months 24-36).

COMPLETE: As described above, we have relied for these studies on the orthotopic model of mesothelioma in immunocompetent mice produced by the IP injection of a syngeneic murine mesothelioma line (40L) in C57/Bl/6 mice (as described above). In this model, intraperitoneal mesothelioma forms 3-4 weeks after the ip injection of 2×10^6 40L cells. This model was developed by Dr. Agnes Kane, who kindly provided this cell line and a second line (7) for these studies [11].

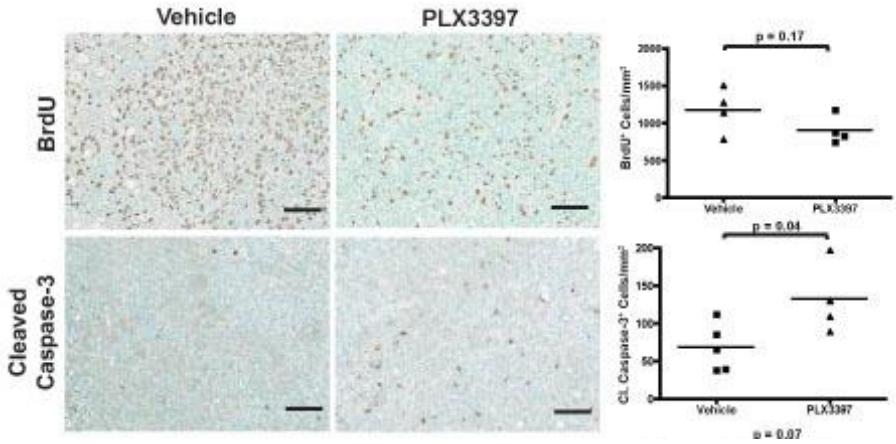
The early results were described in the 2010 Annual Progress Report and showed that macrophage depletion using liposomal clodronate enhanced the response of mesothelioma to treatment with TRAIL plus gemcitabine. These exciting results led us to move to a more clinically relevant treatment regimen, using carboplatin plus pemetrexed, and a more clinically relevant macrophage inhibition regimen, using PLX-3397. The 40L cells have also been transfected with a luciferase construct so that can be monitored by luciferase optical imaging. We have found however that luciferase assays are most useful for screening for the increase in tumor size; the luciferase is not quantitative so actual tumor volume must be measured at postmortem.

In these studies, using the protocol shown in Task 3b above but without the use of chemotherapy, we have now shown that PLX3397 reduces the mesothelioma tumor volume when used alone, even without any chemotherapy (**figure below).**



Monotherapy with PLX3397 reduces the burden of mesothelioma in an orthotopic mouse mesothelioma model.

In addition, we were able to show that the PLX3397 monotherapy also increased the presence of apoptosis in the orthotopic mesothelioma cells, suggesting that survival of the mesothelioma cells was dependent on CSF1, presumably via macrophage effects on tumor cell survival (**see figure below**).



*Monotherapy with PLX3379 led to an increase in apoptosis of the mesothelioma cells, as shown by an increase in cleaved caspase-3. There was no significant change in the proliferation of the tumor cells, as shown by a constant level of BrdU staining. Quantitation was performed on 5 fields of view from 5 independent mouse tumors per treatment group by Aperio ImageScope software. * p < 0.05 by Mann-Whitney test.*

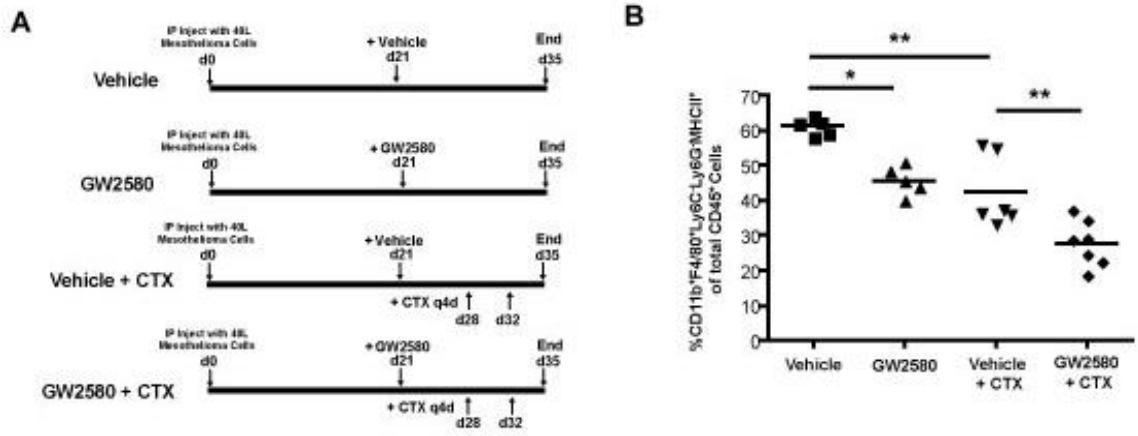
e. Repolarize macrophages in vivo by injections of interferon gamma plus LPS and assaying cytokines of peritoneal ascites and gene expression of peritoneal macrophages obtained by lavage to confirm method of repolarization, and to select best tolerated method for the mice (months 12-24).

Efforts to repolarize the macrophages intraperitoneally were less successful and much less likely to be clinically relevant than the inhibition of macrophages. With the new agents that became available during this award, e.g. PLX3397 and GW2580, we elected

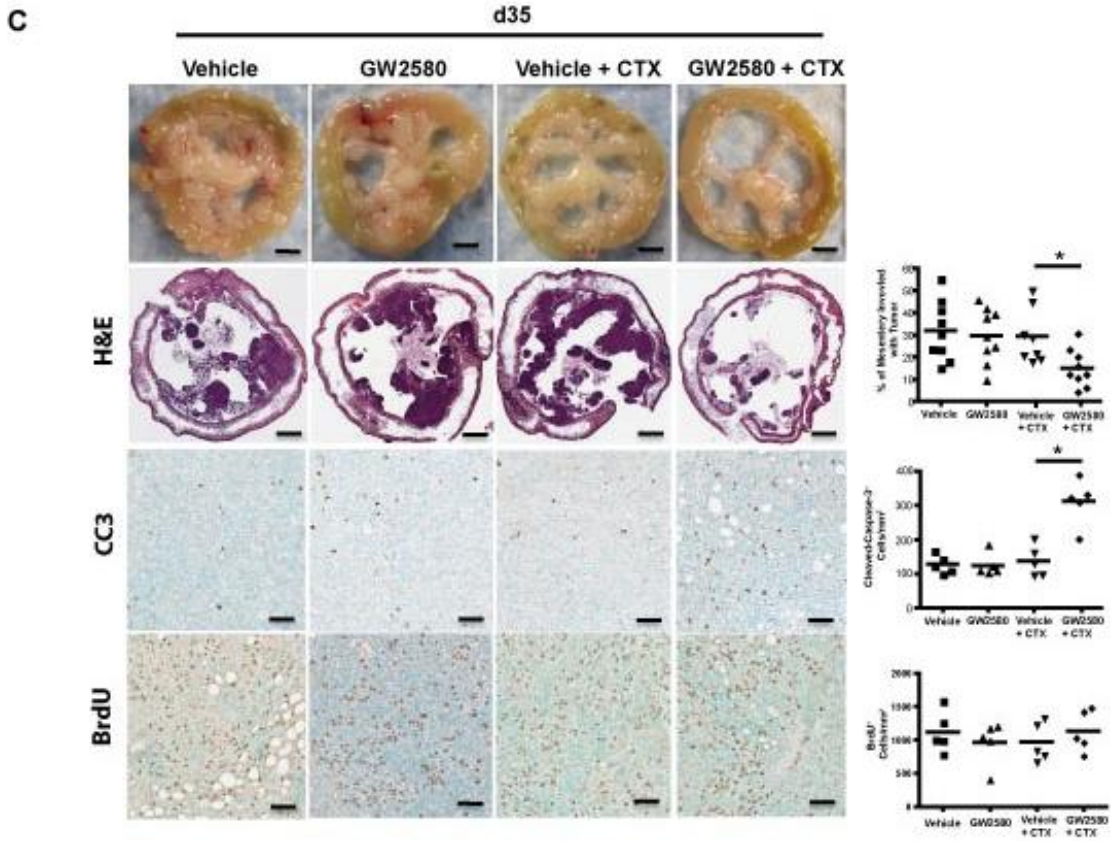
to concentrate our efforts and our mice experiments with the CSF1R antagonists. Such agents are already entering the clinical trials for malignancy and our studies were adapted to move more quickly toward a clinically useful treatment strategy.

f. Determine whether the depletion or repolarization of macrophages in vivo enhances the apoptotic and therapeutic response of mice with mesothelioma to TRAIL plus gemcitabine over 1-4 weeks after treatment (months 24-36).

COMPLETE: These studies have been very successful. GW2580 to inhibit macrophages was effective in enhancing the chemosensitivity of the mesothelioma cells to chemotherapy. Mice with mesothelioma treated with GW2580 showed an increase in apoptotic response to the chemotherapy and a decrease in tumor burden. These studies are consistent with our earlier studies using clodronate to deplete macrophages or using PLX3397 to inhibit macrophages. These studies provide confidence that the macrophage is a promising target in mesothelioma.



The CSF-1R tyrosine kinase inhibitor GW2580 depletes macrophages in the orthotopic model. (A) *Schematic of experimental design. Wild-type C57BL/6 mice were i.p. injected with syngeneic 40L mesothelioma tumor cells at day 0. Tumor-bearing mice were randomized into one of four experimental groups at day 21. Mice were sacrificed at day 35. Cytotoxic chemotherapy (CTX) consisted of carboplatin (50 mg/kg) and pemetrexed (100 mg/kg) administered i.p. GW2580 was incorporated into chow at 800 mg/kg which mice consumed ad libitum. Experiment was performed twice with a minimum of 5 mice per treatment group in each experiment.* ***(B)*** *Effect of CTX and GW2580 on tumor macrophage infiltration. Quantitation of $CD11b^{+}F4/80^{+}Ly6C^{-}Ly6G^{-}MHCII^{+}$ cells (macrophages) as a percentage of total $CD45^{+}$ cells by FACS analysis of whole mesenteric tumors. * $p < 0.05$, ** $p < 0.01$ by Mann-Whitney test.*



The CSF-1R tyrosine kinase inhibitor GW2580 enhances the antitumor effects of cytotoxic chemotherapy in a syngeneic orthortopic murine model of mesothelioma. (C) *Effect of CTX and GW2580 on tumor burden, tumor cell apoptosis, and proliferation. Representative gross images (upper panel) and H&E, cleaved caspase-3 and BrdU stained histological sections of mesenteric tumors. Tumor burden quantitation was performed on 3 serial H&E stained sections 90 microns apart. Cleaved caspase-3 and BrdU quantitation was performed on 5 fields of view from 5 independent mouse tumors per treatment group by Aperio ImageScope software. * $p < 0.05$ by Mann-Whitney test.*

g. Determine whether the depletion or repolarization of macrophages enhances survival of mice with mesothelioma after treatment with vehicle, TRAIL or TRAIL plus gemcitabine (months 24-36).

Survival studies were postponed until the MexTag mice could be introduced into the study. A cohort of these mice has been injected with asbestos intraperitoneally and is being readied for inclusion in a survival study with four conditions: no treatment, GW2580 alone, carboplatin plus pemetrexed alone and GW2580 plus carboplatin plus pemetrexed. The results from these studies, along with complete characterization of the MexTag mice, will be included in a manuscript being prepared for publication.

III. KEY RESEARCH ACCOMPLISHMENTS

Task 1. To determine the functional significance of macrophage phenotype on mesothelioma cell survival.

a. Elucidate the percentage of immune cells (CD45+) in human mesothelioma tumors and correlate immune cell infiltration with histopathologic subtype (months 1-6).
Months 1-12

- Acquired 71 fixed and paraffin-embedded mesothelioma tumor samples
- Prepared mesothelioma tumor tissue microarrays for immunohistochemistry
- Determined the pathologic subtype of each tumor in concert with the thoracic pathologist, Dr. Steven Nishimura
- Quantified each sub-population as a percentage of the total inflammatory cell population using digital imaging software, the Aperio system, optimized for either membrane or nuclear staining
- Correlated presence of inflammatory cell populations with mesothelioma pathologic subtype
- Obtained normal pleural controls by a collaboration with a pulmonary laboratory of Dr. Michael Matthay to obtain normal human pleura from human donor lungs rejected for transplantation

b. Determine the macrophage population (CD14+) as a percentage of the total immune cell population by flow cytometry (months 1-36).
Months 1-12

- Optimized disaggregation protocol for mesothelioma tumors
- Established that the percentage of CD14+ cells in the mesothelioma inflammatory population exceeds that of the other thoracic tumors, lung and esophagus
- Expanded flow cytometry studies to a new high density 14 color system
- Confirmed the high percentage of macrophages using high density flow
- Analyzed 40 tumors so far using 14 color, high density flow cytometry
- Initiated collection of peripheral blood samples from the patients with mesothelioma to compare activation status of cells in tumor compared to periphery
- Included mesothelin in panel of cell surface markers to quantify percentages of mesothelioma cells in tumors

c. Determine the profile of other immune cells within the microenvironment of the mesothelioma tumor using a panel of cell surface markers (months 1-36).
Months 1-12

- Performed immunohistochemical analysis on 71 tumors to identify inflammatory cell infiltrates, e.g. CD45, CD68, CD8, CD4 and CD20

Months 12-24

- Using flow cytometry to evaluate several activation markers on inflammatory cells

d. Confirm macrophage percentages by immunodetection of the same mesothelioma in fixed tissues (CD68+) (months 1-36).
Months 1-12

- Determined the percentages of macrophages in mesothelioma using same tumors studied in immunohistochemistry (by CD68+) and in flow cytometry (by CD14+)

e. Isolate macrophages from human mesothelioma disaggregated into single cells by flow cytometry for use in co-culture spheroids with mesothelioma cells (1-12).
Months 1-12

- Isolated macrophages from fresh mesothelioma tumors with high viability (80-90%) and in sufficient numbers to study
- Produced hybrid spheroids with macrophages isolated from human mesothelioma tumors

Months 13-24

- Determined that macrophages from human mesothelioma acted the same as the peripheral blood monocytic cells (PBMC) so will rely on the PBMC for future studies

f. Determine macrophage functional properties in mesothelioma using fixed tissues, by tissue microarray, and by immunohistochemistry for protein expression to define their M1 or M2 microenvironmental status (months 1-12)

Months 1-24

- Stored all incoming mesothelioma tissues in tissue bank for these studies while technique is being tested in laboratory, once the technique is perfected it will be straightforward to analyze the stored tissues

Months 24-36

- Used the tissues preferentially for PCR which was more consistent and did not require staining of the normal pleura, a structure so thin as to be a poor control for immunohistochemistry

g. Determine the M1 or M2 gene expression signature of macrophages by commercial global chip assay for RNA from tumor tissue or cultures of cells/spheroids for gene profile (months 1-12).

Months 1-12

- Quantified cytokine message in 7 snap frozen mesothelioma tumors
- Compared message to 4 normal pleural samples

Months 13-24

- Extended studies to 25 tumors
- Refined technique of obtaining normal pleural samples
- Extending studies in normal pleura in order to complete statistically valid comparison
- Concluded that expression signature of human mesothelioma compared to normal pleura contains predominantly macrophage markers and Th2 markers

h. Analyze cytokines produced by mesothelioma tumor fragments by commercial cytokine bead assay from human mesothelioma grown as tumor fragments (months 1-12).

Months 1-24

- Determined that cytokines were secreted by tumor fragment spheroids, but were too dilute for accuracy

Months 25-36

- Reduced volume of supernatant until cytokine signal was improved; cytokines produced by tumor fragment spheroids included Th2 cytokines such as IL-4, consistent with genetic expression signature described above

i. Analyze cytokines produced by multicellular spheroids made from either mesothelioma cells alone or mesothelioma cells plus macrophages (THP-differentiated) (months 1-12).

Months 1-12.

- Quantified expression of key M1 or M2 cytokines in multicellular spheroids e.g M1 (TNF, IL-12, IL-6) or M2 (IL-4, IL-10, TGF) compared to housekeeping gene TBP
- Determined that the polarization protocol does polarize hybrid spheroids to an M1 or M2 phenotype

j. Analyze changes in cytokine expression when spheroids (mesothelioma only or mesothelioma plus macrophage) are treated with TRAIL or TRAIL plus gemcitabine (months 1-12).
Months 1-12
- Determined that treatment with TRAIL or TRAIL plus gemcitabine has no significant effect on the M1 or M2 phenotype

Task 2. To determine the functional significance of macrophages as regulators of mesothelioma apoptosis in vitro.

a. Confirm ability of clodronate-liposomes to deplete macrophages from multicellular spheroids made either with THP-1 differentiated macrophage cells or with primary mesothelioma-derived macrophages and determine optimal dose and timing (months 9-14).
Months 1-12
- Obtained and prepared clodronate-liposomes for in vivo use
- Use in vitro systems not initiated in months 1-12

Months 13-24
- Have moved to use of CSF1 receptor antagonists
- PLX3397, a CSF1 receptor inhibitor, reduces the survival of macrophages in vitro
- PLX3397 also reduces the number of macrophages in hybrid spheroids

Months 25-36
- Both PLX3397 and GW2580 reduce the viability of cultured macrophages, primarily those polarized toward a Th2 phenotype.

b. Confirm ability of clodronate-liposomes to deplete macrophages from mesothelioma tumor fragment spheroids with dose and timing established above (months 12-24).
Months 1-12
- Obtained and prepared clodronate-liposomes for in vivo use
- Use in vitro systems not initiated in months 1-12

Months 13-24
- GW2580 over 3 days reduces the number of macrophages found in tumor fragment spheroids

c. Analyze effect of macrophages and of macrophage depletion on apoptosis of mesothelioma cells to treatment with TRAIL or TRAIL plus gemcitabine using multicellular spheroids either with no macrophages, macrophage-depleted or with macrophages (months 12-18).
Months 1-12
- Derived macrophages from peripheral blood and THP monocyte-like cells
- Produced hybrid spheroids with two different mesothelioma cell lines (REN, M28) combined with macrophages derived from 4 different sources (THP, peripheral blood, banked blood, mesothelioma tumors)
- Established an imaging protocol to show that macrophages were viable and well distributed within spheroids
- Determined that macrophages alone (without polarization) had no consistent effect on the mesothelioma cell apoptotic response to treatment

Months 13-24
- Shown that macrophages are necessary for the effect of polarization on apoptotic response

Months 25-35

• Demonstrated that both PLX3397 and GW2580 increased the apoptotic response of mesothelioma cells to carboplatin plus pemetrexed when the mesothelioma cells were in hybrid spheroids that had been polarized toward Th2, again confirming that these inhibitors of macrophage viability and function target the Th2 macrophage

d. Analyze the effect of macrophages and of macrophage depletion on apoptosis of mesothelioma cells in tumor fragment spheroids to treatment with TRAIL or TRAIL plus gemcitabine.
Month 1-12
• Not initiated in months 1-12. Plan to use optimal protocol worked out in Task 2c in multicellular, hybrid spheroids
Month 13-24
• Planning these studies using the PLX3379 compound in next 12 months
Month 25-36
• Have demonstrated that PLX3397 and GW2580 (shown) increase the apoptotic response of the mesothelioma cells within tumor fragment spheroids when the spheroids are polarized to the Th2-phenotype; as in the hybrid spheroids, the CSF1R blockade appears to target the Th2-polarized macrophage leading to an increased chemoresponsiveness of the mesothelioma cells.

e. Confirm ability of M1 cytokines to polarize macrophages to a strong M1 phenotype by exposing multicellular spheroids with macrophages (primary mesothelioma or THP-1) to M1 polarizing agents (interferon gamma plus LPS) and confirming with cytokine and gene expression assays for classics M1 or M2 polarization (months 12-24).
Months 1-24
• Determined that M1 stimulation (IFN gamma plus LPS) does polarize macrophages to an M1 phenotype (e.g. expressing TNF, IL12 and IL6)
• Determined that M2 stimulation (IL4 - M2a or IL10 - M2c) does polarize macrophages to an M2 phenotype (e.g. expressing TGF beta, IL10 and IL4)

f. Polarize macrophages in tumor fragment spheroids using the approach found best above (months 12-24).
Months 1-12
• Not initiated in months 1-12.
Months 13-24
• Polarized macrophages within tumor fragment spheroids from 4 human tumors
• Confirmed with PCR the effective M1 vs M2 polarization of the tumor fragments

g. Determine whether repolarization of macrophages enhances apoptosis of mesothelioma cells in multicellular spheroids (grown with primary mesothelioma macrophages or THP-1 macrophages) when treated with TRAIL plus gemcitabine (months 12-24).
Months 1-12
• Determined that the presence of M1 polarized macrophages consistently enhances mesothelioma cell apoptotic responses to TRAIL plus gemcitabine
• Showed that this conclusion does not depend on the source of macrophages although peripheral blood monocyte appear most consistent
• Showed, in expanded studies, that this pro-apoptotic effect was mediated by soluble factors and could be reproduced by exposing the mesothelioma cells to media conditioned by M1 polarized macrophages
• Determined that the cytokine TNF (an M1 cytokine), but not IL-10 (an M2 cytokine), contributed to the macrophage pro-apoptotic effect

h. Determine whether repolarization of macrophages enhances apoptosis of mesothelioma cells in tumor fragment spheroids (months 12-24).
Months 1-12
- Not initiated in months 1-12

Months 13-24
- Determined that polarization significantly enhances apoptosis induced by carboplatin plus pemetrexed in tumor fragment spheroids from 4 human mesothelioma patients
- Determined that polarization to an M1 phenotype may also increase apoptosis without treatment

Months 25-36
- Determined that Th1 polarization consistently increases the apoptotic response of mesothelioma cells to chemotherapy

Task 3. Define functional significance of macrophage depletion or repolarization on mesothelioma survival in vivo.

a. Characterize immune cell profile of the murine mesothelioma model induced by intraperitoneal asbestos injections in the NF2+/- mouse model (months 0-24).
Months 1-12
- Established an NF2+/- mouse cohort of 20 mice that have completed the required 8 every 3 weeks intraperitoneal asbestos injections for production of mesothelioma
- Initiated a further cohort of 30 mice that are in process of receiving the 8 asbestos ip injections
- Established a second immunocompetent mouse model of mesothelioma using the 40L murine mesothelioma cell line obtained from Dr. Agnes Kane
- Carried out one experiment in the 40L mesothelioma model showing that mesothelioma develops in 4 weeks, as reported by Dr. Kane
- Characterization of immune cell population in these two models (asbestos induced NF2+/- and syngeneic orthotopic 40L) are planned for months 12-36

Months 13-24
- Determined techniques of pleural lavage cytology and Doppler ultrasound to try to identify mesothelioma in the NF2+/- mice
- Concluded that the incidence of mesothelioma in these mice was too unusual and too random to allow useful treatment trials
- Developed a collaboration with Dr. Richard Lake of Perth Australia in order to use the MexTag mesothelin-SV40 mice which develop mesothelioma earlier (20-40 weeks) after only 1-2 intraperitoneal injections of asbestos
- Ordered these mice

Months 25-36
- MexTag mice were injected with asbestos intraperitoneally and are now starting to develop de novo mesothelioma
- Completed the immunophenotyping by 14 multicolor FACS on the immune influx in the orthotopic 40L mouse mesothelioma model; macrophages are the predominant inflammatory cell

b. Deplete in vivo macrophages with intraperitoneal clodronate-liposomes in mice without mesothelioma to establish protocol (months 4-12).
Months 1-12
- Depleted intraperitoneal macrophages successfully using clodronate-embedded liposomes from GFP-macrophage labeled mice by showing loss of GFP signal and loss of cells co-staining with F4/80 and CD11b+

Months 13-24

• Depleted intraperitoneal macrophages using PLX-3397 in the orthotopic 40L mesothelioma model

Months 25-36

• Use both PLX3397 and GW2580 and show that they deplete macrophages in in vivo tumor

c. Deplete in vivo macrophages in mice with mesothelioma to confirm depletion, to establish effect on other immune cell populations, and to confirm lack of toxicity on the mice (months 12-24).

Months 1-12

- Used clodronate-liposomes in mice with 40L orthotopic mesothelioma
- Collected tissues after clodronate treatment to determine whether clodronate used in this schedule depleted all tumor-associated macrophages

Months 13-24

- Found that PLX-3397 is non-toxic to mice over 2 weeks, given in chow
- Determined that PLX-3397 increased CD8 and CD4 positive T cells

Months 25-36

- Showed that PLX3397 depleted tumor associated macrophages in the orthotopic mouse mesothelioma model
- Showed that PLX3397 also increased CD8+ T cells
- Confirmed that PLS3397 was nontoxic to mice, as shown by normal behavior and lack of alteration of key serum biomarkers

d. Determine whether macrophage depletion in mice with mesothelioma enhances apoptotic and therapeutic response of mice with mesothelioma to TRAIL plus gemcitabine over 1-4 weeks after treatment (months 24-36).

Months 1-12

- Carried out one experiment in which mice implanted ip with 40L syngeneic mesothelioma cells were first given clodronate-liposomes (or PBS or PBS-liposomes) intraperitoneally and then treated with nothing, TRAIL alone, gemcitabine alone or TRAIL plus gemcitabine
- Showed that clodronate-treatment itself was associated with a lower tumor burden
- Found that clodronate-treatment increased the efficacy of the treatment with TRAIL plus gemcitabine
- Collected ascites and tissues for determining presence of macrophages, other immune cells, tumor burden and apoptotic cells

Months 13-36

- Found that PLX-3397 when given alone without chemotherapy is able to reduce the burden of mesothelioma
- Found that PLX-3397 when given alone without chemotherapy is able to increase the apoptosis of the tumor cells without altering the proliferation

e. Repolarize macrophages in vivo by injections of interferon gamma plus LPS and assaying cytokines of peritoneal ascites and gene expression of peritoneal macrophages obtained by lavage to confirm method of repolarization, and to select best tolerated method for the mice (months 12-24).

- Not initiated in months 1-36 due to focus on the clinically relevant approach of CSF1 receptor antagonism

f. Determine whether depletion or repolarization of macrophages in vivo enhances the apoptotic and therapeutic response of mice with mesothelioma to TRAIL plus gemcitabine over 1-4 weeks after treatment (months 24-36).

- Not initiated in months 1-24 due to focus on the clinically relevant approach of CSF1 receptor antagonism

Months 24-36

- Showed that GW2580 itself decreased macrophage infiltration but did not reduce tumor burden
- Showed that GW2580 enhanced the effect of chemotherapy on tumor burden
- Showed that GW2580 significantly increased the apoptotic response to chemotherapy

g. Determine whether the depletion or repolarization of macrophages enhances survival of mice with mesothelioma after treatment with vehicle, TRAIL or TRAIL plus gemcitabine (months 24-36).

- Carrying out survival studies using the newly acquired MexTag mice which develop asbestos-induced mesothelioma

IV. REPORTABLE OUTCOMES

A. MANUSCRIPTS (Provided in Appendix B)

Months 1-12

• DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev, 29(2):309-316*, 2010.

Months 13-24

• Barbone D, Ryan J, Kolhatkar N, Chacko AD, Jablons DM, Sugarbaker DJ, Bueno R, Letai AG, Coussens LM, Fennell DA, BROADDUS VC. The Bcl-2 repertoire of mesothelioma spheroids underlies acquired apoptotic multicellular resistance. *Cell Death and Disease* 2, e174, 2011.

• Broaddus VC, Everitt JI, Black B, Kane AB. Non-neoplastic and neoplastic pleural endpoints following fiber exposure. *J Toxicol Environ Health* 14:153-178, 2011.

• Coussens LM, Pollard JW. (2011) Leukocytes in mammary development and cancer. *Cold Spring Harbor Perspectives in Biology*, 3(3) PMID: 21123394

• DeNardo DG, Brennan DJ, Rexhapaj E, Ruffell B, Shiao SL, Madden SF, Gallagher WM, Wadhwani N, Keil SD, Junaid SA, Rugo HS, Hwang ES, Jirstrom K, West BL, Coussens LM. (2011) Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discovery,* 1(1): 54-67.

Featured in:

• *Nature* (2011), 472 303-304. '*Macrophages Limit Chemotherapy"* by, M. De Palma and C.E.Lewis

• Nature Reviews Cancer (2011) 11:3056. '*Bad company*', by N. McCarthy

• Erez M. Coussens LM. Leukocytes as paracrine regulators of metastasis and determinants of organ-specific colonization. *Int J of Cancer* (2011) 128:2536-2544.

Months 25-36:

• Phung YT, Barbone D, BROADDUS VC, Ho M. Rapid generation of in vitro multicellular spheroids for the study of monoclonal antibody therapy. *J Cancer* 2:507-514, 2011.

• Daldrup-Link HE, Golovko D, Ruffell B, DeNardo DG, Castaneda R, Ansari C, Rao J, Tikhomirov GA, Wendland MF, Corot C, Coussens LM. MRI of tumor-associated macrophages with clinically applicable iron oxide nanoparticles. *Clin Cancer Res* 17(17):5695-5704, 2011.

• Shiao SL, Ganesan AP, Rugo HS, Coussens LM. Immune microenvironments in solid tumors: new targets for therapy. *Genes & Dev* 25:2559-2572, 2011.

• Ruffell B, Au A, Rugo HS, Esserman LJ, Hwang ES, Coussens LM. Leukocyte composition of human breast cancer. *Proc. Natl Acad. Sci.,*109(8):2796-2801, 2011

B. ABSTRACTS
Months 1-36 (Provided as Appendix C in this Progress Report)

C. PRESENTATIONS
Symposia and Workshops: International

2011 Broaddus, VC. Invited Expert. 14th World Conference on Lung Cancer (WCLC), International Association for the Study of Lung Cancer, Amsterdam, The Netherlands.

Symposia and Workshops: National

2009 Broaddus, V. Courtney. *Role of macrophages in apoptotic resistance of mesothelioma.* In Workshop on Preclinical Drug and Target Discovery Pipeline. International Mesothelioma Program, Brigham and Womens Hospital, Harvard Medical School, Boston, MA, USA.

2010 Coussens, Lisa M. PLENARY LECTURE. *Regulation of protumor immunity and cancer development.* 2010 *Annual Meeting of the American Association for Cancer Research*, Washington DC USA.

2010 Jablons, David M. *Exploiting emerging biology for the treatment of malignant mesothelioma.* In Symposium entitled: Translational Initiatives in Mesothelioma. American Association of Cancer Research, Washington DC, USA.

2010 Coussens, Lisa M. *Regulation of protumor immunity and cancer development.* IN: *3rd Annual Wyeth Discovery Frontiers in Human Disease Symposium*, New York, NY USA

2010 Coussens LM. *Inflammation and cancer: reprogramming the immune microenvironment as an anti-cancer therapeutic strategy.* NCI, Immunity Inflammation and Cancer Conference, Bethesda, MD.

2011 Broaddus VC. A role for macrophages in a recalcitrant tumor, mesothelioma. In: Cancer, Immunity and Microenvironment Program, UCSF.

2011 Broaddus VC. Malignant mesothelioma: what is the role of radiotherapy? Radiation Oncology Grand Rounds, UCSF.

2012 Broaddus VC. Spheroids and 3D insights into apoptotic resistance; mesothelioma and lung cancer. Harvard Lung Conference, Boston, MA.

Invited Lectures/Seminars: International

2010 Broaddus VC. Invited speaker, International Mesothelioma Interest Group, Kyoto, JAPAN.

2010 Coussens LM. Invited speaker. Nature CNIO Cancer Symposium "Frontiers in Tumour Progression", Madrid, Spain.

2011 Coussens LM. Modulating immune response to improve cancer therapy. Australian Society for Immunology, Australia.

2012 Coussens LM. Inflammation and Cancer: reprogramming the immune microenvironment as an anti-cancer therapeutic strategy. International Symposium of the Collaborative Research Center, Bad Neuenahr Ahrweiller, Germany.

Invited Lectures/Seminars: National

2010 Coussens, Lisa M. *Inflammation and cancer: polarized immune responses regulate cancer development.* Cold Spring Harbor Laboratory, CSH NY USA

2010 Broaddus, V. Courtney. *Current studies under the DOD grant mechanism: macrophages and their contribution to mesothelioma.* Mesothelioma Applied Research Foundation, Washington, DC, USA

2010 Coussens LM. *Inflammation and cancer.* MRS-AACR Joint Conference on Metastasis and the Tumor Microenvironment. Philadelphia, PA

2011 Coussens LM. Modulating immune response to improve therapy for breast cancer. San Antonio Breast Cancer Conference, San Antonio, TX.

2012 Broaddus VC. Manipulating the core apoptotic machinery in mesothelioma. 2nd International Symposium on Lung-sparing Therapies for Malignant Mesothelioma, UCLA, Santa Monica, CA.

2012 Coussens LM. Inflammation and cancer: reprogramming the immune microenvironment as an anti-cancer therapeutic strategy. Cancer Immunotherapy Consortium.

2012 Broaddus VC. Asbestos and its toxic relationship with the pleura: update and future concerns. Medical Grand Rounds, Massachusetts General Hospital, Boston, MA.

Presentations by Coussens or Broaddus Lab Members:

2009 Kohatkar, Nikita. *Macrophages contribute to mesothelioma chemoresistance.* Poster presentation. Annual UCSF Pulmonary Research Retreat, San Francisco, CA.

2010 Kohatkar, Nikita. Targeting macrophages as a novel therapeutic approach for malignant pleural mesothelioma. Invited presentation. American Association for Cancer Research. Washington, DC.

2010 Blakely CM. Targeting macrophages in a preclinical model of mesothelioma. Hematology-Oncology Scientific Retreat, UCSF.

2011 Blakely CM. Macrophage-depletion as a novel therapeutic approach for malignant pleural mesothelioma. Poster presentation at the American Association of Cancer Research (AACR), Orlando, FL

2011 Battula S. Th1-polarized macrophages enhance the apoptotic response to chemotherapy in mesothelioma. Pulmonary Research Retreat, UCSF.

2011 Blakely CM. CSF1 receptor blockade by the tyrosine kinase inhibitor PLX3397 reprograms malignant mesothelioma tumor microenvironments and decreases tumor growth. AACR-NCI-EORTC International Conference, San Francisco, CA.

2012 Battula S. Th1-polarized macrophages enhance the apoptotic response to chemotherapy in mesothelioma. Oral presentation, American Thoracic Society, San Francisco, CA.

2012 Battula S. Macrophages can be manipulated to enhance the apoptotic response to chemotherapy in mesothelioma. Oral presentation, International Mesothelioma Interest Group, Boston, MA.

D. PATENTS AND LICENSES: None

E. DEGREES OBTAINED: None

F. REAGENT DEVELOPMENT:
• Preparation of tumor tissue microarrays prepared with 71 mesothelioma tumors
• Preparation of tumor tissue microarrays stained for CD68, CD4, CD8 and a multitude of inflammatory markers
• Generation of hybrid multicellular spheroids with macrophages derived from THP cell lines
• Generation of hybrid multicellular spheroids with macrophages derived from peripheral blood monocytes
• Generation of hybrid multicellular spheroids with macrophages derived from macrophages isolated from fresh human mesothelioma
• Collection of peripheral blood from patients with mesothelioma
• Collection of frozen mesothelioma tissue from patients at the time of surgery to be used for RNA extraction and qPCR

G. FUNDING APPLIED FOR BASED ON WORK SUPPORTED BY THIS FUNDING:

AWARDED
• Melissa Wheeler (UCSF Pharm D student in Coussens' lab)
NIH/NCRR/OD UCSF-CTSI Grant Number TL1 RR024129

• Sailaja Battula, PhD (Postdoctoral student in Broaddus' lab)
NIH Training Grant Postdoctoral Fellowship (2011-2013)

PENDING

• Barbone, Dario. Mesothelioma Applied Research Foundation. 2012
• Barbone D. SPORE Mechanism Career Development Award. 2012

IN PREPARATION

• Coussens LM, Kindler H, Broaddus VC. NIH / NCI RO1, Multi-PI

H. EMPLOYMENT/RESEARCH OPPORTUNITIES APPLIED FOR:

• Kolhatkar, Nikita. Accepted to a postdoctoral research program in immunology at The University of Washington, Seattle, WA. 2010

V. CONCLUSION

Inflammation is now a recognized as promoting solid tumor growth and survival. The inflammatory cells release cytokines and soluble mediators that *directly* promote growth and survival of the malignant cells, and *indirectly* support the tumor by inducing angiogenesis or by suppressing effective cytotoxic T-cell functions. Each tumor now appears to have a unique inflammatory cell milieu. From our studies to date, mesothelioma appears to have an innate inflammatory profile, with a predominance of macrophages and neutrophils, whereas the other thoracic malignancies studied have a more adaptive profile.

The macrophage is the major inflammatory cell type in mesothelioma and, as such, may play a powerful role in mesothelioma development, maintenance and resistance to chemotherapy. Macrophages from these tumors retain plasticity and can be polarized to an M1 or an M2 phenotype. Macrophages, when polarized to an M1 phenotype, alter the sensitivity of the mesothelioma cells to therapy. This sensitization to chemotherapy does not require contact but can be reproduced by conditioned media from the macrophages. Thus, polarizing the tumor microenvironment to a pro-apoptotic (M1) phenotype is a possible therapeutic strategy.

Another strategy is to deplete or inhibit the tumor-associated macrophages, which at baseline are in a pro-tumor (M2) phenotype. These studies have made progress in the in vivo setting, in which clodronate, and now CSF1 receptor antagonists, are able to reduce macrophages and increase apoptosis. These early in vivo studies show the exciting potential for manipulation of the macrophage in the tumor environment. If depletion of the macrophages can produce a significant improvement in mesothelioma treatment in this one model, then polarization to an M1 phenotype may have even a greater benefit.

At the end of our final funding cycle, we have achieved most of our key aims and plan to continue in this promising area. We have confirmed our initial findings in our application and extended them significantly toward developing new therapeutic strategies targeting the tumor-associated macrophage.

FUTURE DIRECTIONS & PLANS

Dr. Lisa Coussens, Dr. Broaddus and Dr. Battula are continuing the work into the mechanisms of macrophage enhancement of chemosensitivity and in the therapeutic possibilities of anti-CSF1R antagonism. We aim to complete experiments using the asbestos-induced mesothelioma in MexTag mice to confirm our findings in the orthotopic mesothelioma model and carry out survival studies to show that the effect of anti-CSF1R reagents such as GW2580 on decreasing tumor burden also translate to increased survival. A major publication on our findings is in preparation. We also are currently writing a multi-PI NIH RO1 grant application to enable more basic, animal and clinical studies; for the clinical trial design and supervision, we have engaged Dr. Hedy Kindler, Medical Oncologist at the University of Chicago who brings extensive experience in mesothelioma clinical trials.

VI. BIBLIOGRAPHY

1. Dostert, C., et al., *Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica.* Science, 2008. **320**: p. 674-677.
2. Hill, R.J., R.E. Edwards, and P. Carthew, *Early changes in the pleural mesothelium following intrapleural inoculation of the mineral fibre erionite and the subsequent development of mesotheliomas.* J. Exp. Pathol. , 1990. **71**(1): p. 105-118.
3. Fennell, D.A. and R.M. Rudd, *Defective core-apoptosis signalling in diffuse malignant pleural mesothelioma: opportunities for effective drug development.* Lancet Oncol. , 2004. **5**(6): p. 354-362.
4. Pollard, J.W., *Macrophages define the ivasive microenvironment in breast cancer.* J. Leukocyte Biol., 2008. **84**: p. 1-8.
5. Dave, S.S., et al., *Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells.* . N. Engl. J. Med., 2004. **351**: p. 2159-2169.
6. Tsutsui, S., et al., *Macrophage infiltration and its prognostic implications in breast cancer.* Oncol. Rep., 2005. **14**(2): p. 425-431.
7. Johansson, M., D.G. Denardo, and L.M. Coussens, *Polarized immune responses differentially regulate cancer development.* Immunol. Rev., 2008. **222**: p. 145-154.
8. Montovani, A., A. Sica, and M. Locati, *New vistas on macrophage differentiation and activation.* Eur. J. Immunol. , 2007. **37**: p. 14-16.
9. Robinson, C., et al., *MexTAg mice exposed to asbestos develop cancer that faithfully replicates key features of the pathogenesis of human mesothelioma.* Eur J Cancer, 2011. **47**(1): p. 151-61.
10. Robinson, C., et al., *A novel SV40 TAg transgenic model of asbestos-induced mesothelioma: malignant transformation is dose dependent.* Cancer Res, 2006. **66**(22): p. 10786-94.
11. Miselis, N.R., et al., *Targeting tumor-associated macrophages in an orthotopic murine model of diffuse malignant mesothelioma.* Mol. Cancer Ther., 2008. **7**(4): p. 788-799.

VII. APPENDICES

A. Complete academic curriculum vitae for Dr. V. Courtney Broaddus and Dr. Lisa Coussens
B. Publications for months 1-36
C. Abstracts for months 1-36

Prepared: 7/31/12

University of California San Francisco

CURRICULUM VITAE

Name: **V. Courtney Broaddus**

Position: Professor of Medicine, Step 3
Department of Medicine
School of Medicine

Address: Division of Pulmonary & Critical Care Medicine
San Francisco General Hospital
UCSF Box 0841
San Francisco, CA 94110-0841

Voice: (415) 206-3513
Fax: (415) 206-4123
Email:cbroaddus@medsfgh.ucsf.edu

EDUCATION:

1971-1975	Duke University, Durham, NC	B.S.	Summa Cum Laude, Zoology
1975-1979	University of Pennsylvania	M.D.	Medicine
1979-1980	University of Pennsylvania	Intern	Medicine
1980-1982	University of Pennsylvania	Resident	Medicine
1983-1986	University of California, San Francisco	Fellow	Pulmonary/ Critical Care Medicine
2001-2002	Comprehensive Cancer Center, UCSF	Sabbatical	Laboratory of Gerard Evan, Ph.D.
2007	Leadership Development for Physicians in Academic Health Centers		Harvard School of Public Health

LICENSES, CERTIFICATION:

1982	Medical License, California	G-049379
1982	Internal Medicine	American Board of Internal Medicine
1986	Pulmonary Disease	American Board of Internal Medicine

PRINCIPAL POSITIONS HELD:

1986-1988	University of California, SF	Instructor in Residence	Medicine
1988-1995	University of California, SF	Assistant Professor in Residence	Medicine
1995-1997	University of California, SF	Associate Professor in Residence	Medicine
1997-2001	University of California, SF	Associate Professor	Medicine
2001-now	University of California, SF	Professor	Medicine

OTHER POSITIONS HELD CONCURRENTLY:

1998-now	Dept of Medicine	Chief, Division of Pulmonary and Critical Care Medicine, SFGH

HONORS AND AWARDS:

1974	Phi Beta Kappa, Junior Year
1975	Summa Cum Laude
1978	Alpha Omega Alpha, Junior Year
1979	Janet M.Glasgow Memorial Award and Citation
1985	Individual National Research Service Award, NIH
1987	Clinical Investigator Award, NHLBI
1988	American Physiological Society, Elected
1991	Distinction in Teaching Award, Academic Senate, UCSF
1991	Associate, Scientific Staff, Cardiovascular Research Institute, Elected
1992	Pre-tenure Award, UCSF
1999-2002	President, International Mesothelioma Interest Group
2003	Nomination for Most Outstanding Teacher, UCSF Women In Medicine
2005-2012	Best Doctors in America, Selected
2005	Faculty of the Year Award, SFGH Association of Business Officers' Group
2006	Western Society of Clinical Investigation, Elected
2007&2008	Nomination for Subspecialist Consultant of the Year Award, SFGH
2008	Nomination for Wagner Award, International Mesothelioma Interest Group
2010	Michael S. Stulbarg Outstanding Teaching Award, UCSF Pulmonary
2010	Pioneer Award, The Mesothelioma Applied Research Foundation
2012	John F. Murray Distinguished Professorship, Inaugural Recipient

KEYWORDS/AREAS OF INTEREST:

Apoptosis, mesothelioma, macrophages, synergy, 3-dimensional models, TRAIL (TNF-related apoptosis-inducing ligand), pleural disease, pleural effusions, asbestos.

PROFESSIONAL ACTIVITIES

CLINICAL

Attending, Medical Intensive Care Unit, SFGH: I attend 1 month of the year on the ICU, 7 days a week, supervising 8 senior residents and interns.

Attending, Pulmonary Consult Service, SFGH: I attend 1 month each year seeing inpatients with pulmonary problems, 5 days a week, supervising 2 fellows and 1-2 residents/medical students.

PROFESSIONAL ORGANIZATIONS

Memberships
Lung Biology Center, San Francisco General Hospital
Thoracic Oncology Research Group, UCSF Cancer Center
Cardiovascular Research Institute, University of California, San Francisco
Western Society of Clinical Investigation
American Thoracic Society
California Thoracic Society
International Mesothelioma Interest Group
Mesothelioma Applied Research Foundation
International Association for the Study of Lung Cancer (IASLC)

Service to Professional Organizations

1988-1992	ALA/American Thoracic Society	Research Fellowship Review Committee
1989-1991	ALA/American Thoracic Society	Manpower Review Committee
1991-1992	American Thoracic Society	Program Committee,

		Respiratory Structure and Function
1993-1994	American Thoracic Society	Nominating Committee, Respiratory Cell and Molecular Biology
1994-1995	American Thoracic Society	Women's Affairs Committee
1994-1997	American Thoracic Society	Program Committee, Respiratory Cell and Molecular Biology
1995-1997	California Lung Association	Research Fellowship Committee
1995-2001	American Thoracic Society	Long Range Planning Committee, Respiratory Cell and Molecular Biology
1995-1997	American Thoracic Society	Program and Budget Committee
1995-1999	Am Federation for Med Research	UCSF Representative
1997-2003	American Lung Association of CA	Research Administrative Committee
1999-2002	Intl Mesothelioma Interest Group	President
1999-2001	American Thoracic Society	Chair, Long Range Planning Committee, Respiratory Cell and Molecular Biology
2000-2002	American Thoracic Society	Planning on ATS Research Agenda
2000-2002	American Thoracic Society	Assembly Structure Task Force
2003-2004	American Thoracic Society	Education Committee
2003-2007	American Thoracic Society	Scientific Advisory Committee
2005-now	Intl Mesothelioma Interest Group	Member, Board of Directors Scientific Organizing Committee
2006-now	Intl Mesothelioma Interest Group	Chair, Website Subcommittee
2007-2009	American Thoracic Society	Chair, Nominating Committee, Respiratory Cell and Molecular Biology
2007-2008	American Thoracic Society	Member, Search Committee for Editor, Am. Journal of Respiratory Cell Mol. Biol.
2008-2011	Mesothelioma Applied Research Foundation	Member, Scientific Advisory Board
2012-now	International Association for the Study of Lung Cancer (IASLC)	Member, Core Program Committee 15th World Conference, Sydney, Australia
2011-now	Intl Mesothelioma Interest Group	Chair, Organizing Committee 11th International Conference, Boston, MA

SERVICE TO PROFESSIONAL PUBLICATIONS

1990-now Ad hoc referee for:
Oncogene (2 papers in last year)
American Journal of Pathology (1 paper in last year)
New England Journal of Medicine (2 papers in last year)
American Journal of Respiratory and Critical Care Medicine (4 papers in 2 years)
American Journal of Respiratory Cell and Molecular Biology (6 papers in 2 years)

2002-2006 Editor, Murray & Nadel's Textbook of Respiratory Medicine 4th Edition
2007-2010 Editor, Murray & Nadel's Textbook of Respiratory Medicine 5th Edition
2012- Editor-in-Chief, Murray & Nadel's Textbook of Respiratory Medicine, 6th Edition

2005-now Principal Editor,
Website for Murray and Nadel's Textbook of Respiratory Medicine
www.expertconsult.com
1996-1998 Associate Editor,
American Journal of Respiratory Cell and Molecular Biology
2000-2007 Editorial Board, American Journal of Physiology:
Lung Cellular and Molecular Physiology

INVITED PRESENTATIONS

INTERNATIONAL (Selected)

1995 International Chemokine Symposium, Bath, England (Invited speaker)
1996 International Meeting on the Toxicology of Natural and Man-Made Fibrous and Non-Fibrous Particles, Lake Placid, NY (Platform)
1999 International Meeting of the Formosan Medical Association, Taiwan, 1999 (Platform)
2008 Centre for Cancer Research and Cell Biology, Queen's University, Belfast, No. Ireland
2010 International Mesothelioma Interest Group, Kyoto Japan (Invited Speaker)
2011 IASLC/World Conference on Lung Cancer, Amsterdam, Netherlands (Invited Speaker)
2012 2nd Canadian Symposium on Malignant Mesothelioma, Vancouver, CA 2012
2012 2nd International Symposium on Lung-Sparing Therapies for Malignant Pleural Mesothelioma, Los Angeles, CA

American Thoracic Society International Conference. 1986 (Plenary talk); 1988 (Invited speaker, Course on the Academic Pulmonary Physician); 1989 (Invited speaker, Forum on Training and Transition); 1992 (Co-Chair, Poster Symposium); 1993 & 1994 (Co-Chair, Poster Discussion Symposium, Symposium); 1994 (Invited Speaker, Symposium); 1995 (Invited Speaker, Symposium; Meet the Professor Seminar); 1995 & 1997 (Chair, Speaker, Mini-symposium); 1996 (Co-chair, Speaker, Minisymposium); 1997 (Featured speaker, Minisymposium); 1999 (Invited speaker); 2000 (Speaker); 2001 (Co-chair, speaker, Mini-symposium); 2003 (Chair, Symposium); 2004 (Co-chair, Symposium; Meet the Professor Seminar); 2005 (Invited plenary speaker); 2006 (Invited Speaker, Participant in Expert Clinician Panel); 2007 (Co-Chair, Symposium; Presenter, Trudeau Award; Participant in Master Clinician Panel); 2008 (Participant in Master Clinician Panel; Co-Chair Symposium; Presenter); 2009 (Invited Clinical Expert in Pleural Disease; Participant in Master Clinician Panel; Co-Chair, Minisymposium); 2010 (Co-Chair, Minisymposium, Invited Presenter); 2012 (Co-Chair, Minisymposium)

International Mesothelioma Interest Group. San Francisco, 1993 (Invited Speaker); Paris, 1995 (Co-organizer, speaker); United Kingdom, 1999 (Speaker); Brescia, Italy, 2003 (Co-organizer, Speaker); Chicago, 2006 (Organizer Apoptosis Satellite Session, Co-chair, speaker). 2008 (Invited Plenary Speaker, Co-chair of symposium), 2009 (Program Planning Executive Committee, Invited Speaker, Chair of Symposia); 2012 (Program Planning Executive Committee, Invited Speaker, Tumor Microenvironment; Invited Speaker, Apoptosis Resistance).

NATIONAL

1988 Advances in Internal Medicine, San Francisco, CA
1989 Meet the Professor, American College of Physicians, San Francisco, CA
1989 University of Oklahoma Health Science Center, Oklahoma City, OK
1989 St. Luke's-Roosevelt Hospital Center, Columbia University, New York, NY
1990 National Institutes of Health Workshop, Bethesda, MD
1990 Invited Speaker, Scientific Conference on Acute Lung Injury, American Heart Association, Dallas, TX
1990 Advances in Internal Medicine, San Francisco, CA
1991 Advances in Internal Medicine, San Francisco, CA
1994 Chair and Invited Speaker, Cambridge Health Institute Conference on Inflammatory Cytokine Antagonists, Philadelphia, PA
1994 Invited Speaker, University of Pennsylvania, Philadelphia, PA
1994 Gordon Conference on Chemotactic Chemokines
1994 University of Pennsylvania, Philadelphia, PA
1995 Texas Thoracic Society, Austin, TX

1996 Selected Participant, Professional Development Seminar for Senior Women in Medicine, Association of American Medical Colleges, Washington DC
1996 Co-Moderator, Pleural Disease Minisymposium. American College of Chest Physicians, San Francisco, CA
1997 Visiting Pulmonary Scholar, Duke-UNC-NCSU-NIEHS-EPA-CIIT, NC
1998 Visiting Speaker, St. Thomas Hospital, Vanderbilt Univ, Nashville, TN
1998 Invited Speaker, First Annual Symposium on the Pleura, St. Thomas Hospital, Vanderbilt, TN.
1998 Visiting Professor, Medical Grand Rounds, University of Texas at Tyler, TX.
2000 Visiting Professor, Stanford University, Palo Alto, CA
2000 Invited Participant and Co-Chair, American Thoracic Society-National Institute of Environmental Health Science (ATS-NIEHS) Workshop-Toronto, Canada
2002 Visiting Professor, University of Southern California, Los Angeles, CA
2002 Visiting Professor, Yale University, New Haven, CT
2003 Visiting Professor, University of Montana, Center for Environmental Health Sciences, Missoula, MT
2004 Visiting Professor, University of Pennsylvania, Philadelphia, PA
2006 Co-Chair, Minisymposium, Experimental Biology, San Francisco, CA.
2006 Invited Participant and Co-Chair, International Mesothelioma Interest Group, Apoptosis Satellite Symposium, Chicago, IL
2006 Invited Speaker, University of Chicago School of Medicine
2006 Visiting Professor, Northwestern University School of Medicine
2007 Invited Speaker, International Mesothelioma Program, Harvard Medical School/Brigham & Women's Hospital, Boston, MA
2008 Invited Speaker, International Mesothelioma Interest Group, Amsterdam
2009 Invited Speaker, NIH Mesothelioma Symposium, Bethesda, MD.
2009 Invited Speaker, ATS/Hawaii Thoracic Society State of the Art Course, Maui, HI
2009 Invited Outside Reader, PhD Dissertation, Bonnie Lau, Dept of Pathology, Brown University, Providence, RI
2009 Invited Speaker, International Mesothelioma Program, Workshop on Preclinical Drug and Target Discovery, Brigham and Womens Hospital, Boston, MA
2010 Invited Speaker, Mesothelioma Applied Research Foundation, Washington, DC.
2012 Visiting Professor, Harvard Combined Pulmonary & Critical Care Fellowship Program, Boston, MA
2012 Invited Speaker, Medical Grand Rounds, Massachusetts General Hospital, Boston, MA.

REGIONAL AND OTHER INVITED PRESENTATIONS

1988 Medical Grand Rounds, SFGH
1993 Research Conference on Lung Injury, Genentech, So SF
1994 SFGH Cellular and Molecular Medicine Seminar
1995 Medical Grand Rounds, VAMC/ SFGH
1996 Medical Grand Rounds, Moffitt/UCSF
1996 Selected Participant, Senior Women's Conference, University of California San Francisco
1996 Panel Discussant, UCSF Department of Medicine.
1996 Medical Grand Rounds, SFGH
1997 Invited Speaker, UCSF Pulmonary Retreat, Asilomar, CA.

1998 Invited Speaker, UCSF Pulmonary Retreat, Asilomar, CA.
1998 Invited Speaker, Thoracic Oncology Conference, UCSF/Stanford/Mt. Zion
2000 Medical Grand Rounds, Stanford University
2000 Invited speaker, Division of Pulmonary and Critical Care, Stanford Univ.
2000 Invited Speaker, Pulmonary Research Retreat, UCSF
2003 Medical Grand Rounds, SFGH
2003 Invited Speaker, Recent Advances in Pulmonary and Critical Care Medicine
2003 Invited Speaker, Pulmonary Research Retreat, UCSF
2005 Invited Speaker, Thoracic Oncology Research Group, UCSF
2005 Invited Speaker, Radiation Oncology Grand Rounds, UCSF and Mt Zion
2006 Invited Speaker, Thoracic Oncology Research Group, UCSF
2006 Invited Speaker, Pulmonary Research Retreat, UCSF
2008 Invited Speaker, Dean's Seminar Series, SFGH
2009 Invited Speaker, Pulmonary Research Retreat, UCSF
2011 Invited Speaker, Cancer, Immunity & Microenvironment Program, UCSF
2011 Invited Speaker, Radiation Oncology Grand Rounds, UCSF

GOVERNMENT and OTHER PROFESSIONAL SERVICE:

1994 Ad Hoc Member, Comparative Medicine Review Committee, NIH
1999 Ad Hoc Reviewer, National Heart, Lung, and Blood Institute
2001 Ad Hoc Reviewer, National Cancer Institute
2002 Invited Member, NIH Center for Scientific Review, Special Emphasis Panel, Experimental Therapeutics Panel (ZRG1 ET-1).
2004 Site Reviewer, Program Project Grant Review Committee, NCI. MGH/Brigham and Women's, Boston, MA
2005 Invited Member, National Asbestos Research Working Group National Health and Medical Research Council, Australia
2006 Invited Participant, NHLBI Strategic Plan for the Division of Lung Diseases
2009 Reviewer, FY09 Peer-reviewed Medical Research Program, AIBS, US Army Medical Research and Materiel Command
2009 Expert Panel Member, NIEHS Asbestos Mechanism of Action Workshop
2010 Ad Hoc Reviewer, Special Emphasis Panel, Respiratory Integrative Biology and Translational Research Study Section

UNIVERSITY AND PUBLIC SERVICE

UNIVERSITY SERVICE

UCSF CAMPUS-WIDE

1996-1998 Member, Chancellor's Award for the Advancement of Women Committee
2003-now Member, Academic Senate Committee on Research, UCSF
2005 Chair, Academic Senate Task Force Reviewing University of California Policy on Human Subject Injury
2006-2007 Member, Reconvened SFGH Subcommittee of the Chancellor's Advisory Committee on the Long Range Planning Amendment
2006 & 2007 Faculty Presenter, Inaugural Faculty Welcoming Week, Chancellor's Council on Faculty Life, "Building a Research Career"
2007 Small group leader, Junior faculty retreat, Striving & Thriving in the Academic World, Chancellor's Advisory Committee on the Status of Women

SCHOOL OF MEDICINE

1988-1989 Internship Selection Committee, Univ. of Calif, San Francisco
2005 Member, Stewardship Review Committee, Chair of Dermatology, UCSF

2005	Member, Ad Hoc Subcommittee on Faculty Misconduct, Office of Academic Affairs
2006- 2007	Member, Search Committee for Chair, Department of Medicine
2006- 2007	Member, Select Subcommittee of Search Committee for Chair, Department of Medicine, UCSF
2007	Invited speaker, The Senior Faculty Career Challenge, Dean's Office of School of Medicine. "Strategies for revitalizing divisional goals/function"
2007- 2010	Member, Scholarships and Awards Committee, UCSF School of Medicine

DEPARTMENT OF MEDICINE

1988-1989	Member, Bylaws Committee, San Francisco General Hospital
1989	Member, Program Planning Committee Recent Advances in Pulmonary & Critical Care Medicine (8th Annual), University of California, San Francisco
1989-2000	Member, Critical Care Committee, San Francisco General Hospital
1990-1991	Chair, Program Planning Recent Advances in Pulmonary & Critical Care Medicine (9th and 10th Annual), Univ of California, San Francisco
1990-present	Member, Steering Committee of the Pulmonary Research Group, University of California, San Francisco (elected)
1993-present	Member, Pulmonary Fellowship Selection Committee, University of California, San Francisco
1994-present	Member, Pulmonary Research Group Retreat Planning Committee
1994-1996	Member, Search Committees for Chief of Surgical Research and Chief of Rheumatology, San Francisco General Hospital
1996-1998	Member, Task Force on Diversity, University of California, SF
1996-1999	Member, Search Committee for Joint Appointment in Radiology/Pulmonary and Critical Care Medicine, San Francisco General Hospital
1996-2002	Member, Promotions Subcommittee, Academic Senate, University of California, San Francisco
1996-1997	Member, Executive Committee, Department of Medicine, SFGH
1996-2002	Board Member, UCSF/Macy's Center for Creative Therapies, San Francisco General Hospital
1997	Member, Search Committee, first Associate Chair for Biomedical Research, Department of Medicine, UCSF
1997-1998	Member, Search Committee for Chief of Cardiology, SFGH
1998-1999	Member, Search Committee for Chief of Gastroenterology, SFGH
1998-1999	Member, Search Committee, Chief, Pulmonary and Critical Care Med, UCSF
1998-2000	Member, General Clinical Research Center Advisory Committee
1999-2000	Member, Search Committee for Thoracic Surgeon, SFGH
2000	Member, Search Committee for Chief of Radiology, SFGH
2000	Member, Search Committee for Manager, Department of Medicine, SFGH
2003-2004	Chair, Search Committee , Chief of Pulmonary & Critical Care Medicine, Veteran's Administration Medical Center, UCSF
2005-2006	Member, Search Committee for Chest Radiologist, SFGH
2005	Member, John Carbone Chair Nominating Committee
2006-	Member, Search Committee for Scientist, Surgical Research Laboratory
2006-2007	Member, Search Committees for Gastroenterology FTE
2006-current	Chair, Committee to Establish John F. Murray Distinguished Professorship
2008-now	Member, Search Committee for Lung Biology Center Physician-Scientist
2008-now	Member, Search Committee for Chief,

Division of Pulmonary & Critical Care Medicine, UCSF
2009-now Chair, Search Committee for Pulmonary Faculty Member, SFGH/UCSF.
2009-now Member, Recruitment and Retention Workgroup, Department of Medicine Strategic Plan
2011 Member, Search Committee for Associate Chair of Research
2011 Chair, Search Committee for Director, Medical ICU, SFGH
2011 Member, Search Committee for Hospital Director of Critical Care Medicine, SFGH

PUBLIC SERVICE

1997 Presenter on Asbestos-Related Diseases, Gloria R. Davis Academic Middle School
Asbestos Exposure & Risk Assessment, S.F. Department of Public Health
1997 Coordinator, Cigarette Smoke Demonstration, Take Your Daughters to Work Day
2005-7 Lecturer on Avian and Mammal Lungs, San Francisco Day School
2008-9 Fundraiser, Annual Fund, International High School, San Francisco.
2009-11 Class Captain, Annual Fund, International High School, San Francisco.

TEACHING AND MENTORING

Other Courses
1990-now Pulmonary Physiology Seminars (2 hours/year)
1990 Faculty Leader, Preparing Interns for Residency
1990-now Medical Service Conferences
1991 Women's Medical Student Association Retreat
1993-2003 Lecturer to Medical Residents (Parnassus, SFGH, VAMC)
1987-2002 Speaker, Recent Advances in Pulmonary & Critical Care Medicine
1998-now Summer Seminar Series and Practical Sessions on Pleural Disease
2004 Faculty Coach and Presenter for Junior Faculty, Mid-Term Appraisal for Faculty at UCSF
2004 Annual PIBS/BMS Course, Ethics and the Responsible Conduct of Research
2005 Lecturer to Medical Residents (Parnassus, SFGH, VAMC)
2005-2007 Workshop Leader, Mid-term Appraisal for Faculty at UCSF

Predoctoral Students Supervised

Dates	*Name*	*Position while supervised*	*Current position*
1995-1997	Sudha Rani Narasimha	Medical Student	Medical Resident, UCLA
1997-1999	Jack Wu	Undergraduate	Medical resident Cook County, Chicago, IL
2002-2004	Kevin Lee	Undergraduate	Medical student
2009- 2010	Nikita Kolhatkar	Graduate	current

Graduate Students Supervised

Dates	*Name*	*Position while supervised*	*Current position*
2005-6	Dario Barbone	Graduate student	Postdoctoral scholar, UCSF
2008-9	Bonnie Lau	Graduate student	MD/PhD Brown University

Postdoctoral Fellows Supervised

Dates	*Name*	*Position while supervised*	*Current position*
1989-1991	Alice M. Boylan, MD	Pulmonary research fellow	Associate Professor of Medicine Medical Univ of South Carolina

1991-1993	Rex Yung, MD	Pulmonary research fellow	Associate Professor of Medicine Johns Hopkins Univ.
1993-1995	Hans Folkesson, PhD	Postdoctoral research fellow	Associate Professor of Physiology Northeastern Ohio University, OH
1995-1996	Jamie Bigelow, MD	Pulmonary research fellow	Pulmonologist St. Francis Hospital, SF
1996-1997	Evaldo Marchi, MD	Visiting research fellow	Professor of Surgery Sao Paolo, Brazil
1998-2000	Tom Geiser, MD	Postdoctoral research fellow	Associate Professor of Medicine University Hospital, Bern, SW
2000-2001	Masa Ishigaki, MD PhD	Visiting research fellow	Associate Professor, Japan
2000-2003	Claire Vivo, PhD	Postdoctoral research fellow	Research Scientist Ordway Research Institute, NY
2003-2004	Ki Up Kim, MD	Visiting research fellow	Professor of Medicine, Chief of Pulmonary Division Soonchunhyang University Hosp. Seoul, South Korea
2004-2005	Lorriana Leard, MD	Pulmonary research fellow	Assistant Prof of Medicine, UCSF Associate Director, UCSF Fellowship Program
2003-2006	Keith Abayasiriwardana, PhD	Postdoctoral research fellow	Senior Scientist, Vaccine Research, Pfizer
2006- 2008	Tsung-Ming Yang, MD	Postdoctoral research fellow	Assistant Professor, Chiayi Chung-Gung Memorial Hosp Chiayi, Taiwan
2008-on	Eunice Kim, MD	Pulmonary Fellow	Pulmonary Research Fellow
2008-on	Denitza Blagev, MD	Pulmonary Fellow	Pulmonary Research Fellow
2008-on	Joyce Lee, MD	Pulmonary Fellow	Pulmonary Research Fellow
2009-on	Joshua Galanter, MD	Pulmonary Fellow	Pulmonary Research Fellow
2006-now	Dario Barbone, PhD	Postdoctoral Research Fellow	Postdoctoral Research Fellow
2010-now	Sailaja Battula, PhD	Postdoctoral Research Fellow	Postdoctoral Research Fellow

PROTEIINFORMAL TEACHING

1987-now: Attending Rounds, Pulmonary Consult Service, SFGH
(1 month/year with 2 fellows, 1 medical student and/or resident)

1987-now: Attending Rounds, Medical ICU Service, SFGH
(1.5 month/year with 4 3rd year residents and 4 interns/ informal and formal teaching)

1999-2002 Attending Rounds, Medicine Service, SFGH

FACULTY MENTORING

I have selected some representative examples of mentoring relationships from recent years.

Dates	*Name*	*Position While Mentoring*	*Role*	*Current Position*
2000-2002	David Morris, MD	Division chief	Informal advisor	Head Respiratory Research Roche, Palo Alto, CA
2000-2005	Robert Jasmer, MD	Division chief	Advisor	Physician, private practice
2004-now	Payam Nahid, MD	Division chief	Advisor, Reviewed grants	Assoc Prof, UCSF
2004-now	Mary Gray, MD	LBC Assoc Director	Recruited into LBC, collaborator/advisor	Assoc Prof, UCSF
2002-now	Laura Koth, MD	LBC Assoc. Dir.	Career advisor	Asst Prof, UCSF
2004-now	Lorriana Leard, MD	Lab head/Div.chief	Advisor, formal mentor	Asst Prof, UCSF
2005-2007	Dana McClintock, MD	Career committee	Selected formal mentor	Physician, academic practice

2005-now	Harold Collard, MD	Division chief	Advisor, reviewed grants	Asst Prof, UCSF
2006-2010	Janet Diaz, MD	Division chief	Advisor	Asst Prof, UCSF
2010-now	Antonio Gomez, MD	Division chief	Advisor	Asst Prof, UCSF

TEACHING AWARDS AND NOMINATIONS:

1991 Distinction in Teaching Award, Academic Senate, UCSF
2003 Nomination for Most Outstanding Teacher, UCSF Women In Medicine
2007 & 2008 Nominations for Subspecialist Consultant of the Year Award, SFGH Dept of Medicine
2010 Michael S. Stulbarg Outstanding Teaching Award, Pulmonary & Critical Care Medicine, UCSF

SUMMARY OF TEACHING HOURS:

2009-2010 Total anticipated hours of teaching: 540
Formal class or course teaching hours: 20
Informal teaching hours: 250 Mentoring hours: 270

RESEARCH AND CREATIVE ACTIVITIES
RESEARCH AWARDS AND GRANTS

ACTIVE:

Supporting Agency: Peer-Reviewed Medical Investigator-Initiated Research Program – Department of Defense
Grant Title: *The Role of Macrophage-induced Inflammation in Mesothelioma.*
Role on project : Principal Investigator
Time Commitment to Project: 3 Calendar Months Effort (25%)
Total Funding Period: 07/01/2009 – 6/30/2012 **Direct Dollars: / yr 1**
/ yr 1-3
Project Overlap: None
Goals: To demonstrate the functional significance of macrophages as promoters of tumor cell survival in mesothelioma and determine whether tumor-associated macrophages can be repolarized to enhance mesothelioma cell apoptosis.
Specific Aims: 1. To determine the functional significance of macrophage phenotype in mesothelioma. 2. To determine the functional significance of macrophages as regulators of mesothelioma apoptosis in vitro. 3. To define the functional significance of macrophage depletion or repolarization on mesothelioma survival in vivo.

Supporting Agency: Simmons Mesothelioma Foundation
Total Funding Period: 7/1/2012 – 6/30/14
Grant Title: *The Role of Macrophage-induced Inflammation in Mesothelioma*
Role on project: Principal Investigator
Time Commitment to Project: 30% **Direct Dollars**: / yr

Program Overlap: None
Goals: To demonstrate the functional significance of macrophages as promoters of tumor cell survival in mesothelioma and determine whether tumor-associated macrophages can be repolarized to enhance mesothelioma cell apoptosis.

Specific Aims: 1. To determine the functional significance of macrophage phenotype in mesothelioma. 2. To determine the functional significance of macrophages as regulators of mesothelioma apoptosis in vitro. 3. To define the functional significance of macrophage depletion or repolarization on mesothelioma survival in vivo.

Past

NIH Institutional National Research Service Award (HL07185)	1983-1985
NIH Individual National Research Service Award (HL07271)	1985-1986
Academic Senate Committee on Research Grant Origin of pleural effusions in volume-loaded sheep	1986
Academic Senate Committee on Research Grant Origin of pleural effusions in hydrostatic pulmonary edema	1988
NHLBI Pulmonary Vascular SCOR (HL19155) (PI) Dynamics of pleural liquid turnover in health and disease.	1986-1991
American Lung Association Research Grant, Formation of pleural effusions in pleural inflammation.	1987-1989
Clinical Investigator Award, NHLBI (KO8 HL01893) Comparative physiology of the normal and inflamed pleura.	1987-1992
NHLBI Pulmonary Vascular SCOR (HL19155) (Co-Investigator) Mechanisms of acute pleural and lung injury.	1991-1993

Pretenure Award, University of California, San Francisco Mechanisms of interaction of asbestos and mesothelial cells.	1992-1993
Corvas International, San Diego, CA. (Co-Investigator) The role of rabbit IL-8 in neutrophil-mediated inflammation.	1993-1994
Genentech (PI) The role of rabbit IL-8 in neutrophil-mediated inflammation.	1993-1994
RO1 ES06331 (NIEHS) (PI) Molecular interactions of asbestos and mesothelial cells.	1994-1998
Genentech (PI) The role of rabbit IL-8 in sepsis.	1995-1997
Research Evaluation and Allocation Committee Grant, UCSF The role of fiber internalization in mediating the toxic effects of asbestos on mesothelial cells.	1996-1997
Principal Investigator, Tobacco-related Disease Research Program Programmed cell death in cigarette-induced lung disease.	1998-2001
RO1 ES08985 (NIEHS) (PI) Protective role of apoptosis in asbestos pleural injury.	1997-2002
RO1 ES08985 (NIEHS) (PI) Supplement for microarray studies.	2000-2002
Peterson Family Foundation (PI) Role of Akt/mTOR in mesothelioma.	2005-2007
Buzzi Foundation, Italy Role of PI3K/Akt/mTOR pathway in resistance to apoptosis.	2005-2007
RO1 CA95671 (NIH/NCI) (PI) Amplification of TRAIL-induced apoptosis in mesothelioma.	2003-2009
Mesothelioma Applied Research Foundation Award. Antibody development against mesothelioma.	2007-2009

PEER REVIEWED PUBLICATIONS:

1. BROADDUS C, Dake M, Stulbarg MS, Blumenfeld W, Hadley K, Golden JA, Hopewell PC. Bronchoalveolar lavage and transbronchial biopsy for the diagnosis of pulmonary infections in patients with the acquired immunodeficiency syndrome. Ann Intern Med 102:747-752, 1985.

2. Wiener-Kronish JP, Goldstein R, Matthay RA, Biondi JW, BROADDUS VC, Chatterjee K, Matthay MA. Lack of association of pleural effusion with chronic pulmonary arterial and right atrial hypertension. Chest 92:967-970, 1987.

3. BROADDUS VC, Wiener-Kronish JP, Berthiaume Y, Staub NC. Removal of pleural liquid and protein by lymphatics in awake sheep. J Appl Physiol 64:384-390, 1988.

4. Berthiaume Y, BROADDUS VC, Gropper MA, Tanita T, Matthay MA. Alveolar liquid and protein clearance from normal dog lungs. J Appl Physiol 65:585-593, 1988.

5. Wiener-Kronish JP, BROADDUS VC, Albertine KH, Gropper MA, Matthay MA, Staub NC. Relationship of pleural effusions to increased permeability pulmonary edema in anesthetized sheep. J Clin Invest 82:1422-1429, 1988.

6. BROADDUS VC, Wiener-Kronish JP, Staub NC. Clearance of lung edema into the pleural space of volume-loaded anesthetized sheep. J Appl Physiol 68:2623-2630, 1990.

7. BROADDUS VC, Araya M, Carlton DP, Bland RD. Developmental changes in pleural liquid protein concentration in sheep. Am Rev Resp Dis 143:38-41, 1991.

8. Jacobson MA, Mills J, Rush J, Peiperl L, Seru V, Mohanty PK, Hopewell PC, Hadley WK, BROADDUS VC, Leoung G, Feigal DW. Morbidity and mortality of patients with AIDS and first-episode *Pneumocystis carinii* pneumonia unaffected by concomitant pulmonary cytomegalovirus infection. Am Rev Respir Dis 144:6-9, 1991.

9. BROADDUS VC, Araya M. Liquid and protein dynamics using a new, minimally invasive pleural catheter in rabbits. J Appl Physiol 72:851-857, 1992.

10. Boylan AM, Rüegg C, Hoeffel J, Kim KJ, Hébert CA, Pytela R, Sheppard D, Goldstein IM, BROADDUS VC. Evidence of a role for mesothelial cell-derived interleukin-8 in the pathogenesis of asbestos-induced pleurisy in rabbits. J Clin Invest 89:1257-1267, 1992.

11. BROADDUS VC, Hébert CA, Vitangcol RV, Hoeffel JM, Bernstein MS, Boylan AM. Interleukin-8 is a major neutrophil chemotactic factor in pleural liquid of patients with empyema. Am Rev Respir Dis 146:825-830, 1992.

12. BROADDUS VC, Feigal DW Jr. Starting an academic career: a survey of junior academic pulmonary physicians. Chest 105:1858-1863, 1994.

13. BROADDUS VC, Hoeffel JM, Boylan AM, Sadick M, Chuntharapai A, Kim KJ, Hébert CA. Neutralization of interleukin-8 inhibits neutrophil influx in a rabbit model of endotoxin-induced pleurisy. J Immunol 152:2960-2967, 1994.

14. Boylan AM, Hébert CA, Sadick M, Wong WL, Hoeffel JM, Hartiala KT, BROADDUS VC. Interleukin-8 is a major component of pleural liquid chemotactic activity in a rabbit model of endotoxin pleurisy. Am J Physiol Lung Cell Mol Physiol 267(11):L137-L144, 1994.

15. Folkesson HG, Matthay MA, Hébert CA, BROADDUS VC. Acid aspiration lung injury in rabbits is mediated by interleukin-8 dependent mechanisms. J Clin Invest 96:107-116, 1995.

16. Boylan AM, Sanan DA, Sheppard D, BROADDUS VC. Vitronectin enhances internalization of crocidolite asbestos by rabbit pleural mesothelial cells via the integrin □v□5. J Clin Invest 96:1987-2001, 1995.

17. BROADDUS VC, Yang L, Scavo LM, Ernst JD, Boylan AM. Asbestos induces apoptosis of human and rabbit pleural mesothelial cells via reactive oxygen species. J Clin Invest 98:2050-2059, 1996. (* *identified by the Editors as being of broad interest*)

18. BROADDUS VC, Yang L, Scavo LM, Ernst JD, Boylan AM. Crocidolite asbestos induces apoptosis of pleural mesothelial cells: Role of reactive oxygen species and poly (ADP-ribosyl) polymerase. Environ Health Perspect 105 (Suppl 5):1147-1152, 1997.

19. Narasimhan SR, Yang L, Gerwin BI, BROADDUS VC. Resistance of pleural mesothelioma cell lines to apoptosis: relation to expression of Bcl-2 and Bax. Am J Physiol (Lung Cell Mol Physiol) 275(19): L165-L171, 1998.

20. Ernst JD, Yang L, BROADDUS VC. Preparation and characterization of an endogenously fluorescent annexin for detection of apoptotic cells. Anal Biochem 260:18-23, 1998.

21. Perkins RC, BROADDUS VC, Shetty S, Hamilton S, Idell S. Asbestos upregulates expression of the urokinase-type plasminogen activator receptor on mesothelial cells. Am J Respir Cell Mol Biol 21:637-646, 1999.

22. Miyazaki H, BROADDUS VC, Wiener-Kronish JP, Sawa T, Pittet J-F, Kravchenko V, Mathison JC, Nishizawa H, Hattori S, Yamakawa T, Yamada H, Kudoh I. The effects of two anti-inflammatory pretreatments on bacterial-induced lung injury. Anesthesiology 90:1650-1662, 1999.

23. Modelska K, Pittet J-F, Folkesson HG, BROADDUS VC, and Matthay MA. Acid-induced lung injury: protective effect of anti-interleukin-8 pretreatment on alveolar epithelial barrier function in rabbits. Am J Respir Crit Care Med 160:1450-1456, 1999.

24. Marchi E, Liu W, BROADDUS VC. Mesothelial cell apoptosis is confirmed in vivo by morphologic change in cytokeratin distribution. Am J Physiol Lung Cell Mol Physiol 278: L528-L535, 2000.

25. Levresse V, Renier A, Levy F, BROADDUS VC, Jaurand M-C. DNA breakage in asbestos-treated normal and transformed (TSV40) rat pleural mesothelial cells. Mutagenesis 15(3): 239-244, 2000.

26. Liu W, Ernst JD, BROADDUS VC. Phagocytosis of crocidolite asbestos induces oxidative stress, DNA damage and apoptosis in mesothelial cells. Am J Respir Cell Mol Biol 23(3): 371-378, 2000.

27. Wu J, Liu W, Koenig K, Idell SI, BROADDUS VC. Vitronectin adsorption to chrysotile asbestos increases phagocytosis and toxicity for mesothelial cells. Am J Physiol Lung Cell Mol Physiol 279:L916-L923, 2000.

28. Cambier S, Mu DZ, O'Connell D, Boylen K, Travis W, Liu W, BROADDUS VC, Nishimura SL. A role for the integrin αvβ8 in the negative regulation of epithelial cell growth. Cancer Res 60(24): 7084-7093, 2000.

29. Liu W, Bodle E, Chen JY, Gao M, Rosen GD, BROADDUS VC. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and chemotherapy cooperate to induce apoptosis in mesothelioma cell lines. Am J Respir Cell Mol Biol 25(1):111-8, 2001.

30. Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, Sheppard D, BROADDUS VC, Nishimura SL. The integrin αvβ8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-β1. J Cell Biol 157(3):493-507, 2002.

31. Fjellbirkeland L, Cambier S, BROADDUS VC, Hill A, Brunetta P, Dolganov G, Jablons D, Nishimura SL. Integrin αvβ8-mediated activation of TGF-β inhibits human airway epithelial proliferation in intact bronchial tissue. Am J Pathol 163(2):533-542, 2003.

32. Vivo C, Liu WH, BROADDUS VC. C-Jun N-terminal kinase contributes to apoptotic synergy induced by TRAIL plus DNA damage in chemoresistant, p53 inactive mesothelioma cells. J Biol Chem 278(28):25461-7, 2003.

33. Geiser T, Ishigaki M, van Leer C, Matthay MA, BROADDUS VC. H_2O_2 inhibits alveolar epithelial wound repair in vitro by induction of apoptosis. Am J Physiol Lung Cell Mol Physiol 287(2):L448-53, 2004.

34. BROADDUS VC, Dansen TB, Abayasiriwardana KS, Wilson SM, Finch AF, Swigart LB, Hunt AE, Evan GI. Bid mediates apoptotic synergy between TNF-related apoptosis-inducing ligand (TRAIL) and DNA damage. J Biol Chem 280:12486-12493, 2005.

35. Kim KU, Wilson SM, Abayasiriwardana K, Collins R, Fjellbirkeland L, Xu Z, Jablons DM, Nishimura SL, BROADDUS VC. A novel in vitro model of human mesothelioma for studying tumor biology and apoptotic resistance. Am J Respir Cell Mol Biol 33(6):541-8, 2005.

36. Acencio MM, Vargas FS, Marchi E, Carnevale GG, Teixeira LR, Antonangelo L, BROADDUS VC. Pleural mesothelial cells mediate inflammatory and profibrotic responses in talc-induced pleurodesis. Lung 185 (6):343-348, 2007.

37. Araya J, Cambier S, Markovics JA, Wolters P, Jablons D, Hill A, Finkbeiner W, Jones K, BROADDUS VC, Sheppard D, Barzcak A, Xiao Y, Erle DJ, Nishimura SL. Squamous metaplasia amplifies pathologic epithelial-mesenchymal interactions in COPD. J Clin Invest 117 (11): 3551-3562, 2007.

38. Pespeni MH, Hodnett M, Abayasiriwardana KS, Roux J, Howard M, BROADDUS VC*, Pittet JF*. Sensitization of mesothelioma cells to TRAIL-induced apoptosis by heat stress via the inhibition of the 3-phosphoinositide-dependent kinase 1/Akt pathway. Cancer Res 67(6):2865-2871, 2007.

(* *equal contributors as senior author*)

39. Abayasiriwardana KS, Barbone D, Kim KU, Vivo C, Lee KK, Dansen TB, Hunt AE, Evan GI, BROADDUS VC. Malignant mesothelioma cells are rapidly sensitized to TRAIL-induced apoptosis by low dose anisomycin via Bim. Mol Cancer Ther 6(10):2766-2776, 2007.

40. Hassan R, BROADDUS VC, Wilson S, Liewehr DJ, Zhang J. Anti-mesothelin immunotoxin SS1P in combination with gemcitabine results in increased activity against mesothelin-expressing tumor xenografts. Clin Cancer Res 13(23):7166-7171, 2007.

41. An F, Drummond DC, Wilson S, Kirpotin DB, Nishimura SL, BROADDUS VC, Liu B. Targeted drug delivery to mesothelioma cells using functionally selected internalizing human single chain antibodies. Mol Cancer Ther 7(3):569-78, 2008.

42. Barbone D, Yang TM, Morgan JR, Gaudino G, BROADDUS VC. Mammalian target of rapamycin contributes to the acquired apoptotic resistance of human mesothelioma multicellular spheroids. J Biol Chem 283(19):13021-13030, 2008.

43. Wilson SM, Barbone D, Yang TM, Jablons DM, Bueno R, Sugarbaker DJ, Nishimura S, Gordon GJ, BROADDUS VC. mTOR mediates survival signals in malignant mesothelioma grown as tumor fragment spheroids. Am J Respir Cell Mol Biol 39(5):576-583, 2008.

44. Bidlingmaier S, He J, Wang Y, An F, Feng J, Barbone D, Gao D, Franc B, BROADDUS VC, Liu B. Identification of MCAM/CD146 as the target antigen of a human monoclonal antibody that recognizes both epithelioid and sarcomatoid types of mesothelioma. Cancer Res 69(4); 1570-1577, 2009.

45. Yang TM, Barbone D, Fennell DA, BROADDUS VC. Bcl-2 family proteins contribute to apoptotic resistance in lung cancer multicellular spheroids. Am J Respir Cell Mol Biol 41(1):14-23, 2009.

(* With accompanying editorial)

46. Xiang X, Phung Y, Feng M, Nagashima K, Zhang J, BROADDUS VC, Hassan R, FitzGerald D, Ho M. The development and characterization of a human mesothelioma in vitro 3D model to investigate immunotoxin therapy. PLoS ONE 6(1): e14640, 2011.

47. Iyer AK, Lan X, Zhu X, Su Y, Feng J, Zhang X, Gao D, Seo Y, VanBrocklin HF, BROADDUS VC, Liu B, He J. Novel human single chain antibody fragments that are rapidly internalizing effectively target epithelioid and sarcomatoid mesothelioma. Cancer Res 70(1): 2428-2432, 2011.

48. Iyer AK, Su Y, Feng J, Lan X, Zhu X, Liu Y, Gao D, Seo Y, VanBrocklin HF, BROADDUS VC, Liu B, He J. The effect of internalizing human single chain antibody fragment on liposome targeting to epithelioid and sarcomatoid mesothelioma. Biomaterials 32(10):2605-2613, 2011.

49. Barbone D, Ryan J, Kolhatkar N, Chacko AD, Jablons DM, Sugarbaker DJ, Bueno R, Letai AG, Coussens LM, Fennell DA, BROADDUS VC. The Bcl-2 repertoire of mesothelioma spheroids underlies acquired apoptotic multicellular resistance. Cell Death and Disease 2, e174, 2011.

50. Phung YT, Barbone D, BROADDUS VC, Ho M. Rapid generation of in vitro multicellular spheroids for the study of monoclonal antibody therapy. J Cancer 2:507-514, 2011.

51. Hurwitz JL, Stasik I, Kerr EM, Holohan C, Redmond KM, McLaughlin KM, Busacca S, Barbone D, BROADDUS VC, Gray SG, O'Byrne KJ, Johnston PG, Fennell DA, Longley DB. Vorinostat/SAHA-induced apoptosis in malignant mesothelioma is FLIP/caspase 8-dependent and HR23B-independent. Eur J Cancer 48:1096-1107, 2011

IN REVIEW

Barbone D, Fennell D, BROADDUS VC. Vorinostat eliminates multicellular resistance of mesothelioma 3D spheroids via restoration of Noxa expression. In review.

Busacca S, Chacko AD, Klabatsa A, Arthur K, Sheaff M, Gunasekharan VK, Gorski JJ, El-Tanani M, BROADDUS VC, Gaudino G, Fennell DA. BAK and NOXA are critical determinants of mitochondrial apoptosis induced by bortezomib in mesothelioma. In review.

Yang T-M, Barbone D, BROADDUS VC. Hypoxia inducible factor-1 alpha contributes to apoptotic resistance in lung cancer multicellular spheroids. In review.

NON-PEER REVIEWED PUBLICATIONS AND OTHER CREATIVE ACTIVITIES:

EDITORIALS

1. BROADDUS VC, Light RW. What is the origin of pleural transudates and exudates? Chest 102:658-659, 1992.

2. BROADDUS VC. Asbestos, the mesothelial cell and malignancy: a matter of life or death. Am J Respir Cell Mol Biol 17:657-659, 1997.

3. BROADDUS VC. Apoptosis and asbestos-induced disease – is there a connection? J Lab Clin Med 137(5):314-5, 2001.

4. BROADDUS VC. Diuresis and transudative effusions-changing the rules of the game. Am J Med 110(9):732-5, 2001.

WORKSHOP OR MEETING SUMMARIES

1. Crapo JD, BROADDUS VC, Brody AR, Malindzak G, Samet J, Wright JR; American Thoracic Society. ATS-NIEHS Workshop on lung disease and the environment; Where do we go from here? Am J Respir Crit Care Med 168(2):250-4, 2003.

2. BROADDUS VC. Advances in Mesothelioma Research. Report of the IMIG meeting. International Pleural Newsletter 2 (3): 10-11, 2004.

3. Carbone M, Albelda SM, BROADDUS VC, Flores RM, Hillerdal G, Jaurand, M-C, Kjaerheim K, Pass HI, Robinson B, Tsao A. Meeting Review: 8th International Mesothelioma Interest Group Oncogene 26 (49): 6959-6967, 2007.

4. Boylan A, Broaddus VC. Pleural Disease. In: Respiratory Disease in America: An ATS Fact Book. Schraufnagel, DE Editor 2010.

5. BROADDUS VC, Everitt JI, Black B, Kane AB. Non-neoplastic and neoplastic pleural endpoints following fiber exposure. J Toxicol Environ Health (Critical Reviews Part B): 14:153-178, 2011.

REVIEWS

1. Staub NC, BROADDUS VC, Zylak C, Lai-Fook SJ, Light RW, Vinegar R, Gaensler EA, Sahn SA. Pathophysiology of the pleural space. Am Rev Respir Dis 134:820-821, 1986.

2. BROADDUS VC, Berthiaume Y, Biondi JW, Matthay MA. Hemodynamic management of the adult respiratory distress syndrome. J Intensive Care Med 2:190-213, 1987.

3. BROADDUS C and Staub NC. Pleural liquid & protein turnover in health & disease. Sem in Respir Med 9:7-12, 1987.

4. Wiener-Kronish JP, BROADDUS VC. Interrelationship of pleural and pulmonary interstitial liquid. Ann Rev Physiol 55:209-226, 1993.

5. Matthay MA, BROADDUS VC. Fluid and hemodynamic management in acute lung injury. Sem in Respir Med 15:271-288, 1994.

6. BROADDUS, V.C. Infections in the pleural space: An update on pathogenesis and management. Sem in Respir Crit Care Med 16:303-314, 1995.

7. Marchi E, BROADDUS VC. Mechanisms of pleural liquid formation in pleural inflammation. Curr Opinion in Pulmonary Med 3:305-309, 1997.

8. Nishimura SL, BROADDUS VC. Asbestos-induced pleural disease. Clinics in Chest Medicine 19 (2): 311-329, 1998.

9. Leard LE, BROADDUS VC. Mesothelial cell proliferation and apoptosis. Respirology 9: 292-299, 2004.

10. Mutti L, BROADDUS VC. Malignant mesothelioma as both a challenge and an opportunity. Oncogene 23:9155-9161, 2004.

CHAPTERS

1. BROADDUS VC, Wiener-Kronish JP. Pleural diseases. In: Annual Review of Pulmonary and Critical Care Medicine, 1986-1987. Philadelphia: Hanley & Belfus, 1986, 115-132.

2. BROADDUS VC, Wiener-Kronish JP. Pleural diseases. In: Annual Review of Pulmonary and Critical Care Medicine, 1987-1988. Philadelphia: Hanley & Belfus, 1987, 197-219.

3. Wiener-Kronish JP, BROADDUS VC. Pleural diseases. In: Annual Review of Pulmonary and Critical Care Medicine, 1988-1989. Philadelphia: Hanley & Belfus, 1989, 85-102.

4. Wiener-Kronish JP, Boylan AM, BROADDUS VC. Pleural diseases. In: Annual Review of Pulmonary and Critical Care Medicine, 1991-1992. Philadelphia: Hanley & Belfus, 1991, 93-106.

5. BROADDUS VC. Cardiac diseases. In: Pulmonary Manifestations of Systemic Disease. JF Murray, ed. New York: Marcel Dekker, Inc., 1991; 59:149-190.

6. Light RW, BROADDUS VC. Disorders of the pleura: general principles and diagnostic approach. In: Textbook of Respiratory Medicine. JF Murray, JA Nadel, eds. 2nd edition. Philadelphia: WB Saunders Co., 1994, 2145-2163.

7. BROADDUS VC. Mechanisms of Pleural Liquid Turnover in the Normal State. UptoDate in Pulmonary and Critical Care Medicine (CDROM), S.E. Weinberger, Editor, American Thoracic Society, 1996-2004.

8. BROADDUS VC. Mechanisms of Pleural Liquid Accumulation in Disease. UptoDate in Pulmonary and Critical Care Medicine (CDROM), S.E. Weinberger, Editor, American Thoracic Society, 1996-2004.

9. BROADDUS VC, Hébert, CA. The Role of IL-8 in Inflammatory Diseases. In: Chemoattractant Ligands and Their Receptors. R. Horuk, Editor. CRC Press: New York. 1996, pp. 1-28.

10. Bigelow JM, BROADDUS VC. Empyema and Lung Abscess. In: Pulmonary/Respiratory Therapy Secrets. P.E. Parsons, J.E. Heffner, Editors. Hanley & Belfus, Inc. Medical Publishers, Phila, PA. 1996, pp. 179-185.

11. BROADDUS VC, Hébert CA. Neutralization of IL-8 in *in Vivo* models of lung and pleural injury. In: Methods in Enzymology. Chemokines and Chemokine Receptors. R. Horuk, Editor. Academic Press. Orlando, FL. 1997; 288: 161-181.

12. BROADDUS VC, Light RW. Disorders of the Pleura: General Principles and Diagnostic Approach (Chapter 73). In: Textbook of Respiratory Medicine. JF Murray, JA Nadel, RJ Mason, HA Boushey, eds. 3rd edition. Philadelphia: WB Saunders Co., 2000; pp. 1995-2012.

13. Light RW, BROADDUS VC. Pleural Effusion. (Chapter 74) In: Textbook of Respiratory Medicine. JF Murray, JA Nadel, RJ Mason, HA Boushey, eds. 3rd edition. Philadelphia: WB Saunders Co., 2000; pp. 2013-2041.

14. Light RW, BROADDUS VC. Pneumothorax, Chylothorax, Hemothorax and Fibrothorax. (Chapter 75) In: Textbook of Respiratory Medicine. JF Murray, JA Nadel, RJ Mason, HA Boushey, eds. 3rd edition. Philadelphia: WB Saunders Co., 2000; pp. 2043-2066.

15. Light RW, BROADDUS VC. Tumors of the Pleura. (Chapter 76) In: Textbook of Respiratory Medicine. JF Murray, JA Nadel, RJ Mason, HA Boushey, eds. 3rd edition. Philadelphia: WB Saunders Co., 2000; pp. 2067-2078.

16. BROADDUS VC, Jaurand MC. Asbestos Fibers and the Biology of Mesothelial Cells. In: Mesothelioma. P Chahinian, BWS Robinson, ed. Gordon & Breach Science Publishers, Harwood Academic Publishers 2004.

17. Bigelow JM, BROADDUS VC. Empyema and Lung Abscess. In: Pulmonary/Respiratory Therapy Secrets. 2nd edition. P.E. Parsons, J.E. Heffner, Editors. Hanley & Belfus, Inc. Medical Publishers, Phila, PA. 2001.

18. BROADDUS VC. Transudative pleural effusions. In: Pleural Diseases. Loddenkemper R, Antony V, Editors. European Respiratory Society, Sheffield, UK. Eur Respir Monograph 2002: 22, pp. 157-176.

19. Nahid P, BROADDUS VC. Liquid and Protein Exchange. (Chapter 3) In: Pleural Diseases: an International Textbook. Light RW and Lee G.Y.C. Editors Hodder Arnold, London, England 2003; pp. 35-44.

20. BROADDUS VC. Physiology. (Chapter 6) and Transudates (Chapter 27). In: Derrame Pleural (Translation: Pleural Effusion). Vargas FS, Teixeira LR, and Marchi E, Editors. Roca Publishers, Brazil, 2003; pp. 15-24 and 233-248.

21. BROADDUS VC, Light RW. Pleural effusion (Chapter 68). In: Textbook of Respiratory Disease. RJ Mason, VC Broaddus, JF Murray, JA Nadel, eds. 4th edition. Philadelphia: Elsevier 2005; pp.1913-1960.

22. Boylan AM, BROADDUS VC. Tumors of the pleura (Chapter 70). In: Textbook of Respiratory Disease. RJ Mason, VC Broaddus, JF Murray, JA Nadel, eds. 4th edition. Philadelphia: Elsevier 2005; pp. 1989-2009.

23. BROADDUS VC. Fluid and solute exchange in normal physiological states. In: Textbook of Pleural Diseases, 2nd Edition. Light RW, Lee YGC (eds) London: Hodder, Arnold, 2008:43-48.

24. BROADDUS VC, Light RW. Pleural effusion. In: Murray & Nadel's Textbook of Respiratory Medicine. RJ Mason, VC Broaddus, Martin TR, King TE, Schraufnagel DE, JF Murray, JA Nadel, eds. 5th edition. Philadelphia: Elsevier, 2010: 1719-1763.

25. BROADDUS VC, Robinson BWS. Tumors of the pleura. In: Murray & Nadel's Textbook of Respiratory Medicine. RJ Mason, VC Broaddus, Martin TR, King TE, Schraufnagel DE, JF Murray, JA Nadel, eds. 5th edition. Philadelphia: Elsevier, 2010: 1792-1813.

RESEARCH PROGRAM
DESCRIPTION OF CURRENT RESEARCH

My research program continues to focus on the apoptotic resistance of tumors, malignant mesothelioma and lung cancer. We have focused particularly on mesothelioma, as a highly refractory and chemoresistant tumor, and are applying our findings now to lung cancer. We are investigating the apoptotic signaling involved in bypassing resistance and inducing apoptosis, specifically by combining agonists of the two major apoptotic pathways: the death receptor pathway and the DNA damage/mitochondrial pathway. We have shown that, while each pathway alone fails to induce apoptosis in the tumor cells, the combination will induce synergistic apoptosis. We continue to explore this phenomenon, searching for non-toxic means of stimulating these pathways such as heat stress and JNK stimulators such as anisomycin.

We have incorporated 3-dimensional models in our study of resistance, both of the mesothelioma cell lines grown as multicellular spheroids and of the human mesothelioma tumor itself grown as tumor fragment spheroids. Both these models allow us to test the resistance in a more clinically relevant system. We have found that a major survival pathway, the PI3K/Akt/mTOR pathway, contributes to resistance in these 3-dimensional structures. Ultimately however, we believe that the resistance is manifested at the mitochondria by an altered repertoire of anti- and pro-apoptotic molecules. In an ongoing collaboration with Dr. Dean Fennell of Belfast, No. Ireland and Drs. Raphael Bueno and David Sugarbaker of Brigham & Womens, Boston, we are now exploring the mitochondria as a central integrator of apoptotic signaling. Most recently, we are using histone deacetylator inhibitors to restore expression of pro-apoptotic proteins that reverse multicellular resistance and performing connectivity analysis of the key genes upregulated in 3D to identify drugs that can reverse multicellular resistance.

As an exciting direction using 3-dimensional models, we are collaborating with Dr. Lisa Coussens in studying the interaction of tumor-associated macrophages with the tumor cells. We will use our models, 3D multicellular spheroids and human tumor fragments, to investigate the contribution of macrophages to the apoptotic resistance of the tumor cells. We have found that mesothelioma contains a high number of macrophages, far more than in lung cancer or other tumors; thus, if these macrophages can be eliminated or manipulated to change from a supportive role to an anti-tumor role, this could be of significant therapeutic benefit for this currently incurable tumor. Our current findings are that macrophages (polarized from their M2-protumor phenotype to an M1-antitumor phenotype) can enhance the chemosensitivity of the mesothelioma cells. In vivo studies are ongoing to explore this approach in mice with orthotopic and de novo mesothelioma.

CURRICULUM VITAE
Lisa M. Coussens, Ph.D.
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Fax: (503) 494-4253
Email: coussenl@ohsu.edu

I. EDUCATION:

1976 - 1980	San Francisco State University	B.A.	Biology
1988 - 1993	University of California, Los Angeles	Ph.D.	Biological Chemistry
1993 - 1997	University of California, San Francisco	Post-Doctoral Fellow	Cancer Biology

II. PRINCIPAL POSITIONS HELD:

1981 - 1988	Genentech, Inc., South San Francisco	Research Associate	Molec. & Devel. Biology
1997 - 1999	Univ. of California, San Francisco	Assistant Research Biochemist	Hormone Research Inst.
1999 - 2004	Univ. of California, San Francisco	Assistant Professor, In Residence	Cancer Research Inst. & Dept of Pathology
2004 - 2006	Univ. of California, San Francisco	Associate Professor, In Residence	Cancer Research Inst. & Dept. of Pathology
2006 - 2007	Univ. of California, San Francisco	Associate Professor	Dept. of Pathology & Cancer Research Inst.
2007 - 2011	Univ. of California, San Francisco	Professor	Dept. of Pathology & Cancer Research Inst.
2011 - 2012	Univ. of California, San Francisco	Professor	Dept. of Pathology
2012 - present	Univ. of California, San Francisco	Adjunct Professor	Dept. of Pathology
2011 - present	Oregon Health & Sciences University	Professor and Chair	Cell & Developmental Biology
2011 - present	Oregon Health & Sciences University	Associate Director for Basic Research	Knight Cancer Institute

OTHER POSITIONS HELD CONCURRENTLY:

1989 - 1992	Whittier College, Whittier, CA	Lecturer	Biology Dept
1992	Genentech, Inc., South San Francisco	Scientific Consultant	Dept. of Legal Affairs
2000-2012	Helen Diller Family Comprehensive Cancer Center, UCSF	Co-Director	Mouse Pathology Core
2007-2009	*CANCER RESEARCH*	Senior and Deputy Editor	*Tumor Microenvironment* Section
2009-2012	Helen Diller Family Comprehensive Cancer Center, UCSF	Co-Leader	Program in *Cancer Immunity & Microenvironment*
2009-2012	*CANCER RESEARCH*	Deputy Editor	*Breaking Advances*

III. HONORS AND AWARDS:

1985	Recognition Award	Genentech, Inc.,
1986	Recognition Award	Genentech, Inc.,
1988	Recognition Award	Genentech, Inc.
2000 - 02	Hellman Family Award For Early Career Faculty	Univ. of Calif., San Francisco
2000 - 01	V Foundation Scholar	The V Foundation for Cancer Research
2000 - 03	Edward Mallinckrodt, Jr. Fndt. Award for Medical Research	Edward Mallinckrodt, Jr. Fndt.
2002	Gertrude B. Elion Cancer Research Award	Am. Assoc. for Cancer Research
2006 - 11	Era of Hope Scholar Award	Dept. of Defense, Breast

		Cancer Research Program
2011 - 16	Era of Hope Scholar Expansion Award	Dept. of Defense, Breast Cancer Research Program
2011 - 16	KOMEN Promise Award	Susan G Komen For the Cure Foundation
2012	AACR-Women in Cancer Research Charlotte Friend Memorial Lectureship	Am. Assoc. for Cancer Research –Women in Cancer Research
2012	Mildred Scheel Memorial Lectureship (Inaugural)	German Cancer Aid and Deutsches Krebsforschungszentrum (DKFZ)

IV. PROFESSIONAL ACTIVITIES

1999 - 2012	Member	Graduate Program in BioMedical Sciences (BMS)	Univ. of Calif., San Francisco
1999 - 2012	Member	Helen Diller Family Comprehensive Cancer Center	Univ. of Calif., San Francisco
2000 - 2012	Member	Graduate Program in Biological Sciences (PIBS)	Univ. of Calif., San Francisco
2001 - 2012	Co-Director	Mouse Pathology Core	Helen Diller Family Comprehensive Cancer Center, Univ. of Calif., San Francisco
2004 – 2012	Member	Program in Immunology	Univ. of Calif., San Francisco
2004 – 2007	Senior Editor	***Cancer Research (****Cell, Tumor and Stem Cell Biology* Section)	American Association Cancer Research
2007 – 2009	Deputy Editor	***Cancer Research (****general)*	American Association Cancer Research
2007 – 2009	Senior Editor	***Cancer Research (****Tumor Microenvironment* Section)	American Association Cancer Research
2007 - present	Member	External Scientific Advisory Board	Mason Cancer Center, University of Minnesota
2007 – 2011	Member	External Scientific Advisory Board, U54: *Aging, Tumor Microenvironment and Prostate Cancer*; P.I. Steve Plymate, Univ. of Washington, HMC.	University of Washington
2008 – 2011	Member	Board of Directors (*elected*)	American Association of Cancer Research
2009 - present	Member	External Scientific Advisory Board; Neuroblastoma Program Project Grant	Children's Hospital Los Angeles, Univ. of Southern California
2009 – 2012	Deputy Editor	***Cancer Research (****Breaking Advances section*)	American Association Cancer Research
2009 - 2012	Co-Director	Program in *Cancer Immunity and Microenvironment*	Helen Diller Family Comp. Cancer Center, Univ. of Calif., San Francisco
2010 - 2012	Council Member	*Women in Cancer Research*, Council (elected)	American Association of Cancer Research
2009 - present	Member	External Advisory Committee, P01CA100324, Program in *Motility and Invasion*, John Condeelis, Ph.D., PI, Program Director	Albert Einstein College of Medicine
2011	Member	Scientific Review Board	Starr Cancer Consortium
2012 - present	Faculty member	Program in Molecular & Cellular Biosciences	Oregon Health & Sciences University

2012 - present	Member	OHSU Knight Cancer Institute, Program in Cancer Biology	Oregon Health & Sciences University
2012 - present	Member	Scientific Advisory Board	Koch Inst, for Integrated Cancer Research, Massachusetts Inst. of Tech.
2012 - present	Member	Editorial Board	Cancer Cell

Local, National and International Meetings Organized:

2005 Keystone Symposia, *Inflammation and Cancer*, Co-organizer with Dr. Ray DuBois, Vanderbilt Univ, TN), Breckinridge, CO, USA

2006 5th Annual Timberline Symp. on Epithelial Cell Biology, '*Intrinsic and Microenvironmental Regulation of Epithelial Cancer',* Co-Organizer with Dr. Harold Moses (Vanderbilt University, TN, USA), Timberline, OR, USA

2006 Co-Organizer (with Dr. Lewis Lanier), UCSF HDFCCC Annual Symposium, *'Inflammation & Cancer: Bench to Bedside'.*

2007 Keystone Symposia, *Inflammation and Cancer*, Co-Organizer with Drs. Fran Balkwill (Cancer Research UK) and Glenn Dranoff (Beth Israel Cancer Center, Harvard, MA); Santa Fe, New Mexico, USA

2008 AACR Special Conference: *Inflammation and Cancer*, Co-organizer with Drs. Michael Karin and Larry Marnett. Oahu, Hawaii, USA.

2008 International Society for Biological Therapy of Cancer (ISBTc), *2008 Workshop on Inflammation in Cancer Development,* Co-Organizer with Drs. Michael Karin, (UCSD), Steven Dubinett (UCLA), and George Weiner (WU); San Diego CA USA

2010 Co-Organizer (with Dr. Lewis Lanier), UCSF HDFCCC Program in *Cancer Immunity and Microenvironment* Symposium

2011 AACR Special *Conference: Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy*, Co-Organizer with Drs. Yves DeClerck (USC, Children's Hospital) and Melody Swartz (EPFL)

V. PROFESSIONAL ORGANIZATIONS

Memberships

2000 - 2009	American Society for Matrix Biology
2000 – present	American Association for Cancer Research
2001 – 2008	American Society for Cell Biology
2004 – present	American Society for Investigative Pathology
2004 - 2009	International Protease Society

Service to Professional Organizations

American Association for Cancer Research

2003 Subsection Co-chair (Tumor Progression, Invasion and Metastasis) Cellular, Molecular and Tumor Biology Subcommittee, AACR Program Committee for *94th Annual Meeting*.

2003 Chair and organizer, Educational Session *(*Proteases: Successes and Failures): *94th Annual Meeting*, Washington D.C., USA

2003 Minisymposium Co-chair (Inflammatory Mediators & Cancer): *94th Annual Meeting*, Washington D.C., USA

2004 - 2006 Member, Grants Committee

2005 Minisymposium Co-Chair (Inflammation, Microenvironment and Tumor Progression): *96th Annual Meeting*, Anaheim, CA USA

2005 Session Chair (Inflammation*)*: *AACR Special Conference: Cancer, Proteases and the Microenvironment*, Bonita Springs, Florida. USA

2006 Subsection Co-chair (Tumor Progression, Invasion and Metastasis) of the Tumor Biology Subcommittee, AACR Program Committee for *97th* Annual meeting

2006 Minisymposium Co-Chair (Inflammation and Cancer): *97th Annual Meeting*, Washington DC, USA

2006 Co-Chairperson, Program Committee: *6th Annual Frontiers in Cancer Prevention Research Conference*, December 5-8, 2007, Philadelphia, PA USA.

2006 - 2010 Steering Committee Member: AACR Tumor Microenvironment Working Group (TME/AACR).

2007 Organizer, Education session (Inflammation and Cancer), *98th Annual Meeting*, Los Angeles, USA
2007 Minisymposium Co-Chair (Tumor Microenvironment): *98th Annual Meeting*, Los Angeles, CA USA
2007 Co-Chairperson, Program Committee: *2008 99th Annual Meeting of the AACR*. April 12-16, 2008, San Diego, CA. USA
2008 Program Committee Member, Tumor Microenvironment Subcommittee for *99th Annual Meeting of the AACR*. April 12-16, 2008, San Diego, CA. USA
2007 - 2010 Member, AACR Special Conferences Committee
2008 Co-Organizer Special Conference: *Inflammation and Cancer*, with Drs. Michael Karin and Larry Marnett. Oahu, Hawaii, USA.
2008 - 2011 Member, Board of Directors (elected)
2009 Member, 2009 Education Committee, 2009 100th AACR Annual Meeting, Denver, CO. USA
2009 Organizer and Chair: *Inflammation and Cancer: Novel Mechanisms Regulating Protumor Immunity* Major Symposium, 2009 100th AACR Annual Meeting, Denver, CO. USA
2009 Organizer and Chair: Education Session, *Aspects of the Tumor Microenvironment that Regulate Solid Tumor Development,* 2009 100th AACR Annual Meeting, Denver, CO. USA
2010 Co-Chairperson, Program Committee: *2010 101st Annual Meeting of the AACR*, April 17-21, 2010, Washington, DC USA
2009 Member, Scientific Review Committee for *Stand Up to Cancer Innovative Research Grants*
2009 - 2010 Member, Selection Committee: *2010 Pezcoller Foundation-AACR International Award for Cancer Research*
2010 - 2011 Council Member, *Women in Cancer Research* Council (elected)
2010 Co-Chair, Minisymposium '*The Tumor Microenvironment and Therapeutic Strategies" 2010 101st Annual Meeting of the AACR*, April 17-21, 2010, Washington, DC USA
2010 - 2011 Member, Selection Committee: 2010-2011 *AACR Award for Lifetime Achievement in Cancer Research*
2011 Co-Chair, Minisymposium '*Tumor Microenvironments" 2011 102st Annual Meeting of the AACR*, April 3-6, 2010, Orlando, FL USA
2011 Co-Organizer with Drs. Yves DeClerck (USC, Children's Hospital) and Melody Swartz (EPFL); AACR Special *Conference: Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy*, November 2011, Orlando FL USA
2012 Co-Chairperson, Program Committee: *2012 103rd Annual Meeting of the AACR*, April 3-6, 2010, Chicago, IL USA
2012 Chair, Plenary session: "Tumor Heterogeneity: Challenges and Therapeutic Opportunities" *2012 103rd Annual Meeting of the AACR*, April 3-6, 2010, Chicago, IL USA
2012 Chair, Education session: "Tumor Microenvironment" *2012 103rd Annual Meeting of the AACR*, April 3-6, 2010, Chicago, IL USA
2012 Chair, 2012 Landon Foundation-AACR INNOVATOR Award for International Collaboration in Cancer Research Scientific Review Committee, Dr. Judith Varner, recipient.
2012 Speaker, 2012 AACR Meet the Research Pioneer, *2012 103rd Annual Meeting of the AACR*, April 3-6, 2010, Chicago, IL USA
2012 Organizing Committee for 9th AACR-Japanese Cancer Association International Conference, February 21-25, 2013, Maui, Hawaii.

American Society for Cell Biology

2000 American Society for Cell Biology, photo credits in '*Exploring the Cell'* Ed. W. Wells
2001 Table Leader, Career Discussion Lunch, Women in Cell Biology and Education Committee, 40th Annual Meeting, Washington, DC, USA
2001 Co-chair and Co-organizer, Mini-symposium (Microenvironment/Extracellular Matrix in Development and Disease): 40th Annual Meeting, Washington, DC, USA
2003 Table Leader, Career Discussion Lunch, Women in Cell Biology and Education Committee of the ASCB, *42nd Annual Meeting*, San Francisco, CA, USA
2006 Co-Chair Minisymposium (Cancer Mechanisms*)*: *46th Annual Meeting*, San Diego CA, USA

American Cancer Society
1999 14th Annual Excalibur Round Table, San Francisco, CA, USA
2000 San Mateo County Annual Volunteer Meeting, San Mateo, CA, USA

International Society for Preventive Oncology
2002 Session Chair (Chemoprevention): *6th Annual Meeting*, Pasteur Institute, Paris, France.
2002 Poster Judge (Chemoprevention): *6th Annual Meeting*, Pasteur Institute, Paris, France.

International Proteolysis Society
2007 Member, International Scientific Advisory Committee, 5th General Meeting of the International Proteolysis Society, Rion-Patras, GREECE.
2011 Member, Organizing Committee, 9th General Meeting of the International Proteolysis Society, San Diego CA, USA

International Society for Biological Therapy of Cancer
2008 Co-Organizer, *2008 Workshop on Inflammation in Cancer Development*, San Diego CA, USA

Service to Professional Publications:

2003 - 2005	Associate Editor, ***Cancer Research***
2005 – 2007	Editorial Board, ***Carcinogenesis***
2004 – 2007	Senior Editor, ***Cancer Research (****Cell, Tumor and Stem Cell Biology* Section)
2007 – 2009	Senior Editor, ***Cancer Research (****Tumor Microenvironment* Section)
2007 – 2009	Deputy Editor, ***Cancer Research***
2007	Guest Editor, PNAS Editorial Board
2008	Guest Editor (with Tyler Jacks), *Current Opinion in Genetics & Development*
2008 – 2010	Editorial Board, ***Cancer Microenvironment***
2009 – 2012	Deputy Editor for Breaking Advances, ***Cancer Research***
2012 – present	Editorial Board, ***Cancer Cell***

Ad hoc reviewing
1994 Oncogene;
1995 Am J Pathology; Matrix Biology; J Cell Biology
1999 Am J Pathology; Cancer Letters; Nature Medicine; Nature; PNAS; Cell Motility & the Cytoskeleton; Cancer Research
2000 Am J Pathology; Cancer Research; Genes & Development; Int. J Cancer
2001 J Cell Biology; Int. J of Cancer; EMBO; Neoplasia; Cancer Research
2002 Cancer Research; Am J Pathology; Int. J Cancer; Biological Chemistry; Cancer Cell; Cancer Letters
2003 PNAS; Cancer Research; Int. J of Cancer; J Molecular Medicine; Biological Chemistry; Science; Cancer Cell; Nature Medicine; J Leukocyte Biology; Neoplasia; Am J Pathology
2004 Lancet; Cancer Cell; Cancer Research; American J Pathology; J Cell Biology; Nature Reviews Immunology; Nature Reviews Cancer; PNAS; J Biological Chemistry; Nature; J Exp Med; Int J Cancer
2005 Nature Medicine, Cancer Cell, Cancer Research; Am J Pathology; Cell; Nature; Nature Reviews Immunology; Nature Reviews Cancer; Carcinogenesis
2006 Nature Reviews Cancer; Nature; Nature Medicine; Cell; Cancer Research; Clinical Cancer Research; J Exp Med; Cancer Cell: Am J Pathology; J Cell Biology
2007 Cell; Nature; PNAS: J Cell Biology; Cancer Research; J Exp Med; Breast Cancer Research
2008 Cancer Cell; PNAS; J Immunology; Nature; J Exp Med; Trends in Genetics; Current Opinions in Investigational Drugs
2009 Cancer Cell; Cell; Nature; J Exp Med; J Clin Invest, Cancer Research; Int J Cancer; Oncogene, J Immunology
2010 Nature, J Exp Med, Nature Medicine, J Invest Dermatology, Cell, Cancer Cell, J Clinical Onc; PNAS; J Clin Invest; Cancer Research; Dis Mech Models; Cancer Immuno Immunother;
2011 Nature; Nature Medicine; Cancer Research; Cancer Cell; J Clin Invest; Breast Cancer Research; PlosOne, PNAS; Oncogene; J Exp Med; Oncogene;
2012 PNAS; Can Res; Oncogene; Trends in Immunology; Nature; Clin Can Res; J Exp Med; Cancer Discovery; Immunity; J Cell Physio; JoVE; Immunity; J Clin Invest;

VI. INVITED PRESENTATIONS

Symposia and Workshops: International

1996 *Human Tumor Heterogeneity II*: Cytometric Measurement of Growth Regulation and Genetic Alterations: International Society of Analytical Cytometry. Kananaskas, Alberta, Canada.
1997 *GeneMedicine-Boehringer Mannheim Cancer Alliance: Technology Workshop*. Cancún Mexico.
2001 *2nd Annual International Protease Society*. Freising, Germany.
2002 *6th International Symposium on Predictive Oncology & Intervention Strategies*, Pasteur Institute, Paris, France
2002 **KEYNOTE LECTURE**, *Dutch Cancer Society Annual Symposium*, Luntern, The Netherlands
2002 **KEYNOTE LECTURE**, *Cancer: Genome, Signal & Environment, Takeda Genome Urology International*, Kyoto, Japan
2003 *2nd Annual International Symposium on Epithelial Biology*, Timberline, Oregon USA
2004 10th International Congress of the *Metastasis Research Society*, 'Progress Against Tumor Progression', Genoa Italy
2005 *2005 International Consortium Meeting of the Children's Tumor Foundation: Molecular Biology of NF1, NF2 and Schwannomatosis,* Aspen, CO, USA
2005 *International Symposium on Systems Genome Medicine - Bench to Bedside,* Institute of Medical Sciences University of Tokyo, Tokyo, Japan
2005 *Immunotherapy of Cancer*, XI Annual Symposium of the Danish Cancer Society, Copenhagen, Denmark
2005 *4th General Meeting of the International Proteolysis Society*, Quebec City, Canada
2006 Centro Nacional de Investigaciones Oncológicas (CNIO) Cancer Conference: *Inflammation and Cancer*, Mardid SPAIN
2006 18th Annual Pezcoller Symposium *'Tumor Microenvironment: Heterotypic Interactions'*, Trento ITALY
2006 European Association for Cancer Research (EACR) 1st Annual Meeting, Budapest HUNGARY
2006 XXXIV th Meeting of the International Society for Oncodevelopmental Biology and Medicine (ISOBM: Tumor Biology, Detection and Therapy, Pasadena, CA, USA
2006 37th International Symposium of the Princess Takamatsu Cancer Research Fund '*Cancer Cells and Their Microenvironment*', Tokyo, JAPAN
2007 *4th International Conference on Tumor Microenvironment*, Florence, ITALY
2007 *2nd International Symposium on Cancer Metastasis and the Lymphovascular System*: Basis for Rational Therapy, San Francisco CA USA
2007 CNIO – Nature Symposium on "*Oncogenes and Human Cancer*". The Next 25 Years", Madrid SPAIN
2007 **KEYNOTE LECTURE**, *7th International Symposium on Hodgkin Lymphoma*, Cologne, GERMANY
2007 **CANDLELIGHT LECTURE**, *Inflammation and Cancer: From molecular links to bed side*; Inaugural meeting for the *Istituto Clinico Humanitas*, Milan ITALY
2008 *7th Annual International Congress on the Future of Breast Cancer*, Kauai, Hawaii USA
2008 Cancer Research UK Cambridge Research Institute (CRI) Inaugural Annual Symposium, '*Unanswered Questions in the Tumour Microenvironment'*, Homerton College, Cambridge UK
2008 5th International Kloster Seeon Meeting, *Angiogenesis: Molecular Mechanisms and Functional Interactions*. Kloster Seeon, GERMANY
2008 **CANCER RESEARCH UK LECTURE**, NCRI Cancer Conference, Birmingham UNITED KINGDOM
2009 21ST Lorne Cancer Conference, Lorne AUSTRALIA
2009 6th International Symposium on the Intraductal Approach to Breast Cancer, Santa Monica CA USA
2009 **STATE-OF-THE-ART LECTURE**, International Cancer Conference, *CANCER 2009*, Dublin IRELAND
2009 19th Annual BioCity Symposium, 'Tumor Microenvironment in Cancer Progression", Tirku FINLAND
2009 **KEYNOTE LECTURE**, European Association of Cancer Research, Special Conference on *Inflammation and Cancer*, Berlin GERMANY
2009 7th International Symposium on Minimal Residual Cancer, Athens, GREECE
2009 Tri-Society Annual Conference of the Society for Leukocyte Biology, International Cytokine Society, and the International Society for Interferon and Cytokine Research, Lisbon, Portugal
2009 5th International Conference on Tumor Microenvironment, Versailles, FRANCE
2009 **PRESIDENT'S PLENARY LECTURE**: Italian Cancer Society Annual Meeting, Milano ITALY
2010 **PLENARY LECTURE**, CHUV Research Day, University hospital (CHUV) and the Faculty of Biology and

Medicine, Lausanne, SWITZERLAND.
2010 *NATURE - CNIO Cancer Symposium* on *Frontiers in Tumour Progression*, Madrid SPAIN
2011 Curie Institute Symposium, *Breast Cancer from Biology to Clinics*, Paris FRANCE
2011 EPFL Inaugural Symposium, *Hallmarks and Horizons in Cancer*, Lausanne SWITZERLAND
2011 Joint meeting of the International Cytokine Society and the International Society for Interferon and Cytokine Research, Florence, ITALY
2011 41st Australian Society for Immunology (ASI), Adelaide, South AUSTRALIA
2012 International Symposium of the Collaborative Research Center (ISCRC), *Molecular Basis and Modulation of Cellular Interactions in the Tumor Microenvironment,* Cologne, Germany
2012 Keystone Symposium on *The Role of Inflammation During Carcinogenesis*, Dublin, IRELAND
2012 Federation of Clinical Immunology Societies (FOCIS) 2012, Improving Human Health Through Immunology, Vancouver, BC, CANADA

UPCOMING INVITATIONS
2012 25th International IGB Workshop, CNR, Capri Island, Naples, ITALY
2012 Annual Meeting of the (French) National Institute of Cancer, Plenary session on "*Cancer Immunity and Inflammation*", Paris FRANCE
2012 25th International IGB Workshop, organized by the Institute of Genetics and Biophysics "A. Buzzati-Traverso", CNR, Naples, ITALY
2013 9th AACR-Japanese Cancer Association International Conference; February 21-25, 2013, Maui, Hawaii. USA
2013 Cancer Research Center of Lyon (CRCL), First International CRCL Symposium: *A Focus on Tumor Escape*. Lyon FRANCE
2013 International Conference on Immunochemotherapy, Paris FRANCE

Symposia and Workshops: National
1994 *Current Transgenic Technology*, B & K Universal, San Mateo, CA, USA
1997 *Biology of Proteolysis*, Cold Spring Harbor Laboratory, NY, USA
1997 *Molecular Biology & Pathology of Neoplasia*, AACR, Keystone, CO, USA
1997 *Matrix Metalloproteinases*, Gordon Research Conference, Proctor Academy, New London, NH, USA
1998 *Proteolysis*, Gordon Research Conference, Colby-Sawyer College, New London, NH, USA
1998 *Cellular Targets of Viral Carcinogenesis*, AACR Special Conference. Dana Point, CA, USA
1998 *Mechanisms of Tumor Growth & Invasion Mediated by Proteolysis*, UCSF-Molecular Design Institute. San Francisco, CA, USA
1999 *Tumor Microenvironment*, Education Session, AACR Annual Meeting. Philadelphia, PA, USA
1999 *Matrix Metalloproteinases*, Gordon Research Conference, Colby-Sawyer New London, NH, USA.
2000 *Epithelial-Stromal Interactions & Tumor Progression Workshop*, National Cancer Inst., Bethesda, MD, USA
2000 10th National Conference of the Inflammation Research Association, Hot Springs, VA, USA
2001 *'Meet-the-Expert' Sunrise Session,* AACR Annual Meeting, New Orleans, LA, USA
2002 *Chemotherapy of Experimental & Clinical Cancer*, Gordon Research Conference, Colby Sawyer College, New London, NH, USA
2002 *Proteolytic Enzymes & their Inhibitors*, Gordon Research Conference, Colby Sawyer, New London, NH, USA
2002 *From the Cancer Cell to a Tumor - Tumors as Outlaw Organs,* Schilling Research Conference, The American Cancer Society, Aptos CA, USA
2002 *Cancer Intervention 2002*, Van Andel Research Institute, Grand Rapids, Michigan USA
2002 *Pathobiochemistry B Study Section Workshop,* Natl. Cancer Institute, Hilton Head, SC, USA
2002 *Proteases, Extracellular Matrix and Cancer*, AACR Special Conference, Hilton Head Island, SC, USA
2002 *ECM and Cancer*, Minisymposium, ASCB Annual Meeting, San Francisco, CA, USA
2003 *Matrix Metalloproteinases*, Gordon Research Conference, Big Sky, Montana, USA
2003 *Angiogenesis & Microcirculation*, Gordon Research Conference, Salve Regina, Newport R.I., USA
2003 *Inflammatory Cells and Cancer,* Symposium, American Society of Hematology 2003 Annual Meeting, San Diego, CA, USA

2003 *Validation of a Causal Relationship: Criteria to Establish Etiology*, National Cancer Institute, Cancer Etiology Branch, Washington, DC, USA.
2003 *Functional Imaging of Proteolysis*, Special Session, ASCB Annual Meeting, San Francisco, CA, USA
2004 Scleroderma Research Foundation Annual Scientific Workshop, San Francisco, CA, USA
2004 *Systems Biology of Cancer: The Tumor as an Organ,* Symposium, 95th AACR Annual Meeting. Orlando, FL, USA
2004 *Inflammation and Cancer,* Symposium, 95th AACR Annual Meeting. Orlando, FL, USA
2004 *Remarkable Role of the Microenvironment in Development and Disease Pathogenesis*, Symposium; Experimental Biology 2004, Sponsored by: the Assoc. of Anatomy, Cell Biology and Neurobiology, Washington, D.C., USA.
2004 *Molecular and Cellular Basis of Disease: Structure and Function of the Extracellular Matrix in Disease: Novel Roles and Regulation of MMPs and TIMPs in Disease,* Symposium; Experimental Biology 2004, Sponsored by: the Am. Society of Investigative Pathology, the American Society for Matrix Biology and the North American Vascular Biology organization. Washington, D.C., USA.
2004 Pacific Coast Protease Workshop, Half Moon Bay, CA, USA.
2004 19th Aspen Cancer Conference: *Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention and Cancer Therapy.* Aspen, CO, USA.
2005 Keystone Symposia, *The Role of Microenvironment in Tumor Induction and Progression (J5),* Banff, Alberta CANADA
2005 Keystone Symposia, *Inflammation and Cancer (B8)*, Breckenridge, CO, USA
2005 *Symposium on Inflammation, Repair and Carcinogenesis in Liver, Pancreas and Colon*. UCSF Liver Center and the Program in Gastrointestinal Cancer of the UCSF Cancer Center, Rohnert Park, CA, USA
2005 *In the Forefront of Advances in Cancer Research*, Symposium, 96th AACR Annual Meeting. Anaheim, CA, USA
2005 *Macrophage Symposium*, AMGEN, Seattle, WA, USA
2005 *Immune Response to Cancer Symposium*, 41st Annual Meeting, American Society Clinical Oncology (ASCO), Orlando. FL. USA
2005 *Phagocyte*, Gordon Research Conference, New London, CT, USA
2005 *Mouse Models of Human Cancer Consortium*, Annual Steering Committee Meeting, New Brunswick, NJ USA
2005 *Matrix Metalloproteinases*, Gordon Research Conference, Big Sky, Montana, USA
2005 *Annual Buffalo Regional Conference on Immunology*, Buffalo, NY, USA
2005 2005 Montagna Symposium on *'Tissue repair - molecular mechanisms and clinical challenges'*, Salishan Lodge, OR, USA
2005 4th Annual AACR Conference on *Frontiers in Cancer Prevention Research*, Baltimore MD, USA
2005 AACR Special Conference, *Cancer, Proteases and the Microenvironment*, Bonita Springs, Florida. USA
2006 Timberline Annual Symposium on Epithelial Biology, *Intrinsic and Microenvironmental Regulation of Epithelial Cancer*', Timberline Lodge, Oregon, USA
2006 Keystone Symposium, *Molecular Targets for Cancer Prevention*, Granlibakken Resort, Tahoe City, CA, USA
2006 *Inflammation and Cancer,* Symposium, *97th AACR Annual Meeting*. Washington, D.C., USA
2006 Lineberger Cancer Center's *30th Annual Scientific Symposium*, University of North Carolina, Chapel Hill, North Carolina, USA
2006 **KEYNOTE LECTURE**, *Vanderbilt-Ingram Cancer Center Retreat 2006*, Vanderbilt University, Nashville TN, USA
2006 **TUMOR BIOLOGY PLENARY LECTURE**, *Advances in Neuroblastoma Research 2006*, Los Angeles, CA, USA
2006 *Genetic, Cellular and Microenvironmental Determinants of Tumor Progression and Metastasis: A 'TPM' Workshop Honoring Martin L Padarathsingh, Ph.D.* TPM Study Section Workshop, Natl. Cancer Institute, Georgetown, VA, USA
2006 ASCO/Federation of European Societies Symposium: *Inflammation in Cancer Progression*, 2006 ASCO Annual Meeting, Atlanta, GA, USA

2006 AACR Special Conference, *Mouse Models of Cancer*, Cambridge, MA, USA
2006 AACR Special Conference, *Tumor Immunology: An Integrated Perspective.* Miami, FL, USA
2007 7th AACR-Japanese Cancer Association Joint Conference: *In the Forefront of Basic and Translational Cancer Research*, Waikoloa, Hawaii, USA
2007 Keystone Symposium, *'Mouse Models at the Frontiers of Cancer Discovery'*, Whistler, British Columbia, CANADA
2007 Keystone Symposium '*Inflammation and Cancer'*, Santa Fe, NM, USA
2007 AAAS Annual Meeting, *Healthy Aging: Inflammation and Chronic Diseases'* Symposium, San Francisco, CA USA
2007 Tumor Microenvironment and Tumor-Stromal Interactions Workshop: Sponsored by Biogen Idec Inc., Oncology Discovery Research, San Diego CA USA
2007 American Thoracic Society 2007 International Conference, *San Francisco Science: Inflammation, Immunity and Signaling*. San Francisco, CA USA
2007 22nd Aspen Cancer Conference: Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention and Cancer Therapy, Aspen CO, USA
2007 Gordon Research Conference, *Epithelial Differentiation & Keratinization*, Bryant University, Smithfield, RI, USA
2007 AACR, *Frontiers in Cancer Prevention Research Conference*, Philadelphia, PA, USA
2007 National Cancer Institute Workshop, '*Profiling of Immune Response to Guide Cancer Diagnosis, Prognosis and Prediction of Therapy'*, Bethesda, MD, USA
2008 47th Midwinter Conference of Immunologists, '*Meeting the challenge: Immunobiology in health and disease*', Asilomar, CA USA
2008 AACR-TREC-NCI Conference on *Energy Balance and Cancer: Mediators and Mechanisms*, Lansdowne, VA USA
2008 Keystone Joint Symposium, '*Cell Death in the Immune System / Cell Death and Cellular Senescence'*, Beaver Run Resort in Breckenridge, CO, USA
2008 Keystone Symposium, '*Inflammation, Microenvironment and Cancer'*, Snowbird Resort in Snowbird, Utah, USA
2008 **THE JOHN F. ANDERSON MEMORIAL LECTURE IN MEDICINE**, '*The Linkage between Inflammation and Cancer*', University of Virginia, Charlottesville VA, USA
2008 *Tumor Microenvironment Symposium*, Stony Brook University, Stony Brook. NY. USA
2008 **KEYNOTE LECTURE**, Fox Chase Cancer Center 13th Annual Postdoctoral Fellow and Graduate Student Symposium, Philadelphia, PA USA
2008 DOD BCRP Era of Hope Meeting 2008, Symposium Session: *Immune and Inflammatory Contributions to Breast Cancer*, AND *Era of Hope Spotlight Session*, Baltimore MD, USA
2008 *AACR Centennial Conference: Translational Cancer Medicine 2008: Cancer Clinical Trials and Personalized Medicine;* Hyatt Regency Monterey in Monterey, CA USA
2008 University of Michigan Comprehensive Cancer Center 2008 Fall Symposium, Ann Arbor MI, USA
2008 *AACR Special Conference*, *Chemical and Biological Aspects of Inflammation and Cancer*, Ko Olina Hawaii, USA
2008 International Society for Biological Therapy of Cancer (iSBTc), Workshop on Inflammation in Cancer Development, Westin Horton Plaza San Diego, CA USA
2008 Skirball Symposium, New York University School of Medicine, New York, NY USA
2008 *AACR Special Conference in Cancer Research, Tumor Immunology: New Perspectives*; Miami FL, USA
2009 *1st Conference on Regulatory Myeloid Suppressor Cells*, Clearwater, FL USA
2009 Keystone Symposium, '*Extrinsic Control of Tumor Genesis*, Vancouver, British Columbia CANADA
2009 *Inflammation and Cancer: Novel Aspects of Protumor Immunity,* Major Symposium*,* 100th Annual Meeting AACR, Denver CO USA
2009 2nd Annual Retreat of the CCR-NCI Cancer and Inflammation Program, Gettysburg, PA USA
2009 24th Annual Aspen Cancer Conference, Aspen, CO, USA
2009 2009 Geoffrey Beane Cancer Research Symposium: *Inflammation and Cancer*, Memorial-Sloane Kettering Cancer Center, New York NY USA
2009 AACR Special Conference, Advan*ces in Breast Cancer Research: Genetics, Biology, and Clinical Applications*, San Diego CA USA

2009 NCI's National Tumor Microenvironment Network, Nashville TN USA
2010 Joint Keystone Symposia, *Role of Inflammation in Oncogenesis/Molecular and Cellular Biology of Immune Escape in Cancer*, Keystone CO USA
2010 *3rd Annual Wyeth Discovery Frontiers in Human Disease Symposium*, New York, NY USA
2010 **PLENARY LECTURE**, 2010 *Annual Meeting of the American Association for Cancer Research*, Washington DC USA
2010 *10th Annual Oncology Research Symposium at MIT's Koch Institute for Integrative Cancer Research*. Boston MA USA
2010 *Metastasis and the Tumor Microenvironment*, Short Course, Eppley Institute for Cancer Research, Univ of Nebraska, Omaha, NB USA
2010 *Cancer Cell Biology and Signaling Workshop*, ImClone Systems/Eli Lilly, New York NY, USA
2010 Center for Excellence in Immunology of the National Cancer Institute Symposium, Bethesda MD, USA
2010 *25th Annual Critical Issues in Tumor Microenvironment, Angiogenesis and Metastasis*, Boston MA, USA
2010 Metastasis Research Society-AACR Joint Conference on *Metastasis and the Tumor Microenvironment, Philadelphia, PA USA*
2010 **J. WALTER JUCKETT DISTINGUISHED LECTURE**, University of Vermont Cancer Center Clinical and Translational Research Symposium, *Inflammation & Cancer*, Burlington VT, USA
2010 **KEYNOTE LECTURE**, 2010 Saban Research Institute *Annual Symposium, Honoring Yves DeClerck*, University of Southern California and Children's Hospital Los Angeles, Los Angeles CA, USA
2010 **PLENARY LECTURE**, 2010 American College of Veterinary Pathologists and American Society for Veterinary Clinical Pathology, Concurrent Annual Meetings, Baltimore MD, USA
2011 **KEYNOTE LECTURE,** 11th Annual Meeting of NANT Consortium Investigators. Biology and Therapy of High Risk Neuroblastoma, Redondo Beach CA, USA
2011 2nd International Conference on Immunochemotherapy, entitled "*Immunochemotherapy: Correcting Immune Escape in Cancer*", Philadelphia PA USA
2011 *The Biology of Cancer: Microenvironment, Metastasis & Therapeutics*, Cold Spring Harbor Laboratory Meeting Series. Cold Spring Harbor, NY USA
2011 2nd NCI Tumor Microenvironment Network Junior Investigator Meeting, Cambridge, MA USA
2011 AACR Special Conference: *Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy*, Orlando Florida USA
2011 **PLENARY LECTURE**, San Antonio Breast Cancer Conference, San Antonio, Texas USA
2012 51st Midwinter Conference of Immunologists, Asilomar, CA USA
2012 **PLENARY SESSION** "Tumor Heterogeneity: Challenges and Therapeutic Opportunities" *2012 103rd Annual Meeting of the AACR*, April 3-6, 2010, Chicago, IL USA
2012 2012 Scientific Colloquium of the Cancer Immunotherapy Consortium. *Immune Signatures in the Tumor and Beyond: Toward Predictive and Prognostic Markers*. Baltimore Maryland, USA
2012 AACR Special Conference on Pancreatic Cancer, Lake Tahoe NV, USA

UPCOMING INVITATIONS
2012 CELL Symposium, *Hallmarks of Cancer*, San Francisco CA USA
2012 AACR Special Conference, *Tumor Immunology: Multidisciplinary Science Driving Basic and Clinical Advances*, Miami FL, USA
2013 Society of Leukocyte Biology, *Regulators of Innate Cell Plasticity Effects in Host Defense*" Newport RI, USA
2013 **AMERICAN CANCER SOCIETY BASIC SCIENCE LECTURE**, Society of Surgical Oncology Annual Meeting, Washington D.C., USA

Invited Lectures/Seminars: International
2000 Medical Genome Center, Division of Molecular Medicine, Australian National University, Canberra, A.C.T. AUSTRALIA.
2001 German Cancer Center, Heidelberg, GERMANY.
2001 MERCK Pharmaceutical, Damstedt GERMANY.
2003 University of Toronto, Ontario Cancer Institute & Princess Margaret Hospital, Toronto, Ontario, CANADA

2004 Cancer Research UK, Barts & The London Queen Mary's School of Medicine & Dentistry, John Vane Science Center, Charterhouse Square, London, UNITED KINGDOM
2004 Cancer Research UK, London Research Institute, Lincoln's Inn Fields Laboratories, London, UNITED KINGDOM
2004 University of British Columbia, Department of Biochemistry and Molecular Biology, Vancouver, British Columbia, CANADA
2007 Angiogenesis and Tumor Targeting Research Unit & Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, ITALY
2008 Institute of Cell Biology, ETH Zurich Switzerland
2008 Institute of Cancer and the CR-UK Clinical Centre, Barts & The London School of Medicine and Dentistry, London UNITED KINGDOM
2009 University of South Hampton, UNITED KINGDOM
2009 The Netherlands Cancer Institute, Amsterdam, THE NETHERLANDS
2010 **DISTINGUISHED GUEST LECTURER**, Institute of Cancer, Barts & London School of Medicine. London UNITED KINGDOM
2012 Excellence in Genetics and Immunology Lecture Series, Complex Traits Group at McGill University, Montreal, Qc, CANADA
2012 **MILDRED SCHEEL LECTURESHIP (INNAUGURAL)**, German Cancer Aid, and Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, GERMANY

UPCOMING INVITATIONS
2013 Cambridge Research Institute Distinguished Lecture, Cambridge UK.

Invited Lectures/Seminars: National

1997 Biologic Therapy Research Conference. Univ. of Pittsburgh Medical Center, Pittsburgh, PA, USA
1997 Immunology Seminar Series. Univ. of Pittsburgh Medical Center, Pittsburgh, PA, USA
1999 Axys Pharmaceuticals, South San Francisco, CA, USA
1999 Berlex Pharmaceuticals, Emeryville, CA, USA
1999 Axys Pharmaceuticals, La Jolla, CA, USA
1999 14th Annual Excalibur Round Table, American Cancer Society, San Francisco, CA, USA
1999 Colloquium in Microbiology, Cell and Molecular Biology. San Francisco State Univ., San Francisco, CA, USA
2000 Chiron Corporation, Emeryville, CA, USA
2000 Oral and Pharyngeal Cancer Branch/NIDCR, National Institutes of Health, Bethesda, MD, USA
2000 Fibrogen, Inc., South San Francisco, CA, USA
2000 Scios Inc., Sunnyvale, CA, USA
2000 Molecular Biology Department, University of Southern California, Los Angeles, CA, USA
2001 Dept. of Pediatric Hematology and Oncology, Children's Hospital Los Angeles, Univ. of Southern California, Los Angeles, CA, USA
2001 Jonnson Comprehensive Cancer Center, Univ. of Calif., Los Angeles, Los Angeles, CA, USA
2002 Institute for Engineering and Medicine, Univ. of Pennsylvania, Philadelphia, PA, USA
2002 Oncology Grand Rounds, Univ. of Missouri, Columbia, MO.
2002 Cancer Center, Univ. of California, Davis, Davis CA, USA
2002 AstraZeneca, Waltham, MA USA
2002 Pharmacology Seminar Series, Dept. of Pharmacology, Wayne State Univ., Detroit, MI, USA
2003 Dept. of Biology, Univ. of Calif., San Diego, San Diego, CA USA
2003 Tularik, Inc., South San Francisco, CA USA
2003 Dept. of Cancer Biology's Cancer Metastasis Research Program Seminar Series, M.D. Anderson Cancer Center, Univ. of Texas, Houston, TX, USA
2003 Dept. of Cancer Biology, Stanford University, Stanford, CA, USA
2004 Burnham Cancer Institute, San Diego, CA, USA
2004 The Wistar Cancer Institute, Philadelphia, PA, USA
2004 Regeneron Pharmaceuticals, Inc. Tarrytown, New York, USA
2004 Keynote Lecture: Vanderbilt University Digestive Disease Research Center Retreat, Vanderbilt University, Nashville, TN, USA
2004 Dana Farber Cancer Center, Harvard Medical School, Boston MA, USA

2004 Indiana University, Herman B. Wells Center for Pediatric Research and Clinical Cancer Center, Indianapolis IN, USA
2004 Immunology Graduate Program Seminar, Stanford University, Stanford, CA, USA
2005 Dept. of Nutritional Sciences & Toxicology, Univ. of Calif., Berkeley, Berkeley, CA USA
2005 Rigel, Inc., South San Francisco, CA USA
2005 Dept of Pathology & Lab Medicine, Univ. of California, Los Angeles, Los Angeles, CA USA
2006 Division of Cancer Biology and Angiogenesis in the Department of Pathology at Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA USA
2006 Department of Molecular and Medical Pharmacology, University of California, Los Angeles, Los Angeles, CA USA
2007 *Lymphoma and Myeloma Conference*, M.D. Anderson Cancer Center, Houston, TX, USA
2007 University of Minnesota, Dept. of Lab Medicine and Pathology, Minneapolis, MN, USA
2007 Memorial-Sloan Kettering Cancer Center, Program in Cancer Biology and Aging, New York NY, USA
2007 Abramson Family Cancer Research Institute and Univ. of Pennsylvania, Division of Hematology-Oncology, Philadelphia, PA USA
2007 Albert Einstein College of Medicine, New York NY, USA
2007 Oncology Division Research, Biogen Idec Inc., San Diego, CA USA
2007 Genentech, Inc. Immunology Program. South San Francisco, CA USA
2007 University of Iowa Carver College of Medicine, Dept of Pathology, *Pathology Grand Rounds*, Iowa City, Iowa, USA
2007 Hollings Cancer Center, Medical University of South Carolina, Charleston, SC, USA
2007 University of Michigan, Program in Immunology and Cancer Research Series, Ann Arbor, MI USA
2008 Department of Pathology/UCLA School of Medicine Seminar, Los Angeles CA USA
2008 ANNUAL KEYNOTE LECTURE, Dept of Cancer Biology, Meharry Medical College, Nashville, TN USA
2008 University of California, Davis Cancer Center, Sacramento, CA USA
2008 Department of Immunology, University of Pittsburgh School of Medicine. Pittsburgh, PA, USA
2008 Cancer Biology Series, Ben May Cancer Center, University of Chicago, Chicago, IL, USA
2008 National Cancer Institute Center for Cancer Research Grand Rounds Series in Clinical and Molecular Oncology. Bethesda MD, USA
2009 University of Michigan, Oral Health Sciences Program and Biomedical Engineering Seminar Series, Ann Arbor, MI USA
2009 Department of Pharmacology, Wayne State University, Detroit, MI USA
2009 Molecular Biology Seminar Series, Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Aurora, CO USA
2009 National Institutes of Health/National Cancer Institute, Vascular Biology Seminar Series, Bethesda MD, USA
2009 Genentech, Inc., Molecular Oncology Program. South San Francisco, CA USA
2009 Breast Cancer Network of Strength, California Breast Cancer Organizations, Northern California Affiliate, David CA USA
2009 Fred Hutchinson Cancer Center, Seattle WA USA
2010 Cold Spring Harbor Laboratory, CSH NY USA
2010 Albert Einstein College of Medicine, New York, NY USA
2010 Department of Cell Biology & Physiology Washington University, St Louis, MO USA
2010 Cancer Center Seminar Series at Burnham Institute for Medical Research, San Diego CA, USA
2010 Oncology Seminar Series, MedImmune, Gaithersburg, MD, USA
2010 Immunology Institute Seminar Series, Mt Sinai School of Medicine, NY, NY USA
2010 San Francisco State University, Fall Seminar Series, San Francisco CA USA
2011 McArdle Seminar in Cancer Biology series, Univ of Wisconsin-Madison, USA
2011 Duke University Medical Center, Durham, North Carolina USA
2011 **BORNTREE DISTINGUISHED LECTURE**, Immunology and Infectious Disease Program, Dept of Veterinary and Biomedical Sciences, Pennsylvania State Univ. University Park, PA USA
2011 **HUCK DISTINGUISHED LECTURE**, The Huck Institute, University Park, Pennsylvania State Univ. University Park, PA USA
2011 Weill Cornell Medical College of Cornell University, Center for Vascular Biology, NY USA

2011 **CHARLES I. SIEGAL MEMORIAL LECTURE**, Dana-Farber Cancer Institute and the Dana-Farber Cancer Institute, Boston MA, USA
2011 Tulane Cancer Center, Hematology & Medical Oncology, Tulane Univ. School of Medicine, New Orleans, LA USA
2011 Northwestern University Breast Cancer Research Program and Breast Cancer Research Seminar Series, Northwestern University, Chisago IL, USA
2011 Novartis Institutes for Biomedical Research, Emeryville CA USA
2011 FivePrime Therapeutics, South San Francisco, CA USA
2011 Abbott Biotherapeutics, Redwood City CA USA
2012 Brown Foundation Institute of Medicine, Univ. of Texas, Health Science Center at Houston, Houston TX USA
2012 Baylor College of Medicine, Houston TX USA
2012 Harvard Medical School's (HMS) Committee on Immunology Seminar Series
2012 Massachusetts General Hospital's (MGH) Seminar Series
2012 University of Rochester, Department of Microbiology and Immunology, Rochester, NY, USA
2012 Becton, Dickinson and Company, San Jose CA, USA

UPCOMING INVITATIONS
2012 Huntsman Cancer Institute of University of Utah, Salt Lake City UT, USA
2012 Eisai, Inc. Andover MA, USA

Invited Lectures/Seminars: OHSU
2012 OHSU Knight Cancer Biology Research Group Meeting, OHSU
2012 OHSU School of Medicine, TEDMED 2012 Live Simulcast

UPCOMING INVITATIONS
2012 OHSU MD/PhD Annual Retreat, McMenamins Edgefield,Troutdale, OR.
2012 OHSU PMCB Annual Retreat, OR

Invited Lectures/Seminars: UCSF
1997 Breast Cancer SPORE Seminar. UCSF
1999 Cancer Research Institute Retreat, Tomales Bay, CA
2000 Chemistry and Cancer: How Chemistry-Based Tools Are Helping Solve Today's Serious Health Problems, Dev. & Alumni Relations, UCSF
2000 Oncology Grand Rounds, Department of Hematology and Oncology, UCSF
2000 PIBS-Cell Biology Seminar Series, UCSF
2000 Pathology and Lab Medicine Grand Rounds, UCSF
2000 BMS Student Pizza Talk, UCSF
2000 Cell Cycle & Dysregulation Club, Comprehensive Cancer Center, UCSF
2000 Comprehensive Cancer Center Retreat, Granlibakken, Tahoe City, CA
2001 BMS Student Pizza Talk, UCSF
2001 Pathology and Lab Medicine Grand Rounds, Departments of Medicine and Pathology, UCSF
2001 UCSF, Cell Biology Retreat, Wilbur Hot Springs, CA, USA
2001 UCSF TETRAD Retreat, Granlibakken, Lake Tahoe, CA, USA
2001 UCSF Cancer Research Institute/BMS Retreat, Granlibakken, Lake Tahoe, CA. USA
2002 Current Topics in Medical Science, UCSF Medical Scientist Training Program (M170.09)
2002 Mouse Models of Human Cancer Program, Comprehensive Cancer Center, UCSF
2002 Cancer Research Institute Retreat, Santa Cruz, CA
2003 PIBS Student Pizza Talk, UCSF
2003 Breast Oncology Program, Comprehensive Cancer Center, UCSF
2003 Comprehensive Cancer Center Faculty Retreat: *Identification and Functional Assessment of Cancer Effectors*, Golden Gate Club, San Francisco CA
2004 BMS Graduate Program Retreat, Granlibakken Tahoe City, CA
2005 BMS Student Pizza Talk, UCSF
2006 *Introduction to Research,* Department of Pathology, UCSF
2008 Division of Experimental Medicine, Divisional Seminar Series, UCSF
2009 Immunology Program, UCSF

2009	Helen Diller Family Comprehensive Cancer Center *Research Symposium*; UCSF	
2010	Bay Area Workshop on Lung Development, Physiology and Cancer, San Francisco CA USA	
2010	UCSF-GIVI Center for AIDS Research (CFAR) Scientific Symposium for 2010: *HIV Infection, Inflammation, and Premature Aging*, San Francisco, CA USA	
2010	Breast Oncology Program Seminar, Helen Diller Family Comprehensive Cancer Center, UCSF	
2011	Breast Oncology Program Annual Retreat, Helen Diller Family Comprehensive Cancer Center, UCSF	
2012	Breast Oncology Program Annual Retreat, Helen Diller Family Comprehensive Cancer Center, UCSF	

VII. GOVERNMENT AND OTHER PROFESSIONAL SERVICE:

GOVERNMENT SERVICE

2003 - 2006	National Institutes of Health, Center for Scientific Review	Ad hoc reviewer (10/2003; 02/2005; 10/2005; 06/2006), Tumor Progression & Metastasis (TPM) Study Section, Oncological Sciences Review group
2003	Division of Cancer Biology, National Cancer Institute: *Microenvironment Think Tank*	Participant and *Reporter*
2003	Division Cancer Etiology, National Cancer Institute: *Validation of A Causal Relationship: Criteria to Establish Etiology Think Tank*	Invited speaker and Participant
2004	National Institutes of Health, National Cancer Institute	Subcommittee C (05/2004) – Basic & Preclinical NCI Initial Review Group, NCI-C RPRB (T2) Angiogenesis
2005	National Institutes of Health, National Cancer Institute	Subcommittee D (02/2005) – Clinical Studies NCI Initial Review Group, NCI-D RPRB Tumor Pathology
2005	National Institutes of Health, Center for Scientific Review-Oncology	Special Emphasis Panel (SEP); ZRG1 ONC (03) M, Developmental Therapeutics
2010	National Institutes of Health, Center for Scientific Review-Neuroscience	Special Emphasis Panel (SEP)/Scientific Review Group 2010/05 ZNS1 SRB-R (47)
2010	National Institutes of Health, Center for Scientific Review-Neuroscience	Special Emphasis Panel (SEP)/Scientific Review Group 2011/01 ZRG1 DTCS-A (81)
2010 - 2011	Department of Defense (DOD), Breast Cancer Research Program (BCRP)	6th Era of Hope conference Technical Planning Committee (TPC)
2011	National Institutes of Health, Center for Scientific Review	Ad hoc reviewer (06/2011), Cancer Immunotherapy & Immunology (CII) Study Section, Oncology 2 - Translational Clinical IRG (OTC) Division of Translational and Clinical Sciences

OTHER PROFESSIONAL SERVICE

1999	Arkansas Science & Technology Authority	Ad hoc Grant Review
2000	McGraw-Hill, '*Biology'* 6th edition, Ed. P.H. Raven and G.B. Johnson	Ad hoc Review, Chapters 17 and 18
2000	Division of Cancer Biology, NCI: *Epithelial-Stromal Interactions & Tumor Progression Workshop*	Invited speaker and Participant
2001	Department of Veterans Affairs	Ad hoc Grant Review, Oncology Review Board
2001	Research Grants Council of Hong Kong	Ad hoc Grant Review
2003	Danish Cancer Society, DENMARK	Ad hoc Grant Review
2004	Division of Gastroenterology and Digestive Disease Research Center, Vanderbilt University, Nashville TN, USA	'*H. pylori-induced Inflammation and Gastric Adenocarcinoma*, PO1 External Advisory Panel
2004	Cancer Research Ireland, Irish Cancer	Ad hoc grant review

	Society	
2004	Dutch Cancer Society	Ad hoc grant review
2004	Vanderbilt University, Nashville TN, USA; SPORE in GI Cancer	Ad hoc reviewer for SPORE Developmental Research Program
2005	Keystone Symposia, *Inflammation and Cancer*	Co-organizer (with Dr. Ray DuBois, Vanderbilt Univ, TN), Breckinridge, CO, USA
2006	5th Annual Timberline Symp. on Epithelial Cell Biology, '*Intrinsic and Microenvironmental Regulation of Epithelial Cancer'*	Co-Organizer (with Dr. Harold Moses, Vanderbilt University, TN, USA), Timberline, OR, USA
2006	Keystone Symposia Cancer Study Group for 2009 programming	Study group member
2007	Keystone Symposia, *Inflammation and Cancer*	Organizer (with Drs. Fran Balkwill (Cancer Research UK) and Glenn Dranoff (Beth Israel Cancer Center, Harvard, MA) Santa Fe, New Mexico, USA
2008	AACR Special Conference on '*Inflammation and Cancer'*	Co-Organizer (with Drs. Michael Karin and Larry Marnett)
2007 - present	Masonic Cancer, University of Minnesota Center; Douglas Yee, M.D., Director	External Scientific Advisory Board Member
2007 - 2011	University of Washington, Seattle WA, USA	Member, External Scientific Advisory Board, CA U54 TMEN: *Significance of Microenvironment for Prostate Cancer Initiation and Progression*; P.I. Stephen R Plymate, Univ. of Washington School of Medicine.
2007 - 2011	Albert Einstein College of Medicine of Yeshiva University, New York, NY USA	Member, External Scientific Advisory Board, CA U54 TMEN: *Novel Methods for Detection Cell Interactions in the Tumor Microenvironment*; P.I. John S. Condeelis, Albert Einstein College of Medicine.
2008	International Society for Biological Therapy of Cancer (iSBTc), *2008 Workshop on Inflammation in Cancer Development*	Co-Organizer (with Drs. Michael Karin, Steven Dubinett, George Weiner)
2009	GlaxoSmith Kline	Member, Tykerb Post-ASCO KOL Advisory Board
2009	University of Southern California, Children's Hospital	Member, External Scientific Advisory Board, Neuroblastoma, Program Project grant (P01), PI: Robert Seeger, M.D.,
2009 - 2010	Cancer Prevention and Research Institute of Texas (CPRIT)	Member, Scientific Review Committee; Basic Cancer Biology Review Committee
2011	Starr Cancer Consortium Scientific Review Board	Member, Scientific Review Committee
2011	AACR Special Conference on '*Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy''*	Co-Organizer (with Drs. Yves DeClerck and Melodie Shwartz)
2012 - present	Koch Institute for Integrated Cancer Research, Massachusetts Inst. of Tech.	Member, External Advisory Board
2012 - present	Melvin and Bren Simon Cancer Center, Indiana University	Member, External Advisory Board

VIII. UNIVERSITY AND PUBLIC SERVICE

UNIVERSITY SERVICE

Oregon Health and Sciences University

2012 Member, Search Committee, Dept. of Urology and Knight Cancer Institute

2012	Chair, Search Committee, Depts of Cell & Developmental Biology, Molecular and Microbial Immunology
2012	Co-Chair, Search Committee, Dept. of Cell and Developmental Biology and Knight Cancer Institute
2012	Co-Chair, Search Committee, Dept of Medicine. Division of Hematology & Oncology and Knight Cancer Institute
2012	Chair, V Foundation Scholar nomination committee

University of California; System wide

1992-1993	Graduate Student Representative, Dept. of Biological Chemistry Faculty Council, UCLA
2004	*ad hoc* Member External Advisory Panel; Jonsson Comprehensive Cancer Center, University of California, Los Angeles, Los Angeles CA, USA
2009	Member, Site Visit Programmatic Review Group, Department of Pathology & Laboratory Medicine, UCLA School of Medicine. Graduate Council of the UCLA Academic Senate.

University of California, San Francisco; CAMPUS-WIDE

1997	Presentation, Donor Seminar, UCSF Development Office
1998	Presentation, Donor Seminar, UCSF Development Office
2000 – 2004	Member, Steering Committee, Ovarian Cancer Program Project Grant
2000 – 2005	Member, Scholarships and Awards Committee, Academic Senate, School of Medicine
2002 – present	Member, BioMedical Sciences Graduate Program (BMS) Executive Committee
2002 – 2004	Member, Medical Scientist Training Program Executive Committee
2004 - 2006	Member, Search Committee, Director of Molecular Imaging, Dept. of Radiology, Committee Chair: Ron Arenson, M.D. no successful recruitment
2004 – 2006	Member, BioMedical Sciences Graduate Program (BMS); Admissions Committee
2004	Organizer, BioMedical Sciences Graduate Program Retreat, Granlibakken, N. Lake Tahoe, CA USA
2005 - 2006	Member, Tissue Engineering Ladder-rank Faculty Search Committee, Dept. of Surgery. Committee Chair: Nancy Boudreau, Ph.D. Successful recruitment of Valerie Weaver, Ph.D.
2005 - 2009	Member, Ethel and Jane Sokolow Memorial Cancer Endowment Lectureship Committee.
2006	Member, Cancer Faculty Search Committee, Anatomy Dept., Committee Chair: Zena Werb, Ph.D. Successful recruitment of Jeroen Roose, Ph.D.
2006	Member, Faculty Advisory Committee for 2007 Journalist Seminar on *Inflammation and Disease*. Sponsored by Associate Vice Chancellor Barbara J. French
2007	Member, committee to select recipient of Dean's Postdoctoral Prize Lecture.
2007	Member, Faculty Search Committee for Restorative Neurosurgery and Stem Cell Neurobiology, VA Medical Center/UCSF NeuroSurgery. Committee Chair: Linda Noble, Ph.D.; Status: not filled.
2009	Member, Committee to choose 1st Bonnie J. and Anthony Addario Endowed Chair in Thoracic Oncology, School of Medicine, UCSF. Recipient: Thierry Jahon, M.D.
2010	Member, 2010 Selection Committee for the Hellman Family Early-Career Faculty Awards.

University of California, San Francisco, Helen Diller Family Comprehensive Cancer Center

1999	Member, Cancer Center Research Building Space Review Policy Committee
1999 – 2002	Member, Mt Zion Animal Barrier Facility Committee
1999 – 2005	Member, Cancer Center Friday Seminar Series Committee
2000	Organizer and Chair, MZ Cancer Center Research Building Annual Retreat
2001	Member, 'Star Performance Award' selection committee
2001	Presentation, Evelyn Herman Reception, UCSF Development Office
2001 – 2002	Member, Cancer Center Research Building, 'Cancer Center Faculty Working Group'
2001 – 2006	Member, Mouse Models of Human Cancer Working Group
2002 – 2003	Member, UCSF Mt Zion campus, Animal Protocol Review Committee
2002	Member, ACS IRG grant review committee
2002 – 2006	Steering Committee Member, Mouse Models of Human Cancer
2003	Member, Review Committee, UCSF Comprehensive Cancer Center Stewart Trust Award
2003 – 2009	Chair, UCSF Mt Zion Campus Animal Protocol Review Committee

2003 Member, Search Committee: Associate Director for Administration, UCSF Comprehensive Cancer Center (Erica Weber, recruited)
2004 Member, Review Committee, UCSF Comprehensive Cancer Center Stewart Trust Award
2006 Co-Organizer, UCSF CCC Annual Symposium, *'Inflammation & Cancer: Bench to Bedside'.*
2008 Chair, Committee to nominate Postdoctoral scholar for AACR 2008 Annual Meeting, Inaugural "Future Leaders, New Directions" Special Symposium. Nominee: Laura Soucek, Ph.D. (awarded)

University of California, San Francisco, Cancer Research Institute
2001 – 2002 Member, Cancer Research Institute Membership Subcommittee

University of California, San Francisco, Department of Pathology
2003 Member, Committee to recommend faculty for the *Robert E. Smith Endowed Chair in Experimental Pathology*
2004 Member, Search Committee, Ladder rank faculty, Physician-Scientist, Anatomic Pathology. Successful recruitment of Jay Debnath, M.D., Ph.D.
2007 Member, Search Committee, Ladder-rank faculty, Physician-Scientist, Pathology and Neuropathology. Committee Chair: Michael D Prados, M.D.; Status: open.
2008 Member, Search Committee, Ladder-rank faculty, Physician-Scientist, Experimental Pathology. Committee Chair: Benedict Yen, M.D.; Status: open
2009-2011 Member, Academic Merit and Promotions Committee; Pathology Dept.

University (other)
2002 Guest Instructor, Graduate Oncology, University of Missouri, Columbia, Missouri USA
2003 Guest Instructor, Cancer Biology, Stanford University, Stanford, CA USA
2004 Guest Instructor, Immunology, Stanford University, Stanford, CA USA
2008 Guest Instructor, *Exploring the Tumor microenvironment*, Postgraduate course, ISREC, Lausanne University's Biochemistry and Biology Departments, and the Lausanne Branch of the Ludwig Institute, Lausanne Switzerland. Course Organizers, Ivan Stamenkovic and Michel Aguet

PUBLIC SERVICE:
1990 Lecturer, Science Academy Of Whittier, Summer Institute. Whittier College, Whittier, CA
1991 Organizer and Lecturer, Science Academy Of Whittier, Summer Institute. Whittier College, Whittier, CA.
1993 Lecturer, Joslyn Community Center. Claremont, CA.
1994 Provided elementary educators with science-related supplies (photos, slides, fixed tissue samples).
1995 Co-Coordinator Hormone Research Institute, 'Take Our Daughters To Work Day', Univ. of Calif., San Francisco
2002 Photo credits and interviewed for '*Misdiagnosis: Failure of Promising Cancer Treatment Starts Soul Searching by Researchers & Drug Companies',* in: *San Francisco Chronicle,* May 12, 2002.
2003 Interviewed for article '*Body's First Defense May Be Root of Diseases',* in: *The Washington Post,* February 20, 2003
2003 Interviewed for article '*The Body on Fire*', in: U.S. News & World Report, October 20, 2003
2004 Interviewed for comments in: *Science News*, '*Early Warming: Inflammatory protein tied to colon cancer risk*" February 7, 2004, Vol 165.
2004 Interviewed for article '*The Fires Within'*, in: *TIME* Magazine, February 23, 2004
2004 Interviewed for comments on AACR Annual Meeting in: *Oncology Times*, '*Exercise Reduces Inflammatory Response, May also Reduce Cancer Risk*', Robert H Carlson, 26(11):33-34, June10, 2004
2004 Interviewed for article *'Inflammation and Cancer: The Link Grows Stronger',* in: *Science*, 306, 966-968 (2004)
2005 Interviewed for article *'Quieting a Body's Defenses',* in: *Newsweek*, Special Edition, Summer 2005
2006 Interviewed for "Expert Commentary" by *BreastLink.org,* on article "Association Between Circulating White Blood Cell Count and Cancer Mortality." *Archives of Internal Medicine*, January 23, 2006; 166:188-194.
http://www.breastlink.org/index.php?module=announce&ANN_user_op=view&ANN_id=208
2007 UCSF Research Perspectives 2007 – Inflammation as Cause and Consequences of Disease, Media

Event for Journalists, September 27, 2007, UCSF Mission Bay Campus

2007 On-Air radio interview by Dave Iversen, KQED *FORUM*, September 28, 2007 San Francisco CA USA

2012 Delta Kappa Gamma Society International, Winter Keynote Lecture; January 23, 2012, Fairfield, CA USA

2012 Continuing Education Webinar, Project LEAD, Center for NBCC Advocacy Training, National Breast Cancer Coalition

IX. TEACHING AND MENTORING

Formal Scheduled Classes for OHSU Students:

Qtr	Academic Yr	Course No. & Title	Teaching Contribution	Units	Class Size
S	2011/12	CELL 616; Advanced Topics in Cancer Biology	Lecture; *Tumor Microenvironment*	3	20

Formal Scheduled Classes for UCSF Students:

Qtr	Academic Yr	Course No. & Title	Teaching Contribution	Units	Class Size
W	1997/98	IDS 100; Histology Laboratory	*Neoplastic Skin Histopathology*; Laboratory lecture & instruction	10	150
W	1998/99	IDS 100; Histology Laboratory	*Neoplastic Skin Histopathology;* Laboratory lecture & instruction	10	150
W	1999/00	IDS 100; Histology Laboratory	*Neoplastic Skin Histopathology*; Laboratory lecture & instruction	10	150
S	1999/00	BMS 297A; Molecular Biology & Pathology of Neoplasia	*Animal Models of Cancer Laboratory*; Laboratory lecture & instruction	3	15
S	2000/01	BMS 297A; Molecular Biology & Pathology of Neoplasia	*Animal Models of Cancer Laboratory*; Laboratory lecture & instruction	3	15
W	2000/01	BMS 225; Tissue and Organ Biology	Lecture and laboratory instruction	3	15
S	2000/01	BMS 260; Cell Biology	Discussion group leader	1	6
F/W	2001/02	IDS 101; Prologue	Laboratory Instructor	9	30
W	2001/02	BMS 225; Tissue and Organ Biology	Lecture and laboratory instruction	3	15
W	2001/02	IDS 103; Cancer Block	*Invasion & Metastasis*; Lecturer	7	150
S	2001/02	BMS 260; Cell Biology	Discussion group leader	1	7
F	2002/03	BMS 260; Cell Biology	Discussion group leader	1	6
W	2002/03	IDS 103; Cancer Block	*Invasion & Metastasis*; Lecturer	7	150
F/W	2002/03	IDS 101; Prologue	Laboratory Instructor	9	30
F	2003/04	BMS 260; Cell Biology	Discussion group leader	1	6
S	2003/04	BMS 225B, Tissue and Organ Biology	Lecturer and Laboratory Instructor	1.5 - 5	tbd
W	2003/04	Biochem 297; Molecular Biology & Pathology of Neoplasia	*Angiogenesis:* Lecturer	3	30
W	2003/04	BMS 297A Molecular Biology & Pathology of Neoplasia Laboratory	Lecturer and Laboratory Instructor, *Animal Models of Neoplasia*	1	10
S	2003/04	BMS 225B; Tissue & Organ Biology	Lecturer: Cancer I & Cancer II	1.5 - 5	16
F	2004/05	BMS 260; Cell Biology	Discussion group leader	1	6
F	2005/06	BMS 260; Cell Biology	Discussion group leader	1	7
W	2006/07	Biochem 297; Molecular Biology & Pathology of Neoplasia	*Inflammation and Cancer*: Lecturer	3	30
W	2008/09	BMS230; Cellular & Molecular Biology of	Course Co-Director	3.5	22

		Cancer			
W	2008/09	BMS230; Cellular & Molecular Biology of Cancer	Lecturer: *Cancer Microenvironments; Inflammation and Cancer*	3.5	22
W	2010/11	BMS230; Cellular & Molecular Biology of Cancer	Course Co-Director	3.5	22
W	2010/11	BMS230; Cellular & Molecular Biology of Cancer	Lecturer: *Tumor cell heterogeneity*; *Cancer Microenvironments; Inflammation and Cancer*	3.5	22

Postgraduate and Other Courses:

1989	M204, *Biochemistry Lab* Univ. of Calif., Los Angeles	Student Teaching Assistant for quarter long course (100 medical students)
1989	Biology 250, *Human Heredity*; Dept. of Biology Whittier College, Whittier CA	Organized and taught entire lecture-based course (30 undergraduate students)
1990	Biology 350 & 350L, *Molecular Genetics*; Dept. of Biology, Whittier College, Whittier CA	Organized and taught entire lecture and laboratory course (16 undergraduate students)
1990	M204, *Biochemistry Lab* Univ. of Calif., Los Angeles	Student Teaching Assistant for quarter long course (100 medical students)
1990	Biology 250, *Human Heredity*; Dept. of Biology Whittier College, Whittier CA	Organized and taught entire lecture-based course (30 undergraduate students)
1992	Biology 350 & 350L, *Molecular Genetics*; Dept. of Biology, Whittier College, Whittier CA	Organized and taught entire lecture and laboratory course (16 undergraduate students)
2003	Graduate *Oncology*, University of Missouri, Columbia, MS, USA	Invited Guest Lecturer: Lecture syllabus & delivered 2-hr lecture for course (15 students, graduate, medical & postgraduate fellows
2003	Graduate Program in Cancer Biology, Stanford Univ., Stanford, CA USA	Invited Guest Lecturer: Delivered 1-hr lecture to graduate students in Cancer Biology Graduate program
2004	Graduate Program in Immunology, Stanford Univ., Stanford, CA USA	Invited Guest Lecturer: Delivered 1-hr lecture to graduate students in Immunology Graduate program
2005	UCSF Dermatology residents' Basic Science Seminar Series	Invited Guest Lecturer: Delivered 1-hr lecture to UCSF Dermatology Residents (11 M.D. and M.D., Ph.D. Residents)
2008	ISREC, Lausanne University's Biochemistry and Biology Dept, and Lausanne Branch of the Ludwig Institute	Guest Instructor: *Exploring the Tumor microenvironment*, postgraduate course. (20 PhD students, 3 hours of instruction)
2009	OOA Course: Tumor Microenvironment; The Netherlands Cancer Institute	Guest Faculty: (4.5 hours of instruction, 25 PhD students)
2010	25th Annual Harvard Tumor Course: Critical Issues In Tumor Microenvironment, Angiogenesis & Metastasis: *From Bench to Bedside & Back*	Faculty member: (2 hours of instruction. 100 students)
2010	Eppley Institute for Research in Cancer, Univ. of Nebraska Medical Center. Short Course in Cancer Biology: Metastasis and the Tumor Microenvironment	Faculty member: (3 hours of instruction, 166 students)
2010	San Francisco State University, Dept of Biology Seminar Series	Guest Faculty: (1 1/2 hour of instruction, 75 students)
2011	26th Annual Harvard Tumor Course: Critical Issues In Tumor Microenvironment, Angiogenesis & Metastasis:	Faculty member: (2 hours of instruction. 100 students)

2012	27th Annual Harvard Tumor Course: Critical Issues In Tumor Microenvironment, Angiogenesis & Metastasis:	Faculty member: (2 hours of instruction. 100 students)

High School and Undergraduate Students Supervised or Mentored:

Dates	Name	Program or School	Faculty Role	Current position
1998	Christopher Tinkle	Undergraduate, Univ. of Texas, Austin, TX, USA	Summer Research Training Program Supervisor	Ph.D. awarded 2008; M.D. awarded 2010; Resident, Rad/Onc, UCSF
2000	Adam Zucker	Undergraduate, Oberlin College, Ohio USA	Supervised Summer work	unknown
2000	Ashkan Hirari	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA, USA	Supervised Summer work	unknown
2001	Jason Reuter	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA	Supervised Summer work	unknown
2002	Destinee Cooper	Undergraduate, Univ. of Calif., Davis USA	Summer Research Training Supervisor	unknown
2006	Sunum Mobin	UCSF Science & Health Education Partnership: High School Intern Program	Summer Research Training Supervisor	unknown
2008-2009	Julia Lam	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA	Independent study (199), Mentor	B.S. awarded 2009
2010	Scott Keil	Undergraduate, The University of Glasgow, Scotland	Summer Research Training Supervisor	The University of Glasgow, Scotland
2010-2011	Heather Chen	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA	Summer Research Training Supervisor	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA
2010	Amy Desalazar	Cupertino High School Cupertino, CA USA	Summer Research Training Supervisor	Cupertino High School
2010-2011	Nikhil Wadhwani	Undergraduate, Sarah Lawrence College, Bronxville, NY USA	Summer Research Training Supervisor	Sarah Lawrence College, Bronxville, NY
2010	Sharfa Junaid	Undergraduate, Univ. of Calif., San Diego, San Diego CA USA	Summer Research Training Supervisor	Undergraduate, Univ. of Calif., San Diego, San Diego CA USA
2010-2011	Jon Lau	University of Nevada, Reno, Reno, Nevada USA	Summer Research Training Supervisor	University of Nevada, Reno, Reno, Nevada USA
2011	Kara Wang	Undergraduate, Pomona College, Claremont, CA USA	Summer Research Training Supervisor	Undergraduate, Pomona College, Claremont, CA USA
2011	Graham Litchman	Undergraduate, San Francisco State Univ, San Francisco CA USA	Summer Research Training Supervisor	Masters student, Univ. of Nevada, Reno
2011	Jessica Wignall	Smith College, Northampton, MA USA	Summer Research Training Supervisor	Smith College, Northampton, MA USA

Predoctoral Students Supervised or Mentored:

Dates	Name	Program or School	Faculty Role	Current position
2000	Jin-Sae Rhee	UCSF MSTP/BMS, graduate student	Rotation Supervisor	PhD awarded 2003, M.D. awarded 2005
2000 - 2003	Jin-Sae Rhee	UCSF M.D., Ph.D.,	Ph.D. supervisor	Pediatrician, Private Sector

2000	Maria Christophorou	UCSF BMS, graduate student	Faculty coach, BMS 297	Ph.D. awarded 2006
2001	Leslie Chu	UCSF BMS, graduate student	Rotation Supervisor	Ph.D. awarded 2005
2001	Rayna Takaki	UCSF BMS, graduate student	Rotation Supervisor	Ph.D. awarded 2006
2001 – 2002	Sophia Bruggerman	University of Nijmegan, The Netherlands	Masters Thesis Supervisor	Ph.D. awarded 2007
2002	Lucy Lebedeva	UCSF PIBS, graduate student	Faculty coach, BMS 297	Ph.D. awarded 2005
2002	Leslie Chu	UCSF BMS, graduate student	Ph.D. Orals committee	Ph.D. awarded 2005
2002	Andre Whitkin	MSTP student, Cornell University USA	Supervised Summer work	unknown
2002	Karin de Visser	The Netherlands Cancer Institute, The Netherlands	Ph.D. Thesis Reading Committee	Ph.D. awarded 2002
2003	Cathy Collins	UCSF MSTP student	MSTP Advisor	PhD awarded 2009 M.D. awarded 2011
2004	Eric Tamm	University of British Columbia, Canada	Doctoral Dissertation External Examiner	Ph.D. awarded 2004
2004	Annie Hsieh	University of Södertörn, Sweden	Masters Thesis Supervisor	unknown
2005	Geoff Benton	UCSF TETRAD/PIBS, graduate student	Ph.D. Orals committee	Ph.D. awarded 2011
2006	Morgan Truitt	UCSF BMS, graduate student	Rotation Supervisor	UCSF BMS PhD graduate student
2006	Danielle Shin	UCSF MSTP student	Rotation Supervisor	Ph.D. awarded 2011
2006-2008	Celeste Rivera	SFSU/UCSF NIH Post-baccalaureate Research Experience Program (PREP) student	M.S. research advisor	M.S. awarded 2010; Research Technician, EPFL Lausanne Switzerland
2007-2009	Leslie Vasquez	SFSU/UCSF NIH Post-baccalaureate Research Experience Program (PREP) student	M.S. research advisor	M.S. awarded 2009; PharmD student, UCSF
2008	Ashley Martin	UCSF BMS, graduate student	Rotation Supervisor	UCSF BMS PhD graduate student
2009	Kay Wiebrands	Master's Student Utrecht University, the Netherlands	Masters Thesis Internship Supervisor	M.S. awarded 2009
2009-2010	David Tawfik	Medical Student III, UCSF	MSIII break year. Dean's Quarterly Research Fellowship; PACCTR Fall Quarter Fellowship;	M.D., awarded 2011; Resident in Internal Medicine, UCLA Harbor Hospital
2009-present	Renee Vanderlaan	UCSF BMS, graduate student	Chair: Thesis Committee	UCSF BMS graduate student, Lab of Matthias Hebrook, Ph.D.
2009-2011	A. Preethi Ganessan, M.D., PhD.	Ph.D. Graduate Student (Cancer Research UK, Univ of Southampton)	Ph.D. supervisor	Pediatric Hematology Resident, Univ of Southampton, UK
2010 - present	Lucia Cottone	Ph.D. graduate student (San Rafaelle Institute, Milan Italy)	Ph.D. supervisor and 2nd supervisor del candidato	Ph.D. student, San Rafaelle Institute, Milan Italy
2011 - present	Conny Hainer	Master's student, Technical University of Berlin, Berlin, Germany	MS supervisor	Master's student, Technical University of Berlin, Berlin, Germany

2011-2012	Melissa Wheeler	PharmD student, UCSF	PharmD research mentor	PharmD student, UCSF
2011-2012	Paul Huynh	PharmD student, UCSF	PharmD research mentor	PharmD student, UCSF
2012	Derek Zachman	OHSU, MD/PhD and PMCB student	Qualifying exam committee	PMCB student
2012-present	Tim Butler	OHSU, Cancer Biology graduate student, Spellman lab	Thesis committee	Cancer Biology graduate student

Postdoctoral Fellows and Residents Directly Supervised or Mentored

Dates	Name	Position & Funding	Faculty Role	Current Position
2000 - 2001	Ernst Lengyel, M.D., Ph.D.	Post-Doc Researcher, Senior Clinical Fellow	Research Supervisor	Assoc. Adj. Prof., Dept. Gyn. & Oncology, UCSF
2000 -2002	Leon Van Kempen, Ph.D.	Post-Doc Researcher, Dutch Cancer Society Postdoctoral Fellowship	Research Supervisor	Assoc. Prof., Univ. of Nijmegen, Dept. of Pathology, The Netherlands
2002 – 2005	Robert Diaz, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Scientist, Roche Pharmaceuticals
2002 – 2005	Karin de Visser, Ph.D.	Post-Doc Researcher, Dutch Cancer Society Postdoctoral Fellowship	Research Supervisor	Research Scientist, The Netherlands Cancer Inst, Amsterdam, The Netherlands
2003 – 2007	Alexandra Eichten, Ph.D.	Post-Doc Researcher, Serono Fndt for the Advancement of Medical Science (2003-2005);	Research Supervisor	Scientist, Regeneron Corp., New York USA
2003 - 2005	Stephen Robinson, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Private sector, United Kingdom
2003 - 2004	H. Jennifer Shen, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	unknown
2005 -2010	David DeNardo, Ph.D.	Post-Doc Researcher; 1) NGA: 5T32CA09043 PI: BISHOP; *Molec. Analysis of Tumor Viruses;* 2) Am Cancer Society Fellowship 2007-2010	Research Supervisor	Assistant Professor, Molecular Oncology and Immunology, Washington University, St Louis, St Louis MS USA
2005 –2007	Nor Eddine Sounni, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Research Scientist, Univ. of Liege, Belgium
2006 –2007	Tingting Tan, M.D., Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Hem/Onc Fellow, Fox Chase Cancer Center, Phil., PA
2006 -2010	Magnus Johansson, Ph.D.	Post-Doc Researcher; Swedish Cancer Society Fellowship 2006-08	Research Supervisor	MBA candidate, Stanford Univ., Palo Alto CA
2006- 2012	Nessrine Affara, Ph.D.	Post-Doc Researcher; AACR-Astellas USA Fndt in Basic Cancer Research 2009-2010: T32 Cancer Biology 2010-2011	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF
2007 -2010	Pauline Andreu, Ph.D.	Post-Doc Researcher; Cancer Research Institute Postdoctoral Fellowship 2008-2011	Research Supervisor	Research Funding Agency in Private Sector, France
2008-present	Brian Ruffell, Ph.D.	Post-Doc Researcher; Dept of Defense Postdoctoral fellowship 2009-2012	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF, OHSU
2009-2011	Stephen Shiao, M.D., Ph.D.	UCSF Radiation Oncology Resident	Research Supervisor	UCSF Radiation Oncology, Chief Resident
2010-2012	Collin Blakeley, M.D.,	UCSF Hematology-	Research	UCSF Hematology-

	Ph.D.	Oncology Fellow; T32 Hem/Onc Training Grant 2011-2012	Supervisor	Oncology Fellowship
2010-present	Anna Wasiuk, Ph.D	Post-Doc Researcher; Coussens grant	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF, OHSU
2012 - present	Andrew Gunderson, Ph.D.	Post-Doc Researcher; Coussens grant	Research Supervisor	Post-Doctoral fellow, Coussens Lab, OHSU
2012 - present	Tina Bose, Ph.D.	Post-Doc Researcher; Coussens grant	Research Supervisor	Post-Doctoral fellow, Coussens Lab, OHSU
2012 - present	Terry Meddler, Ph.D.	Post-Doc Researcher; Coussens grant	Research Supervisor	Post-Doctoral fellow, Coussens Lab, OHSU
2012 - present	Aubie Shaw, Ph.D.	Post-Doc Researcher; Coussens grant	Research Supervisor	Post-Doctoral fellow, Coussens Lab, OHSU
2012 - present	Sushil Kumar, Ph.D.	Post-Doc Researcher; Coussens grant	Research Supervisor	Post-Doctoral fellow, Coussens Lab, OHSU

FACULTY MENTORING

Dates	Name	Position while Mentored	Mentoring Role	Current Position
2001 – 2004	Ernst Lengyel, M.D., Ph.D.	Asst. Adjunct Professor	Research Mentor	Assoc. Prof., Dept. Gyn. & Oncology, Univ. of Chicago, Chicago, IL
2002 – 2007	Darya Soto, M.D.	Asst. Adjunct Professor,	K08 Research Mentor	Private Practice, Burlingame, CA
2005 – 2007	Runi Chattopadhyay, M.D.	Clinical Instructor and Clinical Fellow	Basic Science Mentor, K12	Private Practice, San Francisco, CA
2006 – 2011	Limin Liu, Ph.D.	Assistant Professor	Member, Mentoring Committee	Dept. of Microbiology & Immunology, Sandler Center for Basic Research in Asthma, UCSF
2010 - 2011	Jaynata Debnath, M.D., Ph.D.,	Assistant Professor	Faculty Mentor	Associate Professor, Dept. of Pathology, UCSF

Sabbatical Visitors:

1999 - 2000 Yves DeClerck, M.D. Professor, Univ. of Southern Calif. & Children's Hospital of Los Angeles

SUMMARY OF TEACHING HOURS

Academic Year	Teaching/Mentoring Summary	Hours
1997/98	Total hours of teaching /mentoring:	27
	Formal class or course teaching hours:	2
	Informal teaching hours including prep time:	1
	Mentoring hours:	24
1998/99	Total hours of teaching /mentoring:	71
	Formal class or course teaching hours:	2
	Informal teaching hours including prep time:	1
	Mentoring hours:	68
1999/00	Total hours of teaching /mentoring:	108
	Formal class or course teaching hours:	4
	Informal teaching hours including prep time:	2
	Mentoring hours:	102
2000/01	Total hours of teaching /mentoring:	130
	Formal class or course teaching hours:	16
	Informal teaching hours including prep time:	9
	Mentoring hours:	105

2001/02	Total hours of teaching /mentoring:	201
	Formal class or course teaching hours:	18
	Informal teaching hours including prep time:	19
	Mentoring hours:	164
2002/03	Total hours of teaching /mentoring:	314.5
	Formal class or course teaching hours:	15.5
	Informal teaching hours including prep time:	17
	Mentoring hours:	282
2003/04	Total hours of teaching /mentoring:	402
	Formal class or course teaching hours:	20
	Informal teaching hours including prep time:	28
	Mentoring hours:	354
2004/05	Total hours of teaching /mentoring:	395
	Formal class or course teaching hours:	17
	Informal teaching hours including prep time:	28
	Mentoring hours:	350
2005/06	Total hours of teaching /mentoring:	395
	Formal class or course teaching hours:	17
	Informal teaching hours including prep time:	28
	Mentoring hours:	350
2006/2007	Total hours of teaching /mentoring:	473
	Formal class or course teaching hours:	45
	Informal teaching hours including prep time:	28
	Mentoring hours	400
2008/2009	Total hours of teaching /mentoring:	499
	Formal class or course teaching hours:	51
	Informal teaching hours including prep time:	48
	Mentoring hours	400
2009/2010	Total hours of teaching /mentoring:	499
	Formal class or course teaching hours:	51
	Informal teaching hours including prep time:	48
	Mentoring hours	400
2010/2011	Total hours of teaching /mentoring:	499
	Formal class or course teaching hours:	51
	Informal teaching hours including prep time:	48
	Mentoring hours	400
2011/2012	Total hours of teaching /mentoring:	460
	Formal class or course teaching hours:	20
	Informal teaching hours including prep time:	40
	Mentoring hours	400

X. RESEARCH AND CREATIVE ACTIVITIES

RESEARCH AWARDS AND GRANTS:

CURRENT

BCRF (PI: Rugo) 10/01/12-09/30/13
Source: Breast Cancer Research Foundation directs/yr
Title: Cellular mechanisms of resistance to antiangiogenic and the impact of immune therapy, and molecular characterization of circulating tumor cells (CTCs) in patients with advanced breast cancer
The major goal of this study is to 1) evaluate significance of immune cells in primary mammary carcinomas and lung metastases in the context of response and relapse/resistance to anti-antiangiogenic and/or immune modulating therapy, 2) Investigate the impact of inhibiting immune cell infiltration alone or in combination with antiangiogenic agents or chemotherapy on the growth of primary tumors and metastases, and 3) characterize the expression of a specific set of genes and gene copy number in circulating tumor cells (CTCs) in patients with ABC, including markers of response and resistance to specific types of therapy.
Role: subcontract PI (Aim 1 and 2)

1U54CA163123-01 (multiPI: Coussens, LM; Krummel, M) 09/23/11 – 07/31/16

Source: NIH/NCI directs/yr
Title: Leukocyte Biomarkers for Predicting Human Breast Cancer Outcomes
The major goal of this study is to identify myeloid- and lymphoid-based biomarkers representing either functional mediators of immune cell phenotype or instead reflecting leukocyte composition, and which of these in turn represent predictive variables for predicting breast cancer response to CTX +/- macrophage-depletion therapy
Role: multi P.I.

W81XWH-10-BCRP-EOHS-EXP (PI: Coussens, LM) 09/30/11 – 09/29/16
Era of Hope Scholar Expansion Award
Source: DOD/U.S. Army Medical Research & Materiel Command directs/yr
Title: *Modulating Immune Response to Improve Therapy for Breast Cancer*
The major goal of this study is to test the hypothesis that the immune microenvironment in breast cancer can be effectively manipulated therapeutically to limit breast cancer recurrence and extend overall survival
Role: P.I.

R01 CA155331 (PI: Coussens, LM) 05/01/11 – 03/31/16
Source: NIH/NCI directs/yr
Title: Regulating the Immune Microenvironment in Breast Cancer
The major goal of this study is to evaluate the efficacy of T_H2-blockade as a therapy for reprogramming pro-tumor immune cells in mouse models of mammary carcinogenesis.
Role: P.I

KG111084 (multuPI: Coussens, LM; Hwang S, Rugo H) 07/01/11 – 06/31/16
Source: Komen Foundation, PROMISE Grant directs/yr
Title: *Enhancing Efficacy of Chemotherapy in Triple Negative/Basal-like Breast Cancer by Targeting Macrophages.*
The major goal of this study is to test the hypothesis that that macrophages in triple negative (TN)/basal-like breast cancer potentiate late-stage disease progression and limit long-term survival, and that by either minimizing tumor associated macrophages (TAM) recruitment or reprogramming TAM bioactivity, outcomes for patients with TN/basal-like breast cancer will significantly improve..
Role: multi P.I.

KG110560 (multiPI: Hwang S; Coussens, LM) 07/1/11 – 06/31/14
Source: Komen Foundation, IDEA Award directs/yr
Title: *Immune and Collagen Basis of Breast Cancer Risk*
The major goal of this study is to establish whether immune and collagen status is correlated with density-associated breast cancer risk.
Role: multi P.I.

R01CA140943 (multiPI: Coussens, Boudreau, Daldrup-Link) 07/01/09 –06/30/13
Source: NIH/NCI directs/yr
Title: Improved Imaging and Drug Delivery Using Novel Approaches to Regulate Tissue Perfusion
The major goal of this project is to examine how short-term inhibition of ALK5 in vivo alters hemodynamics and tissue perfusion in mouse models of cancer.
Role: multi P.I.

RO1 CA130980 (PI: Coussens, LM) 07/01/08-06/30/13
Source: NIH/NCI directs/yr
Title: Regulation of Inflammation-Associated Epithelial Cancer Development
The goal of this study is to determine regulatory programs activating chronic inflammation during squamous carcinogenesis
Role: P.I.

RO1 CA132566 (multiPI: Coussens, LM; Jablons DM) 05/01/08-04/30/13
Source: NIH/NCI directs/yr
Title: Inflammation and Lung Carcinogenesis
The goal of this study is to determine how inflammation and Wnt signaling regulate stem cell niche

autonomy during lung carcinogenesis
Role: multi P.I.

1S10OD010348-01 (PI: Coussens, LM) 06/01/2012 – 05/31/2013
Source: NIH directs/yr
Title: Vevo 2100 Ultrasound System
The goal of this shared instrument grant is to purchase a Vevo 2100 Ultrasound System for imaging tumor development in mouse models of cancer for the Mouse Barrier Facility at UCSF.
Role: multi P.I.

PREVIOUS

USPHS 5 T32 CA09056 (PI: Fox, F, UCLA) 07/01/89 –06/30/92
Source: NIH/UCLA directs/yr1
Title: Regulation of *junB* Gene Expression by TGF-Beta directs/yr 1-3
Competitive Pre-Doctoral award to study transcription factor *junB.*

Univ. of Calif., Dissertation Year Fellowship (PI: Coussens, LM, UCLA) 10/1/92 – 09/31/93
Source: University of California, Office of the President directs/yr
Title: *Effects of E1A on TGF-Beta-inducible junB Expression*
Competitive Pre-Doctoral award to study transcription factor *junB.*

USPHS 5 T32 CA09043 (PI: Bishop, KM, UCSF) 10/01/93-06/31/96
Source: NIH/UCSF directs/yr
Title: *Molecular Analysis of Tumor Viruses* directs/yr 1-3
Post-Doctoral fellowship to study mouse model of epithelial carcinogenesis.

American Social Health Association/Pfizer Post-Doctoral Research Fellowship in Sexually Transmitted Diseases (PI: Coussens, LM, UCSF) 10/01/96 – 9/30/98
Source: Private Foundation directs/ yr 1
Title: *Metalloproteinases and Malignant Progression of Squamous Epithelium in K14-HPV16 Transgenic Mice* directs/yr 1-2
Role: Principal Investigator
Competitive Post-Doctoral fellowship to study proteases and tumor development

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P01 CA072006 (PI: Shuman M, UCSF) 06/10/97 – 06/30/03
Source: NIH/NCI directs/yr 1
Title: *Proteases in Cancer Biology and Drug Development* directs/yr 1-5
Project 3 – Proteases in Models of Tumor Initiation/Progression directs/yr 1
Role: Co-Investigator, Project 3 directs/yr 1-5
The major goal of this project is to study the role of proteases in cancer biology. directs/yr 1
Core C – Transgenic Animal Models directs/yr 1-5
Role: Director (year 4 and 5)
The major goal of this Core is to develop and provide protease null and transgenic mice to program projects.

UCSF IRG-97-150-01 (PI: Coussens LM, UCSF) 07/01/99-06/30/00
Source: American Cancer Society directs/yr 1
Title*: Proteases and Genomics in a Mouse Model of Epithelial Cancer* directs/yr 1
Role: Principal Investigator
Pilot project tested role of proteinases as effectors of genomic instability.

UCSF Cell Cycle and Dysregulation Program (PI: Coussens LM, UCSF) 02/01/00-01/31/01
Source: UCSF Comprehensive Cancer Center, Intramural directs/yr 1
Title*: Epithelial Neoplastic Progression and Degradation of Type I Collagen* directs/yr 1
Role: Principal Investigator
Pilot project assessed functional significance of type I collagen metabolism during epithelial carcinogenesis.

Research Evaluation & Allocation Committee (PI: Coussens LM, UCSF) 07/01/00-06/30/01

Source: UCSF Academic Senate directs/yr 1
Title: *Role of Gelatinase B in Maintenance of Genomic Instability* directs/yr 1
Role: Principal Investigator
Pilot project tested the role of MMP9 as an indirect regulator of genomic instability.

UCSF IRG AC-04-02 (PI: Coussens LM, UCSF) 10/01/00-09/30/01
Source: American Cancer Society directs/yr 1
Title: *Regulation of Intracellular Signaling Pathways by Gelatinase B/MMP-9* directs/yr 1
Role: Principal Investigator
Pilot project to study signal transduction pathways regulated by MMP-9.

The V Foundation for Cancer Research (PI: Coussens LM, UCSF) 06/02/00-05/31/02
Source: Private Foundation directs/yr 1
Title*: Gelatinase B and Epithelial Cancer Development* directs/yrs 1-2
Role: Principal Investigator
Pilot project to study role of MMP9 during epithelial carcinogenesis.

Gertrude B. Elion Cancer Research Award (PI: Coussens LM, UCSF) 07/1/01 – 06/30/02
Source: American Association of Cancer Research directs/yr 1
Title: *Functional Role of MMP-2 During Epithelial Carcinogenesis* directs/yr 1
Role: Principal Investigator
Pilot project to study role of MMP-2 during epithelial carcinogenesis.

Univ. of Calif., Cancer Research Coordinating Committee (PI: Coussens LM, UCSF) 07/01/01 – 06/30/02
Source: University of California directs/yr 1
Title: *Gelatinase A/MMP-2 and Epithelial Cancer Development* directs/ yr 1
Role: Principal Investigator
Pilot project to study role of MMP-2 as a potentiator of tumor development.

Hellman Family Award For Early Career Faculty (PI: Coussens LM, UCSF) 11/01/00-09/30/02
Source: UCSF Intramural directs/ yr 1
Title: *Paracrine Regulation of Epithelial Carcinogenesis by MMP-9* directs/yr 1-2
Role: Principal Investigator
Pilot project to identify matrix molecules regulated by MMP-9.

Edward Mallinckrodt, Jr. Foundation (PI: Coussens LM, UCSF) 10/01/00-09/30/03
Source: Private Foundation directs/yr 1
Title: *Regulation of epithelial cancer by gelatinase B/MMP-9* directs/yr 1-3
Role: Principal Investigator
Pilot project to determine how MMP-9 regulates proliferation, VEGF bioavailability and angiogenesis during epithelial carcinogenesis.

P50 CA58207 (PI: Gray, J: UCSF) 03/01/03-02/28/05
Source: NIH/NCI directs/yr 1
Bay Area Breast Cancer Translational Research Program (SPORE) directs/yr 1-2
Title: *Type I Collagen Remodeling and Mammary Carcinogenesis*
Role: Principal Investigator (Developmental Project)
The overall goal of this pilot project was to explore the role of collagen metabolism during mammary carcinogenesis.

DE-FG02-05ER6401 (PI: Franc, B**;** UCSF**)** 03/01/05 – 01/16/06
Source: DOE Medical Applications Grant directs yr 1
Title: Therapeutic Radionuclide Tumor-targeting Strategy for Breast Cancer total directs
Role: Co-Investigator
The specific aim of this project is to develop a radionuclide delivery molecule (RDM) that specifically targets cancer cells that express matrix-metalloproteinase-14 (MMP-14) on their surface and demonstrate delivery of radiolabeled RDM to MMP-14 expressing cells *in vitro* and *in vivo.*

R01 DK067678 (PI: Cher, M: Wayne State University) 07/01/03-06/30/06
Source: NIH/NIDDK directs/yr 1
Title: *Proteases in Prostate Cancer Bone Metastasis* directs/yr 1-4
Role: Subcontract Principal Investigator
The major goal of this subcontract is to assist with the planned experiments by providing mice (protease deficient) of defined genotype for proposed studies to analyze proteases during prostate metastasis to bone *in vivo*.

Opportunity Award, Sandler Family (PI: Coussens, LM; UCSF) 02/15/05 -02/14/07
Source: UCSF Intramural directs yr 1
Title: *B Lymphocytes as Targets for Cancer Prevention* total directs
Role: Principal Investigator
The major goal of this project is to investigate the efficacy of targeting B cells for chemoprevention

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DAMD17-02-1-0693 (PI: Sloane, B; Wayne State University) 08/01/02-07/31/06
Source: Department of Defense directs/yr 1-4
Breast Cancer Center of Excellence directs/yr 1
Title: *Validation of Proteases as Therapeutic Targets in Breast Cancer Functional Imaging of Protease Expression, Activity and Inhibition*
Role: Subcontract Principal Investigator directs/yr 1-4
The goal of this program is to validate proteases as therapeutic targets in breast cancer by functional imaging of protease expression, activity and inhibition.

R01 CA94168 (PI: Coussens, LM: UCSF) 04/01/02-06/31/07
Source: NIH/NCI directs/yr 1
Title: *Regulation of Epithelial Cancer by MMP-9/gelatinase B* directs/yr 1-5
Role: Principal Investigator
The goal of this project is to identify molecules that mediate proliferative and cellular pathways activated by MMP-9.

U54 RR020843 (PI: Smith, J; Burnham Institute) 09/30/04-07/31/06
Source: NIH/National Center for Research Resources directs/yr 1-5
Title: Center on Proteolytic Pathways
Role: Principal Investigator (Driving Biological Problem #1) directs/yr
DBP#1 *Proteolytic Pathways in Acute Vascular Response*

P01 CA72006 (PI: Werb, Z; UCSF) 07/07/03 – 06/30/08
Source: NIH/NCI directs/yr 6
Title: *Proteases in Cancer Biology and Drug Development* directs/yr 6-11
Project 3 - *Proteases in Models of Tumor Initiation/Progression* directs/yr 6
Role: Co-Investigator, Project 3 directs/yr 6-11
The major goal of this project is to study the role of proteases in cancer biology.
Core C - *Transgenic Animal Models* directs/yr 6
Role: Director directs/yr 6-11
The major goal of this Core is to develop and provide protease null and transgenic mice to program projects.

R01 CA98075 (PI: Coussens, LM; UCSF) 07/01/03-06/30/09
Source: NIH/NCI directs/yr 1
Title: *Microenvironmental Regulation of Tumor Progression* directs/yr 1-5
Role: Principal Investigator
The overall goal of this grant is to determine the role of collagen metabolism on epithelial carcinogenesis.

P50 CA58207 (van 't Veer; UCSF) 08/01/92–11/30/12
Source: NIH/NCI
Bay Area Breast Cancer SPORE
Career Development and Developmental Research Award Multi Project PI: Weaver, Hwang, Coussens **(5/1/10-04/30/11)**
Title: Risk to Malignancy and Immune and Collagen Status

The goals of this project are to 1) Determine whether immune infiltrate and collagen heterogeneity exist between and within multiple regions of breast tissue, 2) Evaluate whether malignant progression is associated with a distinct immune infiltrate and if that is reflected by physical state of collagen, and 3) Determine relationship between immune infiltrate, collagen, radiographic density and clinical measures of cancer risk.
Role: Multi P.I.

P50 CA58207 (van 't Veer; UCSF) 08/01/92–11/30/12
Source: NIH/NCI (project expenses only)
Title: Bay Area Breast Cancer SPORE
Career Development and Developmental Research Award, Multi Project PI: Boudreau N; Coussens LM (**5/1/10-04/30/11)**
Title: Macrophage-Mediated Delivery of the Breast Tumor Suppressor HoxD10 via Autologous Transfer to Breast Tumors. The aims of this project are to 1) establish function and optimize introduction of the engineered HoxD10 protein into macrophages and/or monocytes; 2) visualization of modified monocyte/macrophage accumulation in mammary tumors in vivo and 3) analysis of the impact of monocyte/macrophage delivered HoxD10 on breast tumor growth, progression and metastasis in MMTV-PyMT mouse model of mammary carcinogenesis.
Role: Multi P.I.

BC051640 Era of Hope Scholar Award (PI: Coussens, LM; UCSF) 06/01/06 – 05/31/11
Source: DoD, U.S. Army Medical Research and Materiel Command directs yr
Title: Microenvironment Regulation of Mammary Carcinogenesis
The goal of this Scholar Award is to identify leukocytes and their proteases that modify breast carcinogenesis and to develop noninvasive imaging reagents targeting leukocytes to image inflammation.
Role: P.I.

W81XWH-08-PRMRP-IIRA (multiPI: Broaddus, C; Coussens, LM) 07/01/09 –06/30/12
Source: DoD, U.S. Army Medical Research and Materiel Command directs/yr
Title: Role of Macrophage-induced Inflammation in Mesothelioma
The goals of this project are 1) to determine the functional significance of macrophage phenotype in mesothelioma, 2) to determine the functional significance of macrophages as regulators of mesothelioma apoptosis in vitro and 3) to define the functional significance of macrophage depletion or repolarization on mesothelioma survival in vivo.
Role: multi P.I.

XI. PEER-REVIEWED PUBLICATIONS

1. Francke U, de Martinville B, **Coussens L**, Ullrich A. (1983) The human gene for the Beta subunit of nerve growth factor is located on the proximal short arm of chromosome 1. *Science* 222:1248-1251. PMID: 6648531
2. Breakefield X, Castiglione C, **Coussens L**, Axelrod F, Ullrich A. (1984) Structural gene for Beta-nerve growth factor is not defective in familial dysautonomia. *Proc. Natl. Acad. Sci. USA* 81:4213-4216. PMID: 6330750
3. Ullrich A, **Coussens L**, Hayflick J, Dull T, Gray A, Tam A, Lee J, Yarden Y, Libermann T, Schlessinger J, Downward J, Bye J, Whittle N, Waterfield M, Seeburg P. (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309:418-425. PMID: 6328312
4. Ullrich A, Bell J, Chen E, Herrera R, Petruzzelli L, Dull T, Gray A, **Coussens L**, Liao Y-C, Tsubokawa M, Mason A, Seeburg P, Grunfield C, Rosen O, Ramachandran J. (1985) Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature* 313:756-761. PMID: 2983222
5. Lauffer L, Garcia P, Harkins R, **Coussens L**, Ullrich A, Walter P. (1985) Topology of the signal recognition particle receptor in the endoplasmic reticulum membrane. *Nature* 318:334-338. PMID: 2999608
6. Schechter A, Hung M-C, Vaidanathan L, Weinberg R, Yang-Feng T, Francke U, Ullrich A, **Coussens L.** (1985) The *neu* gene: An *erbB*-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. *Science* 229:976-978. PMID: 2992090

7. **Coussens L**, Yang-Feng T, Liao T-C, Chen E, Gray A, McGrath J, Seeburg P, Libermann T, Schlessinger J, Francke U, Levinson A, Ullrich A. (1985) Tyrosine kinase receptor with extensive homology to the EGF receptor shares chromosomal location with *neu* oncogene. *Science* 230:1132-1139. PMID: 2999974
8. **Coussens L**, Van Beveren C, Smith D, Chen E, Mitchell R, Isacke C, Verma I, Ullrich A. (1986) Structural alteration of viral homologue of receptor proto-oncogene *fms* at carboxyl terminus. *Nature* 320:277-280. PMID: 2421165
9. Parker P, **Coussens L**, Totty N, Rhee L, Young S, Chen E, Stabel S, Waterfield M, Ullrich A. (1986) The complete primary structure of protein kinase C—the major phorbol ester receptor. *Science* 233:853-859. PMID: 3755547
10. **Coussens L**, Parker P, Rhee L, Yang-Feng T, Chen E, Waterfield M, Francke U, Ullrich A. (1986) Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. *Science* 233:859-866. PMID: 3755548
11. **Coussens L**, Rhee L, Parker P, Ullrich A. (1987) Alternative splicing increases the diversity of the human protein kinase C family. *DNA* 6:389-394. PMID: 3677994
12. Yarden Y, Kuang W-J, Yang-Feng T, **Coussens L**, Munemitsu S, Dull T, Schlessinger J, Francke U, Ullrich A. (1987) Human proto-oncogene *c-kit*: A new cell surface receptor-tyrosine kinase for an unidentified ligand. *EMBO J*. 6:3341-3351. PMID: 2448137
13. MacDonald R, Pfeffer S, **Coussens L**, Tepper M, Brocklebank C, Mole J, Anderson J, Chen E, Czech M, Ullrich A. (1988) A single receptor binds both insulin-like growth factor II and mannose-6-phosphate. *Science* 239:1134-1137. PMID: 2964083
14. Formby B, Ullrich A, **Coussens L**, Walker L, Peterson C. (1988) Growth hormone stimulates insulin gene expression in cultured human fetal pancreatic islets. *J. Clin. Endo. Metab.* 66:1075-1079. PMID: 3283160
15. Mosthaf L, Grako D, Dull T, **Coussens L**, Ullrich A, McClain D. (1990) Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *EMBO J.* 9:2409-2413. PMID: 2369896
16. **Coussens LM**, Yokoyama K, Chiu R. (1994) Transforming Growth Factor b_1-mediated induction of *junB* is selectively inhibited by expression of Ad.12-E1A. *J. Cell. Physio.* 160:435-444. PMID: 8077281
17. **Coussens LM**, Hanahan D, Arbeit J. (1996) Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. *Am. J. Pathol.* 149:1899-1917. PMCID: PMC1865365
18. **Coussens LM**, Werb Z. (1996) Matrix metalloproteinases and the development of cancer. *Chemistry and Biology* 3:895-904. PMCID: PMC8939708
19. Bergers G, Hanahan D, **Coussens LM.** (1998) Angiogenesis and apoptosis are cellular parameters regulating neoplastic progression in transgenic mouse models of tumorigenesis. *Int. J. Dev. Biol. 42*:995-1002. PMID: 9853830
20. **Coussens LM,** Raymond WW, Bergers G, Laig-Webster M, Behrendtsen O, Werb Z, Caughey, GH, Hanahan D. (1999) Inflammatory mast cells upregulate angiogenesis during squamous epithelial carcinogenesis. *Genes & Development* 13:1382-1397. PMCID: PMC316772
21. McKerrow JM, Bhargava V, Hansell E, Kuwahara T, Matley M, **Coussens LM,** Warren R. (2000) A functional proteomics screen of proteases in colorectal carcinomas. *Molecular Medicine* 6:450-460. PMID: 10952024
22. Bergers G, **Coussens LM.** (2000) Extrinsic regulators of epithelial tumor progression: metalloproteinases. *Curr. Opin. Genetics & Development* 10:120-127. PMID: 10679388
23. **Coussens LM,** Tinkle CL, Hanahan DH, Werb Z. (2000) MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 103:481-490. PMCID: PMC2843102
24. van Kempen LCL, Rhee JS, Dehne K, Lee J, Edwards DR, **Coussens LM**. (2002) Epithelial carcinogenesis: Dynamic interplay between neoplastic cells and their microenvironment. *Differentiation* 70: 610-623. PMID: 12492502
25. **Coussens LM**, Fingleton B, Matrisian LM. (2002) Matrix metalloproteinases and cancer: Trials and tribulations. *Science* 295:2387-2392. PMID: 11923519
26. **Coussens LM**, Werb Z. (2002) Inflammation and cancer. *Nature* 420: 860-867. PMCID: PMC2803035
27. Daniel D, Meyer-Morse N, Bergsland EK, Dehne K, **Coussens LM,** Hanahan D. (2003) Immune enhancement of skin carcinogenesis by $CD4^+$ T cells. *J Exp. Med*, 197:1017-1028. PMCID: PMC2193878
28. van Kempan LCL, Ruitter DJ, van Muijen GNP, **Coussens LM.** (2003) The tumor microenvironment: a

critical determinant of neoplastic evolution. *Euro J Cell Biol,* 82:539-548. PMID: 14703010
29. Rhee JS, Diaz R, Korets L, Hogson G, **Coussens LM**. (2004) TIMP-1 alters susceptibility to carcinogenesis. *Cancer Research* 64:952-961. PMID: 14871825
30. Chantrain CF, Shimada H, Groshen S, Ye W, Shalinsky DR, Werb Z, **Coussens LM**, DeClerck YA. (2004) Stromal matrix metalloproteinase-9 (MMP-9) regulates the vascular architecture in neuroblastoma by promoting pericyte recruitment. *Cancer Research* 64:1675-1686. PMID: 14996727
31. Baluk P, Raymond WW, Ator E, **Coussens LM**, McDonald DM, Caughey GH. (2004) Matrix metalloproteinase-2 and -9 expression increases in mycoplasma-infected airways but is not required for vascular remodeling. *Am J Physiol Lung Cell Mol Physiol*, 287:307-317. PMID: 15075248
32. de Visser KE, Korets LV, **Coussens LM**. (2004) Early neoplastic progression is complement-independent. *Neoplasia* 6: 768-776. PMCID: PMC1531681
33. Jodele S, Chantrain CF, Blavier L, Crooks GM, Shimada H, **Coussens LM**, DeClerck YA. (2005) The contribution of bone marrow-derived cells to the tumor vasculature in neuroblastoma is matrix metalloproteinase-9-dependent. *Cancer Research,* 65: 3200-3208. PMID: 15833851
34. de Visser KE, Korets LV, **Coussens LM**. (2005) De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell* 7:411-423. PMID: 15889249

Featured in:

- *Faculty of 1000 – Exceptional (Factor 6.0).* Authoritative evaluation by R. Abraham, Wyeth Research. http://f1000biology.com/guardpages/evaluation/1015124//
- *Nature (2005*), 435:752-753. 'Inflammation by Remote Control'. A. Mantovani
- *Cancer Cell (*2005), 7: 403-405. 'The role of the immune system in early carcinogenesis: B-ware the double-edged sword'. A. Houghton, H. Uchi, and J.D. Wolchok.
- *Nature Reviews Immunology (2005)*, 5:517. 'B cells lead the way in tumour progression'. K. Minton
- *Nature Reviews Cancer (2005)*, 5:501. 'B cells lead the way in tumour progression'. K. Minton

35. de Visser KE, **Coussens LM**. (2005) The interplay between innate and adaptive immunity regulates cancer development. *Cancer Immunology & Immunotherapy*, 54:1143-1152. PMID: 15889249
36. de Visser KE, Eichten A, **Coussens LM**. (2006) Paradoxical roles of the immune system during cancer development. *Nature Reviews Cancer*, 6:24-37. PMID: 16397525
37. Tlsty TD, **Coussens LM.** (2006) Tumor stroma and regulation of cancer development. *Ann Rev Pathol. Mech of Disease*, 1: 119-150. PMID: 18039110
38. van Kempen LCL, de Visser KE, **Coussens LM**. (2006) Inflammation, Proteases and Cancer. *Euro J Cancer,* 42: 728-734. PMID: 16524717
39. Yuan F, Verhelst HL, Blum G, **Coussens LM**, Bogyo M. (2006) A selective activity-based probe for the papain family cysteine protease dipeptidyl peptidase I/Cathepsin C. *J Am Chem Society*, 128: 5616-5617. PMID: 16637611
40. Junankar SR, Eichten A, Kramer A, de Visser KE **Coussens LM**. (2006) Analysis of immune cell infiltrates during squamous carcinoma development. *J Invest Dermatol*, 126:36-4. PMID: 17069009
41. Tan TT, **Coussens LM**. Humoral immunity, inflammation and cancer. (2007) *Curr Opin Immunology* 19(2), 209-216. PMID: 17276050
42. Johansson M, Tan T, de Visser KE, **Coussens LM**. (2007) Immune cells as anti-cancer therapeutic targets and tools. *J Cellular Biochemistry*, 101: 918-926. PMID: 17265430
43. Schwartz DR, Moin K, Yao B, Matrisian LM, **Coussens LM**, Bugge TH, Fingleton B, Acuff KB, Sinnamon M, Nassar H, Krawetz SA, Linebaug BE, Sloane, BF. (2007) Hu/Mu ProtIn oligonucleotide microarray: dual species array for profiling protease and protease inhibitor gene expression in tumors and their microenvironment. *Mol Cancer Res,* 5:443-454. PMID: 17510311
44. Egeblad M, Shen HCJ, Behonick DJ, Wilmes L, Eichten A, Korets L, Kheradmand F, Werb Z, **Coussens LM.** (2007). Type I collagen is a modifier of matrix metalloproteinase 2 function in skeletal development. *Dev Dynamics,* 36:1683-1693. PMCID: PMC2581616
45. Eichten AE, Hyun WC, **Coussens LM**. (2007) Distinctive features of angiogenesis and lymphangiogenesis determine their functionality during de novo tumor development. *Cancer Research,* 67:5211-5220. PMID: 17545601
46. DeNardo D **Coussens LM**. (2007) Balancing Immune Response: Crosstalk Between Adaptive and Innate Immune Cells During Breast Cancer Progression. *Breast Cancer Res,* 9:212-222. PMCID:

PMC2206719
47. Kopitz C, Gerg M, Bandapalli O, Ister D, Pennington CJ, Hauser S, Flechsig C, Krell HW, Antolovic D, Brew K, Nagase H, Stangl M, Hann von Weyhern CW, Brucher BLDM, Brand K, **Coussens LM**, Edwards DR, Kruger A. (2007) TIMP-1 promotes liver metastasis by induction of HGF-signaling. *Cancer Research,* 67:8615-8623. PMID: 17875701
48. DeNardo DG, Johansson M, **Coussens LM**. (2008) Immune cells as mediators of solid tumor metastasis. *Cancer Metastasis Rev*. 27:11-18. PMCID: PMC2865635
49. Johansson M, DeNardo DG, **Coussens LM**. (2008) Polarized immune responses differentially regulate cancer development. *Immunol Rev*., 222:145-154. PMCID: PMC2494984
50. Kenny H, Kaur S, **Coussens LM**, Lengyel E. (2008) Adhesion of OvCa cells to peritoneum is mediated by MMP-2 cleavage of fibronectin*, J Clin Invest.* 118(4)1367-1379. PMCID: PMC2267016
51. Wolf K, Alexander S, Schacht V, **Coussens LM**, vom Andrian U, van Rheenan J, Deryugina E, Friedl P. (2009) Collagen-based cell migration models in vitro and in vivo. *Seminars Cell Dev Biol.,* 20(8): 931-941. PMID: 19682592
52. Watkins GA, Jones EF, Shell MS, VanBrocklin HV, Pan MH, Hanrahan SM, Feng JJ, He J, Sounni NE, Dill KA, Contag CH, **Coussens LM**, Franc BL. (2009) Development of an optimized activatable MMP-14 targeted SPECT imaging probe. *Bioorganic & Medicinal Chemistry*, 17:653-659. PMCID: PMC2639212
53. DeNardo DG, Baretto JB, Andreu P, Vasquez L, Kolhatkar N, **Coussens LM**. (2009) CD4+ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell*, 16:91-102. PMCID: PMC2778576

Featured in:

- *Faculty of 1000 – Exceptional (Factor 9.0).* Authoritative evaluation by A. Mantovani, Univ. of Milan. http://f1000biology.com/guardpages/evaluation/1163484//
- *Cancer Cell* (2009), 16:81-82. 'Metastasis-Promoting Immunity: When T cells turn to the dark side' by D. Pardoll
- *Nature Reviews Cancer* (2009) 9:609. 'Influencing bad behaviour' *by* N. McArthy
- *Nature Biotechnology* (2009) 27:828. 'Promoting metastasis' by L. DeFrancesco
- *Nature Medicine* (2009) 15:1135. 'Not Immune' by C. Schubert
- *Ranked as a "Top Cancer Papers" between 2008-2011, Nature Medicine, 17:262-265; Nature Medicine 17: 278-279; Nature Medicine, 17:295*

54. Sista AK, Knebel RJ, Tavri SA, Johansson M, DeNardo DG, Boddington SE, Kishore SA, Ansari C, Reinhart V, Coakley FV, **Coussens LM**, Daldrup-Link HE. (2009) Optical Imaging of the peri-tumoral inflammatory response in breast cancer. *J Transl Med*, 7(1): 94-100. PMCID: 2780997
55. Andreu P, Johansson M, Affara NI, Tan TT, Junankar S, Korets L, Lam J, Tawfik D, Pucci F, De Palma M, DeNardo D, de Visser KE, **Coussens LM**. (2010) FcRγ activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell,* 17(2):121-134. PMCID: PMC3082507

Featured in:

- *Cancer Cell* (2010), 17(2) 111-112. '*La mala educacion of tumor-associated macrophages: Diverse pathways and new players',* by A. Mantovani
- *Nature Reviews Immunology* (2010) 10:158 'Antibodies lend support to tumors', by, L. Byrd
- *Faculty of 1000 – Exceptional (Factor 6.4).* Authoritative evaluations by X. Ma (Weill Medical College of Cornell) and F. Kiefer (Max-Planck-Institute, Germany). http://f1000biology.com/article/id/2467956
- *Ranked as a "Top Cancer Papers" between 2008-2011, Nature Medicine, 17:262-265; Nature Medicine 17: 278-279;*
- *Nature Medicine* (2011), 17(3) 285-286. 'B Cells and Macrophages in Cancer: Yin and Yang', by A. Mantovani

56. Dennemarker J, Lohmuller T, Mayerie J, Tacke M, Lerch MM, **Coussens LM**, Peters C, Reinheckel. (2010) Deficiency for cysteine protease cathepsin L promotes tumor progression in mouse epidermis mice. Oncogene, 29(11):1611-1621. PMCID: PMC3082505
57. Sounni NE, Dehne K, vanKempen LCL, Egeblad M, Affara NI, Cuevas I, Wiesen J, Junankar S, Korets L, Lee J, Shen J, Morrison C, Overall CM, Krane SM, Werb Z, Boudreau N, **Coussens LM**. (2010) Stromal regulation of vessel stability by MMP14 and TGFβ. *Disease Model. Mech.* 3:317-332. PMCID: 2860851
58. Okamoto J, Hirata T, Chen Z, Zhou HM, Mikami I, Li H, Beltran A, Johansson M, **Coussens LM**,

Clement G, Shi Y, Zhang F, Koizumi K, Shimizu K, Jablons DM, He B. (2010) EMX2 is epigenetically silenced and suppresses growth in human lung cancer. *Oncogene,* 29(44):5969-5975. PMCID: PMC3090446

59. Doedens A, Stockman C, Rubenstein M, Liao D, DeNardo D, **Coussens LM**, Karin M, Goldrath A, Johnson R. (2010) Macrophage expression of HIF1α suppresses T cell function and promotes tumor progression. *Cancer Res,* 70(90): 7465-7475. PMCID: PMC2948598
60. Ursini-Siegel J, Cory S, Zuo D, Hardy WR, Rexhepaj E, Lam S, Schade B, Jirstrom K, Bjur E, Piccirillo CA, DeNardo D, **Coussens LM**, Brennan DJ, Gallagher WM, Park M, Pawson T, Hallett M, Muller WJ (2010) Receptor tyrosine kinase signaling in breast cancer cells inhibits the adaptive immune response to favor a pro-tumorigenic state. *Cancer Res,* 70(20): 7776-7787. PMID: 20924104
61. Ruffell B, DeNardo DG, Affara NI, **Coussens LM**. (2010) Lymphocytes in cancer development: Polarization towards pro-tumor immunity. *Cytokine Growth Factor Reviews, 21:3-10.* PMCID: 2834837
62. DeNardo DG, Andreu P, **Coussens LM**. (2010) Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev, 29(2):309-316*, PMCID: 2865635
63. Shiao SE, **Coussens LM.** (2010) The tumor-immune microenvironment and response to radiation therapy. *J Mammary Gland Biol Neoplasia*, 15:411-421. PMCID: PMC3011087
64. Masset A, Maillard C, Sounni NE, Jacobs N, Bruyere F, Delvenne P, Tacke M, Reinheckel T, Foidart JM, **Coussens LM**, Noel A. (2011) Unimpeded skin carcinogenesis in K14-HPV16 transgenic mice deficient for plasminogen activator inhibitor. *Int J Cancer*, 128:283-293. PMCID: PMC3084626
65. Erez N, **Coussens LM**. (2011) Leukocytes as paracrine regulators of metastasis and determinants of organ-specific colonization. *Int. J Cancer.* 128:2536-2544. PMCID: PMC3084629
66. **Coussens LM**, Pollard JW. (2011) Leukocytes in mammary development and cancer. Cold Spring Harbor Perspectives in Biology, 3(3) PMID: 21123394
67. DeNardo DG, Brennan DJ, Rexhapaj E, Ruffell B, Shiao SL, Madden SF, Gallagher WM, Wadhwani N, Keil SD, Junaid SA, Rugo HS, Hwang ES, Jirstrom K, West BL, **Coussens LM**. (2011) Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discovery,* 1(1): 54-67. PMCID: PMC3203524

Featured in:

- *Nature* (2011), 472 303-304. '*Macrophages Limit Chemotherapy"* by, M. De Palma and C.E. Lewis
- Nature Reviews Cancer (2011) 11:3056. '*Bad company*', by N. McCarthy

68. Benavides F, Perez C, Blando J, Contreras O, Shen J, **Coussens LM**, Fischer SM, Kusewitt DF, Digiovanni J, Conti CJ (2011) Protective role of cathepsin L in mouse skin carcinogenesis. *Mol Carcinog*. May 2, doi: 10.1002/mc.20782 [Epub ahead of print] PMCID: PMC3155742
69. Barbone D, Ryan J, Kolhatkar N, Chacko A, Jablons DM, Sugarbaker DJ, Bueno R, Latei AG, **Coussens LM**, Fennel DA, Broaddus VC. (2011) The Bcl-2 repertoire of mesothelioma spheroids underlies acquired apoptotic multicellular resistance. *Cell Death & Disease,* Jun 23;2:e174. doi: 10.1038/cddis.2011.58. PMCID: PMC3169000
70. Schioppa T, Moore R, Thompson R, Rosser E, Kulbe H, Nedospasov S, Mauri C, **Coussens LM**, Balkwill F. (2011) B regulatory cells and the tumor-promoting actions of TNF-(alpha) during squamous carcinogenesis*, Proc. Natl Acad. Sci*. USA, 108(26):10662-10667. PMCID: PMC3127875
71. Daldrup-Link H, Golovko D, DeNardo DG, Ruffel D, Castenada R, Wendland M, Corot C, **Coussens LM**. (2011) MR imaging of tumor-associated macrophages with clinically-applicable iron oxide nanoparticles. *Clin Can Res*. 17(17): 5695-5704. PMCID: PMC3166957
72. Ruffell B, Au A, Rugo HS, Esserman LJ, Hwang ES, **Coussens LM**. (2011) Leukocyte complexity in human breast cancer. *Proc. Natl Acad. Sci.,109(8):2796-2801.* PMCID: PMC3287000
73. Shiao S, Ganesan AP, Rugo HS, **Coussens LM**. (2011) Immune microenvironments in solid tumors: New targets for therapy. *Genes and Development, 25(24): 2559-2572*. PMCID: PMC3248678
74. Ruffell B, Affara NI, **Coussens LM**. (2012) Differential macrophage programming in the tumor microenvironment. *Trends Immunol*. *33*, 119-125. PMCID: PMC3294003
75. Hanahan D, **Coussens LM**. (2012) Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment, *Cancer Cell*, 21: 309-322. PMID: 22439926

Manuscripts Submitted or In Revision:

- Affara NI, Ruffell B, Johansson M, Fujikawa K, DeNardo DG, Chen HI, Wadhani N, Steinhoff M, Truitt M,

Olson P, Hanahan D, Li Y, Gong Q, Wiesen JF, Kim G, Tempero M, Balkwill F, Irving B, Coussens LM. CD20 as a target for therapy in solid tumors. *In revision (Nature)*

XII. PATENTS

1. U.S. Patent Application Serial No. 10/567,873
   Title: *Novel Indications for Transforming Growth Factor-Beta Regulators*.
   Inventors: **Lisa M. Coussens** and Zena Werb
   Application published on August 28, 2008 as U.S. Patent Publication #2008-0206219-A1
   International filing date: August 9, 2004. Application Filing Number: 60/493,643; Docket Number: 23540-09361/PCT; International Publication Number WO 2005/013915 A2; International Application Number: PCT/US2004/025902
2. DeNardo D, Brennan D, **Coussens LM**. *Phenotyping Tumor-Infiltrating Leukocytes* (PCT/US2010/042654). US patent application: 13/314,072, EP application: 10802804.4, AU application: 2010276324. PCT application pending: PCT/US2011/063812.

XIII. NON-PEER REVIEWED PUBLICATIONS AND EDITORIALS:

Review Articles, Symposium Proceedings and Editorials

1. Ullrich A, Gray A, Berman C, **Coussens LM,** Dull T. (1983) Sequence homology of human and mouse Beta-nerve growth factor subunit genes. *CSHSQ* 48:435-442. PMID: 6327169
2. Ullrich A, Riedel H, Yarden Y, **Coussens LM**, Gray A, Dull T, Schlessinger J, Waterfield MD, Parker PJ. (1986) Protein kinases in cellular signal transduction: Tyrosine kinase growth factor receptors and protein kinase C. *CSHSQ* 51:713-724. PMID: 3472757
3. Chen E, **Coussens LM**, Liao Y-C, Smith D, Yang-Feng T, McGrath J, Van Beveren C, Verma IM, Libermann TA, Schlessinger J, Francke U, Levinson A, Ullrich A. (1986) Structural features of growth factor receptors with oncogenic potential. *Biochem. Soc. Symp*. 52:65-82. PMID: 3579970
4. **Coussens LM,** Werb Z. (2001) Inflammatory cells and cancer: Think Different! *J. Exp. Med.* 193:F23-F26. PMCID: PMC2193419
5. Rhee JS, **Coussens LM**. (2002) RECKing MMP functions: Implications for cancer development. *Trends in Cell Biology*, *12:* 209-211. PMID: 12062160
6. van Kempen LCL, **Coussens LM.** (2002) MMP9 potentiates pulmonary metastasis formation. *Cancer Cell*, 2: 251-252. PMID: 12398887
7. DeClerck YA, Mercurio AM, Stack MS, Chapman HA, Zutter MM, Muschel RJ, Raz A, Matrisian L, Sloane BF, Noel A, Hendrix MJ, **Coussens LM**, Padarathsingh M. (2004) Proteases, Extracellular Matrix and Cancer: A workshop of the PathB study section. *Am J Pathol,* 164:1131-1139. PMID: 15039201
8. Balkwill F, **Coussens LM**. (2004) Inflammation and cancer – the missing link? *Nature* 431:405-406. PMID: 15385993
9. Robinson S, **Coussens LM**. (2005) Soluble mediators of inflammation during tumor development. *Adv Cancer Research* 93:159-187. PMID: 15797447
10. Fingleton B, **Coussens LM**. (2005) Host-tumor interactions influencing cancer progression. *Drug Discovery Today: Disease Mechanisms*, 2:199-204
11. De Clerck YA, Weissman BE, Yu D, Parsons R, Bar-Eli M, Roy-Burman P, Seewaldt VL, Cress AE, Languino LR, Batra SK, Tang CK, Sheng S, Chen WT, Chellappan S, Cheng SY, Ladisch S, McCarthy JB, **Coussens LM**, Cohen MB. (2006) Tumor Progression and Metastasis from Genetic to Microenvironmental Determinants: A Workshop of the Tumor Progression and Metastasis NIH Study Section in Honor of Dr. Martin L. Padarathsingh, May 31, 2006, *Cancer Biol Ther*. 5:1588-1599. PMID: 17224636
12. **Coussens LM.** Inflammation, Proteolysis and Cancer Development. *In:* Cancer Microenvironments. Extended Abstracts for the 37th International Symposium of the Princess Takamatsu Cancer Research Fund, Tokyo, 2006. (H Esumi, HL Moses, S Hirohashi, K Miyazono, ED). Princess Takamatsu Research Fund. Tokyo Japan, pp. 33-35.
13. Affara NI, **Coussens LM**. (2007) IKKα at the crossroads of inflammation and metastasis. *Cell,* 129: 25-26. PMID: 17418780
14. **Coussens LM**, Jacks T. (2008) Genetic and cellular mechanisms of oncogenesis. Editorial Overview. *Curr Opin Genet Dev*, 18:1-2. PMID: 18440220

15. DeNardo DG, Johansson M, **Coussens LM**. (2008) Inflaming gastrointestinal oncogenic programming. *Cancer Cell*, 14: 7-9. PMID: 18598939
16. Yahui-Beltran A, **Coussens LM**, Jablons DM. (2009) Respiratory homeostasis and exploitation of the immune system for lung cancer vaccines. *US Oncology*. 5(1):40-47. PMID: 22368692
17. Demaria S, Pikarsky E, Karin M, **Coussens LM**, Chen YC, El-Omar EM, Trinchieri G, Dubinett SM, Mao JT, Szabo E, Krieg A, Weiner GJ, Fox BA, Coukos G, Wang E, Abraham RT, Carbone M, Lotze MT. (2010) Cancer and inflammation: promise for biologic therapy. *J Immunother*. 33(4):335-351. PMCID: PMC2941912
18. Ruffell B, **Coussens LM** (2011) Histamine restricts cancer: Nothing to sneeze at. *Nat Med* 17, 43-44. PMCID: 2494984
19. Swartz MA, Iida N, Roberts EW, Sangaletti S, Wong MH, Yull FE, **Coussens LM**, DeClerck YA. (2012) Conference Report: Tumor microenvironment complexity: Emerging roles in cancer therapy. *Cancer Research,* 72(10):2473-2480. PMID: 22414581
20. Daldrup-Link H. & **Coussens LM**. (2012) MR imaging of tumor-associated macrophages. *OncoImmunology* **1**(4), 507-509.

Book Chapters

1. Breakefield XO, Orloff G, Castiglione CM, Axelrod FB, **Coussens LM**, Ullrich A. (1983) Genetic linkage analysis in familial dysautonomia using a DNA probe for Beta-nerve growth factor. In: Biochemical and Clinical Aspects of Neuropeptides: Synthesis Processing and Gene Structure (Koch, G., Richter, D., Eds.) Academic Press, San Francisco, pp. 113-128.
2. Breakefield XO, Castiglione CM, **Coussens LM**, Axelrod FB, Ullrich A. (1984) Linkage analysis in familial dysautonomia using polymorphisms in DNA sequence: A beginning. In: Cellular and Molecular Biology of Neuronal Development (Black, I.B., Ed.) Plenum Press, New York, pp. 309-328.
3. Ullrich A, **Coussens LM**, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Schlessinger J, Downward J, Whittle N, Waterfield MD. Seeburg PH (1985) Structure of human epidermal growth factor receptor and expression of normal and variant mRNAs in epidermoid carcinoma cells. In: Cancer Cells 3: Growth Factors and Transformation, Cold Spring Harbor Laboratory, New York, pp. 1-9.
4. Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, Gray A, **Coussens LM**, Liao Y-C, Tsubokawa M, Grunfield C, Rosen OM, Ramachandran J. (1986) Structure of the human insulin receptor precursor. In: Mechanisms of Insulin Action (Belfrage, P., Donner, J., and Stralfors, P., Eds) Elsevier Science Publishers (Biomedical Division), New York, pp. 1-11.
5. Riedel H, Yarden Y, **Coussens LM**, Lee J, Dull TJ, Gray A, Schlessinger J, Ullrich A. (1986) Structural and functional analysis of cell surface receptors with tyrosine kinase activity. In: Cell Cycle and Oncogenes (Tanner, W., and Gallwitz, D., Eds.) Springer Verlag, Heidelberg, pp. 157-171.
6. Werb Z, Vu TH, Rinkenberger JL, **Coussens LM.** (1999) Matrix-degrading proteases and angiogenesis during development and tumor formation. *In:* Proteases and Protease Inhibitors in Cancer. (L. Matrisian, N. Brunner, and K. Dano, Eds). APMIS 107:11-18. PMID: 10190275
7. Sternlicht MD, **Coussens LM**, Vu T, Werb Z. (2000) Biology and regulation of the matrix metalloproteinases. *In:* Matrix Metalloproteinase Inhibitors in Cancer Therapy. (NJ Clendeninn and K Apperlt, Eds). Humana Press, Totowa NJ, USA, pp. 1-38.
8. **Coussens LM**, Shapiro SD, Soloway PD, Werb Z. (2001) Models for gain-of-function and loss-of-function of MMPs: Transgenic and gene targeted mice. *In:* Methods in Molecular Biology: Matrix Metalloproteinases. (I. Clark, Ed.). Humana Press, Totowa, NJ, USA. pp. 149-179. PMID: 11217297
9. de Visser KE, **Coussens LM.** (2004) Inflammation and matrix metalloproteinases: Implications for cancer development. *In*: Cancer and Inflammation. (Morgan D, Forssman U, and Nakada M, Eds) Birkhauser Publishing, Basel, Switzerland. pp. 71-97.
10. Diaz RJ, Eichten AE, de Visser KE, **Coussens LM**. (2005) Matrix metalloproteinases: Mediators of Tumor-Host Cell Interactions. *In:* Cancer Growth and Progression, Vol. 15. 2nd Edition (Hans E. Kaiser, Series Ed), Integration/Interaction of Oncologic Growth, (Gary Meadows, Ed), Springer, The Netherlands. pp. 81-126.
11. Eichten A, Shen HCJ, **Coussens LM**. (2005) Three-dimensional visualization of blood and lymphatic vasculature in tissue whole mounts using confocal microscopy. *In:* Current Protocols in Cytometry, Unit 12.5, John Wiley & Sons, Inc., Canada. pp 12.5.1-12.5.11. PMID: 18770816

12. de Visser KE, **Coussens LM**. (2006) The inflammatory tumor microenvironment and its impact on cancer development. *In:* Infection and Inflammation: Impacts on Oncogenesis (T Dittmar, KS Zaenker, A Schmidt, Ed) Karger Pub. Basel Switzerland, vol 13, pp 118-137 PMID: 16627962
13. Eichten AE, de Visser KE, and **Coussens LM**. (2008) Macrophages in tumor development and metastasis. *In:* Cancer Growth and Progression, 2nd Edition (Hans E. Kaiser and Aejaz Nasir, Ed), Selected Aspects of Cancer Progression: Metastasis, Apoptosis and Immune Response. (Ronald H. Goldfarb, Ed), Springer, The Netherlands. *Vol 11, pp115-137.*
14. De Palma M, **Coussens LM.** (2008) Immune cells and inflammatory mediators as regulators of tumor-associated angiogenesis. *In:* Angiogenesis: an Integrative Approach from Science to Medicine (W.D. Figg, J Folkman, Ed.) Springer Science, New York, NY USA, *pp 225-238.*
15. Affara NI, **Coussens LM.** (2008) Proteolytic pathways: Intersecting cascades in cancer development. *In:* The Cancer Degradome-Proteases and Cancer Biology, 1st Edition (Dylan Edwards, Gunilla Hoyer-Hansen, Francesco Blasi, Bonnie F. Sloane, Eds), Springer Science and Business Media, *pp 157-182.*
16. Affara NI, Andreu P, **Coussens LM.** (2009) Delineating protease function during cancer development. *In:* Methods in Molecular Biology, Proteases and Cancer. (Thomas Bugge and Toni Antalis, Eds.) Humana Press, *539:1-32*. PMID: 19377975
17. de Visser KE, **Coussens LM**. (2011) Utilizing mouse models of human cancer for assessing immune modulation of cancer development. *In:* Mouse Models for Cancer Research. (Jeffrey Green and Thomas Ried, Eds.). Springer Science, *Chapter 21, 443-463.*

Published Abstracts:

1. Laig-Webster M., **Coussens LM,** Caughey GH. (1998) A general method for genotyping mast cell-deficient KIT^{W}/KIT^{Wv} mice by allele-specific polymerase chain reaction. *FASEB J*, 12:A895
2. McDonald DM, Thurston G, Murphy TJ, **Coussens L**, Baluk P. (1998) Wild-type FVB/N mice are unusually susceptible to *Mycoplasma pulmonis* respiratory tract infection. *FASEB J* 12(5) 4559 Part 2.
3. Baluk P, **Coussens LM**, Werb Z, Raymond WW, Caughey GH, McDonald DM. (1999) Gelatinase B is not essential for microvascular remodeling in airway inflammation. *Am. J. Resp. Crit. Care Med.* 159:A353
4. van Kempen L, Rhee JS, Reuter J, Krane S, **Coussens LM**. (2001) To digest or not digest: Collagen Type I remodeling during squamous carcinogenesis. *Mol. Biol. Cell.* 12:269a.
5. van Kempen L, Rhee JS, Reuter J, Krane S, **Coussens LM**. (2002) To digest or not digest: Collagen Type I remodeling during squamous carcinogenesis. *Proceedings American Association for Cancer Research*, 43:535.
6. Chantrain C, Shimada H, **Coussens L**, Shalinsky D, Brekken J, De Clerck YA. (2002) Role of matrix metalloproteinase-2 and -9 in neuroblastoma angiogenesis and metastasis. *Proceedings American Association for Cancer Research*, 43:539.
7. Machamer JE, van Kempen LCL, **Coussens LM**. (2002) Endothelial cell migration through collagen is independent of collagenolysis. The International Symposium on Laboratory Automation and Robotics 2002, Boston, MA. Published online: http://198.199.168.17:8080/islar/proceedings.nsf/
8. van Kempen LCL, Lee J, Krane S, **Coussens LM**. (2002) Type I collagen remodeling functionally regulates vascular permeability and epithelial carcinogenesis. *Mol. Biol. Cell*. 13: 4a
9. Diaz RJ, van Kempen L, Dehne K, Schmuth M, Krane S, Albertson D, **Coussens LM**. (2004) Type I collagen remodeling regulates keratinocyte malignant potential. *Proceedings American Association for Cancer Research*, 45:792
10. de Visser KE, **Coussens LM**. (2004) The interplay between adaptive and innate immunity promotes cancer development. *Proceedings American Association for Cancer Research*, 45:403.
11. Eichten A, **Coussens LM**. (2004) Microenvironmental regulation of lymphatic metastasis development. *Proceedings American Association for Cancer Research*, 45:335.
12. **Coussens LM**. (2004) ECM architecture regulates tissue homeostasis and disease pathogenesis. *FASEB J*, 18:A21-A21.
13. **Coussens LM**. (2004) Inflammation and cancer. *Toxicologic Pathology, 32:732.*

XIV. RESEARCH PROGRAM:

The Coussens lab focuses on the role of immune cells and their mediators as critical regulators of cancer (squamous cancer of the skin, non small cell lung cancer, mesothelioma, breast and pancreas cancer)

development. During the early development of cancer, many physiological processes occur in the vicinity of 'young tumor cells' that are similar to processes that occur during embryonic development and to healing of wounds in adult tissue, e.g., leukocyte recruitment and activation (inflammation), angiogenesis (development of new blood supply) and tissue remodeling. During tumor development; however, instead of initiating a 'healing' response, activated leukocytes provide growth-promoting factors that help tumors grow. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. To address these issues, we have taken several approaches to investigate mechanisms involved in: *i.* induction and maintenance of chronic inflammatory microenvironments in premalignant, malignant and metastatic tissues, *ii.* role of leukocyte in regulating tissue remodeling, angiogenesis, immune suppression and cancer development, and *iii.* development of novel non-invasive imaging reagents to monitor immune response in tissues/tumors. The long-term goal of this work is to translate basic observations made in the mouse, toward rational design of novel therapeutics whose aim will be to block and/or alter rate-limiting events critical for solid tumor growth, maintenance or recurrence in humans. Currently, we are actively utilizing transgenic mouse models of solid tumor development (non-small cell lung cancer, non-melanoma squamous and breast cancer, pancreatic adenocarcinoma, and mesothelioma) to reveal the functional roles of adaptive and innate leukocytes during tumor development, and to identify new targets for anti-cancer therapy. These experimental studies are conducted in parallel with evaluation of representative human cancer specimens to affirm that mechanisms revealed in the experimental setting represent fundamental parameters of multi-stage cancer development in humans.

XV. MOST SIGNIFICANT PUBLICATIONS

1. de Visser KE, Korets LV, **Coussens LM**. (2005) De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell,* 7:411-423.

Role: This research was the first to demonstrate a protumor role for B cells and humoral immunity in solid tumor development. Using the K14-HPV16 mouse model of squamous carcinogenesis, we reported that adoptive transfer of B lymphocytes or serum from HPV16 mice into T and B cell-deficient/HPV16 mice reinstated necessary parameters for full malignancy, e.g., chronic inflammation, angiogenic vasculature, hyperproliferative epidermis. These findings support a model in which acquired immunity is essential for establishing chronic inflammatory states that promote de novo carcinogenesis. This research initiated a paradigm shift as it provided the first evidence linking humoral immunity and solid tumor development, thus revealing provocative new targets for anti-cancer therapy. This manuscript was the “featured article” in its issue of *Cancer Cell*, and was the subject of several invited review articles (Houghton et al., *Cancer Cell* 2005; Montavani, *Nature* 2005), as well as being featured in ‘Research Highlights’ in *Nature Reviews Cancer* and *Nature Reviews Immunology* (Minton, 2005). 100% of the research supporting this manuscript was conceived and conducted in my laboratory. Dr. de Visser wrote drafts of the manuscript under Dr. Coussens’ direct supervision.

2. DeNardo DG, Baretto JB, Andreu P, Vasquez L, Kolhatkar N, **Coussens LM.** (2009) $CD4^+$ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell,* 16:91-102.

Role: Infiltration of T lymphocytes in human breast cancers has been recognized by pathologist for decades, however their functional role has been undetermined. We utilized a transgenic mouse model of mammary carcinogenesis and demonstrated a tumor-promoting role for T_H2-$CD4^+$ T lymphocytes that elicit pro-tumor, as opposed to cytotoxic bioactivities of tumor-associated macrophages and enhancement of pro-metastatic epidermal growth factor receptor signaling programs in malignant mammary epithelial cells. This work revealed a novel pro-tumor regulatory program involving components of the acquired and cellular immune systems that effectively collaborate to promote pulmonary metastasis of mammary adenocarcinomas, and identified new cellular targets, namely $CD4^+$ T effector cells and IL-4 for anti-cancer therapy. This manuscript appeared as the “featured article” in the August 4th 2009 issue of *Cancer Cell*, and was the subject of an invited “Preview” article (Pardoll, *Cancer Cell* 2009), and was featured in the research highlights section of *Nature Reviews Cancer* (McCarthy, 2009*).* In 2011, this manuscript was ranked as a *“Top Cancer Papers” between 2008-2011, Nature Medicine, 17:262-265; Nature Medicine 17: 278-279; Nature Medicine, 17:295.* 100% of the research supporting this manuscript was conceived and conducted in my laboratory. Dr. DeNardo wrote drafts of the manuscript under Dr. Coussens’ direct supervision.

3. Andreu P, Johansson M, Affara NI, Tan TT, Junankar S, Korets L, Lam J, Tawfik D, Pucci F, De Palma M, DeNardo D, de Visser KE, **Coussens LM**. (2010) FcRγ activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell,* 17(2):121-134.

Role: This work presented novel findings demonstrating functional significance of Fcγ receptors and humoral immunity as potentiators of squamous carcinogenesis. While myeloid cells and some T cell subsets have been implicated in neoplastic progression and cancer development, the tumor-promoting capabilities of B lymphocytes have remained unclear. Using the HPV16 transgenic mouse model of inflammation-associated squamous carcinogenesis, we previously reported that B and T cell-deficient HPV16 mice failed to progress beyond a benign hyperplastic state due to deficient activation of chronic inflammatory programs in early neoplastic skin (deVisser at al, *Cancer Cell* 2005). In Andreu et al., we revealed revealed that B cells potentiate squamous carcinogenesis via humoral immunity, where immunoglobulins (Ig) in the form of immune complexes (IC) activate Fcγ receptor-mediated signaling pathways on resident and recruited myeloid cells. Activation of these programs on resident mast cells initially leads to peripheral blood leukocyte recruitment into neoplastic skin and activation of angiogenic vasculature. The subsequent chronic inflammation that ensues in part maintains angiogenic support but also supports neoplastic keratinocyte hyperproliferation and progression to dysplastic/carcinoma *in situ* states and subsequent malignant conversion and carcinoma development. These novel findings have clinical significance in that they imply that anti-cancer strategies targeting B cells, Ig or FcRγ may harbor therapeutic efficacy in limiting risk of malignant conversion in patients suffering from chronic inflammatory diseases, or in patients harboring premalignant lesions whose molecular and/or immunologic characteristics favor tumor development. This manuscript appeared as the "featured article" in the March 2010 issue of *Cancer Cell*, and was the subject of an invited "Preview" article (Mantovani, *Cancer Cell* 2010), and was featured in the research highlights section of *Nature Reviews Immunology* (Byrd, 2010*).* In 2011, this manuscript was *Ranked as a "Top Cancer Papers"* between 2008-2011 by Nature Medicine (17:262-265; 17: 278-279), and subsequently reviewed as a 'News and Views in *Nature Medicine* (2011), 17(3) 285-286. '*B Cells and Macrophages in Cancer: Yin and Yang*', by A. Mantovani. 100% of the research supporting this manuscript was conceived and conducted in my laboratory. Drs. Andreu, Johansson and Affara wrote drafts of the manuscript under Dr. Coussens' direct supervision.

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4. Sounni NE, Dehne K, vanKempen LCL, Egeblad M, Affara NI, Cuevas I, Wiesen J, Junankar S, Korets L, Lee J, Shen J, Morrison C, Overall CM, Krane SM, Werb Z, Boudreau N, **Coussens LM**. (2010) Stromal regulation of vessel stability by MMP14 and TGFβ. *Disease Model. Mech.* 3:317-332.

Role: In patients with locally advanced solid tumors, first-line treatment is often neo-adjuvant or pre-operative chemotherapy, which helps shrink tumors before surgery, allowing for more conservative surgical approaches and reducing the potential for developing systemic disease. However, despite aggressive chemotherapy, long-term survival for many patients remains poor, in part owing to limitations with the targeting and accumulation of cytotoxic drugs in tumor tissue. The vasculature of solid tumors is abnormal, both in terms of vessel architecture and the dynamics of blood flow. Permeable heterogeneous vessel walls allow the leakage of proteins and fluid that, coupled with the inefficiency of lymphatic drainage, could be exploited to develop novel, enhanced drug delivery strategies that are therapeutically selective and improve clinical outcome. This work describes a previously unappreciated role for transforming growth factor beta (TGFβ) in regulating vascular stability and vessel permeability in solid tumors. Using mouse models, we demonstrated an endogenous pathway that regulates normal vascular permeability, which is controlled by perivascular collagen, the metalloproteinase enzyme MMP14, and TGFβ. In wild-type mice, inhibitors of either MMP14 or TGFβ signaling induce blood vessel permeability. Conversely, enhanced MMP14 or TGFβ activity in the mouse epidermis decreases leakage across cutaneous vessels. This pathway remains functional during tumor progression, as acute blockade of either MMP14 or TGFβ signaling transiently alters vessel stability, 'opening' vascular beds and promoting intravenous delivery of high molecular weight compounds to the tumor. This implying that delivery of standard therapeutic agents or diagnostic molecular imaging agents to tumor tissue may be enhanced by transient blockade of the TGFβ pathway. If so, this could advance disease therapy and/or diagnostic imaging, not only in cancer medicine, but also in fibrotic disorders such as scleroderma and kidney failure. Research in support of this manuscript spanned over 7 years and many post doctoral fellows and technicians. Dr. Coussens, who wrote the manuscript with input from Drs. Boudreau and Werb, conceived of the initial hypothesis and ideas towards successful completion of this project.

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5. DeNardo DG, Brennan DJ, Rexhapaj E, Ruffell B, Shiao SL, Madden SF, Gallagher WM, Wadhwani N, Keil SD, Junaid SA, Rugo HS, Hwang ES, Jirstrom K, West BL, **Coussens LM**. (2011) Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discovery,* 1(1): 54-67.

Role: Local control of malignant breast tumors by cytotoxic therapies is critical for long-term survival of breast cancer patients. This study is the first to provide evidence that common cytotoxic agents induce alterations in the immune microenvironment, including recruitment of immune-suppressive macrophages that facilitate resistance to chemotherapy and promote metastasis. These data provide rationale for selective blockade of CSF1R signaling pathways in combination with cytotoxic therapies to combat breast cancer, and potentially other tumor types where macrophages foster tumor development. This work provided the foundation for a KOMEN Promise grant award that enables ongoing mouse modeling, as well as an investigator-initiated phase Ib/II clinical trial to evaluate a CSF1R kinase inhibitor in combination with taxol-based chemotherapy in women with recurrent triple negative breast cancer. This manuscript was the subject of an invited "Preview" article (De Palma and Lewis, *Nature* 2011), and was featured in the research highlights section of *Nature Reviews Cancer* (McCarthy, 2011*).* 100% of the research supporting this research was conceived and conducted in Dr. Coussens' laboratory. Dr. DeNardo wrote drafts of the manuscript under Dr. Coussens' supervision.

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Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity

David G. DeNardo · Pauline Andreu · Lisa M. Coussens

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Abstract Tumor-associated myeloid cells have been implicated in regulating many of the "hallmarks of cancer" and thus fostering solid tumor development and metastasis. However, the same innate leukocytes also participate in anti-tumor immunity and restraint of malignant disease. While many factors regulate the propensity of myeloid cells to promote or repress cancerous growths, polarized adaptive immune responses by B and T lymphocytes have been identified as regulators of many aspects of myeloid cell biology by specifically regulating their functional capabilities. Here, we detail the diversity of heterogeneous B and T lymphocyte populations and their impacts on solid tumor development through their abilities to regulate myeloid cell function in solid tumors.

Keywords Cancer · Inflammation · Lymphocyte · Macrophage · Metastasis

1 Introduction

Virchow first described leukocyte infiltration of solid tumors in the 1800s; however, only recently have we begun to understand the diverse regulatory roles played by immune cells during cancer development. Historically, leukocytes found in and around developing tumors were thought to represent an attempt by the host to eradicate neoplastic cells. Indeed, some leukocytes, including $CD8^+$ cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, do play a critical role in restraining tumor development [1]. However, we now appreciate that the significance of these anti-tumor programs can be thwarted by other subsets of leukocytes that instead foster tumor development [2–5]. Immune-competent mouse models of human cancer have enabled a detailed evaluation of the tumor-promoting capacity of several subsets of myeloid cells, including mast cells (MCs), monocytes, granulocytes/neutrophils, and macrophages, as well as some subsets of lymphocytes [6, 7]. However, depending on their differentiation status and immune microenvironment, subpopulations of these same cells can also support tumor rejection and response to anti-cancer therapy [2, 8, 9], thus indicating that pro- and anti-tumor programming of leukocytes is dynamic. In this review, we discuss recent insights into the role of B and T lymphocytes as "gatekeepers" of myeloid cell bioactivity (Fig. 1) and how recognition of these dynamic interactions reveals novel opportunities for anti-cancer therapy.

2 Paradoxical role of $CD4^+$ T lymphocytes in solid tumor development

In contrast to $CD8^+$ CTLs that play well-defined roles in hindering cancer development, the functional significance of $CD4^+$ T lymphocytes in tumor progression appears more paradoxical. For example, retrospective evaluation of colon and lung carcinomas revealed that extensive infiltration of tumors by $CD4^+$ T cells correlates with favorable clinical outcome, whereas in breast and renal cancers exhibiting similar infiltrations instead correlates with decreased overall

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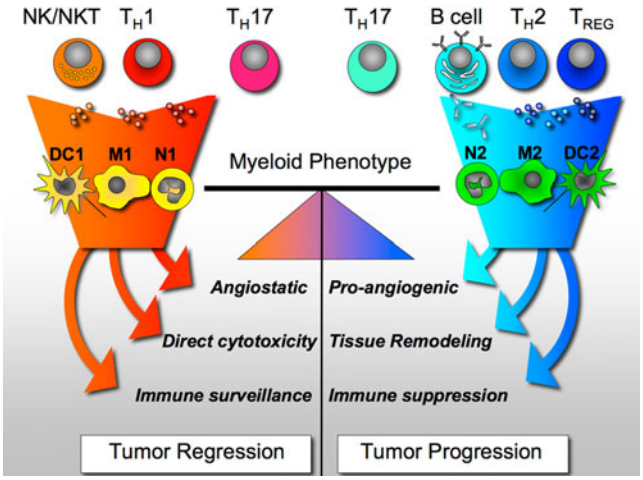

Fig. 1 Adaptive immune responses control tumor-associated myeloid cell bioactivity and tumor progression. Polarized responses by adaptive immune cells alter the balance between pro- and anti-tumor myeloid cell bioactivities. When the host's response to neoplastic cell growth results in the production of T_H1 cytokines by $CD4^+$ T lymphocytes and NK cells, myeloid cells in turn induce programs promoting tumor regression and/or dormant disease. However, when these adaptive immune responses include chronic B lymphocyte activation and IgG production in combination with T_H2 and T_{REG} lymphocyte activation, programs of immune suppression, angiogenesis, tissue remodeling, and invasion are favored in myeloid cells and contribute to tumor progression and metastasis

survival [10–13]. Analysis of mouse models of human cancer have provided some clarity for these disparate findings and revealed that etiology and organ specificity matters with regards to how $CD4^+$ T cells aid or constrain tumor progression. Schreiber and colleagues demonstrated that, whereas $CD4^+$ T cell deficiency enhances methylcholanthrene (MCA)-initiated sarcoma development [14], carcinoma development on the other hand is inhibited following two-stage squamous carcinogenesis [15, 16]. Similarly, in a mouse model of skin and cervical carcinoma development where oncogenes from human papilloma virus type 16 (HPV16) are expressed under the control of the keratin 14 promoter, skin carcinoma formation is modestly attenuated by $CD4^+$ T cell deficiency, whereas cervical carcinoma development is significantly enhanced [17, 18]. Together, these observations demonstrate that immune responses accompanying tumor development are organ dependent and, based on the neoplastic microenvironment, engage either pro- or anti-tumor immune programs. The heterogeneity of $CD4^+$ T cell subsets that accumulate in tissues may be at the heart of these paradoxical findings.

3 $CD4^+$ T lymphocyte heterogeneity

$CD4^+$ T cells represent a highly heterogeneous population of cells that develop along different functional lineages depending upon polarizing cytokine signals during activation by antigens [19]. Classically, $CD4^+$ T lymphocyte subsets include T_H1 and T_H2 lineages that are characteristically fostered by exposure to interleukin (IL)12 and IL4, respectively. Following activation, $CD4^+$ T cells assuming the T_H1 fate secrete interferon (IFN)γ, tumor necrosis factor (TNF)α, IL2, and IL12 [20]. Through the production of these cytokines, T_H1 cells regulate immune surveillance programs by up-regulating antigen processing and presentation on major histocompatability complex (MHC) I and II molecules by professional antigen presenting cells (APCs) and as such can regulate the duration and magnitude of $CD8^+$ CTL responses [21]. In addition, following strong antigen-specific activation, T_H1 cells can directly kill tumor cells by releasing high levels of IFNγ, TNFα and cytolytic granules. Thus, when present, T_H1 responses can directly and indirectly regulate anti-tumor programs that restrain cancer development.

In contrast, T_H2 $CD4^+$ T cells express high levels of IL4, -5, -6, -10, and -13 that, together, alter adaptive immunity by inducing T cell anergy, inhibiting T cell-mediated cytotoxicity as well as fostering humoral immune responses directed by B cells [22, 23]. The T_H2 cytokines IL4 and IL13 are important mediators of $CD4^+$ T cell functionality *in vitro*, T_H2 $CD4^+$ T cells inhibit apoptosis and induce proliferation of breast carcinoma cells; *in vivo*, IL4 emanating from $CD4^+$ T cells fosters breast cancer growth [24, 25]. Consistent with these findings, a high ratio of T_H2^+ to T_H1^+ cells correlates with parameters of clinical disease progression, such as increased tumor size and grade and lymph node metastasis of breast cancers [26].

Adding to this T_H1 versus T_H2 paradigm, CD4 lineages have recently expanded to include a T_H17 subset that is differentiated by a combination of IL6 and transforming growth factor (TGF)β and mediate their effects through secretion of IL17, -21, and -22, [27–29]. T_H17 cells are thought to play an important role in protection against some extracellular pathogens and in regulating auto-immune disease [30, 31]. As such, T_H17 cells have been implicated in the development of inflammation-associated colonic tumors in response to pathogenic bacteria [32]. T_H17 cell infiltration has also been observed in patients with colon, ovarian, prostate, and hepatocellular carcinoma where high numbers of IL17-producing cells correlates with poor prognosis [33–35]. In mouse models of non-small cell lung cancer (NSCLC), IL17 enhanced tumor growth by promoting development of angiogenic vasculature [36, 37]. In contrast, in a B16 melanoma model, IL17 depletion rendered mice more susceptible to metastasis [38], a phenotype that was blocked by adoptive transfer of tumor-specific T_H17 cells that fostered immune surveillance by $CD8^+$ CTLs and dendritic cells (DCs) [38]. Together, these experimental findings indicate that the role

of T_H17 cells in regulating aspects of cancer development may also be context dependent.

In addition to T_H1, T_H2, and T_H17 $CD4^+$ effectors, populations of $CD4^+FoxP3^+$ T regulatory (T_{REG}) cells are also thought to play a considerable role in regulating tumor immunity. In human cancers, increased prevalence of $CD4^+FoxP3^+$ T_{REG} cells correlates with increased survival for follicular lymphoma [39], while it instead correlates with poor prognosis in pancreatic ductal carcinoma [40], non-small cell lung cancer [41], renal cell carcinoma [11], and breast carcinoma [42]. Suppression of the anti-tumor activities of $CD8^+$ CTLs, NKs, and DCs is at the heart of how T_{REG} cells control tumor development [43, 44]; however, the multitude of mechanisms whereby they regulate anti-tumor programs suggest the existence of distinct tissue-specific subpopulations of T_{REG} cells, each endowed with or capable of various bioeffector activities [45–47]. Mechanistic studies have revealed that T_{REG} cells support pro-tumor immunity not only by increasing local levels of immunosuppressive cytokines including TGFβ, IL35, and IL10 but also by direct cytolytic effects through production of perforin and granzyme. In addition, T_{REG} cells can disrupt the metabolic activity of cyclic adenosine monophosphate (cAMP) transfer, as well as inhibit APC function by inducing binding of CTLA-4 to CD80/86 [44, 48].

Thus, while it is now clear that a spectrum of $CD4^+$ T cell subtypes are present in human tumors of essentially all types, the role they play in promoting or repressing tumor development likely has to do with the type of $CD4^+$ T cell subtype that is either recruited to or accumulates within each distinct tumor microenvironment. These in turn then regulate anti-tumor programs by professional cytotoxic cells ($CD8^+$ T CTLs and NK cells), as well as regulating pro-tumor properties of a diverse array of myeloid cell subtypes as discussed below.

4 Myeloid heterogeneity and tumor development

Innate immune cells of myeloid origin, e.g., granulocytes (neutrophils, basophils, and eosinophils), DCs, macrophages, NK cells, and MCs, are also prominent components of pre- and malignant tissues and functionally contribute to cancer development by releasing a myriad of cytokines, chemokines, matrix metalloproteinases, serine proteases, DNA-damaging molecules (reactive oxygen species), histamine, and other bioactive mediators that regulate tissue remodelling and angiogenesis [49–53], suppress anti-tumor immunity [54–56], and enhance tumor cell survival, migration, and metastasis [57, 58].

Nucleated hematopoietic cells that have been directly implicated in tumor angiogenesis include MCs [51], tumor-associated macrophages (TAMs) [5, 23, 59], Tie2-expressing monocytes [50, 60], neutrophils [52], DC precursors [61], and myeloid immune suppressor cells [62, 63]. Other hematopoietic cell types, such as platelets [64], eosinophils [65], and hematopoietic progenitors [66], also participate in angiogenic processes, but it remains to be established whether they can directly promote tumor angiogenesis, rather than having a broader function in supporting tissue inflammation and remodelling.

In contrast, these same myeloid cell lineages also foster tumor rejection by inducing angiostatic programs, enhancing CTL and NK responses, and directly inducing tumor cell death [13]. As an example of these paradoxical roles, studies from several laboratories have reported that TAMs enhance angiogenesis and metastasis of malignant mammary tumors [25, 67, 68]. In contrast, TAMs exposed to toll-like receptor (TLR) ligands and/or IFNγ directly lyse mammary tumor cells, increase antigen presentation, and secrete angiostatic proteins such as CXCL10 and 11 [8, 9 , 69]. These distinct bioactivities are mirrored in neutrophils, MCs, and DCs and may be due to the inherent plasticity of myeloid lineage cells regulated by local factors present in distinct tissue and/or organ microenvironments.

The bioactive states of macrophages, as well as other myeloid cells, have been classified according to T_H1 and T_H2 nomenclature, referred to as M1 (classical) or M2 (alternative) activation, respectively [2, 70, 71] (Fig. 1). M1 macrophages are regulated by T_H1 cytokines including IFNγ, TNFα, and granulocyte monocyte-colony stimulating factor (GM-CSF) that enhance macrophage cytotoxic activity, production of pro-inflammatory cytokines, and antigen presentation capacity [70, 71]. In contrast, tissue macrophages can achieve various alternatively activated M2 states following exposure to T_H2 cytokines, including IL4 or IL13 (M2a), potentiation by immune complexes and TLR ligands (M2b) or immunosuppressive cytokines including IL10 or TGFβ and/or glucocorticoid hormones (M2c) [70].

The general hallmarks of M2 macrophages include high levels of IL10, IL1Ra, IL1 decoy receptor CCL17 and CCL22 secretion, high expression of mannose, scavenger and galactose-type receptors, low expression of IL12, as well as poor APC capability. Intriguingly, although these alternative activation states (M2a, b, and c) share many phenotypic characteristics, they are distinct and induce individual context-dependent environmental responses. For example, induction of an M2c phenotype by IL10 results in highly immune suppressive macrophages that can also produce matrix components such as versican or PTX3. In contrast, T_H2 cytokine induction of M2a TAMs induces expression of fibronectin, as well as catabolism of *L*-arginine by arginase that in turn leads to increased collagen synthesis and matrix remodeling [70, 72]. Our own work has revealed that IL4 and/or IL13 activation of macro-

phages induces production of growth factors including epidermal growth factor, TGFβ, and basic fibroblast growth factor that together regulate invasive, angiogenic, and immune-suppressive programs [25] (unpublished data). Both M2a and M2b macrophages down-regulate the pro-inflammatory cytokines IL1, IL6, and TNFα [73], whereas induction of M2b macrophages by immune complexes induces these same inflammatory cytokines in addition to IL10 and also likely enhances vascular responses such as endothelial migration and vessel dilation [74]. Thus, M2-polarized cells promote scavenging of debris, angiogenesis, and remodeling and repair of wounded/damaged tissues. Parallel and non-redundant activity states have also been defined for DCs (i.e., DC1, DC2) [75] and neutrophils (i.e., N1, N2) [76] (Fig. 1).

To address pro- versus anti-tumor capabilities of these opposing states, Hagemann and colleagues demonstrated that by "reprogramming" M2 TAMs through deletion of IKKβ macrophage phenotype could be switched from immunosuppressive to actively promoting immune surveillance, as reflected by decreases in IL10 and arginase-1 expression and increased IL12 and nitric oxide production together resulting in decreased ovarian tumor growth through recruitment and activation of NK cells [67, 77]. Again, organ specificity and/or etiology may play a role in regulating how reprogramming can be achieved. In a mouse model of squamous carcinogenesis, we recently reported that FcγR signaling in myeloid cells directly regulates whether myeloid cells enhance or repress cancer development, which correlate with their unique gene expression signatures that reflect M1 versus M2 and DC1 versus DC2 programs [74]. These data indicate the significance of reprogramming myeloid cell phenotypes to affect tumor outcome. The major question that arises with regards to this capability then becomes what are the cellular and molecular programs in tissues and/or tumors that regulate the bioactive state of these important myeloid cells and how recognition of these can be translated into anti-cancer therapy.

5 T lymphocytes as regulators of anti-tumor macrophages

Establishment of an immune reaction in homeostatic tissue typically involves activation of NK cells in response to stress signals or infectious agents, whom by their production of IFNγ in turn prime macrophages towards an M1 state, culminating in enhanced presence of macrophages with cytotoxic capability [71]. However, production of IFNγ by NK cells is generally transient and therefore insufficient to sustain M1 macrophage polarization; thus, IFNγ-producing T_H1 cells are critical for immune responses requiring sustained M1 macrophage bioactivities. In tumors, studies by Corthay and colleagues demonstrated that T_H1 cell regulation of locally activated M1 macrophages were significant and fostered rejection of myeloma and lymphomas in the absence of CTL responses [69, 78]. Moreover, expression of IL12 and IFNγ by T_H1 cells can combine to enhance anti-tumor responses by NK and NK-T cells by up-regulating expression of NK receptors such as NKG2D (in response to IL12) and expression of NK receptor ligands such as RAE1 on target cells (in response to IFNγ) [79]. T_H1 responses then in turn favor anti-tumor NK and macrophage responses that eliminate neoplastic cells. However, while T_H1 cells are antigen specific, tumoricidal macrophages exert indiscriminate cell killing activity. Thus, multiple immunosuppressive programs have evolved to eliminate the adverse autoimmune pathologies, such as rheumatoid arthritis, that are associated with over-activation of these M1 responses [80]; unfortunately, many of these immunosuppressive programs are usurped by developing cancers.

6 T lymphocytes as regulators of pro-tumor myeloid cells

In contrast to induction of tumor-immune surveillance programs by T_H1 cells, T_{REG} and T_H2 cells have the capacity to induce alternative activation states of macrophages, DCs, and neutrophils involved in promoting cancer development. Studies of human T_{REGs} have demonstrated their ability to block classical activation of macrophages and instead foster immunosuppressive myeloid phenotypes through the production of IL10 and TGFβ [81]. Similar biology may apply to neutrophils, as recently reported by Fridlender and colleagues who found that loss of TGFβ signaling through ALK4/5 inhibition resulted in recruitment of N1-polarized neutrophils with tumoricidal bioactivities [76]. While TGFβ in the tumor microenvironment is produced by multiple cell types, these data may indicate that T_{REG} cells suppress N1 tumoricidal responses through production of TGFβ and, as such, favor pro-tumor N2 or immature monocyte (IMC) phenotypes. Our own work has demonstrated that $CD4^+$ T_H2 cells in mammary tumors promote M2 responses in TAM and IMCs that in turn enhance pulmonary metastasis [25]. Together, these data indicate that the balance of T_H1 versus T_H2/T_{REG} responses regulates the pro- versus anti-tumor programming of tumor-associated myeloid cells.

7 B lymphocytes as regulators of myeloid cells during cancer development

B lymphocytes constitute a central component of humoral immunity and not only serve in antibody production but also in antigen presentation and cytokine secretion. In

particular, B lymphocyte expression of MHC and co-stimulatory molecules as well as secretion of pro-inflammatory cytokines are critical for regulating $CD4^+$ and $CD8^+$ T cell activation, expansion, antigenic spreading, and memory T lymphocyte formation. The heterogeneity of B lymphocyte responses has been recently recognized and diverse B cell subtypes with either pro-immune or regulatory properties have been identified *in vivo*. Precisely, regulatory B lymphocytes (B_{REG}), which include various subtypes of IL10-producing cells, have been identified in the context of autoimmune diseases and exert anti-inflammatory functions [82, 83]. However, a role for B_{REG} cells in cancer has not been fully elucidated.

B lymphocytes in general have only recently gained recognition for representing significant components of tumor immunity [84]. B cell involvement in solid tumor development was initially described in syngeneic allograft murine tumor models in combination with genetic or antibody-mediated B cell depletion. In these studies, B cell-deficient mice (μMT) exhibited resistance to several types of syngeneic tumors, including EL4 thymoma, MC38 colon carcinoma, and B16 and D5 melanoma [85, 86], whereas partial B cell depletion resulted in significantly reduced tumor burden in a transplantable model of colorectal cancer [87]. A tumor-promoting role for B cells in solid tumor development was also revealed in transgenic mice expressing tumor necrosis factor (TNF)-receptor-associated factor 3 (TRAF3) in lymphocytes [88]. $TRAF3^+$ lymphocytes induce humoral immune responses leading to chronic inflammation and a significantly elevated incidence of squamous cell carcinomas [88]. These experimental findings indicate that, in the absence of an initiating oncogenic event, B lymphocyte-mediated chronic inflammation is sufficient to foster solid tumor formation. In contrast, an opposite and anti-tumor immune surveillance role for B lymphocytes has also been demonstrated in a syngeneic melanoma model where deletion of mature B cells by anti-CD20 IgG significantly enhanced tumor growth and metastasis [80], suggesting that the role of B lymphocytes in tumor progression, like $CD4^+$ T lymphocytes, may be context dependent and driven by individual B lymphocyte subtype specificity.

Mechanistically, B cells and humoral immunity can act to modulate solid tumor development by regulation of diverse effector pathways involving secretion of pro-inflammatory, as opposed to regulatory, cytokines, e.g., IL10, TGFβ, inhibition of CTL activity [89], perturbation of T_H1/T_H2 $CD4^+$ T cell lineages [90, 91], as well as differential recruitment and activation of innate immune cells [89, 92].

Recently, using a transgenic mouse model of inflammation-associated carcinogenesis, i.e., K14-HPV16 mice [93], we revealed a novel pathway by which B lymphocytes enhance squamous carcinogenesis and demonstrated the significance of the B cell/immunoglobulin/FcγR signaling axis. We found that B cells and humoral immunity fostered cancer development by activating Fcγ receptors on resident and recruited myeloid cells [74]. Stromal accumulation of autoantibodies in premalignant skin, through their interaction with activating FcγRs, regulated recruitment, composition, and bioeffector functions of leukocytes, in particular subsets of tumor-promoting polarized myeloid cells in neoplastic tissue which in turn enhanced neoplastic progression and subsequent carcinoma development [74]. A similar pro-tumor role for B cells was recently reported by Ammirante and colleagues who found that B cells are critical for growth of castration-resistant prostate cancer metastasis, not through production of immunoglobulins or regulation of FcγR signaling but instead by delivery of lymphotoxin that in turn activates IKK-α and STAT3 in prostate cancer cells and subsequently stimulates metastasis by an NF-κB-independent, cell-autonomous mechanism [94]. These findings together with other experimental studies support a model in which B cells, through various mechanisms, including humoral immunity, activating FcγRs and IKK, are required for establishing chronic inflammatory programs that promote *de novo* carcinogenesis.

8 Conclusions

While many factors regulate the propensity of immune cells to promote or repress solid tumor development, polarized adaptive immune responses by B and T lymphocytes can specifically regulate multiple pro-tumor properties of myeloid cells that in turn control many of the "hallmarks" of cancer development [95, 96]. Thus, recognition of the soluble molecules that mediate these important paracrine interactions may represent critical targets to evaluate for anti-cancer therapy. Importantly, targeting of pro-tumor pathways that neutralize M2-type macrophage and/or T_H2-type $CD4^+$ T cell responses and therein foster M1 or T_H1-type immunity may enhance sensitivity to cytotoxic therapies, including chemo- and radiation therapy, whose durability may be limited by the longevity of the anti-tumor immune responses that they induce.

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References

1. Dunn, G. P., Old, L. J., & Schreiber, R. D. (2004). The immunobiology of cancer immunosurveillance and immunoediting. *Immunity, 21*(2), 137–148.

2. Ostrand-Rosenberg, S. (2008). Immune surveillance: a balance between protumor and antitumor immunity. *Current Opinion in Genetics & Development, 18*(1), 11–18.
3. Karin, M., Lawrence, T., & Nizet, V. (2006). Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell, 124*(4), 823–835.
4. de Visser, K. E., Eichten, A., & Coussens, L. M. (2006). Paradoxical roles of the immune system during cancer development. *Nature Reviews Cancer, 6*, 24–37.
5. Balkwill, F., Charles, K. A., & Mantovani, A. (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell, 7*(3), 211–217.
6. Mantovani, A., Allavena, P., Sica, A., & Balkwill, F. (2008). Cancer-related inflammation. *Nature, 454*(7203), 436–444.
7. De Palma, M., & Coussens, L. M. (2008). Immune cells and inflammatory mediators as regulators of tumor angiogenesis. In W. D. Figg & J. Folkman (Eds.), *Angiogenesis: An integrative approach from science to medicine* (pp. 225–238). Springer: New York.
8. Stout, R. D., & Bottomly, K. (1989). Antigen-specific activation of effector macrophages by IFN-gamma producing (TH1) T cell clones. Failure of IL-4-producing (TH2) T cell clones to activate effector function in macrophages. *Journal of Immunology, 142*(3), 760–765.
9. Watkins, S. K., Egilmez, N. K., Suttles, J., & Stout, R. D. (2007). IL-12 rapidly alters the functional profile of tumor-associated and tumor-infiltrating macrophages *in vitro* and *in vivo*. *Journal of Immunology, 178*(3), 1357–1362.
10. Wakabayashi, O., Yamazaki, K., Oizumi, S., Hommura, F., Kinoshita, I., Ogura, S., et al. (2003). CD4(+) T cells in cancer stroma, not CD8(+) T cells in cancer cell nests, are associated with favorable prognosis in human non-small cell lung cancers. *Cancer Science, 94*(11), 1003–1009.
11. Siddiqui, S. A., Frigola, X., Bonne-Annee, S., Mercader, M., Kuntz, S. M., Krambeck, A. E., et al. (2007). Tumor-infiltrating Foxp3-CD4+CD25+ T cells predict poor survival in renal cell carcinoma. *Clinical Cancer Research, 13*(7), 2075–2081.
12. DeNardo, D. G., Johansson, M., & Coussens, L. M. (2008). Immune cells as mediators of solid tumor metastasis. *Cancer Metastasis Reviews, 27*(1), 11–18.
13. Ruffell, B., Denardo, D. G., Affara, N. I., & Coussens, L. M. (2010). Lymphocytes in cancer development: polarization towards pro-tumor immunity. *Cytokine & Growth Factor Reviews, 21*, 3–10.
14. Koebel, C. M., Vermi, W., Swann, J. B., Zerafa, N., Rodig, S. J., Old, L. J., et al. (2007). Adaptive immunity maintains occult cancer in an equilibrium state. *Nature, 450*(7171), 903–907.
15. Girardi, M., Glusac, E., Filler, R. B., Roberts, S. J., Propperova, I., Lewis, J., et al. (2003). The distinct contributions of murine T cell receptor (TCR)gammadelta+ and TCRalphabeta+ T cells to different stages of chemically induced skin cancer. *Journal of Experimental Medicine, 198*(5), 747–755.
16. Girardi, M., Oppenheim, D., Glusac, E. J., Filler, R., Balmain, A., Tigelaar, R. E., et al. (2004). Characterizing the protective component of the alphabeta T cell response to transplantable squamous cell carcinoma. *Journal of Investigative Dermatology, 122*(3), 699–706.
17. Daniel, D., Chiu, C., Giraud, E., Inoue, M., Mizzen, L. A., Chu, N. R., et al. (2005). CD4+ T cell-mediated antigen-specific immunotherapy in a mouse model of cervical cancer. *Cancer Research, 65*, 2018–2025.
18. Daniel, D., Meyer-Morse, N., Bergsland, E. K., Dehne, K., Coussens, L. M., & Hanahan, D. (2003). Immune enhancement of skin carcinogenesis by CD4+ T cells. *Journal of Experimental Medicine, 197*(8), 1017–1028.
19. Zhou, L., Chong, M. M., & Littman, D. R. (2009). Plasticity of CD4+ T cell lineage differentiation. *Immunity, 30*(5), 646–655.
20. Munk, M. E., & Emoto, M. (1995). Functions of T-cell subsets and cytokines in mycobacterial infections. *European Respiratory Journal. Supplement, 20*, 668s–675s.
21. Romagnani, S., Parronchi, P., D'Elios, M. M., Romagnani, P., Annunziato, F., Piccinni, M. P., et al. (1997). An update on human Th1 and Th2 cells. *International Archives of Allergy and Immunology, 113*(1–3), 153–156.
22. Parker, D. C. (1993). T cell-dependent B cell activation. *Annual Review of Immunology, 11*, 331–360.
23. Pollard, J. W. (2004). Tumour-educated macrophages promote tumour progression and metastasis. *Nature Reviews. Cancer, 4*(1), 71–78.
24. Aspord, C., Pedroza-Gonzalez, A., Gallegos, M., Tindle, S., Burton, E. C., Su, D., et al. (2007). Breast cancer instructs dendritic cells to prime interleukin 13-secreting CD4+ T cells that facilitate tumor development. *Journal of Experimental Medicine, 204*(5), 1037–1047.
25. DeNardo, D. G., Baretto, J. B., Andreu, P., Vasquez, L., Kolhatkar, N., Tawfik, D., et al. (2009). CD4+ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell, 16*(2), 91–102.
26. Chin, Y., Janseens, J., Vandepitte, J., Vandenbrande, J., Opdebeek, L., & Raus, J. (1992). Phenotypic analysis of tumor-infiltrating lymphocytes from human breast cancer. *Anticancer Research, 12*(5), 1463–1466.
27. Wilson, C. B., Rowell, E., & Sekimata, M. (2009). Epigenetic control of T-helper-cell differentiation. *Nature Reviews. Immunology, 9*(2), 91–105.
28. Weaver, C. T., Harrington, L. E., Mangan, P. R., Gavrieli, M., & Murphy, K. M. (2006). Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity, 24*(6), 677–688.
29. Dong, C. (2008). TH17 cells in development: an updated view of their molecular identity and genetic programming. *Nature Reviews. Immunology, 8*(5), 337–348.
30. Ouyang, W., Kolls, J. K., & Zheng, Y. (2008). The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity, 28*(4), 454–467.
31. Bettelli, E., Korn, T., Oukka, M., & Kuchroo, V. K. (2008). Induction and effector functions of T(H)17 cells. *Nature, 453*(7198), 1051–1057.
32. Wu, S., Rhee, K. J., Albesiano, E., Rabizadeh, S., Wu, X., Yen, H. R., et al. (2009). A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nature Medicine, 15*(9), 1016–1022.
33. Miyahara, Y., Odunsi, K., Chen, W., Peng, G., Matsuzaki, J., & Wang, R. F. (2008). Generation and regulation of human CD4+ IL-17-producing T cells in ovarian cancer. *Proceedings of the National Academy of Sciences of the United States of America, 105*(40), 15505–15510.
34. Sfanos, K. S., Bruno, T. C., Maris, C. H., Xu, L., Thoburn, C. J., DeMarzo, A. M., et al. (2008). Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing. *Clinical Cancer Research, 14*(11), 3254–3261.
35. Zhang, J. P., Yan, J., Xu, J., Pang, X. H., Chen, M. S., Li, L., et al. (2009). Increased intratumoral IL-17-producing cells correlate with poor survival in hepatocellular carcinoma patients. *Journal of Hepatology, 50*(5), 980–989.
36. Numasaki, M., Watanabe, M., Suzuki, T., Takahashi, H., Nakamura, A., McAllister, F., et al. (2005). IL-17 enhances the net angiogenic activity and *in vivo* growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis. *Journal of Immunology, 175*(9), 6177–6189.
37. Numasaki, M., Fukushi, J., Ono, M., Narula, S. K., Zavodny, P. J., Kudo, T., et al. (2003). Interleukin-17 promotes angiogenesis and tumor growth. *Blood, 101*(7), 2620–2627.

38. Martin-Orozco, N., Muranski, P., Chung, Y., Yang, X. O., Yamazaki, T., Lu, S., et al. (2009). T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity, 31*(5), 787–798.
39. Carreras, J., Lopez-Guillermo, A., Fox, B. C., Colomo, L., Martinez, A., Roncador, G., et al. (2006). High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. *Blood, 108*(9), 2957–2964.
40. Hiraoka, N., Onozato, K., Kosuge, T., & Hirohashi, S. (2006). Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clinical Cancer Research, 12*(18), 5423–5434.
41. Petersen, R. P., Campa, M. J., Sperlazza, J., Conlon, D., Joshi, M. B., Harpole, D. H., Jr., et al. (2006). Tumor infiltrating Foxp3+ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. *Cancer, 107*(12), 2866–2872.
42. Merlo, A., Casalini, P., Carcangiu, M. L., Malventano, C., Triulzi, T., Menard, S., et al. (2009). FOXP3 expression and overall survival in breast cancer. *Journal of Clinical Oncology, 27*(11), 1746–1752.
43. Trzonkowski, P., Szmit, E., Mysliwska, J., Dobyszuk, A., & Mysliwski, A. (2004). CD4+CD25+ T regulatory cells inhibit cytotoxic activity of T CD8+ and NK lymphocytes in the direct cell-to-cell interaction. *Clinical Immunology, 112*(3), 258–267.
44. Strauss, L., Bergmann, C., Szczepanski, M., Gooding, W., Johnson, J. T., & Whiteside, T. L. (2007). A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment. *Clinical Cancer Research, 13*(15 Pt 1), 4345–4354.
45. Fehervari, Z., & Sakaguchi, S. (2004). CD4+ Tregs and immune control. *Journal of Clinical Investigation, 114*(9), 1209–1217.
46. Feuerer, M., Hill, J. A., Mathis, D., & Benoist, C. (2009). Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nature Immunology, 10*(7), 689–695.
47. Cao, X., Cai, S. F., Fehniger, T. A., Song, J., Collins, L. I., Piwnica-Worms, D. R., et al. (2007). Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity, 27*(4), 635–646.
48. Tang, Q., & Bluestone, J. A. (2008). The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nature Immunology, 9*(3), 239–244.
49. Okamoto, R., Ueno, M., Yamada, Y., Takahashi, N., Sano, H., Suda, T., et al. (2005). Hematopoietic cells regulate the angiogenic switch during tumorigenesis. *Blood, 105*(7), 2757–2763.
50. De Palma, M., Venneri, M. A., Galli, R., Sergi Sergi, L., Politi, L. S., Sampaolesi, M., et al. (2005). Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell, 8*(3), 211–226.
51. Coussens, L. M., Raymond, W. W., Bergers, G., Laig-Webster, M., Behrendtsen, O., Werb, Z., et al. (1999). Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes & Development, 13*(11), 1382–1397.
52. Nozawa, H., Chiu, C., & Hanahan, D. (2006). Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America, 103*(33), 12493–12498.
53. Takakura, N. (2006). Role of hematopoietic lineage cells as accessory components in blood vessel formation. *Cancer Science, 97*(7), 568–574.
54. Zou, W. (2005). Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nature Reviews. Cancer, 5*(4), 263–274.
55. Blankenstein, T. (2005). The role of tumor stroma in the interaction between tumor and immune system. *Current Opinion in Immunology, 17*(2), 180–186.
56. Bronte, V., Cingarlini, S., Marigo, I., De Santo, C., Gallina, G., Dolcetti, L., et al. (2006). Leukocyte infiltration in cancer creates an unfavorable environment for antitumor immune responses: a novel target for therapeutic intervention. *Immunological Investigations, 35*(3–4), 327–357.
57. Condeelis, J., & Pollard, J. W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell, 124*(2), 263–266.
58. Wyckoff, J., Wang, W., Lin, E. Y., Wang, Y., Pixley, F., Stanley, E. R., et al. (2004). A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Research, 64*(19), 7022–7029.
59. Lewis, C. E., & Pollard, J. W. (2006). Distinct role of macrophages in different tumor microenvironments. *Cancer Research, 66*(2), 605–612.
60. De Palma, M., & Naldini, L. (2006). Role of haematopoietic cells and endothelial progenitors in tumour angiogenesis. *Biochimica et Biophysica Acta, 1766*(1), 159–166.
61. Coukos, G., Benencia, F., Buckanovich, R. J., & Conejo-Garcia, J. R. (2005). The role of dendritic cell precursors in tumour vasculogenesis. *British Journal of Cancer, 92*(7), 1182–1187.
62. Yang, L., Debusk, L. M., Fukuda, K., Fingleton, B., Green-Jarvis, B., Shyr, Y., et al. (2004). Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell, 6*(4), 409–421.
63. Serafini, P., Borrello, I., & Bronte, V. (2006). Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Seminars in Cancer Biology, 16*(1), 53–65.
64. Kisucka, J., Butterfield, C. E., Duda, D. G., Eichenberger, S. C., Saffaripour, S., Ware, J., et al. (2006). Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage. *Proceedings of the National Academy of Sciences of the United States of America, 103*(4), 855–860.
65. Puxeddu, I., Alian, A., Piliponsky, A. M., Ribatti, D., Panet, A., & Levi-Schaffer, F. (2005). Human peripheral blood eosinophils induce angiogenesis. *International Journal of Biochemistry & Cell Biology, 37*(3), 628–636.
66. Takakura, N., Watanabe, T., Suenobu, S., Yamada, Y., Noda, T., Ito, Y., et al. (2000). A role for hematopoietic stem cells in promoting angiogenesis. *Cell, 102*(2), 199–209.
67. Hagemann, T., Lawrence, T., McNeish, I., Charles, K. A., Kulbe, H., Thompson, R. G., et al. (2008). "Re-educating" tumor-associated macrophages by targeting NF-kappaB. *Journal of Experimental Medicine, 205*(6), 1261–1268.
68. Qian, B., Deng, Y., Im, J. H., Muschel, R. J., Zou, Y., Li, J., et al. (2009). A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. *PLoS One, 4*(8), e6562.
69. Corthay, A., Skovseth, D. K., Lundin, K. U., Rosjo, E., Omholt, H., Hofgaard, P. O., et al. (2005). Primary antitumor immune response mediated by CD4+ T cells. *Immunity, 22*(3), 371–383.
70. Mantovani, A., Sica, A., & Locati, M. (2007). New vistas on macrophage differentiation and activation. *European Journal of Immunology, 37*(1), 14–16.
71. Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. *Nature Reviews. Immunology, 8*(12), 958–969.
72. Wynn, T. A. (2004). Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nature Reviews. Immunology, 4*(8), 583–594.
73. Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., & Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology, 25*(12), 677–686.

74. Andreu, P., Johansson, M., Affara, N. I., Pucci, F., Tan, T., Junankar, S., et al. (2010). FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell, 17*, 121–134.
75. Kapsenberg, M. L., & Kalinski, P. (1999). The concept of type 1 and type 2 antigen-presenting cells. *Immunology Letters, 69*(1), 5–6.
76. Fridlender, Z. G., Sun, J., Kim, S., Kapoor, V., Cheng, G., Ling, L., et al. (2009). Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell, 16*(3), 183–194.
77. Hagemann, T., Wilson, J., Burke, F., Kulbe, H., Li, N. F., Pluddemann, A., et al. (2006). Ovarian cancer cells polarize macrophages toward a tumor-associated phenotype. *Journal of Immunology, 176*(8), 5023–5032.
78. Corthay, A. (2007). CD4+ T cells cooperate with macrophages for specific elimination of MHC class II-negative cancer cells. *Advances in Experimental Medicine and Biology, 590*, 195–208.
79. Newman, K. C., & Riley, E. M. (2007). Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. *Nature Reviews. Immunology, 7*(4), 279–291.
80. Szekanecz, Z., & Koch, A. E. (2007). Macrophages and their products in rheumatoid arthritis. *Current Opinion in Rheumatology, 19*(3), 289–295.
81. Tiemessen, M. M., Jagger, A. L., Evans, H. G., van Herwijnen, M. J., John, S., & Taams, L. S. (2007). CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. *Proceedings of the National Academy of Sciences of the United States of America, 104*(49), 19446–19451.
82. Dilillo, D. J., Matsushita, T., & Tedder, T. F. (2010). B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. *Annals of the New York Academy of Sciences, 1183*, 38–57.
83. Mizoguchi, A., & Bhan, A. K. (2006). A case for regulatory B cells. *Journal of Immunology, 176*(2), 705–710.
84. Tan, T. T., & Coussens, L. M. (2007). Humoral immunity, inflammation and cancer. *Current Opinion in Immunology, 19* (2), 209–216.
85. Inoue, S., Leitner, W. W., Golding, B., & Scott, D. (2006). Inhibitory effects of B cells on antitumor immunity. *Cancer Research, 66*(15), 7741–7747.
86. Shah, S., Divekar, A. A., Hilchey, S. P., Cho, H. M., Newman, C. L., Shin, S. U., et al. (2005). Increased rejection of primary tumors in mice lacking B cells: inhibition of anti-tumor CTL and TH1 cytokine responses by B cells. *International Journal of Cancer, 117*(4), 574–586.
87. Barbera-Guillem, E., Nelson, M. B., Barr, B., Nyhus, J. K., May, K. F., Jr., Feng, L., et al. (2000). B lymphocyte pathology in human colorectal cancer. Experimental and clinical therapeutic effects of partial B cell depletion. *Cancer Immunology and Immunotherapy, 48*(10), 541–549.
88. Zapata, J. M., Llobet, D., Krajewska, M., Lefebvre, S., Kress, C. L., & Reed, J. C. (2009). Lymphocyte-specific TRAF3 transgenic mice have enhanced humoral responses and develop plasmacytosis, autoimmunity, inflammation, and cancer. *Blood, 113*(19), 4595–4603.
89. Qin, Z., Richter, G., Schuler, T., Ibe, S., Cao, X., & Blankenstein, T. (1998). B cells inhibit induction of T cell-dependent tumor immunity. *Nature Medicine, 4*(5), 627–630.
90. Schreiber, H., Wu, T. H., Nachman, J., & Rowley, D. A. (2000). Immunological enhancement of primary tumor development and its prevention. *Seminars in Cancer Biology, 10*(5), 351–357.
91. Wijesuriya, R., Maruo, S., Zou, J. P., Ogawa, M., Umehara, K., Yamashita, M., et al. (1998). B cell-mediated down-regulation of IFN-gamma and IL-12 production induced during anti-tumor immune responses in the tumor-bearing state. *International Immunology, 10*(8), 1057–1065.
92. Barbera-Guillem, E., May, K. F., Jr., Nyhus, J. K., & Nelson, M. B. (1999). Promotion of tumor invasion by cooperation of granulocytes and macrophages activated by anti-tumor antibodies. *Neoplasia, 1(5), 453–460.*
93. Coussens, L. M., Hanahan, D., & Arbeit, J. M. (1996). Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. *American Journal of Pathology, 149*(6), 1899–1917.
94. Ammirante, M., Luo, J. L., Grivennikov, S., Dedospasov, S., & Karin, M. (2010). B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. *Nature, 464*, 302–306.
95. Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57–70.
96. Colotta, F., Allavena, P., Sica, A., Garlanda, C., & Mantovani, A. (2009). Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis, 30*(7), 1073–1081.

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The Bcl-2 repertoire of mesothelioma spheroids underlies acquired apoptotic multicellular resistance

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Three-dimensional (3D) cultures are a valuable platform to study acquired multicellular apoptotic resistance of cancer. We used spheroids of cell lines and actual tumor to study resistance to the proteasome inhibitor bortezomib in mesothelioma, a highly chemoresistant tumor. Spheroids from mesothelioma cell lines acquired resistance to bortezomib by failing to upregulate Noxa, a pro-apoptotic sensitizer BH3-only protein that acts by displacing Bim, a pro-apoptotic Bax/Bak-activator protein. Surprisingly, despite their resistance, spheroids also upregulated Bim and thereby acquired sensitivity to ABT-737, an inhibitor of anti-apoptotic Bcl-2 molecules. Analysis using BH3 profiling confirmed that spheroids acquired a dependence on anti-apoptotic Bcl-2 proteins and were 'primed for death'. We then studied spheroids grown from actual mesothelioma. ABT-737 was active in spheroids grown from those tumors (5/7, ~70%) with elevated levels of Bim. Using immunocytochemistry of tissue microarrays of 48 mesotheliomas, we found that most (33, 69%) expressed elevated Bim. In conclusion, mesothelioma cells in 3D alter the expression of Bcl-2 molecules, thereby acquiring both apoptotic resistance and sensitivity to Bcl-2 blockade. Mesothelioma tumors *ex vivo* also show sensitivity to Bcl-2 blockade that may depend on Bim, which is frequently elevated in mesothelioma. Therefore, mesothelioma, a highly resistant tumor, may have an intrinsic sensitivity to Bcl-2 blockade that can be exploited therapeutically.

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Resistance to apoptosis may underlie the chemoresistance of tumors.[1,2] Apoptotic resistance and chemoresistance, however, may not be fully reflected in two-dimensional (2D) cell cultures (monolayers). Indeed, when cancer cells are grown as three-dimensional (3D) spheroids, they acquire multicellular resistance that mimics the chemoresistance observed *in vivo* and can effectively recapitulate some of the complexity of solid tumors.[3–5]

Understanding multicellular resistance may provide key insights into effective therapies for recalcitrant solid tumors such as human malignant mesothelioma,[6] which usually presents at a late stage as a thick tumor mass.[7] At this time, few effective therapeutic options are available for mesothelioma patients and survival remains poor. Our group[8] and others[9] have found that mesothelioma spheroids acquire multicellular resistance to a variety of treatments.[8,9] We decided to focus on resistance to bortezomib (PS-341, Velcade), an agent that has shown promise in pre-clinical studies of mesothelioma[10,11] and is now being evaluated in clinical trials. Although it is not yet known whether mesothelioma will exhibit resistance to bortezomib, bortezomib resistance, while rare in hematologic cancers,[12] has been a common problem in solid tumors.[13]

Bortezomib, a proteasome inhibitor, is thought to induce apoptosis via the alteration of expression of Bcl-2 proteins.[14] Thus, resistance to bortezomib may also manifest itself in changes in the balance of the pro- and anti-apoptotic Bcl-2 molecules. If so, new experimental approaches, such as Bcl-2 homology domain 3 (BH3)-profiling, may be used to uncover the anti-apoptotic barriers mediating resistance. With BH3-profiling, the cellular dependence on anti-apoptotic proteins can be decoded based on mitochondrial sensitivity to a panel of BH3 peptides.[15] In addition, using mechanistic approaches such as these, it has been seen that anti-apoptotic resistance mechanisms may coexist with a high level of pro-apoptotic potential, a situation termed 'primed for death'. Tumors identified as 'primed for death' may respond to inhibition of the anti-apoptotic defenses with small molecules such as ABT-737, an inhibitor of Bcl-2/X_L/w. Probing of the anti-apoptotic strategies of 3D spheroids may reveal vulnerabilities that can be sought in tumors as well.

Here, we have investigated the multicellular resistance and the apoptotic priming that develops in mesothelioma cells grown in 3D spheroids. By using Bcl-2 inhibitors and BH3-profiling, we have probed the anti-apoptotic defenses of multicellular spheroids and also of human mesothelioma

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Keywords: mitochondria; 3D; chemotherapy; BH3-profiling; bortezomib
Abbreviations: TFS, tumor fragment spheroid; 2D, two-dimensional; 3D, three-dimensional; EPP, extrapleural pneumonectomy; TMA, tissue microarray; R8, 8 arginine-repeat peptide; BH3, Bcl-2 homology domain 3
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grown as tumor fragment spheroids (TFS). This study presents for the first time key insights into the apoptotic repertoire of 3D spheroids and proposes ways to detect the pro-apoptotic potential within mesothelioma and to exploit it therapeutically.

Results

Mesothelioma cells acquire resistance to bortezomib when grown as 3D spheroids. We first studied two mesothelioma cell lines, M28 and REN, previously reported to be sensitive to bortezomib-induced apoptosis[10] and known to form spheroids.[8] Monolayers and spheroids were treated with increasing doses of bortezomib (1–100 nM) for 48 h. Monolayers displayed an $IC50^{(48h)}$ of approximately 18 nM (M28) and 9 nM (REN), whereas spheroids exhibited resistance, with an approximate $IC50^{(48h)}$ of 100 nM (M28 and REN) (Figure 1a).

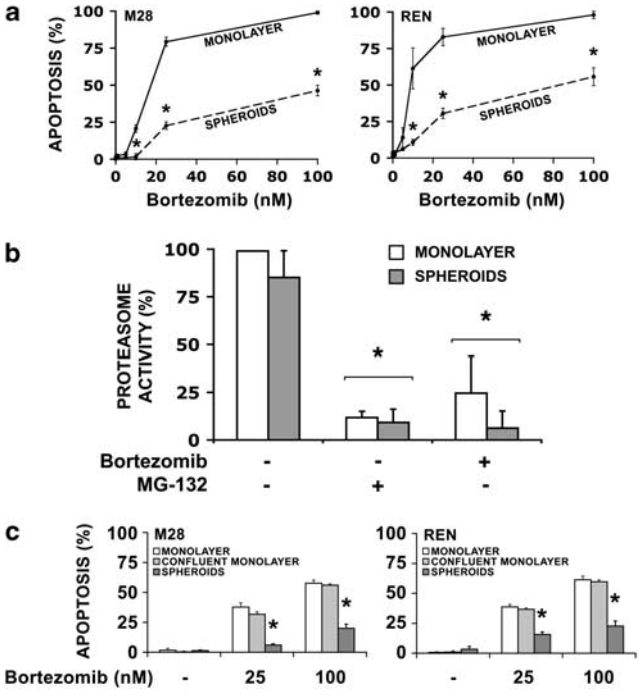

Figure 1 Spheroids grown from mesothelioma cell lines acquire multicellular resistance to bortezomib. (**a**) M28 and REN monolayers and spheroids were treated with bortezomib 1, 5,10, 25 or 100 nM for 48 h. Apoptosis was measured in Hoechst-stained cells; cells with signs of nuclear condensation were considered apoptotic. Spheroids grown from both cell lines acquired a marked multicellular resistance to bortezomib even at the highest concentrations. (*$P<0.05$ spheroids compared with monolayers, $n=3$). (**b**) REN monolayers and spheroids were treated with bortezomib (100 nM) or MG-132 (10 μM), a potent proteasome inhibitor, for 4 h before cells were lysed and analyzed for proteasomal activity. Monolayers and spheroids showed similar proteasomal activity at baseline and were both equally inhibited by bortezomib or MG-132 after 4 h. (*$P<0.05$ compared with untreated control, $n=3$). (**c**) M28 and REN monolayers at standard confluence (70%), completely confluent monolayers (obtained by plating twice the number of cells to achieve at least 100% confluence) and spheroids were treated with bortezomib (25 or 100 nM). Completely confluent monolayers showed an apoptotic response comparable to monolayers and did not acquire multicellular resistance. (*$P<0.05$ spheroids compared with monolayers and confluent monolayers, $n=3$)

Resistance was not due to differences of proteasome activity at baseline or after bortezomib. We found that the proteasomal chymotryptic activity of monolayers and spheroids of REN cells was equal at baseline and equally inhibited following exposure to bortezomib or to another proteasome inhibitor, MG-132, for 4 h (Figure 1b).

Resistance was not a function of high cell density, as has been proposed.[16] We found that completely confluent monolayers did not acquire resistance to bortezomib (Figure 1c), but were as responsive to bortezomib as the subconfluent monolayers.

In spheroids, pro-apoptotic Noxa is not upregulated by bortezomib whereas Bim is high. The proteasome recycles >80% of the total protein content of cells[17] and thereby mediates the activity of key cellular functions such as apoptosis. Bortezomib-induced apoptosis has been attributed to the accumulation of the pro-apoptotic BH3-only protein Bim and the induction of other pro-apoptotic sensitizers such as Noxa.[14,18–20] We asked whether bortezomib might alter these apoptotic triggers differently in spheroids and, if so, whether such differences might underlie spheroids' acquired multicellular resistance.

Using immunoblotting, we analyzed the expression of key Bcl-2 proteins in monolayers and spheroids before and after treatment with bortezomib (Figure 2). In monolayers exposed to bortezomib, Noxa was strongly upregulated. In spheroids exposed to bortezomib, however, Noxa failed to increase.

In addition, spheroids upregulated Bim expression at baseline and following bortezomib (Figure 2). Intriguingly, spheroids, despite their increased apoptotic resistance, expressed more pro-apoptotic Bim than did monolayers. Because Noxa functions indirectly by displacing Bim from anti-apoptotic Mcl-1, we asked whether addition of Noxa to the spheroids could restore apoptotic sensitivity.

A Noxa peptide reduces multicellular resistance acquired by spheroids. To confirm that Noxa contributed to the apoptosis induced by bortezomib in the monolayers, we reduced Noxa expression by siRNA. Silencing successfully reduced bortezomib-induced Noxa upregulation, without alteration of other Bcl-2 molecules, Mcl-1 or Bim (Figure 3a). The reduction in Noxa significantly reduced apoptosis in monolayers following bortezomib, suggesting an important role for Noxa in mediating bortezomib-induced apoptosis (Figure 3a). We then attempted to restore Noxa in spheroids using a $Noxa^{BH3}$ peptide bound to 8 arginine residues ($R8\text{-}Noxa^{BH3}$), a modification that aids diffusion of the peptide across cell membranes.[21,22] Although an R8 control peptide had no activity, $R8\text{-}Noxa^{BH3}$ enhanced the apoptotic response of the spheroids, thereby significantly reducing the multicellular resistance of spheroids to bortezomib (Figure 3b). In addition, $R8\text{-}Noxa^{BH3}$ alone induced apoptosis in REN spheroids. To determine whether $R8\text{-}Noxa^{BH3}$ peptide had off-target effects, we ablated Mcl-1 by siRNA. Because Noxa activity requires Mcl-1, we would expect $R8\text{-}Noxa^{BH3}$ to have no activity in the absence of Mcl-1. Without Mcl-1, $R8\text{-}Noxa^{BH3}$ lost all apoptotic activity indicating that, at

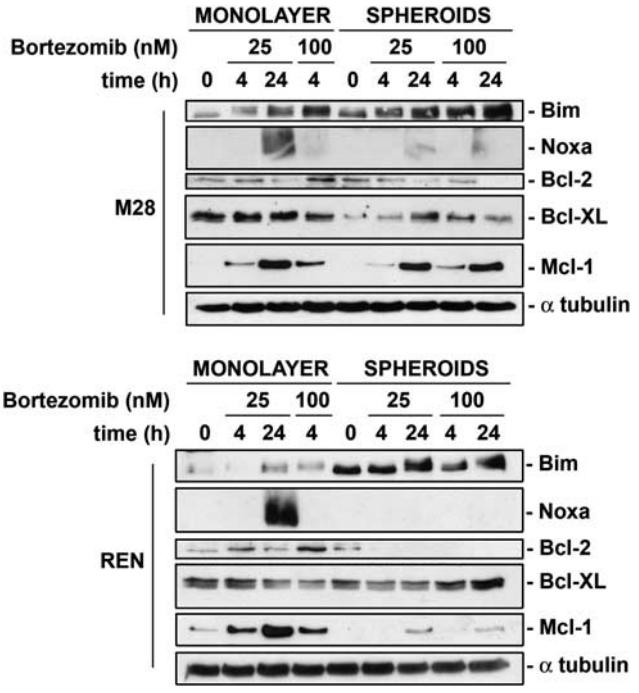

Figure 2 Spheroids fail to upregulate Noxa after bortezomib but have a high expression of Bim. M28 and REN monolayers and spheroids were treated with bortezomib (25 or 100 nM) for 4 or 24 h. Whole cell lysates (50 μg) were resolved on SDS-PAGE and immunoblotted for a panel of pro-/anti-apoptotic Bcl-2 proteins. Both M28 and REN monolayers upregulated Noxa after bortezomib (25 nM) at 24 h. (Owing to the high degree of apoptosis, monolayers treated with 100 nM bortezomib at 24 h were not collected.) Spheroids, however, failed to upregulate Noxa, even after bortezomib 100 nM at 24 h. Interestingly, at baseline and during exposure to bortezomib, spheroids expressed more Bim protein than monolayers. (Representative of three experiments)

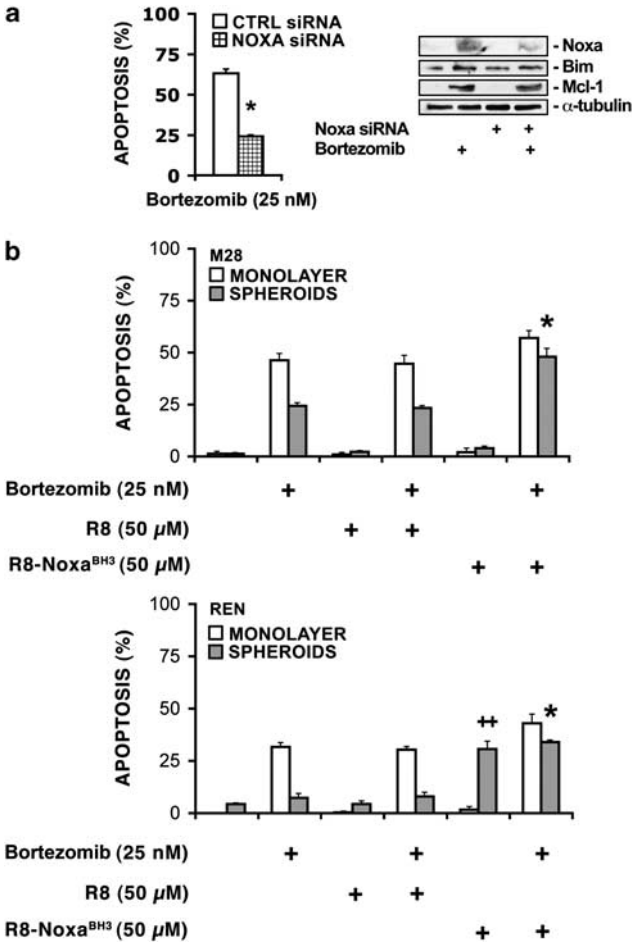

Figure 3 A Noxa peptide sensitizes spheroids to bortezomib-induced apoptosis. (**a**) Noxa was silenced in REN cells and, 24 h later, cells were plated as monolayers and treated with bortezomib (25 nM). After 4 h, cells were collected and lysed to verify the efficacy of Noxa siRNA (see immunoblot). No changes in other Bcl-2 proteins, Mcl-1 and Bim, were detected upon Noxa silencing, with or without bortezomib. After 24 h, Hoechst-stained cells were counted for the presence of apoptosis. Noxa siRNA significantly reduced the apoptosis induced by bortezomib. ($^*P<0.05$, $n=3$). (**b**) M28 and REN monolayers and spheroids were treated with bortezomib (25 nM), a cell-permeable R8-Noxa^{BH3} peptide (50 μM) or a control R8 peptide (50 μM), either alone or in combination, for 24 h. R8-Noxa^{BH3}, but not the R8 peptide, eliminated the acquired multicellular resistance of spheroids to bortezomib without affecting monolayers. Interestingly, REN spheroids, but not M28, were sensitive to R8-Noxa^{BH3} alone. ($^*P<0.05$ compared with untreated and R8 control; $^{++}P<0.05$ spheroids *versus* monolayers, $n=3$)

least in these cells at the concentrations used, Noxa peptide activity was specifically targeted to Mcl-1 (Supplementary Figure 1).

The activity of the Noxa peptide suggested that the lack of Noxa upregulation in spheroids contributed to the multicellular resistance to bortezomib. Nevertheless, Noxa, as a BH3-only sensitizer, depends on the presence of a pro-apoptotic Bax/Bak-activator such as Bim. The high expression of Bim in the spheroids suggested that the spheroids were primed for apoptotic death and thus vulnerable to other approaches designed to release Bim.

ABT-737 reduces multicellular resistance of spheroids. We asked whether ABT-737, a small molecule that inhibits Bcl-2/X_L/w, would be effective in spheroids by unmasking the pro-apoptotic potential revealed by the R8-Noxa^{BH3} peptide. Indeed ABT-737 (1 μM) increased the apoptotic response to bortezomib of spheroids as well as of monolayers and reversed the multicellular resistance of spheroids (Figure 4). Moreover, ABT-737 alone induced apoptosis of both M28 and REN spheroids, confirming that spheroids are 'primed for death'[15] and dependent on their anti-apoptotic defenses for survival. To confirm the role of Bim in the activity of ABT-737, we reduced Bim expression using siRNA. Knockdown of Bim abolished the ability of ABT-737 by itself to induce apoptosis in spheroids and significantly reduced its ability to enhance bortezomib-induced apoptosis (Figure 5a).

Unlike Bcl-2/X_L/w, Mcl-1 is not inhibited by ABT-737 and is known to mediate resistance to ABT-737.[23,24] We ablated Mcl-1 using siRNA to determine its contribution to resistance in spheroids. Ablation of Mcl-1 increased the apoptotic responses to ABT-737 and to bortezomib (Figure 5b). In fact, inhibition of both Bcl-2/X_L/w (by ABT-737) and Mcl-1 (by siRNA) increased the apoptosis in M28 and REN spheroids, to 100 and 65% of total cells, respectively, even in the absence of bortezomib.

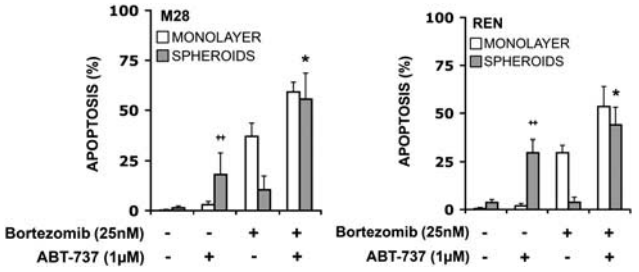

Figure 4 ABT-737 eliminates multicellular resistance to bortezomib. M28 and REN monolayers and spheroids were treated with bortezomib (25 nM) and ABT-737 (1 μM), either alone or in combination, for 24 h. ABT-737 eliminated the acquired multicellular resistance of spheroids to bortezomib. Interestingly, when given alone, ABT-737 was able to induce apoptosis in spheroids but not in monolayers. (*$P<0.05$ compared with bortezomib alone; $^{++}P<0.05$ spheroids *versus* monolayers, $n=3$)

ABT-737 has been shown in some settings to alter levels of Bcl-2 proteins,[25–27] including Mcl-1. To address this issue, we grew REN cells as monolayers and spheroids and exposed them to ABT-737 alone or to ABT-737 together with bortezomib for 6 h. During that time, selected to avoid the changes induced by apoptosis, we found no significant change in the levels of two Bcl-2 proteins, Mcl-1 and Bim (Supplementary Figure 2).

To determine whether ABT-737 or bortezomib acts on the benign counterpart of mesothelioma, we studied normal mesothelial cells obtained from benign human ascites. Because benign mesothelial cells *in vivo* exist as single cell monolayer and *in vitro* do not form spheroids as readily as do malignant cells, we studied them as monolayers. Normal mesothelial cells did not undergo apoptosis following bortezomib, ABT-737 alone or bortezomib plus ABT-737 (Supplementary Figure 3A). The normal cells also did not express Bim, even following bortezomib treatment for 6 h (Supplementary Figure 3B).

Spheroids are 'primed for death'. Mesothelioma cells in 3D spheroids thus appeared to acquire apoptotic resistance along with an underlying sensitivity to apoptosis. We used BH3-profiling to confirm these findings in four cell lines (two epithelioid lines, M28 and REN and two sarcomatous lines, SARC and VAMT). For these studies, cells from monolayers and spheroids were disaggregated identically to single cells for flow cytometry and probed with a panel of BH3 peptides or small molecules such as ABT-737 for the loss of mitochondrial potential indicative of apoptosis.[15]

Cells derived from monolayers and spheroids were equally sensitive to the Bim BH3 peptide, confirming an equal ability to activate the apoptotic program (Figure 6). However, compared with cells from monolayers, cells from spheroids were more sensitive to sensitizer BH3-only molecules, Bad and Hrk, targeting Bcl-2 and Bcl-X_L, respectively. In addition, cells from spheroids were confirmed to be more sensitive to ABT-737, which had no effect on the cells from monolayers.

Because cells from spheroids of the two additional mesothelioma cell lines, VAMT and SARC, were found to be sensitive to pro-apoptotic sensitizers by BH3-profiling, we

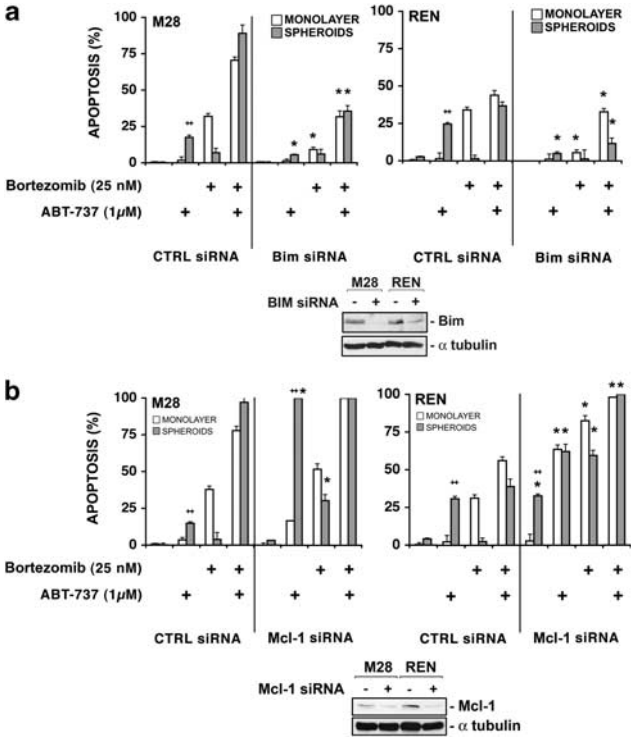

Figure 5 ABT-737 sensitization of spheroids to bortezomib is dependent on Bim and aided by ablation of Mcl-1. (**a**) Bim was ablated in M28 and REN cells and, 24 h later, cells were plated to generate monolayers and spheroids, exposed to bortezomib 25 nM and/or ABT-737 1 μM for 24 h and lysed and probed for Bim at 72 h after siRNA (see immunoblot insert), the time when cells were counted for the presence of apoptosis. The absence of Bim in spheroids completely abolished the apoptotic effect of ABT-737 when given alone and significantly reduced the effect when given with bortezomib. (*$P<0.05$ compared with control siRNA, $^{++}P<0.05$ spheroids *versus* monolayers, $n=3$). (**b**) Mcl-1 was ablated, as performed for Bim above (see immunoblot insert). The absence of Mcl-1 reduced the acquired multicellular resistance of spheroids to bortezomib. The absence of Mcl-1 also increased the apoptotic response to ABT-737 when given alone or in combination with bortezomib. (*$P<0.05$ compared with control siRNA, $^{++}P<0.05$ spheroids *versus* monolayers, $n=3$)

anticipated that they would also show sensitivity to BH3-only sensitizers when tested in cell culture. Indeed, spheroids of both cell lines were sensitive to ABT-737- and R8-Noxa^{BH3}-induced apoptosis, even in the absence of bortezomib (Figure 7a). When spheroids from all four cell lines were compared, the M28 spheroids appeared to have less sensitivity, with no response to R8-Noxa^{BH3} given alone (see also Figure 3b). We then confirmed that the expression levels of Bim increased in each cell line when grown as spheroids. Of interest, the increase in Bim levels as estimated by the ratio of densitometry values was less in M28 (1.9-fold increase) when compared with the more sensitive lines (3.2–4.5-fold increase) (Figure 7b and Supplementary Figure 4)

ABT-737 has activity against human mesothelioma grown as TFS. The sensitivity of multicellular spheroids to Bcl-2 blockade with ABT-737 raised the intriguing possibility that human mesothelioma tumor cells in their 3D environment might also be sensitive. Using a human tumor

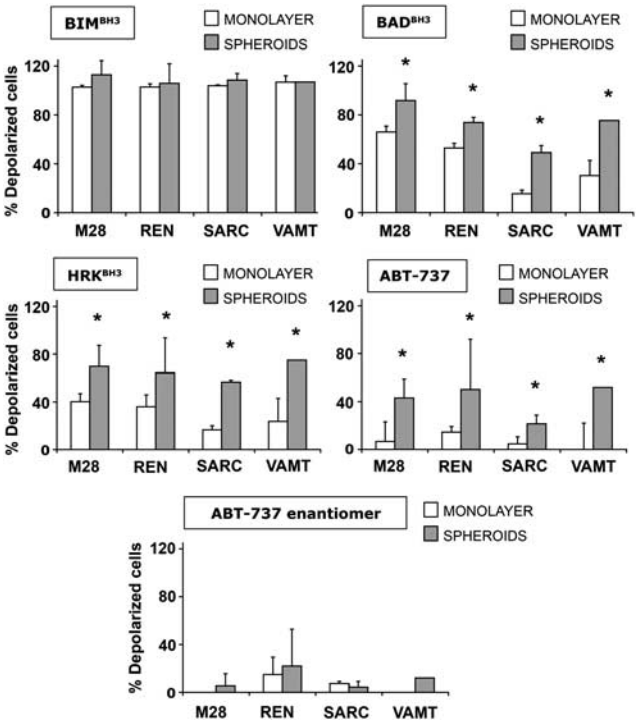

Figure 6 Spheroids are 'primed for death'. BH3-profiling was performed on cells disaggregated from monolayers and spheroids grown from M28, REN, SARC and VAMT cells. The cells were gently permeabilized as described[39] and exposed to BH3 peptides (100 μM) for 90 min before JC1 was added for 30 min and depolarization measured as a percentage of the total. Cells from spheroids and monolayers were equally depolarized by Bim^{BH3}, the positive control, confirming a functioning apoptotic apparatus. However, cells disaggregated from spheroids were more sensitive to Bad^{BH3}, Hrk ^{BH3} and ABT-737 than cells from monolayers, confirming that cells in 3D acquired apoptotic priming. Cells showed no response to an ABT-737 inactive enantiomer, used as negative control. (*$P<0.05$ compared with monolayers, $n=3$)

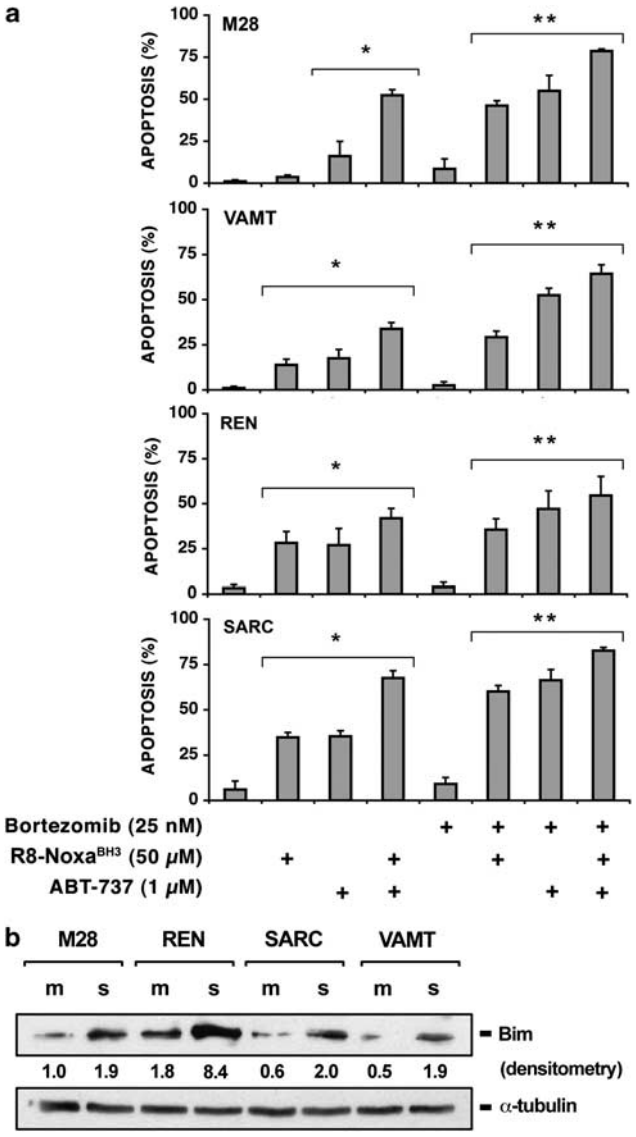

Figure 7 Apoptotic priming of spheroids increases with elevated Bim expression. (**a**) Spheroids grown from M28, VAMT, REN and SARC cell lines were treated with R8-Noxa^{BH3} (50 μM), ABT-737 (1 μM), bortezomib (25 nM) or their combination for 24 h. Whereas R8-Noxa^{BH3} alone failed to induce apoptosis in M28 spheroids, ABT-737 alone induced apoptosis in the spheroids generated from all four cell lines. Nevertheless, when given with bortezomib, either R8-Noxa^{BH3} or ABT-737 was effective in increasing apoptosis of spheroids. (*$P<0.05$ compared with untreated spheroids, **$P<0.05$ compared with bortezomib alone, $n=3$). (**b**) Immunoblot analysis of Bim expression in M28, REN, SARC and VAMT monolayers and spheroids with densitometry shown for Bim, standardized for α-tubulin expression. Immunoblot and densitometry values are representative of three separate experiments. In all four cell lines, spheroids expressed increased Bim levels. Compared with monolayers, spheroids of M28 displayed a 1.9-fold increase in Bim levels whereas spheroids of REN, SARC and VAMT showed a 4.5-, 3.2- and 4.0-fold, increase, respectively

fragment model we previously developed for *ex vivo* studies,[5] we tested spheroids grown from seven mesothelioma tumors for responsiveness to ABT-737. In tumor fragments from five of the tumors, ABT-737 did sensitize the mesothelioma cells to bortezomib-induced apoptosis (Figures 8a and b). In two cases, there was no response to ABT-737 ($<5\%$ apoptosis), either when ABT-737 was given alone or together with bortezomib. We then asked whether the level of Bim expression could explain the different responses to ABT-737 in these tumors. Using immunohistochemistry of a tissue microarray (TMA) comprising the original tumor samples from which the TFS were derived, we found that the responsive tumors showed high Bim expression compared with normal pleura, whereas the unresponsive tumors expressed Bim at low or undetectable levels, comparable to normal pleura (Figure 8c). The possibility that Bim could represent a predictive biomarker for ABT-737 activity in mesothelioma prompted us to investigate the frequency of Bim overexpression in mesothelioma tumors. Using immunohistochemistry of 48 mesothelioma samples and 5 normal pleural samples included on a tumor TMA and assessed using an image analysis system, we found that 33 (69%) of mesothelioma tumors expressed Bim at levels higher than in the normal tissues. Elevated Bim expression was found in most tumors of the epithelioid (20 of 22) and mixed subtype (12 out of 21) but was uncommon in the sarcomatous subtype (only 1 of 5).

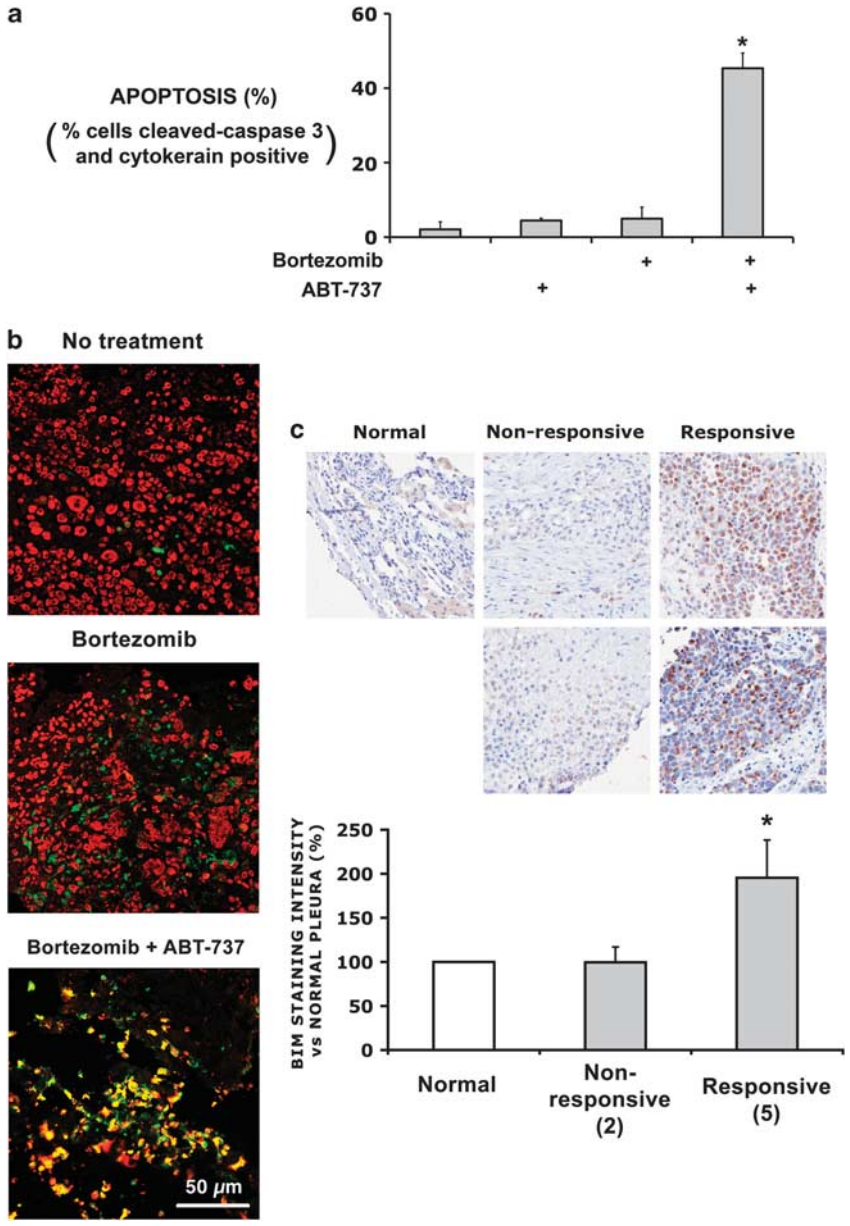

Figure 8 ABT-737 increases activity of bortezomib against mesothelioma cells in spheroids derived from tumors with high expression of Bim. (**a**) TFS generated from seven tumors were treated with bortezomib (50 nM), ABT-737 (2.5 μM) or their combination for 24 h. In the responsive tumors ($n=5$) (shown), ABT-737 increased apoptosis when given together with bortezomib. In unresponsive tumors ($n=2$) (not shown), ABT-737 with or without bortezomib had no effect (apoptosis $<5\%$), ($^*P<0.05$ compared with no treatment, bortezomib alone or ABT-737 alone, $n=5$). (**b**) Confocal images representative of the responsive tumor fragment spheroids treated with bortezomib (50 nM) with or without ABT-737 (2.5 μM) for 24 h. Pan-cytokeratin (red) was used to identify mesothelioma cells within the spheroids and cleaved caspase-3 (green) to identify apoptosis. The merged color (yellow) identifies apoptotic mesothelioma cells. The mesothelioma cells are resistant to ABT-737 alone (not shown) and to bortezomib alone. The addition of ABT-737 to bortezomib, however, effectively increased apoptosis of the human mesothelioma cells. (**c**) Using a TMA generated with tissue from the original seven tumors tested for apoptosis along with normal pleural tissue as control, Bim expression was determined by immunohistochemistry and quantified using an imaging analysis system (ScanscopeXT, Aperio). Every sample was represented three times on the slide. Normal pleura and the two tumors not responsive to ABT-737 and bortezomib showed comparable, very low amounts of Bim. Conversely, the responsive tumors showed strong Bim staining. Intensity of staining was expressed as percentage compared with normal pleura. ($^*P<0.05$ compared with normal and non-responsive)

Discussion

Understanding how cancer cells attain chemoresistance can provide clues for designing new therapeutic strategies. Because cancer cells in 3D cultures acquire a multicellular resistance that resembles the chemoresistance seen in solid tumors *in vivo*, 3D models may be a useful platform for investigating novel therapeutic approaches. In this study, as in previous studies in our laboratory, we investigated resistance mechanisms using 3D models generated from cell lines that we then tested for relevance in tumor grown *ex vivo*.[8,28] We were particularly interested in applying this approach to

bortezomib, a therapy that has shown less efficacy in solid tumors than in non-solid tumors such as multiple myeloma, perhaps because of resistance mechanisms seen only in 3D. Although 2D models have been used to elucidate resistance to bortezomib,[29] here we show for the first time the additional multicellular resistance mechanisms that develop in 3D. Compared with the same cells in 2D, mesothelioma cells in 3D express a different balance of Bcl-2 pro- and anti-apoptotic molecules and acquire a dual nature: on the one hand, they become resistant to bortezomib; on the other hand, they become dependent on anti-apoptotic Bcl-2 defenses and therefore sensitive if the anti-apoptotic mechanisms are neutralized. In the *ex vivo* human mesothelioma tumor model, we found that blockade of the Bcl-2 anti-apoptotic molecules enhanced the apoptotic response to bortezomib, at least in tumors with overexpression of the pro-apoptotic BH3-only molecule Bim. The response of the *in vitro* and *ex vivo* models to inhibition of anti-apoptotic Bcl-2 members supports a new, clinically relevant approach to this highly chemoresistant tumor.

Mesothelioma cells in 3D acquired a resistance to bortezomib that was initially identified by an inability to upregulate Noxa, the pro-apoptotic BH3-only molecule that interacts with Mcl-1 to release Bim.[30] In many systems, Noxa has been shown to mediate bortezomib activity,[31–33] a role we confirmed in the mesothelioma monolayers. In spheroids, the addition of a R8-Noxa^{BH3} peptide was able to restore bortezomib-induced apoptosis to the same level as in the monolayers, suggesting that the lack of Noxa upregulation contributed to the bortezomib resistance in 3D. Because Noxa acts indirectly by releasing Bim, we suspected that the effect of Noxa depended on Bim and, as expected, the ablation of Bim decreased the activity of Noxa. However, we were surprised at the high level of Bim in the 3D spheroids, especially given their chemoresistance. It suggested that the Bim was sequestered and held in check by anti-apoptotic buffering and that, despite apoptotic resistance, spheroids would be poised for apoptosis if the Bim could be released. Indeed, the small molecule, ABT-737, an inhibitor of Bcl-2/X_L/w, was able to undermine the bortezomib resistance of spheroids and, in some cell lines, to induce apoptosis in spheroids by itself. In fact, when used as single agents in the resistant spheroids, ABT-737 was more effective than bortezomib.

By BH3-profiling, we confirmed that the cells disaggregated from the spheroids had acquired sensitivity to ABT-737 and to peptides that displace Bim from Bcl-2 and Bcl-X_L. Of interest, the apoptotic priming was acquired as the malignant cells moved to 3D, a feature that has not previously been described. Analogous to what has been proposed for cancer in general, spheroids acquired resistance to apoptosis along with a latent pro-apoptotic potential that could be harnessed therapeutically.[34]

Mcl-1 can confer resistance to ABT-737 as well as to bortezomib,[31–33] and can be a major resistance factor in 3D spheroids, as we have previously shown in lung cancer.[35] Interestingly, in our studies in mesothelioma, the presence of Mcl-1 did not prevent the response to ABT-737 although the reduction of Mcl-1 (by R8-Noxa^{BH3} or by Mcl-1 siRNA) did increase the response to ABT-737, as has been previously shown.[23] Thus, Mcl-1 appeared to blunt, but not block, the activity of ABT-737 and the combined inactivation of both Mcl-1 and the Bcl-2/X_L/w was more effective than inhibition of either one alone. Mcl-1 also appeared to have a smaller role when the increase in Bim levels was lower. For example, R8-Noxa^{BH3} was less effective than ABT-737 in the cell line with the lowest increase in Bim (M28). Therefore, we speculate that Bim may be preferentially sequestered by Bcl-2/X_L/w, as has been described,[36] with additional buffering by Mcl-1 as Bim increases. Nonetheless, Mcl-1 represents a potential barrier to the use of ABT-737 clinically. Thus, the effectiveness of ABT-737 in combination with bortezomib or other therapies may be enhanced by agents that reduce Mcl-1.[23,25,26]

Why Bim increases in the 3D spheroids is not yet known; nonetheless, the finding that Bim increases in mesothelioma cells in 3D represents a unique and potentially important observation. Bim was essential for the response to ABT-737, as demonstrated by the Bim siRNA experiments, and the level of Bim correlated with sensitivity to ABT-737 in the multicellular spheroids and in the tumor fragments. Bim may thus be a useful predictive biomarker for the response of mesothelioma to ABT-737 together with bortezomib. Almost 70% of mesotheliomas were found to express Bim at levels higher than in normal tissue. Interestingly, Bim overexpression was frequent in the more chemosensitive subtype (epithelioid, 90%) and uncommon in the more chemoresistant subtype (sarcomatoid, 20%). Unleashing the pro-apoptotic molecule Bim using small molecule inhibitors such as ABT-737 may enhance the effectiveness of current chemotherapy in this recalcitrant tumor.

TFS were developed to investigate therapeutic approaches in a clinically relevant *ex vivo* setting.[5] When tested in TFS, ABT-737 was also effective, but only in spheroids derived from tumors with a high expression of Bim. Thus, by virtue of studies in these complementary 3D models, we have identified a potential therapy, ABT-737, and identified a possible biomarker for predicting response to ABT-737 in this tumor, Bim. Further studies will be necessary to confirm the validity of these predictions. Nonetheless, we think that agents able to upregulate Bim, such as vorinostat,[37] could be potent adjuncts to current chemotherapies.

There are potential clinical benefits to targeting tumors at the level of their anti-apoptotic defenses. Most targeted therapies have focused on impairing signaling pathways on which cancers depend for survival.[38] However, the efficacy of these approaches may be impaired by intracellular signaling redundancy, crosstalk, adaptation and differing levels of activating/silencing mutations. Direct inhibitors of anti-apoptotic defenses could bypass the need to inhibit multiple pathways by moving to a distal level at which multiple signals are integrated. Our data suggest that such an approach may be effective in the different mesothelioma subtypes, against a variety of apoptotic therapies, and without injury to normal tissues.

In sum, using 3D culture models, we have shown that multicellular resistance to bortezomib is mediated by alterations in the Bcl-2 repertoire, which provides a druggable target specific to the malignant cells. Inhibition of the anti-apoptotic buffers in association with chemotherapy represents a promising strategy for the treatment of mesothelioma.

Materials and Methods

Cell cultures and reagents. The human mesothelioma cell lines M28, REN, VAMT and SARC were cultured in DMEM supplemented with 10% FBS and 100 IU/ml penicillin–streptomycin in a 37 °C humidified incubator with 5% CO_2 (full DMEM). Primary human mesothelial cells were cultured from ascites fluid from patients without infection or malignancy according to a protocol approved by the UCSF Committee on Human Research. TFS were generated as previously described [28]. Tumor samples were obtained from extrapleural pneumonectomy (EPP) or pleurectomy procedures performed by DJS and RB at Brigham and Women's Hospital in Boston, MA, USA.

R8-Noxa^{BH3} (RRRRRRRR-EVECATQLRRFGDKLNFRQKL) and R8 (RRRRRRRR) peptides were from Genscript (Piscataway, NJ, USA). Bortezomib (Velcade) was from Millennium Pharmaceuticals (Cambridge, MA, USA). ABT-737 was generously provided from Abbott Pharmaceuticals (Abbott Park, IL, USA).

Generation and treatment of spheroids

Multicellular spheroids. Multicellular spheroids were generated in non-adsorbent round-bottomed 96-well plates. The 96-well plates were coated with a 1 : 24 dilution of polyHEMA (120 mg/ml) (#P3932, Sigma-Aldrich, St. Louis, MO, USA) in 95% ethanol and dried at 37 °C for 48 h. Before use, plates were sterilized by UV light for 30 min. For generation of multicellular spheroids, 10 000 cells were added into each well of polyHEMA-coated 96-well plate. The plates were briefly spun for 5 min at 800 r.p.m. and then placed in a 37 °C humidified incubator with 5% CO_2 for 24 h. For generation of monolayers, 180 000 cells were added into each well of six-well plates.

Tumor fragment spheroids. TFS were grown from seven tumor samples obtained from surgical resection performed at the Brigham and Women's Hospital (Boston, MA, USA). A part of the tumor was fixed in 10% formalin (Fisher Scientific, Fair Lawn, NJ, USA) and embedded in paraffin. For spheroid culture, tumor tissue was diced finely with scalpels to pieces smaller than 1 mm in diameter that were suspended in medium in 10-cm plates coated with 0.8% agar (Agar Noble, #A5431, Sigma-Aldrich) in full DMEM. The volume of overlay media was 15 ml, and half the volume of the overlay media was changed twice a week. The agar-coated plates were regularly observed using an inverted phase microscope during the incubation period, up to 4 weeks. Spheroids were collected at different time points, treated as described in figure legends, fixed in 10% formalin and embedded in paraffin for immunostaining.

Treatment. Before treatment, 18 multicellular spheroids or 20–30 TFS were transferred to each well of a polyHEMA-coated 24-well plate to match the numbers of cells plated as monolayers (180 000 cells per well). The spheroids and monolayers were treated with apoptotic agents in full DMEM with or without inhibitors (and the appropriate DMSO vehicle control) for 24 h.

Immunoblotting. After treatment, monolayers and spheroids were lysed in boiling lysis buffer (2.5% SDS, Tris-HCl 250 mM pH 7.4). The concentration of total protein was evaluated with a colorimetric assay (DC protein assay from Bio-Rad, Hercules, CA, USA). In all, 50 μg of cell lysates were loaded in reducing conditions (0.2 M Tris, pH 6.8, 5% SDS, 3% glycerol, 0.01% bromophenol blue and 200 mM DTT). After separation in SDS-PAGE (5 to 15% acrylamide) and transfer to PVDF (Immobilon, Millipore, Billerica, MA, USA), membranes were blocked with a protein-free TBS blocking buffer (Pierce, Rockford, IL, USA) and gently agitated with antibodies diluted in 5% non-fat dry milk or 5% BSA, as appropriate, at 4 °C overnight. Secondary antibodies were from Amersham (Piscataway, NJ, USA). Chemiluminescence was detected with the enhanced SuperSignal West Pico Substrate (Pierce). The antibodies against Bcl-2 (#2872) and Bcl-X_L (#2764) were from Cell Signaling Technology (Beverly, MA, USA). The Mcl-1 (sc-819) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the Bim antibody (#559685) was from BD Pharmingen (San Jose, CA, USA), and the Noxa antibody was from Calbiochem (#OP180, San Diego, CA, USA). The α-tubulin antibody (#T-6074) was from Sigma-Aldrich.

Proteasome activity assay. Proteasome activity in REN spheroids and monolayers was measured using a commercial 20S proteasome activity assay kit (APT280, Millipore Corporation). In brief, spheroids and monolayers were lysed with proteasome activity buffer (50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl and 1% Triton X-100). The lysates were incubated with the fluorogenic substrate LLVY-AMC, a fluorophore 7-amino-4-methylcoumarin (AMC) bound with LLVY peptide, at 37 °C for 2 h. LLVY is a substrate recognized and cleaved by the 20S proteasome. After LLVY chymotryptic cleavage by the 20S proteasome, AMC is released and emits fluorescence that can be read by using a 380 nm excitation and a 460 nm emission filter in a fluorometer.

RNA interference. Cells (4×10^6) were pelleted and resuspended in 100 μl of nucleofection buffer (solution T, Amaxa Biosystems, Cologne, Germany) with 1.5 μg of the appropriate siRNA duplex (Ambion, Austin, TX, USA). A non-targeting siRNA sequence was utilized as control in all experiments. This suspension was transferred to a sterile cuvette and nucleofected using program T-20 on a Nucleofector II device (Amaxa Biosystems). After 30-min recovery in complete DMEM medium, the cells were plated and allowed to grow for 24 h. Cells were then trypsinized, counted and plated as monolayers and spheroids for 24 h, and exposed to apoptotic stimuli. The siRNA sequences were: Bim (5′-ACUUACAUCAGAAG GUUGCtt-3′), Mcl-1 (5′-CCAGUAUACUUCUUAGAAAtt-3′) and Noxa (5′-GAAAU GUGUCAAUAAUUACtt-3′), non-targeting control (5′-GCAACCUUCUGAUGUAA GUtt-3′).

BH3 profiling. Experiments were performed at the Dana Farber Cancer Institute (Boston, MA, USA) by JA Ryan and AG Letai as previously published.[39] Monolayers and spheroids formed from equal numbers of cells were disaggregated in an identical fashion to single cells. The cells were gently permeabilized as described[39] and exposed to BH3 peptides (100 μM) for 90 min before JC1 was added for 30 min and depolarization measured as a percentage of the total.

Hoechst staining for measurement of apoptosis in multicellular spheroids. Monolayers and spheroids were disaggregated by exposure to trypsin for the same period of time, washed with ice-cold PBS, and then fixed with 2.5% glutaraldehyde (Sigma-Aldrich). The cells were then stained with 8 μg/ml of Hoechst 33342 (Molecular Probes, Invitrogen, Carlsbad, CA, USA) and placed on slides. For each condition, at least 300 cells were counted in triplicate by investigators blinded to the experimental conditions. Cells with distinctive signs of nuclear condensation were considered apoptotic.

Confocal analysis for measurement of apoptosis in TFS. In all, 5 μm paraffin sections were deparaffinized with Xylene (2×5 min), 100% EtOH (2×2 min), 95% EtOH (2×2 min), 70% EtOH (2×2 min), 50% EtOH (1×2 min) and ddH20 (2×2 min). Endogenous peroxidases were blocked with a solution of 250 ml MeOH + 5 ml 30% H_20_2 for 20 min. Antigens were retrieved in citrate buffer (Citra #HK087-5K, Biogenex, Fremont, CA, USA) in a microwave oven set to high for 7–8 min. Sections were blocked with a 5% normal goat serum, 2.5% BSA in PBS for 30–45 min in humidified chamber at RT. Primary antibodies for cleaved caspase 3 (1:100, #AB3623, Chemicon, Billerica, MA, USA) and pan-cytokeratin (1 : 100, Progen, Heidelberg, Germany; clone GP14) were incubated in a humidified chamber at 4 °C overnight. The secondary antibodies donkey anti-rabbit AlexaFluor 488 (Pierce; #31821) and anti-guinea pig AlexaFluor 633 (Invitrogen; #A-21105), both 1 : 200, were incubated for 30–45 min at RT in a humidified chamber. Slides were washed three times in PBS for 3 min and mounted with Vectashield. In a blinded fashion, the investigators examined images of doubly stained slides. Apoptotic mesothelioma cells were considered cells that had merged red (pan-cytokeratin) and green (cleaved caspase 3) and were expressed as a percentage of the total cells (DAPI-stained nuclei). For each condition, 3–10 spheroids were counted until a total of 300 DAPI-stained cells were visualized.

Bim immunohistochemistry of TMAs. Two TMAs were studied. One included the seven mesothelioma tumors that were studied as TFS for their apoptotic response and two normal pleural samples. The second included 48 mesothelioma tumors and 5 normal pleural samples. All samples were obtained without identifiers from surgeries performed at the Brigham and Women's Hospital and at UCSF Medical Center and were fixed in formalin and assembled in a TMA. Each sample was embedded in triplicate. The histopathology was determined on separate formalin-fixed, hematoxylin- and eosin-stained sections of the tumor. In all, 5 μm paraffin sections of the TMA were processed as described for the confocal analysis of apoptosis. Bim antibody (1 : 150, 4 °C overnight) was visualized with a HRP/DAB Envision + Kit (Dako, Carpinteria, CA, USA; #K4010). Intensity of Bim staining was measured by the Positive Pixel Count algorithm on a ScanscopeXT system (Aperio Technologies, Inc., Vista, CA, USA), which determined the average intensity of pixels within the tissue region of interest.

Statistical analysis. Data are expressed as mean ± 1 S.D. of at least three different experiments. Statistical significance was evaluated by one- or two-way

analysis of variance, and Tukey's test was performed to detect where the differences lay (GraphPad Prism v 4.0, GraphPad Software, Inc., La Jolla, CA, USA). A *P*-value <0.05 was considered significant.

Conflict of Interest

The authors declare no conflict of interest.

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1. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002; **108**: 153–164.
2. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
3. Smalley KS, Lioni M, Herlyn M. Life isn't flat: taking cancer biology to the next dimension. *In Vitro Cell Dev Biol Anim* 2006; **42**: 242–247.
4. Pampaloni F, Reynaud EG, Stelzer EH. The third dimension bridges the gap between cell culture and live tissue. *Nat Rev Mol Cell Biol* 2007; **8**: 839–845.
5. Kim KU, Wilson SM, Abayasiriwardana KS, Collins R, Fjellbirkeland L, Xu Z *et al.* A novel *in vitro* model of human mesothelioma for studying tumor biology and apoptotic resistance. *Am J Respir Cell Mol Biol* 2005; **33**: 541–548.
6. Fennell DA, Gaudino G, O'Byrne KJ, Mutti L, van Meerbeeck J. Advances in the systemic therapy of malignant pleural mesothelioma. *Nat Clin Pract Oncol* 2008; **5**: 136–147.
7. Fennell DA, Rudd RM. Defective core-apoptosis signalling in diffuse malignant pleural mesothelioma: opportunities for effective drug development. *Lancet Oncol* 2004; **5**: 354–362.
8. Barbone D, Yang T, Morgan J, Gaudino G, Broaddus V. Mammalian target of rapamycin contributes to the acquired apoptotic resistance of human mesothelioma multicellular spheroids. *J Biol Chem* 2008; **283**: 13021–13030.
9. Daubriac J, Fleury-Feith J, Kheuang L, Galipon J, Saint-Albin A, Renier A *et al.* Malignant pleural mesothelioma cells resist anoikis as quiescent pluricellular aggregates. *Cell Death Differ* 2009; **16**: 1146–1155.
10. Sartore-Bianchi A, Gasparri F, Galvani A, Nici L, Darnowski JW, Barbone D *et al.* Bortezomib inhibits nuclear factor-kappaB dependent survival and has potent *in vivo* activity in mesothelioma. *Clin Cancer Res* 2007; **13**: 5942–5951.
11. Gordon GJ, Mani M, Maulik G, Mukhopadhyay L, Yeap BY, Kindler HL *et al.* Preclinical studies of the proteasome inhibitor bortezomib in malignant pleural mesothelioma. *Cancer Chemother Pharmacol* 2008; **61**: 549–558.
12. Shah JJ, Orlowski RZ. Proteasome inhibitors in the treatment of multiple myeloma. *Leukemia* 2009; **23**: 1964–1979.
13. Scagliotti GV, Germonpre P, Bosquee L, Vansteenkiste J, Gervais R, Planchard D *et al.* A randomized phase II study of bortezomib and pemetrexed, in combination or alone, in patients with previously treated advanced non-small-cell lung cancer. *Lung Cancer* 2010; **68**: 420–426.
14. Fennell DA, Chacko A, Mutti L. BCL-2 family regulation by the 20S proteasome inhibitor bortezomib. *Oncogene* 2008; **27**: 1189–1197.
15. Certo M, Del Gaizo Moore V, Nishino M, Wei G, Korsmeyer S, Armstrong SA *et al.* Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* 2006; **9**: 351–365.
16. Butcher DT, Alliston T, Weaver VM. A tense situation: forcing tumour progression. *Nat Rev Cancer* 2009; **9**: 108–122.
17. Dalton WS. The proteasome. *Semin Oncol* 2004; **31**(6 Suppl 16): 3–9; discussion 33.
18. Fribley AM, Evenchik B, Zeng Q, Park BK, Guan JY, Zhang H *et al.* Proteasome inhibitor PS-341 induces apoptosis in cisplatin-resistant squamous cell carcinoma cells by induction of Noxa. *J Biol Chem* 2006; **281**: 31440–31447.
19. Perez-Galan P, Roue G, Villamor N, Montserrat E, Campo E, Colomer D. The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status. *Blood* 2006; **107**: 257–264.
20. Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND *et al.* ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* 2007; **129**: 1337–1349.
21. Futaki S. Oligoarginine vectors for intracellular delivery: design and cellular-uptake mechanisms. *Biopolymers* 2006; **84**: 241–249.
22. Kloss A, Henklein P, Siele D, Schmolke M, Apcher S, Kuehn L *et al.* The cell-penetrating peptide octa-arginine is a potent inhibitor of proteasome activities. *Eur J Pharm Biopharm* 2009; **72**: 219–225.
23. van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE *et al.* The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* 2006; **10**: 389–399.
24. Yecies D, Carlson NE, Deng J, Letai A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. *Blood* 2010; **115**: 3304–3313.
25. Bhat UG, Pandit B, Gartel AL. ARC synergizes with ABT-737 to induce apoptosis in human cancer cells. *Mol Cancer Ther* 2010; **9**: 1688–1696.
26. Kang MH, Wan Z, Kang YH, Sposto R, Reynolds CP. Mechanism of synergy of N-(4-hydroxyphenyl)retinamide and ABT-737 in acute lymphoblastic leukemia cell lines: Mcl-1 inactivation. *J Natl Cancer Inst* 2008; **100**: 580–595.
27. Hauck P, Chao BH, Litz J, Krystal GW. Alterations in the Noxa/Mcl-1 axis determine sensitivity of small cell lung cancer to the BH3 mimetic ABT-737. *Mol Cancer Ther* 2009; **8**: 883–892.
28. Wilson SM, Barbone D, Yang TM, Jablons DM, Bueno R, Sugarbaker DJ *et al.* mTOR mediates survival signals in malignant mesothelioma grown as tumor fragment spheroids. *Am J Respir Cell Mol Biol* 2008; **39**: 576–583.
29. Zhang L, Littlejohn JE, Cui Y, Cao X, Peddaboina C, Smythe WR. Characterization of bortezomib-adapted I-45 mesothelioma cells. *Mol Cancer* 2010; **9**: 110.
30. Han J, Goldstein LA, Hou W, Rabinowich H. Functional linkage between NOXA and Bim in mitochondrial apoptotic events. *J Biol Chem* 2007; **282**: 16223–16231.
31. Gomez-Bougie P, Wuilleme-Toumi S, Menoret E, Trichet V, Robillard N, Philippe M *et al.* Noxa up-regulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. *Cancer Res* 2007; **67**: 5418–5424.
32. Woo HN, Seo YW, Moon AR, Jeong SY, Choi EK, Kim TH. Effects of the BH3-only protein human Noxa on mitochondrial dynamics. *FEBS Lett* 2009; **583**: 2349–2354.
33. Baou M, Kohlhaas SL, Butterworth M, Vogler M, Dinsdale D, Walewska R *et al.* Role of NOXA and its ubiquitination in proteasome inhibitor-induced apoptosis in chronic lymphocytic leukemia cells. *Haematologica* 2010; **95**: 1510–1518.
34. Lowe SW, Cepero E, Evan G. Intrinsic tumour suppression. *Nature* 2004; **432**: 307–315.
35. Yang TM, Barbone D, Fennell DA, Broaddus VC. Bcl-2 family proteins contribute to apoptotic resistance in lung cancer multicellular spheroids. *Am J Respir Cell Mol Biol* 2009; **41**: 14–23.
36. Chen S, Dai Y, Pei XY, Grant S. Bim upregulation by histone deacetylase inhibitors mediates interactions with the Bcl-2 antagonist ABT-737: evidence for distinct roles for Bcl-2, Bcl-xL, and Mcl-1. *Mol Cell Biol* 2009; **29**: 6149–6169.
37. Dai Y, Chen S, Venditti CA, Pei XY, Nguyen TK, Dent P *et al.* Vorinostat synergistically potentiates MK-0457 lethality in chronic myelogenous leukemia cells sensitive and resistant to imatinib mesylate. *Blood* 2008; **112**: 793–804.
38. Reinhardt HC, Jiang H, Hemann MT, Yaffe MB. Exploiting synthetic lethal interactions for targeted cancer therapy. *Cell Cycle* 2009; **8**: 3112–3119.
39. Ryan JA, Brunelle JK, Letai A. Heightened mitochondrial priming is the basis for apoptotic hypersensitivity of CD4+ CD8+ thymocytes. *Proc Natl Acad Sci USA* 2010; **107**: 12895–12900.

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NON-NEOPLASTIC AND NEOPLASTIC PLEURAL ENDPOINTS FOLLOWING FIBER EXPOSURE

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Exposure to asbestos fibers is associated with non-neoplastic pleural diseases including plaques, fibrosis, and benign effusions, as well as with diffuse malignant pleural mesothelioma. Translocation and retention of fibers are fundamental processes in understanding the interactions between the dose and dimensions of fibers retained at this anatomic site and the subsequent pathological reactions. The initial interaction of fibers with target cells in the pleura has been studied in cellular models in vitro and in experimental studies in vivo. The proposed biological mechanisms responsible for non-neoplastic and neoplastic pleural diseases and the physical and chemical properties of asbestos fibers relevant to these mechanisms are critically reviewed. Understanding mechanisms of asbestos fiber toxicity may help us anticipate the problems from future exposures both to asbestos and to novel fibrous materials such as nanotubes. Gaps in our understanding have been outlined as guides for future research.

TRANSLOCATION AND RETENTION OF FIBERS IN THE PLEURA

Anatomy and Physiology of the Pleura

The parietal pleura lines the chest wall and the superior surface of the diaphragm and the visceral pleura covers the lungs (Figure 1). The pleural space in humans contains a small amount of fluid (0.1–0.2 ml/kg body weight) that is a filtrate from the underlying systemic circulation (Owens & Milligan, 1995; Broaddus, 2008). This space (10–20 μm wide) is lined by a single layer of mesothelial cells resting on a basement membrane and underlying connective tissue and blood vessels. The major routes of drainage of fluid, protein, particulates, and cells from the pleural space are through the lymphatic stomata that open between mesothelial cells on the parietal pleural lining (Hammar, 1994; Wang, 1975; Broaddus et al., 1988).

Effusions, an accumulation of excess liquid in the pleural space, are common features of a multitude of diseases. Transudative effusions, those not associated with inflammation or injury, usually develop due to increased

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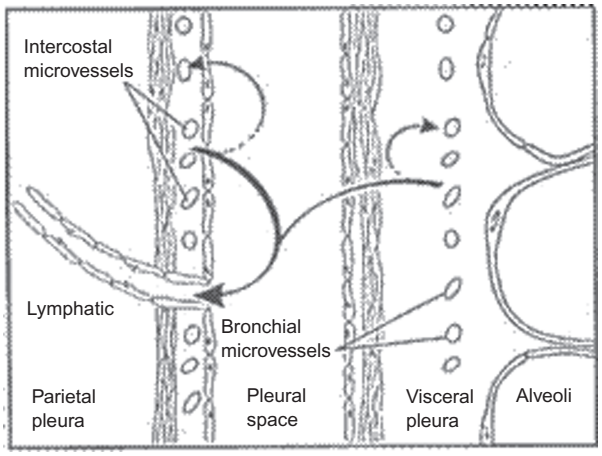

FIGURE 1. Fluid turnover and lymphatic drainage from the pleural space. In the normal pleural space (shown here), as in other interstitial spaces of the body, liquid slowly filters from systemic capillaries and is absorbed via lymphatics (solid arrows). In the pleural space, the capillary filtrate from systemic capillaries moves across a permeable pleural membrane toward the lower pressure pleural space and is absorbed via the parietal pleural lymphatics. From there, liquid moves via lymphatic propulsion to the central veins. When interstitial edema forms in the adjacent lung, some of that excess liquid moves across the visceral pleura into the pleural space. Asbestos fibers may follow similar routes from the lung to the pleura and are thought to lodge in the parietal pleura preferentially at sites of lymphatic drainage.

hydrostatic pressure. In congestive heart failure, the most common cause of transudative effusions, increased pulmonary venous pressure leads to fluid accumulation in the interstitial spaces of the lung; the fluid then moves toward the lower pressure pleural space and leaks across the visceral pleura into the pleural space (Broaddus et al., 1990; Owens & Milligan, 1995). In the setting of inflammation or injury of the lung, pleura, or other organs, exudative effusions may form; these effusions contain elevated levels of protein due to the increased leakage across capillaries with increased permeability (Mutsaers et al., 2004). Excess fluid in any part of the body may find its way to the pleural space via the interstitial tissues along pressure gradients and by moving across the permeable pleural membranes. The normal and pathological paths by which liquid, cells, and particles enter and exit the pleural space suggest pathways by which asbestos fibers may also enter and exit or fail to exit the pleural space. The study of the physiology of the pleural space is challenging; even when using laboratory animal studies, analyses of the pleural space are limited by the narrowness of the space and the difficulty in sampling without inducing inflammation or injury.

Pathways Leading to Translocation of Fibers to the Pleura

The route of translocation of fibers from the lungs to the visceral pleura, into the pleural space, and to the parietal pleura is unknown. It is postulated that asbestos fibers may migrate to the lung interstitium and visceral pleura by a paracellular route or by direct penetration across injured alveolar epithelial cells (Miserocchi et al., 2008). Fibers may be transported to the pleural space via the lymphatics and bloodstream (Oberdörster et al., 1983). Fibers may translocate by themselves or within macrophages. Although studies of asbestos fiber movement have not been possible due to technical limitations, it is likely that asbestos fibers translocate to the pleural space passively in the same manner as interstitial fluid. This process may be enhanced by lung inflammation induced by asbestos fibers or by mixed dust exposures that increase interstitial fluid accumulation and thus fluid movement along the interstitial spaces to the pleural space (Miserocchi et al., 2008).

There are thus few studies that investigated the translocation of fibers from the lung into the pleural space. Even in the few existing studies, data from animal studies may have limited

relevance for humans because of the different visceral pleural anatomy in rodents. In the rodent, the visceral pleura is "thin," consisting mostly of a mesothelial layer and basement membrane lying directly over the alveoli. There is little submesothelial connective tissue and no pleural vasculature. In sheep and humans and other large mammals, the visceral pleura is "thick" and has a significant submesothelial connective tissue space, containing nerves and systemic blood vessels (Figure 1). In contrast to the visceral pleura, the parietal pleura in different species has a constant and similar anatomy (Figure 1; Light & Broaddus, 2010). Thus, due to differences in the visceral pleura, one might postulate a difference between rodents and humans in the movement of the fibers into the pleural space. Due to similarities in the parietal pleura, one might suggest that the localization, accumulation, and actions of fibers in the parietal pleura might be similar.

These questions have been almost impossible to address using current technology but it is hoped that new tools and imaging techniques such as nuclear magnetic resonance (NMR) spectroscopy or two-photon microscopy can be developed to provide data on (1) how fibers distribute in the lungs and pleura, (2) the ultimate destination of fibers, (3) how fiber movement is enhanced, and (4) whether fibers are translocated and retained differently in animals and humans. Such techniques could be invasive, using labeled fibers that could be traced, for use in animal studies, and noninvasive for clinical studies of those exposed to asbestos. This imaging information on fiber localization would enhance diagnosis and follow-up of subjects exposed to asbestos, such as in directing where to sample tissues to assess fiber dosimetry, and how to determine preneoplastic biomarkers (Greillier et al., 2008) that might lead to intervention and prevention of non-neoplastic and neoplastic pleural diseases.

Pleural Fiber Dosimetry in Rodents

Translocation and retention of fibrous particulates from initial sites of pulmonary deposition to extrapulmonary sites are believed to be important aspects of their potential toxicity (Dodson et al., 2003; Suzuki & Kohyama, 1991). Pathologic tissue responses such as edema, inflammation, or fibrosis might potentially affect translocation and retention of particulates in the body, as well as properties of particles themselves including dose, dimensions and biopersistence. Although similarities exist between animal models and humans concerning physiological processes such as interstitial fluid dynamics and lymphatic flow, there are also anatomical differences such as in visceral pleural thickness (Tyler, 1983), as well as physiological differences such as of macrophage size and function, that need to be taken into account when comparing across animal species and when extrapolating from animals to humans (Jarabek et al., 2005; Maxim & McConnell, 2001). Rodents and humans also differ in particle respirability (Mossman et al., 2011) and this limits the use of rodent models for human risk assessment based on fiber dimensions (Lippmann & Schlesinger, 1984; Lippmann et al., 1980).

Biopersistence of fibers in the lung parenchyma also influences the fiber dose that is ultimately translocated to the pleura. Biopersistence in the lung is dependent on (1) site and rate of deposition, (2) pulmonary clearance parameters, (3) solubility in lung fluids, (4) breakage rate and patterns, and (5) rates of fiber translocation and retention. Surface chemistry and diameter are important determinants of solubility. Much of the knowledge base concerning the role of biopersistence is actually derived from studies of synthetic vitreous fibers (Bernstein, 2007; Oberdörster, 2000).

Fiber characteristics also affect clearance from the lung and translocation to the pleura. Macrophage-mediated particle clearance in the lung is likely to influence translocation of particles to interstitial sites. There are important interspecies differences in particle clearance, as well as in biological effects of high pulmonary concentrations of particles, in humans and in different animal species (Bermudez et al., 2002; Oberdörster, 2002). In addition, the method of dose administration in experimental

animals is shown to influence pleural pathology outcomes following particle exposure. In silica-exposed rats, pleural granulomas developed in animals following inhalation, but not after instillation, and the different response was likely due to differences in kinetics of particle delivery and lymphatic clearance (Henderson et al., 1995).

The effects of asbestos may be altered when asbestos is mixed with other particulates, a situation common in occupational and environmental exposures. Studies by Davis and colleagues (1991) showed that coexposure of rats to amosite asbestos and to quartz increased the incidence of amosite-induced pleural mesothelioma, presumably by elevation in fiber dosimetry and translocation through the visceral pleura. Recent studies by Bernstein and colleagues (2008) demonstrated that coexposure of chrysotile asbestos together with nonfibrous particulates decreased fiber retention in the lungs of rats, perhaps by increasing macrophage recruitment and macrophage-mediated clearance or by inducing more inflammatory fluid movement to the pleura.

Fiber translocation in rodents appears to be rapid and may be responsible in some cases in particular for pathologic outcomes. In rats, short chrysotile asbestos fibers are found in the pleural space within a week following intratracheal instillation (Viallat et al., 1986). Similarly, crocidolite fibers were detected in the pleural space 1 wk following inhalation (Choe et al., 1997). In another study in rats, short fibers (<5 μm length) were found 5 d after inhalation exposure to a synthetic vitreous fiber (refractory ceramic fiber) aerosol (Gelzleichter et al., 1996). In studies in rats and hamsters involving chronic inhalation of synthetic vitreous fibers as well as of amosite and chrysotile asbestos used as reference materials, significant interspecies differences in pleural pathology were seen (Mast et al., 1994; McConnell, 1994). Subsequent short-term mechanistic studies of translocation showed that the Syrian golden hamster, a species prone to development of pleural fibrosis and mesothelioma following synthetic vitreous fiber exposure, displayed greater translocation of fibers to the pleura than did similarly exposed rats (Gelzleichter et al., 1999). The greater translocation in the Syrian golden hamster may thus have accounted for its greater susceptibility to fiber-induced toxicity.

Pleural Fiber Dosimetry in Humans

There is virtually no knowledge of the kinetics of fiber translocation and retention in the human pleura and there are few pleural fiber burden studies in occupationally exposed workers. In addition, due to loss of anatomical orientation after ashing or digestion of target tissues, it is not known where fibers reside intracellularly or extracellularly. Fibers are identified within mesothelial cells (Davis, 1974; Fasske, 1986; Lee et al., 1993) but, due to technical limitations, no comprehensive studies have quantified intracellular fiber burden at the microscopic level. Although analytical transmission electron microscopy (TEM) with x-ray energy-dispersive analysis is the gold standard for quantitation and identification of asbestos fibers in tissue, in vivo studies would be enhanced greatly by nondestructive imaging approaches that could detect the presence of fibers, their chemical composition, or even the cellular response without destroying anatomical relationships.

A major data gap in understanding mechanisms of asbestos-related pleural disease is the paucity of information available to determine the dose of asbestos fibers that is deposited and retained in the pleural membranes. This overview describes the technical complexity and limitations associated with quantitation of human lung and pleural fiber burdens, as well as summarizing available data.

Roggli (1990, 1992), Roggli and Sharma (2004), and Dodson and Atkinson (2006) reviewed the numerous variables and technical considerations associated with quantitation of tissue fiber burdens in general. Their major conclusions and caveats include:

1. The source of tissue samples ranged from pleural biopsies obtained during diagnostic thoracoscopy (Boutin & Rey, 1993); to surgical specimens including needle biopsies, wedge biopsies, or pneumonectomy or pleural decortication samples; and to pleural and lung tissues obtained during autopsy examination (Roggli & Sharma, 2004).
2. Regardless of the source of tissue, sampling is a potential source of error since there is significant variation in anatomical distribution of fibers, especially in the parietal pleura (Roggli, 1992; Boutin et al., 1996; Mitchev, et al., 2002).
3. Tissues may be contaminated during surgical resection or at autopsy due to fibers present in fixatives, in specimen containers, on surgical gloves, or on dissecting instruments (Roggli & Sharma, 2004).
4. Light microscopy is inadequate for identification and counting of asbestos fibers. Dodson and Atkinson (2006) recommend analytical transmission electron microscopy in combination with x-ray energy-dispersive analysis and selected area diffraction techniques for specific mineralogical identification. Both coated and uncoated fibers, as well as particulates, should be analyzed and quantitated (Dodson & Atkinson, 2006).
5. A systematic approach to counting fibers of all dimensions and analysis of lung fiber burdens needs to be used, as described by the European Respiratory Society (DeVuyst et al., 1988).
6. Appropriate control populations need to be used because there is significant variability in human lung fiber burdens (Roggli, 1990). A systematic analysis of lung asbestos fiber burdens in workers with asbestos-related disease, people with asbestos exposure in households or in buildings, and control cases revealed a wide range of counts with considerable overlap between workers, other asbestos-exposed cases, and controls (Roggli & Sharma, 2004).
7. The criteria used to define and count asbestos fibers need to be stated explicitly. Some investigators only count fibers longer than 5 μm; however, the majority of asbestos fibers in human tissue samples are shorter than 5 μm (Dodson & Atkinson, 2006).
8. Tissue preparation techniques may introduce artifacts due to tissue drying or traumatic disruption of fiber bundles (Dodson & Atkinson, 2006).

Finally, although quantitation of human lung and pleural asbestos fiber burden is the only technique available to assess the dose delivered to and retained at the target tissue, there are additional considerations in interpretation of these data. Tissue fiber burden depends on the time since cessation of exposure. In addition, the fiber burden and the types of fibers in the lung may not reflect the fiber burden in the pleura. For example, shorter uncoated fibers are more readily cleared from the lungs; however, while these fibers may be decreasing in the lungs, they may be accumulating in the pleura and extrapulmonary sites (Holt, 1981) and be associated with development of disease at these sites (Dodson & Hammer, 2006; Dodson & Atkinson, 2006).

It is important to note that the lungs of normal control cases evaluated at autopsy contain significant numbers of commercial and noncommercial asbestos fibers, as well as other particulate and fibrous minerals. This is noteworthy especially in lungs from those who resided in urban settings (Table 1). By comparing lung fiber burdens between those with pleural mesothelioma and those without, investigators showed that, although there is overlap, there is an increased risk for mesothelioma, with an elevated lung burden of certain fibers, such as crocidolite, amosite, and tremolite; due to its lower biopersistence, chrysotile may not be reliably analyzed by autopsy studies (Table 2).

In contrast to these and other studies of fiber burdens in the lung, only a few studies have reported asbestos fiber burdens in the pleura. In those few studies that analyzed pleural fiber burden, the results from lung and

TABLE 1. Asbestos Fiber Content in Lung Tissue of an Urban Population

Fiber type	Fiber number/g wet lung
Chrysotile asbestos fibers	130.0×10^3
Antigorite	2.5×10^3
Noncommercial amphiboles:	
Tremolite	15.0×10^3
Actinolite	5.1×10^3
Anthophyllite	3.7×10^3
Commercial amphiboles:	
Amosite and crocidolite	1.1×10^3

Note. Analysis of 21 urban cases using analytical transmission electron microscopy with analysis of all fibers longer than 1 μm revealed these average fiber numbers/g wet lung. (Churg & Warnock, 1980).

TABLE 2. Lung Fiber Burdens in Malignant Mesothelioma Patients

Fiber type	Percent of patients with fibers	Percent of controls with fibers
Chrysotile	80	67
Tremolite	20	11
Crocidolite	59	16
Amosite	81	40
Other:		
Mullite	98	98
Iron	88	65
Rutile	83	79
Muscovite	61	65
Silica	55	65

Note. In a study of young persons (age 50 yr or less at the time of diagnosis), the lungs of 69 patients who had died with malignant pleural mesothelioma and 57 controls selected from the national work-related disease surveillance system in the United Kingdom were analyzed by electron microscopy for fiber distribution. Increased odds ratios for mesothelioma were found for crocidolite, amosite, and tremolite; the contribution of chrysotile was less clear due to low biopersistence. Nonasbestos fibers probably made no contribution to mesothelioma in this study (McDonald et al., 2001).

pleura differed, perhaps due to the technical problems described earlier, and appeared to indicate that pleura has a predominance of short chrysotile fibers. Sebastien et al. (1980) concluded that lung fiber burden could not be used as an accurate reflection of pleural fiber burden. In their parietal pleural samples, most of the asbestos fibers were short chrysotile fibers. Gibbs et al. (1991) also reported lower asbestos counts in the visceral pleura than in matched lung samples from the same patients and found mostly short chrysotile asbestos fibers.

Dodson et al. (1990) analyzed lung tissue, lymph nodes, and pleural plaques obtained at autopsy from eight shipyard workers in Italy. Data showed both chrysotile and amphibole asbestos fibers in the lungs; however, chrysotile asbestos fibers were the most frequent type of asbestos found in pleural plaques. Most fibers in the lymph nodes and pleural plaques were shorter than 5 μm, although some fibers longer than 8 μm were present at these sites. More recently, Suzuki and his coworkers (2005) compared asbestos fiber burdens of human mesothelioma tissues obtained following bulk tissue digestion or ashing of 25-μm tissue sections using high-resolution analytical electron microscopy. The majority of fibers were $\leq$5 μm long and 92.7% were $\leq$0.25 μm wide. Chrysotile asbestos fibers were identified most frequently in a total of 168 cases of human malignant mesotheliomas obtained from biopsy or autopsy specimens (Suzuki et al., 2005). In an earlier study, Suzuki and Yuen (2001) detected only short, thin chrysotile asbestos fibers in 25.7% of the lungs and in 77.4% of the mesothelial tissues of patients with malignant mesothelioma. These tissue samples were obtained from cases throughout the United States that were sent to Dr. Suzuki for pathological review and were systematically analyzed using histology, immunohistochemistry, and electron microscopy, in some cases, over a 15-yr period. As summarized succinctly by Dumortier et al. (1998) in a letter to the editor in 1998, the size and type of asbestos fibers associated with development of diffuse malignant pleural mesothelioma remain controversial (Mossman et al., 2011; Case et al., 2011; Aust et al., 2011).

One possible explanation for the confusion in pleural sampling came from a pioneering study carried out by Boutin et al. (1996). Using video-assisted fiber-optic thoracoscopy in eight asbestos-exposed patients and six unexposed cases, Dr. Boutin and colleagues (1996) sampled specific anatomic regions of the parietal pleura identified as collecting spots for inorganic particulates and fibers that

translocate to the pleural spaces. These regions are called "black spots" due to localized accumulation of carbon particles and are sites of lymphatic drainage located in the lower coastal regions of the parietal pleura and on the superior dome of the diaphragm. Using transmission electron microscopy, Boutin et al. (1996) identified numerous amphibole as well as chrysotile fibers at black spots, and 22.5% were ≥ 5 μm long. The mean asbestos fiber concentration in the 8 exposed cases was $12.4 \pm 9.8 \times 10^6$ fibers/g dry lung tissue, $4.1 \pm 1.9 \times 10^6$ fibers/g black spots on the parietal pleura, and $0.5 \pm 0.2 \times 10^6$ fibers/g normal parietal pleura, using bleach digestion of lung tissue and low-temperature ashing of pleural tissue samples. Evidence indicated that asbestos fibers accumulate in focal areas of the parietal pleura and that these "black spots" are the most likely anatomic origin of diffuse malignant mesothelioma. In a subsequent study of black spots analyzed from 150 consecutive autopsies of urban residents in Brussels, Belgium, the histopathological appearance of black spots showed chronic inflammation with lymphocytes, plasma cells, and macrophages with a variety of particulates and fibers both intracellularly and extracellularly (Mitchev et al., 2002). Of note, there was no anatomic relationship between black spots and parietal pleural plaques. Black spots were present in 92.7% of these cases; these lesions were more numerous in older cases and in males. Evidence indicated that these cases may have had greater exposure to coal dust used for home heating and in industry. In this case series, asbestos bodies >1000/g dry lung were found in 15 of 97 cases studied; unfortunately, pleural samples were not analyzed for the presence of asbestos fibers (Mitchev et al., 2002). The discrepancy between studies of pleural fiber burden and distribution may thus be explained by the inhomogeneity of fiber deposition in the parietal pleura. Since this important observation of the localization of pleural fibers in black spots, almost no studies addressed pleural fiber burden to clarify which fibers are present and which fibers are associated most closely with asbestos-induced pleural disease, whether neoplastic or non-neoplastic.

Knowledge and Data Gaps in Fiber Translocation and Dosimetry

In considering the data existing on the subject of fiber translocation and dosimetry, there are numerous gaps in the knowledge base that may be amenable to newer methods.

(a) There is a significant lack of understanding of the contributions of the various potential routes of fiber translocation, including direct interstitial transport, macrophage-mediated transport, lymphatic transport, and hematogenous transport. There is little known about which fibers move out from the lung to the pleura and at what rate, and which accumulate in the pleura. There is a need to improve our understanding of the kinetics of fiber translocation and pathogenic pleural responses following mixed asbestos fiber exposure and with coexposure to other particulates.

(b) There are gaps in understanding the role of fiber dose, dimension, and type in the induction of pleural lesions. There is a lack of understanding of the relationship between pleural fiber burden and disease in mixed fiber dust exposure. To date, experimental animal studies only examined relatively limited size fractions of fibers, due to limited respirability in rodent studies (Lippmann & Schlesinger, 1984; Lippmann et al., 1980).

(c) There is a need to study the role of short, thin fibers in the induction of pleural lesions. Most pleural disease is believed to be due to amphibole exposure (Churg, 1982; Roggli et al., 2002), and most disease was ascribed to long, thin fibers, but there is still much uncertainty concerning the contributions to disease of short, thin fibers that predominate in pleural fiber burden studies (Dodson et al., 2003; Suzuki et al., 2005; Mossman et al., 2011; Aust et al., 2011; Case et al., 2011). While the preponderance of evidence shows that long, thin fibers are the most pathogenic, there

is little understanding of how dose, surface properties, and biopersistence of short, thin fibers affect the exposure-response relationship with respect to non-neoplastic pleural outcomes in mixed exposures. This need is made more urgent with recent findings concerning the pleural effects of engineered fibrous nanomaterials such as instilled (Poland et al., 2008) and inhaled (Ryman-Rasmussen et al., 2009) carbon nanotubes in mice.

(d) There is a significant lack of information correlating kinetics with pathological outcomes in the pleura following experimental fiber exposures in laboratory animals. Maxim and McConnell (2001) suggested that humans and rats are similar in pathological responses to fibers with respect to pulmonary fibrosis outcomes; as yet, there are no comparable data for pleural fibrosis.

(e) There is need to develop fiber size separation methods to enable mechanistic studies of characterized fiber preparations. This will allow an understanding of the role of fiber size and dimension on cellular targets of pleural disease.

(f) In general, it is not understood how inhalation of fibers leads ultimately to pleural disease. To date there have been few inhalation studies with well-characterized aerosols of different asbestos fiber types in experimental animals. Most inhalation bioassays have been long-term hazard assessment studies in animal models; otherwise, studies have relied on short-term instillation studies in rodents or in vitro studies. The cost, complexity, and specialized requirements of inhalation studies with fibers have not allowed routine state-of-the-art fiber inhalation exposures in support of mechanistic studies.

INTERACTION OF FIBERS WITH TARGET CELLS IN THE PLEURA

Cellular Interactions

For reasons yet to be fully elucidated, fibrous particulates have an unusual affinity for the visceral and parietal pleura, and these tissues are sites for inflammatory, fibroproliferative, and neoplastic diseases in humans and in experimental animals. Non-neoplastic asbestos-associated diseases of the pleura in humans include benign asbestos-related pleural effusion, pleural plaques, diffuse pleural thickening, and rounded atelectasis (Chapman et al., 2003; Nishimura & Broaddus, 1998). Pleural fibrotic and inflammatory lesions develop in rodents in response to inhaled fibers, but these have not been categorized into separate lesion types as is the case in humans and often have not been described separately from pulmonary parenchymal fibrosis by toxicologic pathologists. It is noteworthy that significant pleural lesions similar to human pleural fibrotic lesions were found in chronic rodent inhalation bioassays with synthetic vitreous fibers as well as with asbestos fibers (McConnell et al., 1999).

Mesothelial cells, resident and elicited inflammatory cells, and pleural fibroblasts are believed to be important effector cells in the pathogenesis of asbestos-induced non-neoplastic pleural diseases. Mesothelial progenitor cells may also participate in pleural repair and disease (Herrick & Mutsaers, 2004). There have been numerous studies both in vivo in experimental animals and in vitro using human and animal cell culture systems that review the cellular interactions in pleural tissues and in the pleural space (Chapman et al., 2003; Mutsaers et al., 2004, 2006; Robledo & Mossman, 1999). It is known that following inhalation and instillation of asbestos fibers into the lung, there are rapid alterations in both resident and elicited populations of pleural inflammatory cells and mesothelial cells. Pleural inflammatory cell changes were produced in rats in association with translocation of asbestos fibers (Choe et al., 1997) or following particulate-induced pulmonary inflammation itself (Lehnert et al., 1985). Changes were noted in mesothelial cells of the visceral pleura at early time points following asbestos instillation and inhalation (Adamson, 1997; Dodson & Ford, 1985), and interactions between pleural inflammatory cells and

mesothelial cells are believed to be important in the development of fiber-induced pleural injury and disease. Rat and rabbit pleural mesothelial cells are known to release chemoattractants for inflammatory cells following exposure to asbestos (Boylan et al., 1992; Hill et al., 2003; Tanaka et al., 2000), and pleural macrophage-derived mediators can modulate mesothelial cell function (Baumann et al., 1993, 1996).

Mesothelial Cell Biology and Fiber Interactions

During the past two decades, there has been a great increase in our knowledge of the importance of the mesothelial cell in fiber-induced pleural disease. It has become apparent that these cells play a central dynamic role in the control of injury and repair processes that take place in the pleural and other serosal tissues (Mutsaers et al., 2004). Mesothelial cells are a unique cell type originating from mesoderm and vested with a number of important specialized functions including release of pro- and anti-inflammatory and other immunomodulatory mediators; secretion of factors that promote deposition and clearance of fibrin; and synthesis of growth factors and extracellular matrix proteins that aid in serosal repair (Jantz & Antony, 2008). Asbestos may injure pleural mesothelial cells either by direct or indirect mechanisms, including (1) injury by free radicals, (2) inflammasome activation, (3) alterations of intracellular signaling pathways, (4) release of cytokines and chemokines, (5) physical disruption of chromosomes, (6) alterations in growth factors, and (7) changes in coagulation and fibrinolysis pathways (Manning et al., 2002; Mutsaers et al., 2004; Robledo & Mossman, 1999). Mesothelial cells internalize the fibers via integrins or other receptors, and uptake of the fibers was found in some studies to be necessary for adverse effects of the fibers such as reactive oxygen species (ROS) generation, DNA damage, and apoptosis (Liu et al., 2000). Reactive oxygen species derived directly from the surface chemistry of fibers themselves (Fubini, 1997) as well as from cellular responses are believed to be important in both neoplastic and non-neoplastic asbestos-associated pleural disease (Janssen-Heininger et al., 2008; Shukla et al., 2003a). Although the limitations of cell culture systems for particulate studies have been well described, it is recognized that much of the mechanistic understanding of how fibers interact with mesothelial cells and produce fiber-induced effects derives from in vitro experiments (Donaldson et al., 2009).

Knowledge and Data Gaps Concerning Pleural Cell Biology and Asbestos Fiber Exposure

(a) Fibers may translocate to the pleural space and are postulated to induce pleural disease by direct interaction with pleural cells. However, some pleural conditions such as inflammation or fibrosis may be influenced by fibers and their actions in the neighboring lung. The relative contribution of direct fiber exposure versus indirect signaling effects on mesothelial cells is not known.

(b) There is still much to know about how fiber mineralogy, dimensions, physicochemical properties, and biopersistence contribute to induction and progression of pleural lesions.

(c) There is need for better development of biomarkers of pleural disease and assessment of pleural changes in experimental animal models. Although bronchoalveolar lavage fluid (BALF) analysis has been routinely utilized in experimental studies of the lung, pleural lavage has not been routinely used to assess changes in the pleural space. Advancing understanding of pleural disease will require better use of pleural endpoints in acute and chronic studies of fiber exposure.

(d) The major target of asbestos in the pleural space is thought to be the mesothelial cell. The contribution of the inflammatory pleural cells including macrophages is less well understood. In addition, there is a need to develop additional understanding of possible mesothelial progenitor cells

in asbestos-associated injury and disease (Herrick & Mutsaers, 2004).

(e) The thoracoscopic study by Boutin et al. (1996) demonstrating the focal accumulation of fibers in "black spots" of the parietal pleura provided important new insights. Similar studies might advance the understanding of pleural fiber burden and disease. By obtaining biopsy samples from those undergoing thoracoscopy or thoracotomy, one could determine the locations of fibers and the genetic changes at sites of asbestos deposition, and investigate biomarkers of asbestos toxicity. A systematic analysis using analytical transmission electron microscopy could quantitate the dimensions and types of mineral particles and fibers that are translocated to and retained in the pleura (both visceral and parietal) of control individuals and patients with asbestos-related pleural diseases. Such studies may also help identify whether fibers are located predominately intracellularly or extracellularly, and identify the target cells.

(f) The role of the specific arms of the inflammatory response can now be studied using mice with genetically engineered deletion of specific cell types or inflammatory cytokines. Such studies can be used to indicate the role of inflammation in producing the various fiber-induced pleural diseases and whether particular inflammatory mechanisms might be a therapeutic target.

(g) Noninvasive techniques for assessing fiber burden or the tissue reaction to fibers would be of inestimable value in investigating the natural history of pleural reactions in animals and in humans over the decades of tumor development. Novel imaging techniques could ultimately serve as a tool for following those at risk and testing strategies for intervention.

Biological Mechanisms Responsible for Non-Neoplastic Pleural Disease

It has generally been accepted from studies of animal models of asbestos fiber exposure that inflammatory changes in the lung and pleura precede subsequent fibroproliferative and mesothelial cell proliferative responses. The early pioneering intracavitary instillation and implantation studies of Freidrich Pott and coworkers (Pott, 1980; Pott et al., 1974) and Merle Stanton and colleagues (Stanton et al., 1969, 1977, 1981) revealed that the physical properties of fibers such as dimension are important in the pathogenesis of asbestos-associated disease of the serosal tissues (Case et al., 2011; Aust et al., 2011).

Since those initial studies, much has been learned about other physicochemical properties of inhaled particles believed to be important in their disease-inducing abilities such as the surface properties relevant for oxidant generation (Fubini, 1997) and the chemical properties relevant for biopersistence (Bernstein et al., 2001, 2005; Bernstein, 2007; Mossman et al., 2011).

It is noteworthy that up until now biopersistence studies focused on the lung parenchyma. There are few pleural fiber burden or pleural biopersistence investigations in either experimental animals or humans.

Fiber Type and Potency

In rodent studies in which high concentrations of fibers were instilled or implanted in the pleural space, all mineralogical forms of asbestos fibers were produced pleural fibrosis and malignant mesothelioma. In an inhalation study in rats using well-characterized aerosols and state-of-the-art methods to assess retained lung burdens, Bernstein et al. (1995) found that chrysotile exposure failed to induce pleural lesions despite producing severe pulmonary fibrosis (asbestosis) and lung tumors (Mast et al., 1994). This appears to correlate with human epidemiologic studies because recent analysis suggests that most asbestos-associated mesotheliomas are due to amphibole exposure (Berman & Crump, 2008; Mossman et al., 2011).

While there is a general lack of understanding of comparative pleural potency of different asbestos fiber types, a reanalysis of previous asbestos fiber inhalation studies in

rats compared size, shape, and mineralogy with lung tumor and mesothelioma outcomes (Berman et al., 1995). In this study, multivariate measures of exposure were identified that described the lung tumor responses in 13 previous asbestos (chrysotile, amosite, crocidolite, tremolite) inhalation experiments in AF/HAN rats. Due to limitations in the characterization of asbestos fiber dimensions in the original studies, new exposure measures were developed from samples of the original dusts that were regenerated and analyzed by transmission electron microscopy using a direct transfer technique. Structures contributing to lung cancer risk appeared to be long ($\geq$20 μm) and thin ($\leq$0.4 μm) fibers. The analysis did not find significant mineralogical differences in potency across asbestos types for pulmonary tumors but noted that amphibole asbestos was more potent than chrysotile in induction of malignant mesothelioma.

Proposed Mechanisms for Asbestos-Associated Non-Neoplastic Pleural Lesions in Rodents

Although the rodent visceral pleura differs markedly from that of humans in thickness and anatomy (Tyler, 1983), the rodent parietal pleura and the resident pleural inflammatory cells are similar to those of humans (Everitt et al., 1997; Gelzleichter et al., 1996). In rodents, asbestos and synthetic vitreous fiber exposure by inhalation or instillation resulted in pleural inflammatory and fibrotic changes (McConnell et al., 1999). In animal models of asbestos-induced lung cancer and mesothelioma, inflammation and fibrosis always preceded the development of oncogenic outcomes (Greim et al., 2001), and these processes may share some mechanistic underpinnings. Similarly, it is worthy of note that in the rodent fiber inhalation bioassays conducted to date, pleural inflammatory and fibroproliferative lesions were accompanied by pulmonary parenchymal changes. Recently, there have been a number of chronic studies that suggested that the Syrian golden hamster may be particularly susceptible to the development of pleural mesothelioma as well as of pleural fibroproliferative changes following asbestos and synthetic vitreous fiber inhalation (Everitt et al., 1997; Gelzleichter et al., 1999; Mast et al., 1994; McConnell et al., 1999).

As described earlier, mesothelial cell responses to translocated fibers and/or responses to inflammatory mediators released from lung parenchymal and pleural cells are believed to be important in the pathogenesis of pleural fibrosis and asbestos-associated pleurisy (Mutsaers et al., 2004; Robledo & Mossman, 1994). Mesothelial cells are known to phagocytize asbestos fibers (Boylan et al., 1995), a step that may then lead to oxidant-induced signaling pathways, altered cell proliferation, apoptosis (Liu et al., 2000; Shukla et al., 2003b), necrosis (Yang et al., 2010), and release of chemokines and cytokines that mediate pleural inflammation (Jantz & Antony, 2008). A variety of growth factors are associated with pleural fibrosis, especially transforming growth factor (TGF)-β1 (Decologne et al., 2007; Mutsaers et al., 2006).

Proposed Mechanisms for Asbestos-Associated Non-Neoplastic Pleural Disease in Humans

The nonmalignant manifestations of asbestos in the pleura include benign asbestos pleurisy, pleural plaques, diffuse pleural fibrosis, and rounded atelectasis (ATS Official Statement, 2004; Nishimura & Broaddus 1998). Although these nonmalignant pleural diseases may themselves produce symptoms, especially diffuse pleural fibrosis, these are important clinically because the symptoms identify people who have had significant exposure to asbestos and often mimic and require diagnostic workups to exclude malignancy. These diseases are also important for research by giving insight into fiber toxicology and pathogenesis and by identifying groups for which development of biomarkers and early intervention for diagnosis or treatment are warranted.

Benign asbestos pleurisy may develop as early as 10 yr after exposure (ATS, 2004). Because it usually produces no apparent

symptoms and is often detected incidentally, the incidence is unclear. The benign pleural effusion may be bloody, thus leading to concern for underlying malignancy. The effusion may last for months, may be unilateral or bilateral, and may recur. The effusion may antedate diffuse pleural fibrosis (Lillis et al., 1988), although the reason for this association is not known.

Pleural plaques are the most common pleural manifestations of asbestos exposure and represent evidence of clinically significant exposure, retention, and biologic response to fibers. In general, plaques develop 20–30 yr after initial exposure (Nishimura & Broaddus, 1998; ATS, 2004). Plaques are usually located on the parietal pleura or on the dome of the diaphragm and appear as circumscribed areas of collagen deposition without inflammation. Plaques may be associated with decreases in lung function and symptoms of dyspnea, but most individuals with pleural plaques alone display no apparent symptoms and no obvious impaired lung function. Although localized and unilateral pleural thickening may have other causes such as prior tuberculosis, trauma, or talc instillation, multiple and bilateral pleural plaques, particularly when calcified, are considered to be pathognomonic for asbestos or erionite exposure (Nishimura & Broaddus, 1998). Of note, those subjects without plaques may also have significant asbestos exposure; it is not known why some exposed individuals form plaques and others do not.

Plaques are biomarkers for asbestos or erionite exposure and of elevated fiber burden in the lung (Churg, 1982; Kishimoto et al., 1989; Roggli & Sanders, 2000). It is not known how pleural plaques correlate with pleural fiber burden. Different fiber types may play a role in plaque formation: Plaques have been associated with the presence of high aspect ratio amphiboles in the lung (Churg, 1982, 1983, 1994), but in at least one study, only chrysotile fibers were found in the plaques themselves (Churg, 1982).

The biologic response to asbestos fibers in individuals with pleural plaques may differ from those without plaques. In animal studies pleural plaques were found to be a consequence of the cellular inflammatory response to asbestos (Sahn & Antony, 1984). In any case, pleural plaques represent a marker of exposure to asbestos and therefore a marker of increased risk for asbestos-related disease, and perhaps may be used to select patients for focused clinical trials to assess other biomarkers of risk and to identify prevention strategies. In general, understanding more about the formation of pleural plaques and their significance as a biomarker of exposure and of enhanced risk would provide insight into pathological mechanisms and suggest possible interventions in exposed populations.

Rounded atelectasis is thought to be a consequence of any type of pleuritis and pleural fibrosis and represents a folding of the lung within a region of pleural thickening. It may resemble a mass and thus raise concern for lung cancer. Little is known regarding the pathogenesis of this entity (Hillerdal, 1989).

Diffuse pleural thickening is a diffuse, not circumscribed, thickening of the pleura that develops approximately 30 yr following exposure (Nishimura & Broaddus, 1998; ATS, 2004). Unlike pleural plaques, diffuse thickening mostly affects the visceral pleura. Diffuse pleural thickening is associated with clinically significant ventilatory impairment, pulmonary restriction, and low lung volumes. Diffuse pleural thickening may coexist with pleural plaques, and may be associated with a higher fiber burden than is found with pleural plaques alone (Stephens et al., 1987). The relationship of diffuse pleural thickening to asbestos-induced fibrosis of the lungs is not known. The types of asbestos fibers likely to produce diffuse pleural thickening are not known. In one study, the fibers found in the pleura were short chrysotile fibers, while the fibers in the lungs were longer and thinner amphiboles (Gibbs et al., 1991). As with other non-neoplastic asbestos-induced pleural disease, diffuse pleural thickening raises concerns for underlying mesothelioma. If lung function is severely compromised, the patient may undergo decortication or removal of the pleura; nevertheless, removal of the thickened pleura may not improve lung function

due to accompanying fibrosis of the underlying lung.

These non-neoplastic pleural pathologies are particularly common in those exposed to amphibole fibers in Libby, MT (Peipins et al., 2003), suggesting that these fibers may exert unique toxicity for the pleura. Libby amphibole is a mixture of winchite, richterite, and tremolite, in decreasing order of abundance (Meeker et al., 2003). In 1980, a morbidity study was carried out on workers who had used Libby vermiculite as an inert carrier for various types of lawn-care products (Lockey et al., 1984). Libby vermiculite was found to contain asbestiform minerals. In the workers exposed to Libby vermiculite, workplace exposures were associated with bloody pleural effusions and localized pleural thickening. A follow-up study of the worker cohort 25 years after discontinuation of Libby vermiculite mining in 1980 demonstrated an elevated prevalence of pleural changes, increasing from 2% in 1980 to 29% (80 of 280 workers) in 2005 (Rohs et al., 2005). Of workers with a low lifetime cumulative fiber exposure (CFE) of only <2.2 fibers/cc-yr, as many as 20% displayed pleural changes. A significant CFE response relationship was demonstrated between percent pleural changes, which ranged from 7 to 54%, and the lowest to the highest CFE quartile. The mean CFE (SD) related to localized pleural thickening, diffuse pleural thickening, and interstitial fibrosis in vermiculite workers with no historical exposure to commercial asbestos was 3.45 (4.95), 8 (5.32), and 11.37 (6.82) fiber-cc/yr, respectively (Rohs et al., 2005). This relationship was confirmed by Whitehouse (2004), who demonstrated progressive loss of lung function in Libby residents with and without reported occupational exposure who had predominantly pleural changes. Studies of Libby miners and millers demonstrated an association between Libby amphibole exposure and increased incidence of nonmalignant respiratory disease mortality at a CFE of less than 4.5 fiber/cc-yr (Sullivan, 2007). In summary, studies of workers exposed to the Libby amphibole indicate the propensity for these amphiboles to induce pleural disease and nonmalignant respiratory morbidity and mortality at relatively low lifetime cumulative fiber exposure levels.

Knowledge and Data Gaps in Nonmalignant Pleural Disease in Humans

Unanswered questions regarding nonmalignant pleural disease include:

(a) Pleural plaques have been associated with long amphibole fibers in the lung and with short chrysotile fibers in the pleura. Thus, it is not known which types of asbestos fibers induce pleural plaques and how pleural plaques correlate with pleural (not lung) fiber burden.
(b) The fiber burden and fiber types in the pleura of those with pleural disease have not been documented. Fiber burden in the lung may not correlate with that in the pleura: Low counts in the lung may be associated with high counts in the pleura if fibers have translocated to the pleura; fiber types found in the lung may be the ones that are retained at this site, whereas different fibers may translocate to the pleura and induce disease there. Autopsy studies might be used to compare lung and pleural fiber burdens and relative distribution of different fiber types and sizes in these different locations.
(c) Most studies of pleural fiber burden reported the presence of short chrysotile fibers, and yet the role of these short chrysotile fibers in pleural disease has not been established. Because most pleural disease has been attributed to high aspect ratio amphibole fibers, it is not known whether the short chrysotile fibers are pathogenic, either alone or by enhancing the toxicity of longer amphibole fibers, or whether they are acting as bystanders. These fibers may be located outside the area of interest, corresponding to the "black spots" where pathogenic fibers are located. Further animal studies using well-characterized short chrysotile fibers in the pleural space would be valuable in addressing this important issue.

(d) Although the incidence and severity of pleural disease following exposure to Libby amphibole is high, it is not yet known whether it is actually higher than after exposure to other asbestos types. If Libby amphibole is particularly toxic for the human pleura, the mechanism is not known; perhaps Libby fibers are more readily translocated and retained in the pleura or the Libby fibers that reach the pleura are particularly toxic. In vitro and in vivo studies using Libby amphibole can address these important questions.

(e) The genetic determinants of the individual responses to asbestos are not known–genetic factors may determine susceptibility to non-neoplastic and neoplastic pleural disease following asbestos exposure. Similarly, it is not known whether genetic differences explain why some individuals develop a fibrotic response while others have a neoplastic response. Genetic studies of affected individuals and their families would be valuable to address this issue.

(f) New mechanistically oriented, short-term testing strategies need to be developed to assess pathogenicity of fiber and particulate preparations, not only with respect to carcinogenicity but also for fibrotic and inflammatory changes in the pleura. The reason for the propensity of the Syrian golden hamster to develop pleural disease needs further investigation.

(g) There is a need to compare nanoparticle-induced lung and pleural changes with asbestos-associated pleural diseases to identify specific physicochemical determinants of toxicity.

(h) The role of inflammation in the development of pleural fibrosis is not understood. Studies are needed in the role of inflammatory cells and of profibrotic cytokines such as TGF-beta. The role of specific receptors such as the Nalp3 inflammasome and its contribution to chronic inflammatory states (Dostert et al., 2008) need additional investigation.

BIOLOGICAL MECHANISMS RESPONSIBLE FOR NEOPLASTIC PLEURAL DISEASE

Fiber Type and Potency

The most potent risk factors for diffuse malignant mesothelioma are environmental or occupational exposure to erionite, asbestos fibers, and vermiculite that contains noncommercial amphiboles (Institute of Medicine, 2006). Based on a recent meta-analysis of the epidemiologic evidence (Berman & Crump, 2008), amphibole asbestos is more potent than chrysotile asbestos in inducing diffuse malignant mesothelioma. This difference in potency was attributed to the greater biopersistence of amphibole asbestos in lungs in comparison with chrysotile asbestos (Bernstein & Hoskins, 2006). Development of diffuse malignant mesothelioma following exposure to chrysotile asbestos is attributed to contamination of some chrysotile deposits with tremolite, a naturally occurring amphibole (Institute of Medicine, 2006).

Biopersistence in the lungs is a key physicochemical property of crystalline mineral fibers and is associated with induction of fibrosis, lung cancer, and malignant mesothelioma in rodent models (ILSI, 2005). Biopersistence in the pleura has not been studied extensively. Boutin and Rey (1993) recovered asbestos fibers in parietal pleural samples of asbestos workers during thoracoscopy. It is likely that long asbestos fibers accumulate at the parietal pleural membrane because they cannot be efficiently cleared through lymphatic stomata. Earlier studies reported low pleural asbestos fiber burdens in asbestos workers (Gibbs et al., 1991). More recent studies recovered large numbers of short asbestos fibers from the lungs and pleural tissues of asbestos-exposed patients (Dodson et al., 2003, 2005, 2007; Suzuki et al., 2005). Mechanistic studies conducted in cell culture associated exposure to long asbestos fibers with activation of the EGF receptor and intracellular signaling pathways leading to cell proliferation (Pache et al., 1998; Mossman et al., 2011). Asbestos fibers were

also found to interfere physically with the mitotic apparatus (Hei et al., 2000; Huang et al., 2011).

Fibers may be altered secondarily in the lungs or pleura. Depending on their chemical composition, surface area, and crystalline structure, asbestos fibers may leach, split, or break (ILSI, 2005). The kinetics of fiber alteration and clearance from the pleural space has not been investigated. Secondary modification of surface properties including binding of phospholipids, acquisition or depletion of cations, and protein adsorption in the pleura may also modify toxicity (Fubini & Mollo, 1995).

Proposed Mechanisms for Asbestos-Induced Mesothelioma

Asbestos and erionite fibers were shown to induce genotoxicity directly. Chronic rodent studies established an association between persistent inflammation and carcinogenicity induced by inhalation of crystalline mineral fibers (ILSI, 2005). Chronic inflammation triggered in response to biopersistent fibers may amplify the genotoxicity of asbestos fibers in pleural target cells (Figure 2). Following internalization by phagocytosis, asbestos fibers trigger macrophage activation and generation of reactive oxygen and nitrogen species, leading

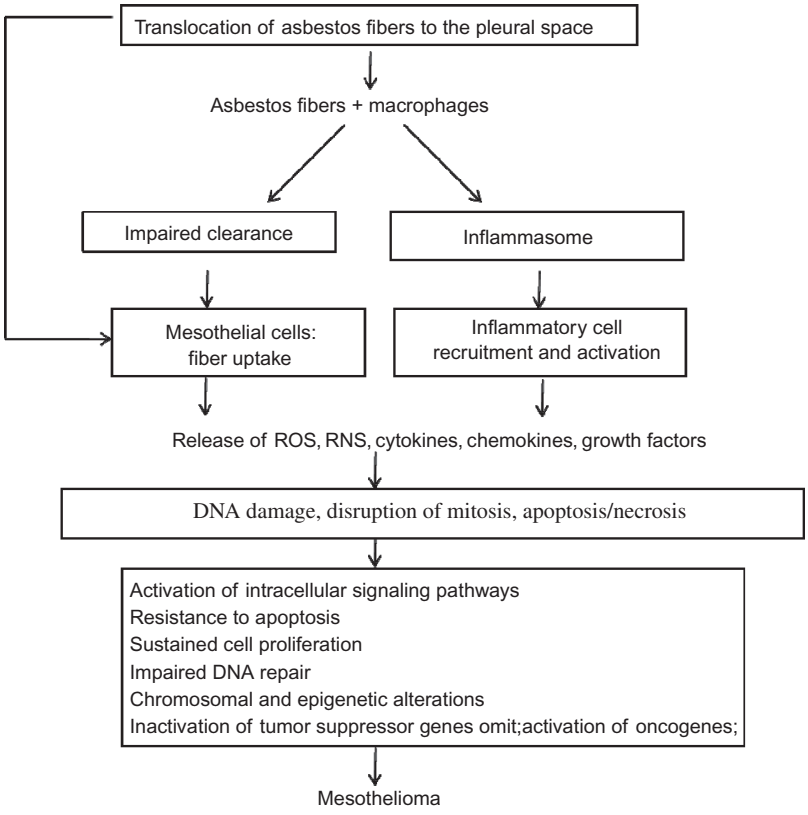

FIGURE 2. Proposed mechanisms for asbestos-induced mesothelioma. Asbestos fibers are thought to lead to mesothelioma via mechanisms as outlined in this algorithm. Asbestos fibers enter the pleural space, where they interact with pleural macrophages and mesothelial cells and induce an influx of inflammatory cells. These early interactions result in release of reactive oxygen and nitrogen species (ROS, RNS), cytokines, and growth factors that may mediate indirect effects on mesothelial cells. The fibers may also act directly on mesothelial cells by inducing DNA damage, interrupting chromosomal segregation, or inducing apoptosis or necrosis. Such direct and indirect actions lead to chronic stimulation and injury of the mesothelium that may proceed over decades by a multistep path to cancer. Key steps in the development of cancer include genetic and epigenetic alterations leading to sustained cell proliferation, resistance to apoptosis, and inactivation of tumor suppressor genes.

to tissue injury. Recent studies in genetically engineered mice suggest a central role for the NALP3 inflammasome in rapid release of active IL-1β (Cassel et al., 2008; Dostert et al., 2008), a cytokine that triggers recruitment of additional inflammatory cells and release of cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-6, IL-8) that perpetuate inflammation in response to biopersistent asbestos fibers (Shukla et al., 2003a). TNF-α also activates the nuclear factor (NF)-κB pathway in mesothelial cells, allowing these cells to survive and proliferate in the presence of asbestos-induced DNA damage (Yang et al., 2006).

Amphibole asbestos fibers contain surface redox-active iron (Fe) that generates ROS leading to lipid peroxidation, protein oxidation, and DNA damage in lung and pleural target cells (Manning et al., 2002). Erionite fibers may secondarily acquire Fe that catalyzes generation of ROS (Hardy & Aust, 1995; Aust et al., 2011). Secondary deposition of endogenous Fe may enhance redox activity or disrupt Fe homeostasis in the lungs or pleura producing oxidative stress (Ghio et al., 2008). In response to chronic oxidative stress, intracellular signaling pathways trigger activation of transcription factors, stimulation of cell proliferation, and resistance to apoptosis (Albrecht et al., 2004; Mossman et al., 2011).

Asbestos in cell culture (Broaddus et al., 1996; Berube et al., 1996) and in some animal studies (Marchi et al., 2000) was found to induce apoptosis in mesothelial cells, and ROS may contribute to this early apoptosis (Broaddus et al., 1996); at later times, DNA or chromosomal damage may also trigger apoptosis. Presumably, it is the cells that have inherent resistance to apoptosis or acquire resistance that will survive the initial and ongoing damage to initiate multistep acquisition of genetic abnormalities that characterize tumor development (Broaddus, 1997). Mesothelial cells (or other progenitor cells) may acquire resistance by inherent overexpression of anti-apoptotic molecules or more likely by upregulation of these molecules, e.g., the Bcl-2 or the IAP family. Mesothelial cells with other preexisting abnormalities in DNA damage-induced signaling or in mitochondrial function may not undergo apoptosis and may persist despite asbestos-induced toxicity (Upadhyay & Kamp, 2003).

Mesothelial cells may have inherent or acquired activation of prosurvival pathways, from either the exposure to asbestos with upregulation of growth factor receptors (Pache et al., 1998) or downstream pathways (MAPK, ERK, Akt/mTOR) or another survival mechanism (Jimenez et al., 1997; Altomare et al., 2005), as proposed with SV40 infection (Kroczynska et al., 2006). Inflammation may also initiate an environment that itself promotes prosurvival mechanisms. Inflammation may also be induced by the fibers themselves, by an influx of cells of the innate immune system or by asbestos-induced necrosis (Yang et al., 2010). The microenvironment including the presence of inflammatory cells, endothelial cells, and fibroblasts, along with the formation of a three-dimensional shape itself, supports the resistance to apoptosis (Barbone et al., 2008; Daubriac et al., 2009). Critically important survival mechanisms that inhibit apoptosis, if understood, might be a target for intervention (Heintz et al., 2010).

This chronic inflammatory environment may contribute to acquired, heritable genetic, or epigenetic alterations leading to inactivation of tumor suppressor genes, activation of oncogenes, and altered regulation of cell cycle and DNA repair pathways (Kratzke and Gazdar, 2005). Specific genetic, epigenetic, and chromosomal alterations are characteristic of diffuse malignant mesothelioma (Murthy and Testa, 1999; Apostolou et al., 2005). Oxidants generated directly by redox-active asbestos fibers or indirectly following phagocytosis may also induce DNA and chromosomal damage (Jaurand, 1996; Hei et al., 2000; Huang et al., 2011). An indirect mechanism associated with persistent inflammation was proposed for altered gene methylation profiles characteristic of human malignant pleural mesotheliomas (Christensen et al., 2009). These genetic and epigenetic alterations may select for mesothelial cells that are able to survive and proliferate

in a chronic inflammatory environment (Huang et al., 2011).

KNOWLEDGE AND DATA GAPS FOR THE BIOLOGIC MECHANISMS FOR NEOPLASTIC PLEURAL DISEASE

(a) Workers are usually exposed to mixed dusts contaminated with asbestos fibers. It is unknown whether asbestos-related pleural diseases are potentiated by exposure to other dusts such as vermiculite, crystalline silica, or metals in the occupational environment.

(b) The role of SV40 virus as a cofactor with asbestos fibers in the development of diffuse malignant mesothelioma is controversial (Gazdar et al., 2002). Mechanistic studies in cell cultures and in rodents suggest that SV40 viral oncoproteins induce mesothelial cell transformation and diffuse malignant mesothelioma, although human epidemiological studies do not support a causal association (Weiner & Neragi-Miandoab, 2009). Additional epidemiological studies using specific serological markers for SV40 virus infections are needed (Kean et al., 2009).

(c) The physical and chemical properties of mineral fibers associated with carcinogenicity include surface chemistry and reactivity, surface area, fiber dimensions, and biopersistence. The relative importance of these different properties with respect to carcinogenic potency is uncertain and may depend on the geological source of the fibrous mineral and its associated contaminants. Commercial asbestos fibers and erionite fibers have been most widely studied. Noncommercial amphibole fibers, other naturally occurring asbestiform fibers, and newly engineered fibrous nanomaterials (Jaurand et al., 2009; Sanchez et al., 2009) need to be well characterized and their potential for translocation and persistence in the pleura must be determined.

(d) The potential for any natural or engineered fibrous material with physicochemical properties similar to asbestos fibers to induce persistent inflammation and promote the development of diffuse malignant mesothelioma needs to be investigated before the fibers are widely used.

(e) Various direct and indirect mechanisms were proposed for the induction of diffuse malignant mesothelioma by asbestos fibers. These mechanisms may interact at multiple stages during the long latent period associated with this malignancy. The relative importance of these different mechanisms in tumor development and progression is unknown. The ability of different fiber types to induce specific genetic and epigenetic alterations characteristic of diffuse malignant mesothelioma needs to be determined (Andujar et al., 2007).

(f) Chronic rodent inhalation assays are expensive, technically demanding, and not suitable for mechanistic studies because only a minority of rats develop diffuse malignant mesothelioma following inhalation. Current screening assays for fiber toxicity use short-term in vitro or in vivo assays; however, it is difficult to extrapolate from acute, high-dose exposures to chronic or repeated, low-dose exposures in vivo. A new toxicologic screening strategy needs to be developed and validated to assess potential carcinogenicity of naturally occurring mineral fibers and engineered fibrous nanomaterials.

(g) Mesothelial cells are mobile and appear to be able to detach and relocate at other sites in the pleural space (Foley-Comer et al., 2002). Mesothelioma is also associated with mobile spheroids, clumps of malignant cells floating in the pleural fluid; such spheroids appear to remain viable and resistant to apoptosis (Barbone et al., 2008; Daubriac et al., 2009). It is not known whether this mobility may allow preneoplastic cells to move from areas of asbestos accumulation to other areas where mesothelioma may develop. If so, and if new technologies are developed that will allow one to distinguish malignant mesothelial cells from reactive benign mesothelial cells, pleural fluid could be sampled for preneoplastic mesothelial

cells in order to identify individuals at risk for developing diffuse malignant mesothelioma.

(h) New immunohistochemical and molecular markers of preneoplastic and neoplastic lesions would improve early diagnosis and therapy of diffuse malignant mesothelioma (Husain et al., 2009).

(i) Populations exposed to asbestos or asbestiform fibers from Libby MT, workers in certain trades, those exposed on 9/11 at Ground Zero, or those with known high exposure (e.g., those with bilateral pleural plaques) need to be followed in epidemiologic studies that include noninvasive studies of biomarkers and imaging with the potential for more invasive studies using pleuroscopy or pleural lavage. In this way, knowledge can be gained about the natural history of asbestos-induced pleural disease in order to understand preneoplastic changes and ultimately permit early diagnosis or prevention studies.

REFERENCES

Adamson, I. Y. 1997. Early mesothelial cell proliferation after asbestos exposure: in vivo and in vitro studies. *Environ. Health Perspect*. 105:1205–1208.

Albrecht, C., Borm, P. J., and Unfried, K. 2004. Signal transduction pathways relevant for neoplastic effects of fibrous and non-fibrous particles. *Mutat Res* 553: 23–35.

Altomare, D. A., You, H., Xiao, G. H., Ramos-Nino, M. E., Skele, K. L., De Rienzo, A., Jhanwar, S. C., Mossman, B. T., Kane, A. B., and Testa, J. R. 2005. Human and mouse mesotheliomas exhibit elevated AKT/PKB activity, which can be targeted pharmacologically to inhibit tumor cell growth. *Oncogene* 24:6080–6089.

American Thoracic Society. (ATS) 2004. Diagnosis and initial management of nonmalignant diseases related to asbestos. *Am. J. Respir. Crit. Care Med*. 170:691–715.

Andujar, P., Lecomte, C., Renier, A., Fleury-Feith, J., Kheuang, L., Daubriac, J., Janin, A., and Jaurand, M. C. 2007. Clinico-pathological features and somatic gene alterations in refractory ceramic fibre-induced murine mesothelioma reveal mineral fibre-induced mesothelioma identities. *Carcinogenesis* 28:1599–1605.

Apostolou, S., Balsara, B. R., and Testa, J. R. 2005. Cytogenetics of malignant mesothelioma. Malignant mesothelioma. In *Advances in pathogenesis, diagnosis and translational therapies*, eds. H. I. Pass, N. J. Vogelzang, and M. Carbone, vol, 6, pp. 101–111. New York: Springer Science and Business Media.

Aust, A. E., Cook, P. M., and Dodson, R. F. 2011. Morphological and chemical mechanisms of elongated mineral particle toxicities. *J. Toxicol. Environ. Health B*, 14:40–75.

Barbone, D., Yang, T. M., Morgan, J. R., Gaudino, G., and Broaddus, V. C. 2008. Mammalian target of rapamycin contributes to the acquired apoptotic resistance of human mesothelioma multicellular spheroids. *J. Biol. Chem*. 283:13021–13030.

Baumann, M. H., Heinrich, K., Sahn, S. A., and Strange, C. 1993. Pleural macrophages differentially alter mesothelial cell growth and collagen production. *Inflammation* 17:1–12.

Baumann, M. H., Strange, C., Sahn, S. A., and Kinasewitz, G. T. 1996. Pleural macrophages differentially alter pleural mesothelial cell glycosaminoglycan production. *Exp. Lung Res*. 22:101–111.

Berman, D. W., and Crump, K. S. 2008. A meta-analysis of asbestos-related cancer risk that addresses fiber size and mineral type. *Crit. Rev. Toxicol*. 38:49–73.

Berman, D. W., Crump, K. S., Chatfield, E. J., Davis, J. M., and Jones, A. D. 1995. The sizes, shapes, and mineralogy of asbestos structures that induce lung tumors or mesothelioma in AF/HAN rats following inhalation. *Risk Anal*. 15:181–195.

Bermudez, E., Mangum, J. B., Asgharian, B., Wong, B. A., Reverdy, E. E., Janszen, D. B., Hext, P. M., Warheit, D. B., and Everitt, J. I. 2002. Long-term pulmonary responses of three laboratory rodent species to subchronic inhalation of pigmentary titanium dioxide particles. *Toxicol. Sci*. 70:86–97.

Bernstein, D. M. 2007. Synthetic vitreous fibers: A review toxicology, epidemiology

and regulations. *Crit. Rev. Toxicol*. 37: 839–886.

Bernstein, D. M., Chevalier, J., and Smith, P. 2005. Comparison of Calidria chrysotile asbestos to pure tremolite: Final results of the inhalation biopersistence and histopathology examination following short-term exposure. *Inhal. Toxicol*. 17:427–449.

Bernstein, D. M., Donaldson, K., Decker, U., Gaering, S., Kunzendorf, P., Chevalier, J., and Holm, S. E. 2008. A biopersistence study following exposure to chrysotile asbestos alone or in combination with fine particles. *Inhal. Toxicol*. 20:1009–1028.

Bernstein, D. M., and Hoskins, J. A. 2006. The health effects of chrysotile: current perspective based upon recent data. *Regul. Toxicol. Pharmacol*. 45:252–264.

Bernstein, D. M., Riego Sintes, J. M., Ersboell, B. K., and Kunert, J. 2001. Biopersistence of synthetic mineral fibers as a predictor of chronic inhalation toxicity in rats. *Inhal. Toxicol*. 13:823–849.

Bernstein, D. M., Thevenaz, P., Fleissner, H., Anderson, R., Hesterberg, T. W., and Mast, R. 1995. Evaluation of the oncogenic potential of man-made vitreous fibres: The inhalation model. *Ann. Occup. Hyg*. 39:661–672.

BéruBé, K. A., Quinlan, T. R., Fung, H., Magae, J., Vacek, P., Taatjes, D. J., and Mossman, B. T. 1996. Apoptosis is observed in mesothelial cells after exposure to crocidolite asbestos. *Am. J. Respir. Cell Mol. Biol*. 15:141–147.

Boutin, C., Dumortier, P., Rey, F., Viallat, J. R., and DeVuyst, P. 1996. Black spots concentrate oncogenic asbestos fibers in the parietal pleura: Thoracoscopic and mineralogic study. *Am. J. Respir. Crit. Care Med*. 153:444–449.

Boutin, C., and Rey, F. 1993. Thoracoscopy in pleural malignant mesothelioma: A prospective study of 188 consecutive patients: Part I. Diagnosis. *Cancer* 72:389–393.

Boutin, C., Schlesser, M., Frenay, G., and Germain, S. 1997. Malignant mesothelioma. Diagnosis and treatment. *Rev. Pract*. 47:1333–1339.

Boylan, A. M., Ruegg, C., Kim, K. J., Hebert, C. A., Hoeffel, J. M., Pytela, R., Sheppard, D., Goldstein, I. M., and Broaddus, V. C. 1992. Evidence of a role for mesothelial cell-derived interleukin-8 in the pathogenesis of asbestos-induced pleurisy in rabbits. *J. Clin. Invest*. 89:1257–1267.

Boylan, A. M., Sanan, D. A., Sheppard, D., and Broaddus, V. C. 1995. Vitronectin enhances internalization of crocidolite asbestos by rabbit pleural mesothelial cells via the integrin alpha v beta 5. *J. Clin. Invest*. 96:1987–2001.

Broaddus, V. C. 1997. Asbestos, the mesothelial cell and malignancy: A matter of life or death. *Am. J. Respir. Cell Mol. Biol*. 17: 657–659.

Broaddus, V. C. 2002. Transudative pleural effusions. In *Pleural diseases*, European Respiratory Monograph 22, eds. R. Loddenkemper and V. Anthony, pp. 157–176. Sheffield, UK: European Respiratory Society Journals

Broaddus, V. C. 2008. Physiology: fluid and solute exchange in normal physiological states. In *Textbook of pleural diseases*, eds. R. Light and Y. Lee, pp. 43–48. London: Hodder Arnold.

Broaddus, V. C., Wiener-Kronish, J. P., Berthiaume, Y., and Staub, N. C. 1988. Removal of pleural liquid and protein by lymphatics in awake sheep. *J. Appl. Physiol*. 64:384–390.

Broaddus, V. C., Wiener-Kronish, J. P., and Staub, N. C. 1990. Clearance of lung edema into the pleural space of volume-loaded, anesthetized sheep. *J. Appl. Physiol*. 68:2623–2630.

Broaddus, V. C., Yang, L., Scavo, L. M., Ernest, J. D., and Boylan, A. M. 1996. Asbestos induces apoptosis of human and rabbit pleural mesothelial cells via reactive oxygen species. *J. Clin. Invest*. 98:2050–2059.

Case, B. W., Abraham, J., Meeker, G., Pooley, F. D., and Pinkerton, K. E. 2011. Applying definitions of "asbestos" to environmental and "low-dose" exposure levels and health effects, particularly malignant mesothelioma. *J. Toxicol. Environ. Health B*. 14: 3–39.

Cassel, S. L., Eisenbarth, S. C., Iyer, S. S., Sadler, J. J., Colegio, O. R., Tephly, L. A., Carter, A. B., Rotham, P. B., Flavell, R. A., and Sutterwala, F. S. 2008. The Nalp3 inflammasome is essential for the development of silicosis. *Proc. Natl. Acad. Sci. USA* 105:9035–9040.

Chapman, S. J., Cookson, W. O. C., Musk, A. W., and Lee, Y. C. 2003. Benign asbestos pleural diseases. *Curr. Opin. Pulm. Med*. 9:266–271.

Choe, N., Tanaka, S., Xia, W., Hemenway, D. R., Roggli, V. L., and Kagan, E. 1997. Pleural macrophage recruitment and activation in asbestos-induced pleural injury. *Environ. Health Perspect*. 105:1257–1260.

Christensen, B. C., Marsit, C. J., Houseman, E. A., Godleski, J. J., Longacker, J. L., Zheng, S., Yeh, R. F., Wrensch, M. R., Wiemels, J. L., Karagas, M. R., Bueno, R., Sugarbaker, D. J., Nelson, H. H., Wiencke, J. K., and Kelsey, K. T. 2009. Differentiation of lung adenocarcinoma, pleural mesothelioma, and nonmalignant pulmonary tissues using DNA methylation profiles. *Cancer Res*. 69:6315–6321.

Churg, A. 1982. Asbestos fibers and pleural plaques in a general autopsy population. *Am. J. Pathol*. 109:88–96.

Churg, A. 1983. Current issues in the pathologic and mineralogic diagnosis of asbestos-induced disease. *Chest* 84:275–280.

Churg, A. 1994. Deposition and clearance of chrysotile asbestos. *Ann. Occup. Hyg*. 38:625–633.

Churg, A., and Warnock, M. L. 1980. Asbestos fibers in the general population. *Am. Rev. Respir. Dis*. 122:669–678.

Daubriac, J., Fleury-Feith, J., Kheuang, L., Galipon, J., Saint-Albin, A., Renier, A., Giovannini, M., Galateau-Sallé, F., and Jaurand, M. C. 2009. Malignant pleural mesothelioma cells resist anoikis as quiescent pluricellular aggregates. *Cell Death Differ*. 16:1146–1155.

Davis, J. M. G. 1974. An electron microscope study of the response of mesothelial cells to the intrapleural injection of asbestos dust. *Br. J. Exp. Pathol*. 55:64–70.

Davis, J. M., Jones, A. D., and Miller, B. G. 1991. Experimental studies in rats on the effects of asbestos inhalation coupled with the inhalation of titanium dioxide or quartz. *Int. J. Exp. Pathol*. 72:501–525.

Decologne, N., Kolb, M., Margetts, P. J., Menetrier, F., Artur,Y., Garrido, C., Gauldie, J., Camus, P., and Bonniaud, P. 2007. TGF-beta1 induces progressive pleural scarring and subpleural fibrosis. *J. Immunol*. 179:6043–6051.

DeVuyst, P., Karjalainen, A., Dumortier, P., Pairon, J., Monso, E., Brochard, P., Teschlet, H., Tossavainen, A., and Gibbs, A. 1998. Guidelines for mineral fibre analyses in biological samples: Report of the ERS Working Group. *Eur. Respir. J*. 11:1416–1426.

Dodson, R. F., and Atkinson, M. A. L. 2006. Measurements of asbestos burden in tissues. *Ann. NY Acad. Sci*. 1076:281–291.

Dodson, R. F., and Hammar, S. P. 2006. Pleural mesothelioma in a woman whose documented past exposure to asbestos was from smoking asbestos-containing filtered cigarettes: The comparative value of analytical transmission electron microscopic analysis of lung and lymph node tissue. *Inhal. Toxicol*. 18:679–684.

Dodson, R. F., Atkinson, M. A., and Levin, J. L. 2003. Asbestos fiber length as related to potential pathogenicity: a critical review. *Am. J. Ind. Med*. 44:291–297.

Dodson, R. F., Graef, R., Shepherd, S., O'Sullivan, M., and Levin, J. 2005. Asbestos burden in cases of mesothelioma from individuals from various regions of the United States. *Ultrastruct. Pathol*. 29: 415–433.

Dodson, R. F., Shepherd, S., Levin, J., and Hammar, S. P. 2007. Characteristics of asbestos concentration in lung as compared to asbestos concentration in various levels of lymph nodes that collect drainage from the lung. *Ultrastruct. Pathol*. 31: 95–133.

Dodson, R. F., Williams, M. G., Jr., Corn, C. J., Brollo, A., and Bianchi, C. 1990. Asbestos content of lung tissue, lymph

nodes, and pleural plaques from former shipyard workers. *Am. Rev. Respir Dis*. 142: 843–847.

Dodson, R. F., and Ford, J. O. 1985. Early response of the visceral pleura following asbestos exposure: An ultrastructural study. *J. Toxicol. Environ. Health* 15: 673–686.

Dodson, R. F., O'Sullivan, M. F., Huang, J., Holiday, D. B., and Hammar, S. P. 2000. Asbestos in extrapulmonary sites: omentum and mesentery. *Chest* 117:486–493.

Donaldson, K., Borm, P. J., Castranova, V., and Gulumian, M. 2009. The limits of testing particle-mediated oxidative stress in vitro in predicting diverse pathologies; Relevance for testing of nanoparticles. *Part. Fibre Toxicol*. 6:13.

Dostert, C., Pétrilli, V., Van Bruggen, R., Steele, C., Mossman, B. T., and Tschopp, J. 2008. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* 320:674–677.

Dumortier, P., De Vuyst, P., and Rey, F. 1998. Letter to the editor: RE main asbestos type in pleural mesothelioma. *Am. J. Ind. Med*. 33:94–95.

Everitt, J. I., Gelzleichter, T. R., Bermudez, E., Mangum, J. B., Wong, B. A., Janszen, D. B., and Moss, O. R. 1997. Comparison of pleural responses of rats and hamsters to subchronic inhalation of refractory ceramic fibers. *Environ. Health Perspect*. 105: 1209–1213.

Fasske, E. 1986. Pathogenesis of pulmonary fibrosis induced by chrysotile asbestos. *Virchows Arch*. 408:329–346.

Foley-Comer, A. J., Herrick, S. E., Al-Mishlab, T., Prêle, C. M., Laurent, G. J., and Mutsaers, S. E. 2002. Evidence for incorporation of free-floating mesothelial cells as a mechanism of serosal healing. *J. Cell. Sci*. 115:1383–1389.

Fubini, B. 1997. Surface reactivity in the pathogenic response to particulates. *Environ. Health Perspect*. 105:1013–1020.

Fubini, B., and Mollo, L. 1995. Role of iron in the reactivity of mineral fibers. *Toxicol. Lett*. 82–83:951–960.

Gazdar, A. F., Butel, J. S., and Carbone, M. 2002. SV40 and human tumours: myth, association or causality? *Nat. Rev. Cancer* 2:957–964.

Gelzleichter, T. R., Bermudez, E., Mangum, J. B., Wong, B. A., Everitt, J. I., and Moss, O. R. 1996. Pulmonary and pleural responses in Fischer 344 rats following short-term inhalation of a synthetic vitreous fiber. I. Quantitation of lung and pleural fiber burdens. *Fundam. Appl. Toxicol*. 30:31–38.

Gelzleichter, T. R., Bermudez, E., Mangum, J. B., Wong, B. A., Janszen, D. B., Moss, O. R., and Everitt, J. I. 1999. Comparison of pulmonary and pleural responses of rats and hamsters to inhaled refractory ceramic fibers. *Toxicol. Sci*. 49:93–101.

Gelzleichter, T. R., Bermudez, E., Mangum, J. B., Wong, B. A., Moss, O. R., and Everitt, J. I. 1996. Pulmonary and pleural responses in Fischer 344 rats following short-term inhalation of a synthetic vitreous fiber. II. Pathobiologic responses. *Fundam. Appl. Toxicol*. 30:39–46.

Ghio, A. J., Stonehuerner, J. G., Richards, J. H., Crissman, K. M., Roggli, V. L., Piantadosi, C. A., and Carraway, M. S. 2008. Iron homeostasis and oxidative stress in idiopathic pulmonary alveolar proteinosis: A case-control study. *Respir Res*. 9:10–18.

Gibbs, A. R., Stephen, M., Griffith, D. M., Blight, B. J., and Pooley, F. D. 1991. Fibre distribution in the lungs and pleura of subjects with asbestos related diffuse fibrosis. *Br. J. Ind. Med*. 48:762–770.

Greillier, L., Baas, P., Welch, J. J., Hasan, B., and Passioukov, A. 2008. Biomarkers for malignant pleural mesothelioma: current status. *Mol. Diagn. Ther*. 12:375–390.

Greim, H., Borm, P., Schins, R., Donaldson, K., Driscoll, K., Hartwig, A., Kuempel, E., Oberdörster, G., and Speit, G. 2001. Toxicity of fibers and particles. Report of the workshop held in Munich, Germany, 26–27 October 2000. *Inhal. Toxicol*. 13: 737–754.

Hammar, S. P. 1994. The pathology of benign and malignant pleural disease. *Chest Surg. Clin. North Am*. 4:405–430.

Hardy J. A., and Aust, A. E. 1995. The effect of iron binding on the ability of crocidolite asbestos to catalyze DNA single-strand breaks. *Carcinogenesis*. 16: 319–325.

Hei, T. K., Xu, A., Louie, D., and Zhou, Y. L. 2000. Genotoxicity versus carcinogenicity: Implications from fiber toxicity studies. *Inhal. Toxicol*.12(suppl. 3):141–147.

Heintz, N. H., Janssen-Heininger, Y. M., and Mossman, B. T. 2010. Asbestos, lung cancers, and mesotheliomas: from molecular approaches to targeting tumor survival pathways. *Am. J. Respir. Cell Mol. Biol*. 42: 133–139.

Henderson, R. F., Driscoll, K. E., Harkema, J. R., Lindenschmidt, R. C., Chang, I. Y., Maples, K. R., and Barr, E. B. 1995. A comparison of the inflammatory response of the lung to inhaled versus instilled particles in F344 rats. *Fundam. Appl. Toxicol*. 24: 183–197.

Herrick, S. E., and Mutsaers, S. E. 2004. Mesothelial progenitor cells and their potential in tissue engineering. *Int. J. Biochem. Cell Biol*. 36:621–642.

Hill, G. D., Mangum, J. B., Moss, O. R., and Everitt, J. I. 2003. Soluble ICAM-1, MCP-1, and MIP-2 protein secretion by rat pleural mesothelial cells following exposure to amosite asbestos. *Exp. Lung Res*. 29: 277–290.

Hillerdal, G. 1989. Rounded atelectasis. Clinical experience with 74 patients. *Chest* 95:836–841.

Holt, P. F. 1981. Transport of inhaled dust extrapulmonary sites. *J. Pathol*. 133:123–129.

Huang, S. X. L., Jaurand, M. C., Kamp, D. W., Whysner, J., and Hei, T. K. 2011. Role of mutagenicity in mineral fiber-induced carcinogenicity and other diseases. *J. Toxicol. Environ. Health B*, 14:179–245.

Husain, A. N., Colby, T. V., Ordonez, N. G., Krausz, T., Borczuk, A., Cagle, P. T., Chirieac, L. R., Chur, A., Galateau-Salle, F., Gibbs, A. R., Gown, A. M., Hammar, S. P., Litzky, L. A., Roggli, V. L., Travis, W. D., and Wick, M. R. 2009. Guidelines for pathologic diagnosis of malignant mesothelioma: A consensus statement from the International Mesothelioma Interest Group. *Arch. Pathol. Lab. Med*. 133:1317–1331.

Institute of Medicine. 2006. *Asbestos: Selected cancers*. Washington, DC: National Academies Press.

International Life Sciences Institute. 2005. Testing of fibrous particles: Short-term assays and strategies. Report of an ILSI Risk Science Institute Working Group. *Inhal. Toxicol*. 17:497–537.

Janssen-Heininger, Y. M., Mossman, B. T., Heintz, N. H., Forman, H. J., Kalyanaraman, B., Finkel, T., Stamler, J. S., Rhee, S. G., and van der Vliet, A. 2008. Redox-based regulation of signal transduction: principles, pitfalls, and promises. *Free Radical Biol. Med*. 45:1–17.

Jantz, M. A., and Antony, V. B. 2008. Pathophysiology of the pleura. *Respiration* 75:121–133.

Jarabek, A. M., Asgharian, B., and Miller, F. J. 2005. Dosimetric adjustments for interspecies extrapolation of inhaled poorly soluble particles (PSP). *Inhal.Toxicol*. 17: 317–334.

Jaurand, M. C. 1996. Use of in-vitro genotoxicity and cell transformation assays to evaluate the potential carcinogenicity of fibres. *IARC Sci. Publ*. 140:55–72.

Jaurand, M. C., Renier, A., and Daubriac, J. 2009. Mesothelioma: Do asbestos and carbon nanotubes pose the same health risk? *Part. Fibre Toxicol*. 6:16–30.

Jiménez, L. A., Zanella, C., Fung, H., Janssen, Y. M., Vacek, P., Charland, C., Goldberg, J., and Mossman, B. T. 1997. Role of extracellular signal-regulated protein kinases in apoptosis by asbestos and H_2O_2. *Am. J. Physiol*. 273:L1029–L1035.

Kean, J. M., Rao, S., Wang, M., and Garcea, R. L. 2009. Seroepidemiology of human polyomaviruses. *PLoS Pathol*. doi: 10.1371/journal.ppat.1000363.

Kishomoto, T., Ono, T., Okada, K., and Ito, H. 1989. Relationship between number of asbestos bodies in autopsy lung and pleural plaques on chest X-ray film. *Chest* 95: 549–552.

Kratzke, R. A., and Gazdar, A. F. 2005. Oncogenes and tumur suppressor genes in malignant mesothelioma. In *Advances in pathogenesis, diagnosis, and translational therapies*, vol. 8, eds. H. I. Pass, N. J. Vogelzang, and M. Carbone, pp. 124–140. New York: Springer Science & Business Media.

Kroczynska, B., Cutrone, R., Bocchetta, M., Yang, H., Elmishad, A. G., Vacek, P., Ramos-Nino, M., Mossman, B. T., Pass, H. I., and Carbone, M. 2006. Crocidolite asbestos and SV40 are cocarcinogens in human mesothelial cells and in causing mesothelioma in hamsters. *Proc. Natl. Acad. Sci. USA* 103:14128–4133.

Lee, M. M., Green, F. H., Demetrick, D. J., Jiang, X. X., and Schürch, S. 1993. A study of surface property changes in rat mesothelial cells induced by asbestos using aqueous two-phase polymer solutions. *Biochim. Biophys. Acta*.1181:223–232.

Lehnert, B. E., Valdez, Y. E., and Bomalaski, S. H. 1985. Lung and pleural free-cell responses to the intrapulmonary deposition of particles in the rat. *J. Toxicol. Environ. Health* 16:823–839.

Light, R. W., and Broaddus, V. C. 2010. Pleural Effusion and Pleural Tumors. In *Murray and Nadel's Textbook of Respiratory Medicine*, 5th ed. Eds. J. R. Mason, V. C. Broaddus, T. R. Martin, T. E. King, Jr., D. E. Schraufnagel, J. F. Murray, and J. A. Nadel. pp. 1719–1763, 1792–1813, vol. 2. Philadelphia, Elsevier Saunders.

Lillis, R., Lerman, Y., and Selikoff, I. J. 1988. Symptomatic benign pleural effusions among asbestos insulation workers: Residual radiographic abnormalities. *Br. J. Ind. Med*. 45:443–449.

Lippmann, M., and Schlesinger, R. B. 1984. Interspecies comparisons of particle deposition and mucociliary clearance in tracheobronchial airways. *J. Toxicol. Environ. Health*. 13:441–469.

Lippmann, M., Yeates, D. B., and Albert, R. E. 1980. Deposition, retention, and clearance of inhaled particles. *Br. J. Ind. Med*. 37: 337–362.

Liu, W., Ernest, J. D., and Broaddus, V. C. 2000. Phagocytosis of crocidolite asbestos induces oxidative stress, DNA damage and apoptosis in mesothelial cells. *Am. J. Respir. Cell Mol. Biol*. 23:371–378.

Lockey, J. E., Brooks, S. M., Jarabek, A. M., Khoury, P. R., McKay, R. T., Carson, A., Morrison, J. A., Wiot, J. F., and Spitz, H. B. 1984. Pulmonary changes after exposure to vermiculite contaminated with fibrous tremolite. *Am. Rev. Respir. Dis*. 129: 952–958.

Manning, C. B., Vallyathan, V., and Mossman, B. T. 2002. Diseases caused by asbestos: mechanisms of injury and disease development. *Int. Immunopharmacol*. 2:191–200.

Marchi, E., Liu, W., and Broaddus, V. C. 2000. Mesothelial cell apoptosis is confirmed in vivo by morphological change in cytokeratin distribution. *Am. J. Physiol. Lung Cell. Mol. Physiol*. 278:L528–L535.

Mast, R. W., Hesterberg, T. W., Glass, L. R., McConnell, E. E., Anderson, R., and Bernstein, D. M. 1994. Chronic inhalation and biopersistence of refractory ceramic fiber in rats and hamsters. *Environ. Health Perspect*. 102:207–209.

Maxim, L. D., and McConnell, E. E. 2001. Interspecies comparisons of the toxicity of asbestos and synthetic vitreous fibers: A weight-of-the-evidence approach. *Regul. Toxicol. Pharmacol*. 33:319–342.

Meeker, G., Bern, A. M., Brownfield, I. K., Lowers, H. A., Sutley, S. J., Hoefen, T. M., and Vance, J. S. 2003. The composition and morphology of amphiboles from the Rainy Creek complex, near Libby, Montana. *Am. Mineral*. 88:1955–1969.

McConnell, E. E. 1994. Synthetic vitreous fibers—Inhalation studies. *Regul. Toxicol. Pharmacol*. 20:S22–S34.

McConnell, E. E., Axten, C., Hesterberg, T. W., Chevalier, J., Miller, W. C., Everitt, J., Oberdörster, G., Chase, G. R., Thevenaz, P., and Kotin, P. 1999. Studies on the inhalation toxicology of two fiberglasses and amosite asbestos in the Syrian golden hamster. Part II. Results of chronic exposure. *Inhal. Toxicol*. 11:785–835.

McDonald, J. C., Armstrong, B. G., Edwards, C. W., Gibbs, A. R., Lloyd, H. M., Pooley, F. D., Ross, D. J., and Rudd, R. M. 2001. Case-referent survey of young adults with mesothelioma: I. Lung fibre analyses. *Ann. Occup. Hyg*. 45:513–518.

Miserocchi, G., Sancini, G., Mantegazza, F., and Chiappino, G. 2008. Translocation pathways for inhaled asbestos fibers. *Environ. Health* 7:4.

Mitchev, K., Dumortier, P., and De Vuyst, P. 2002. Black spots and hyaline pleural plaques on the parietal pleura of 150 urban necropsy cases. *Am. J. Surg. Pathol*. 26:1198–1206.

Mossman, B. T., Lippman, M., Hesterberg, T. W., Kelsey, K. T., Barchowsky, A., and Bonner, J. C. 2011. Pulmonary endpoints (lung carcinomas and asbestosis) following inhalation exposure to asbestos. *J. Toxicol. Environ. Health B*, 14: 76–121.

Murthy, S. S., and Testa, J. R. 1999. Asbestos, chromosomal deletions, and tumor suppressor gene alterations in human malignant mesothelioma. *J. Cell Physiol*. 180: 150–157.

Mutsaers, S. E., Kalomenidis, I., Wilson, N. A., and Lee, Y. C. 2006. Growth factors in pleural fibrosis. *Curr. Opin. Pulm. Med*. 12: 251–258.

Mutsaers, S. E., Prele, C. M., Brody, A. R., and Idell, S. 2004. Pathogenesis of pleural fibrosis. *Respirology* 9:428–440.

Nishimura, S. L., and Broaddus, V. C. 1998. Asbestos-induced pleural disease. *Clin. Chest Med*. 19:311–329.

Oberdörster, G., Ferin, J., Marcello, N. L., and Meinhold, S. H. 1983. Effects of intrabronchially instilled amosite on lavagable lung and pleural cells. *Environ Health Perspect*. 51:41–48.

Oberdörster, G. 2000. Determinants of the pathogenicity of man-made vitreous fibers (MMVF). *Int. Arch. Occup. Environ. Health* 73:S60–S68.

Oberdörster, G. 2002. Toxicokinetics and effects of fibrous and nonfibrous particles. *Inhal. Toxicol*. 14:29–56.

Owens, M. W., and Milligan, S. A. 1995. Pleuritis abd pleural effusions. *Curr. Opin. Pulm. Med*. 1:318–323.

Pache, J. C., Janssen, Y. M., Walsh, E. S., Quinlan, T. R., Zanella, C. L., Low, R. B., Taatjes, D. J., and Mossman, B. T. 1998. Increased epidermal growth factor-receptor protein in a human mesothelial cell line in response to long asbestos fibers. *Am. J. Pathol*. 152:333–340.

Peipins, L. A., Lewin, M., Campolucci, S., Lybarger, J. A., Miller, A., Middleton, D., Weis, C., Spence, M., Black, B., and Kapil, V. 2003. Radiographic abnormalities and exposure to asbestos-contaminated vermiculite in the community of Libby, Montana, USA. *Environ. Health Perspect*. 111:1753–1759.

Poland, C. A., Duffin, R., Kinloch, I., Maynard, A., Wallace, W. A., Seaton, A., Stone, V., Brown, S., Macnee, W., and Donaldson, K. 2008. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. *Nat. Nanotechnol*. 3:423–428.

Pott, F. 1980. Animal experiments on biological effects of mineral fibres. *IARC Sci. Publ*. 30:261–272.

Pott, F., Huth, F., and Friedrichs, K. H. 1974. Tumorigenic effect of fibrous dusts in experimental animals. *Environ. Health Perspect*. 9:313–315.

Robledo, R., and Mossman, B. 1999. Cellular and molecular mechanisms of asbestos-induced fibrosis. *J. Cell Physiol*. 180: 158–166.

Roggli, V. L., Sharma, A., Butnor, K. J., Sporn, T., and Vollmer, R. T. 2002. Malignant mesothelioma and occupational exposure to asbestos: A clinicopathological correlation of 1445 cases. *Ultrastruct. Pathol*. 26:55–65.

Roggli, V. L. 1990. Human disease consequences of fiber exposures: A review of human pathology and fiber burden data. *Environ. Health Perspect*. 88:295–303.

Roggli, V. L. 1992. Quantitative and analytical studies in the diagnosis of mesotheliomas. *Sem. Diag. Pathol*. 9:162–168.

Roggli, V. L., and Sanders, L. L. 2000. Asbestos content of lung tissue and carcinoma of

the lung: A clinicopathologic correlation and mineral fiber analysis of 234 cases. *Ann. Occup. Hyg*. 44:109–117.

Roggli, V. L., and Sharma, A. 2004. Analysis of tissue mineral fiber content In *Pathology of asbestos-associated disease*, 2nd ed., eds. V. L. Roggl, T. D. Oury, and T. A. Spron, pp. 309–354. New York: Springer-Verlag.

Rohs, A. M., Lockey, J. E., Dunning, K. K., Shukla, R., Fan, H., Hilbert, T., Borton, E., Wiot, J., Meyer, C., Shipley, R. T., Lemasters, G. K., and Kapil, V. 2008. Low-level fiber-induced radiographic changes caused by Libby vermiculite: A 25-year follow-up study. *Am. J. Respir. Crit. Care Med*. 177:630–637.

Ryman-Rasmussen, J. P., Cesta, M. F., Brody, A. R., Shipley-Phillips, J. K., Everitt, J. I., Tewksbury, E. W., Moss, O. R., Wong, B. A., Dodd, D. E., Andersen, M. E., and Bonner, J. C. 2009. Inhaled carbon nanotubes reach the subpleural tissue in mice. *Nat. Nanotechnol*. 4:747–751.

Sahn, S. A., and Antony, V. B. 1984. Pathogenesis of pleural plaques. Relationship of early cellular response and pathology. *Am. Rev. Respir. Dis*. 130:884–887.

Sanchez, V. C., Pietruska, J. R., Miselis, N. R., Hurt, R. H., and Kane, A. B. 2009. Biopersistence and potential adverse health impacts of fibrous nanomaterials: What have we learned from asbestos? *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol*. 1: 511–529.

Sebastien, P., Janson, X., Gaudichet, A., Hirsch, A., and Bignon, J. 1980. Asbestos retention in human respiratory tissues: Comparative measurements in lung parenchyma and in partial pleura. *IARC Sci. Publ*. 30:237–246.

Shukla, A., Gulumian, M., Hei, T. K., Kamp, D., Rahman, Q., and Mossman, B. T. 2003. Multiple roles of oxidants in the pathogenesis of asbestos-induced diseases. *Free Radical Biol. Med*. 34:1117–1129.

Shukla, A., Jung, M., Stern, M., Fukagawa, N. K., Taatjes, D. J., Sawyer, D., Van Houten, B., and Mossman, B. T. 2003. Asbestos induces mitochondrial DNA damage and dysfunction linked to the development of apoptosis. *Am. J. Physiol. Lung Cell. Mol. Physiol*. 285:L1018–L1025.

Stanton, M. F., Blackwell, R., and Miller, E. 1969. Experimental pulmonary carcinogenesis with asbestos. *Am. Ind. Hyg. Assoc. J*. 30:236–244.

Stanton, M. F., Layard, M., Tegeris, A., Miller, E., May, M., Morgan, E., and Smith, A. 1981. Relation of particle dimension to carcinogenicity in amphibole asbestoses and other fibrous minerals. *JNCI* 67:965–975.

Stanton, M. F., Laynard, M., Tegeris, A., Miller, E., May, M., and Kent, E. 1977. Carcinogenicity of fibrous glass: Pleural response in the rat in relation to fiber dimension. *J. Natl. Cancer Inst*. 58: 587–603.

Stephens, M., Gibbs, A. R., Pooley, F. D, and Wagner, J. C. 1987. Asbestos induced diffuse pleural fibrosis: pathology and mineralogy. *Thorax* 42:583–588.

Sullivan, P. A. 2007. Vermiculite, respiratory disease, and asbestos exposure in Libby, Montana: update of a cohort mortality study. *Environ Health Perspect*. 115: 579–585.

Suzuki, Y., and Kohyama, N. 1991. Translocation of inhaled asbestos fibers from the lung to other tissues. *Am. J. Ind. Med*. 19:701–704.

Suzuki, Y., Yuen, S. R., and Ashley, R. 2005. Short, thin asbestos fibers contribute to the development of human malignant mesothelioma: Pathological evidence. *Int. J. Hyg. Environ. Health* 208:201–210.

Suzuki, Y., and Yuen, S. R. 2001. Asbestos tissue burden study on human malignant mesothelioma. *Ind. Health* 39: 150–160.

Suzuki, Y., Yuen, S. R., and Ashley, R. 2005. Short, thin asbestos fibers contribute to the development of human malignant mesothelioma: Pathological evidence. *Int. J. Hyg. Environ. Health* 208:201–210.

Tanaka, S., Choe, N., Iwagaki, A., Hemenway, D. R., and Kagan, E. 2000. Asbestos exposure induces MCP-1 secretion by pleural mesothelial cells. *Exp. Lung Res*. 26: 241–255.

Tyler, W. S. 1983. Comparative subgross anatomy of lungs. Pleurae, interlobular septa, and distal airways. *Am. Rev. Respir. Dis*. 128:S32–S36.

Upadhyay, D., and Kamp, D. W. 2003. Asbestos-induced pulmonary toxicity: role of DNA damage and apoptosis. *Exp. Biol. Med*. 228:650–659.

Viallat, J. R., Raybuad, F., Passarel, M., and Boutin, C. 1986. Pleural migration of chrysotile fibers after intratracheal injection in rats. *Arch. Environ. Health* 41:282–286.

Wang, N. S. 1975. The preformed stomas connecting the pleural cavity and the lymphatics in the parietal pleura. *Am. Rev. Respir*. 110:623–633.

Weiner, S. J., and Neragi-Miandoab, S. 2009. Pathogenesis of malignant pleural mesothelioma and the role of environmental and genetic factors. *J. Cancer Res. Clin. Oncol*. 135:15–27.

Whitehouse, A. C. 2004. Asbestos-related pleural disease due to tremolite associated with progressive loss of lung function: Serial observations in 123 miners, family members, and residents of Libby, Montana. *Am. J. Ind. Med*. 46: 219–225.

Yang, H., Bocchetta, M., Kroczynska, B., Elmishad, A. G., Chen, Y., Liu, Z., Bubici, C., Mossman, B. T., Pass, H. I., Testa, J. R., Franzoso, G., and Carbone, M. 2006. TNF-alpha inhibits asbestos-induced cytotoxicity via a NF-kappaB-dependent pathway, a possible mechanism for asbestos-induced oncogenesis. *Proc. Natl. Acad. Sci. USA* 103:10397–10402.

Yang, H., Rivera, Z., Jube, S., Nasu, M., Bertino, P., Goparaju, C., Franzoso, G., Lotze, M. T., Krausz, T., Pass, H. I., Bianchi, M. E., and Carbone, M. 2010. Programmed necrosis induced by asbestos in human mesothelial cells causes high-mobility group box 1 protein release and resultant inflammation. *Proc. Natl. Acad. Sci. USA* 107: 12611–12616.

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Leukocytes in Mammary Development and Cancer

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Leukocytes in Mammary Development and Cancer

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Leukocytes, of both the innate and adaptive lineages, are normal cellular components of all tissues. These important cells not only are critical for regulating normal tissue homeostasis, but also are significant paracrine regulators of all physiologic and pathologic tissue repair processes. This article summarizes recent insights regarding the trophic roles of leukocytes at each stage of mammary gland development and during cancer development, with a focus on *Murids* and humans.

Mammary gland development can be divided into discrete phases. An initial analge is laid down from the milk-line during embryonic development resulting in a minimal ductal structure emanating from the nipple. Development of this anlage into a ductal tree is reactivated postnatally by exposure to the female sex steroid hormone estradiol-17β (E2), whose synthesis begins upon entry into puberty. In mice, this occurs at about 3 wk of age and is characterized by the formation of terminal end buds (TEB) at the ends of the ducts. These TEBs are clublike multilaminate epithelial structures that are the proliferative engines that drive mammary development. These structures also contain the mammary stem cells whose progeny differentiate into luminal and myoepithelial cells. The TEB structures disappear on their encounter with the edge of the fat pad and turn into terminal end-ducts (TED) that cease proliferation and which are bilaminar. As the primary branches grow out through the fat pad, secondary branches form to generate the mature tree that in mice is completed about 8 wk of age coincident with sexual maturity. At each estrus cycle thereafter, there is further development of the secondary branches and dependent on mouse strain, a degree of lobuloalveolar development. The next major phase of growth is during pregnancy in response to progesterone and prolactin when there is significant secondary branching morphogenesis, and the generation of the milk producing lobuloalveolar structures sprouting from these branches. At the end of the process, the gland is filled with ducts and alveolar structures with

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a commensurate loss of adipocytes. After birth and on suckling, lactation occurs with its effect on the secretory structure of alveoli that flatten to surround a milk-filled lumen. Weaning terminates the lactational process and the gland involutes to re-form a virgin-like structure to begin the cycle again during the next pregnancy (Daniel and Silberstein 1987; Richert et al. 2000; Neville et al. 2002). Every stage of mammary epithelial development is accompanied by changes in the surrounding stroma. This stroma is populated by many immune cells particularly those of the innate system. Although these cells undoubtedly have a role in immunological responses especially during lactation (Paape et al. 2002; Atabai et al. 2007), this review will concentrate on the trophic roles of these hematopoietic cells at each stage of development and during cancer development, with a focus on *Murids* and humans.

PUBERTAL MAMMARY DEVELOPMENT

In early postnatal development, classical experiments revealed that instructive signals arise from stromal cells that define the identity of the mammary epithelial structures (Sakakura 1987). In mice, the rudimentary mammary ductal tree begins to develop with the formation of the multilaminate club-shaped TEBs at their distal end. These TEBs grow out through the fatty stroma, bifurcating to generate the primary ductal tree (Richert et al. 2000).

The stroma of the developing mammary gland is dominated by adipocytes (Neville et al. 1998). However, although these cells are required for mammary epithelial development, they do not appear to define its identity (Landskroner-Eiger et al. 2010). Instead adipocytes provide structural support and their secreted adipokines that influence ductal development. Macrophages are found abundantly adjacent to the nipple area and rudimentary ductal structures at 2 wk of age before mammary development commences (Gouon-Evans et al. 2000). Co-incident with the initiation of development the newly formed TEBs is surrounded by a complex stroma containing fibroblasts, macrophages, mast cells, and eosinophils (Gouon-Evans et al. 2000, 2002; Lilla and Werb 2010) (Figs. 1–4). In contrast, neither basophils nor T and B cells can be detected in the vicinity of the TEBs (Gouon-Evans et al. 2002).

The macrophages have a tendency to accumulate around the shaft of the club and in this vicinity they move rapidly along the sheaf of collagen fibrils that align along the axis of the TEB (Ingman et al. 2006) (Fig. 3). Eosinophils are preferentially located around the head of the TEB and they also concentrate in the cleft that forms as the TEBs bifurcate (Gouon-Evans et al. 2000) (Figs. 1, 4). Mast cells are found in a scattered pattern at the invasive front of the TEBs (Atabai et al. 2007; Lilla and Werb 2010) (Fig. 2). Macrophages and eosinophils persist in these locations through development but disappear as soon as the TEBs turn into TEDs (Gouon-Evans et al. 2000; Lilla and Werb 2010). Thereafter, eosinophils, macrophages and mast cells are not present in significant numbers adjacent to the epithelia until development is restarted during pregnancy (Pollard and Hennighausen 1994; Szewczyk et al. 2000; Gouon-Evans et al. 2002; Lilla and Werb 2010). However, macrophages are found throughout the adipose tissue during this and other stages of mammary development (Gouon-Evans et al. 2000; Schwertfeger et al. 2006a).

The mechanisms whereby these innate immune cells are recruited to the mammary epithelial structures have not been fully elucidated, although the process is clearly triggered by estrogen. Genetic depletion of the chemoattractant eotaxin completely eliminates the recruitment of eosinophils to the mammary gland even though circulating numbers in bone marrow and peripheral blood are normal (Gouon-Evans et al. 2000). Eotaxin is induced in the mammary gland at puberty coincident with eosinophil recruitment strongly suggesting that this is the estrogen-regulated chemoattractant (Gouon-Evans et al. 2000). Studies of mice in which colony stimulating factor-1 (CSF-1) is depleted owing to homozygosity for the *Csf1* null mutation, osteopetrotic, ($Csf1^{op}$) have shown that many tissues but not all are severely depleted of macrophages. The mammary gland is one of these severely affected tissues indicating

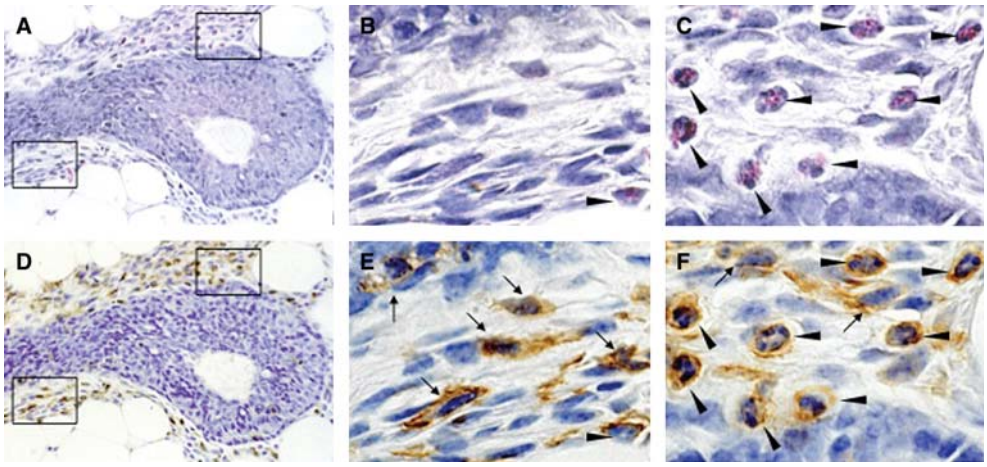

Figure 1. Macrophage and eosinophil recruitment to the terminal end buds of mice. H&E longitudinal sections of terminal end buds at 5 wk of age. Sections were first stained with H&E (*A–C*) and then destained and immunostained using anti-F4/80 antibody followed by a peroxidase detection system for positive signal (brown; *D,E,F*). Note the presence of a dense stroma particularly around the shaft and beginning of the TEB head that consists of fibroblasts and abundant immune cells. This stroma isolates the epithelial compartment from the adipoctyes of the fat pad but is sparser at the growing tip. *B,C*, and *E,F* are high-powered views of *A* and *D*, respectively, and the lower panels boxed in *A* and *D* are shown in *B* and *E* whereas the upper panels are in *C* and *F*. The immunostain indicates the F4/80 positive macrophages (arrows) and eosinophils (filled arrow heads) the latter recognized by their eosinophilic granules and polymorphonuclear structures in *C*. Note the distinct but overlapping localization of macrophages and eosinophils around the bulbous head and shaft of the TEB. (Figure adapted from Gouon-Evans et al. [2000] and reprinted here with permission from The Company of Biologists © 2000.)

an essential requirement for CSF-1 (Pollard and Stanley 1996; Gouon-Evans et al. 2000). However, although CSF-1 is expressed by the mammary epithelium (Ryan et al., 2001), transplantation experiments indicate that this epithelial expression is not required for macrophage recruitment during development (Van Nguyen and Pollard, 2002). This suggests that another epithelial-derived chemoattractant recruits the macrophages to the TEB and that

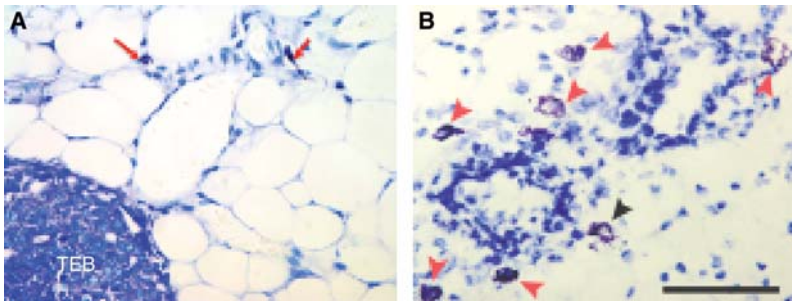

Figure 2. Distribution of mast cells around the terminal end bud of mice. Shown are formalin fixed, paraffin embedded sections stained with toluidine blue in which mast cells are identified by an enzymatic reaction to detect chymase a defining enzyme of these cells. (*A*) Mast cells (red arrows) shown in the fatty stroma in front of an invading terminal end bud (TEB]. (*B*) Many mast cells (red arrows) adjacent to developing mammary epithelium of mice at 5 wk of age. Black arrow shows a mast cell degranulating. (Panel *A* reprinted from Lilla and Werb [2010] and reprinted here with permission from Elsevier © 2010; panel *B* kindly provided by Dr. Zena Werb, USCF). Bar 50 μm.

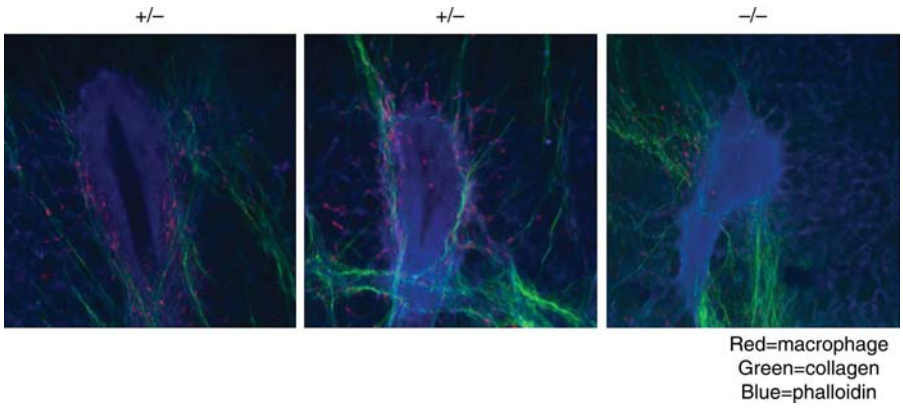

Figure 3. Association of macrophages and collagen fibers with the terminal end bud. Multiphoton imaging of frozen sections of terminal end buds with nuclei stained with DAPI in which the collagen fibers are shown by second harmonic residence and pseudo-colored in green while macrophages are shown by expression of GFP from the *Cs1r*-promoter (data from Sasmono et al. 2003) pseudocolored to red. In *A* and *B*, TEBs from mice heterozygous ($^{+/-}$) for the *Csf1*^{op} allele, whereas *C* is from mice homozygous ($^{-/-}$) for this allele. Note the sheafing of the TEB with collagen 1 containing fibers and the association of macrophages with these fibers. The tubular structure with the visible collagen sheaf running laterally across the image in *B* is a blood vessel. In *C*, the collagen fibers are more disorganized and the TEB is rounder in structure (data from Ingman et al. 2006).

CSF-1 is necessary for their lineage development systemically and for their development from monocytes within the tissue. Interestingly, eosinophils situated at the TEB specifically express the monocyte chemoattractant, CCL-6 (C10), suggesting a cross-talk between these eosinophils and macrophages (Gouon-Evans et al. 2002). However, it is unknown whether CCL-6 is required for macrophage recruitment at this site.

Human mammary development is much less well understood and appears to be less defined as its processes are rather sporadic (Howard and Gusterson 2000). Sexual dimorphism in contrast to the embryonic specification in mice, in humans is initiated by sex hormone secretion at puberty. In females proliferative end budlike structures form and ductal outgrowth occurs that results in the formation of terminal ductal lobular units. According to the individual, variable and extensive branching occurs through puberty that ultimately leads to a structure where primary ducts lead from the nipple to a complex branching pattern of subsidiary ducts that in turn lead to segmental ducts and smaller subsegmental ducts. These subsegmental ducts in turn lead to terminal ducts that lead to blind ended acini. The collection of acini embedded in a complex stroma is referred to as the terminal duct lobular alveolar unit (TDLU). This is thought to be the functional unit of the breast and also thought to be the site of tumor initiation. Strikingly there is great variation in proliferation rates in different TDLUs suggesting the predominance of local factors (Howard and Gusterson 2000). Little is known about hematopoietic cells in human mammary gland during development. Extramedullary hematopoiesis has been described adjacent to the ductal structures in infant breasts until four months of age (Anbazhagan et al. 1991). Macrophages are abundant cells in nipple aspirates from reproductive age women (King et al. 2002). Intriguingly macrophages in the human breast express aromatase suggesting that they may be a local source of estradiol (Mor et al. 1998). A variation in macrophage density therefore could possible explain localized differences in growth of the TDLUs.

Mechanistic studies performed in mice strongly argue for important roles for hematopoietic cells in mammary ductal development. Postnatal ablation of bone marrow cells

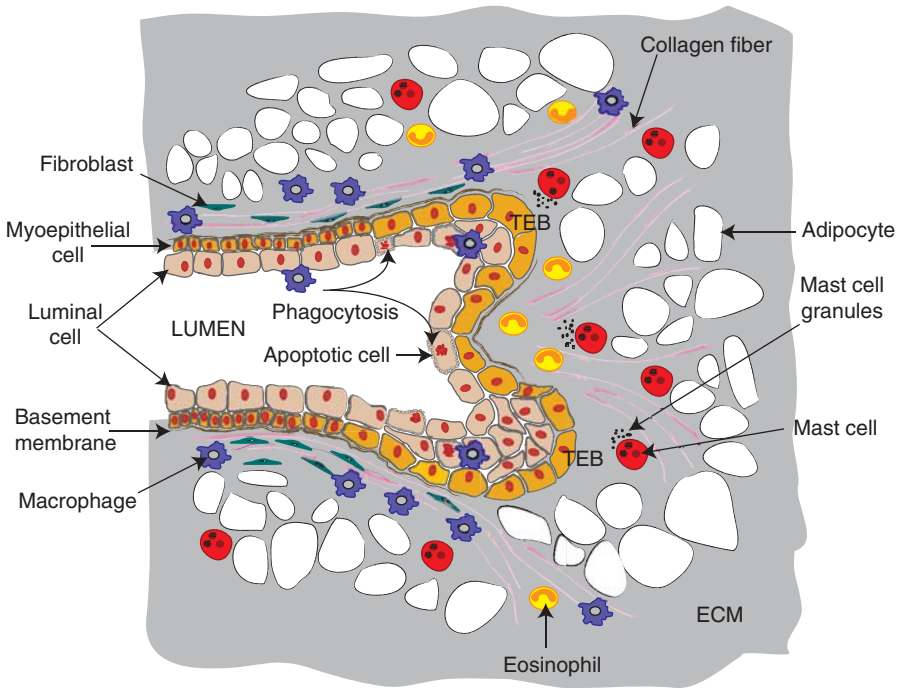

Figure 4. Topography of immune cells in the developing terminal end bud. The diagram shows a schematic of a terminal end bud (TEB) that is bifurcating to give two ductal branches. This TEB is surrounded by a dense fibroblastic stoma and encased by a fibrillar collagen network that is aligned in the direction of the outgrowth through the mammary fat pad that is densely populated by adipocytes. Abundant numbers of innate immune cells are recruited to the TEB and have preferred domains as indicated. Macrophages are enriched around the base and shaft of the TEB but move rapidly up and down the collagen fibers. In addition, they are found in the TEB itself where they phagocytose the apoptotic epithelial cells in the process of lumen formation. Mast cells in contrast, are preferentially located in the stroma in front of the invading TEBs where they provide proteases that enhance TEB invasion. Eosinophils in turn, are found around the bulbous head of the TEB and also are found in the cleft of the bifurcating TEB. Genetic ablation of each of these cell types as described in the text indicates roles for them in the branching morphogenesis of the mammary gland and their combined functions results in a fully branched ducal tree that forms during puberty.

by irradiation before mammary development begins, blocks subsequent mammary development. This developmental block can be overridden by restoration of the bone marrow by transplantation of the irradiated mice, a treatment that results in a complete rescue of the mammary tree. This indicates that irradiation does not irrevocably damage mammary stem cells (Gouon-Evans et al. 2000). In the few mice that survive irradiation without transplantation but who are severely depleted of leukocytes, the mammary gland also fails to develop to any significant extent (Gouon-Evans et al. 2000).

Mice homozygous for the *Csf1* null mutation, osteopetrotic, ($Csf1^{op}$) have inhibited mammary development characterized by fewer numbers of TEBs, reduced branching, and diminished ductal length compared to wild-type mice. Thus in these macrophage-deficient mice, although a ductal tree eventually develops that fills the fat pad, the resulting gland is atrophic (Gouon-Evans et al. 2000). A similar defect was found in the CSF-1 receptor null mutant mice (Dai et al. 2002). The continuous requirement for macrophages in mammary development was shown by transgenic add-back experiments where CSF-1 was expressed exclusively in the mammary epithelium on a tetracycline-regulated system without rescue of any systemic phenotypes. In these transgenic

mice ductal development was rescued in the homozygous *Csf1*^{op} null mice coincident with macrophage recruitment, but only for the duration of the CSF-1 expression (Van Nguyen and Pollard 2002). These data together with expression data showing that the CSF-1 receptor is only expressed in macrophages in the mammary gland, indicates an important role for macrophages in mammary ductal development throughout puberty (Gouon-Evans et al. 2000). This conclusion was reinforced by studies that showed macrophage depletion using a conditional suicide gene approach inhibited mammary epithelial cell proliferation in a model of fibroblast growth factor (FGF) receptor–induced epithelial hyperplasia (Schwertfeger et al. 2006b).

The mammary ductal epithelium can be seeded from a single bipotential stem cell (Shackleton et al. 2006). These stem cells can form an entire mammary epithelial tree on transplantation into a recipient mammary fat pad. The efficiency of this process is dramatically reduced in mice depleted for macrophages either by homozygosity of the CSF-1 null mutation or chemically, using the macrophage-specific liposome-encapsulated clodronate (Gyorki et al. 2009). Thus, macrophages potentiate the stem cell niche that enables engraftment and growth of the stem cells. This data is consistent with effects of macrophage depletion on mammary ductal development because this development is driven by proliferation and differentiation of the stem cells that lie in the TEBs.

Depletion of eosinophils using mice homozygous for a null mutation in the gene encoding the eosinophil chemoattractant, eotaxin, resulted in a decreased numbers of TEBs, and reduced branching although ductal lengths were normal (Gouon-Evans et al. 2000). In contrast, depletion of interleukin (IL)-5, another chemoattractant for eosinophils, did not affect mammary development (Gouon-Evans et al. 2002). However, overrecruitment of eosinophils by transgenic IL-5 expression throughout the mammary gland inhibited TEB formation and ductal branching compared to wild-type mice. This in part, was because of an inhibition of epithelial cell proliferation (Sferruzzi-Perri et al. 2003). The unique position of eosinophils in the normal mammary gland and the preferential effect of their depletion on ductal branching strongly suggest that their role is in regulating branching complexity perhaps by providing inhibitory signals at branch point (Gouon-Evans et al. 2000).

Mast cell depletion in mice carrying a mutation in the W locus (W^{ash}) that encodes the c-kit receptor required for the formation of these cells results in a reduced number of TEBs and branches as well as defective TEB outgrowth caused by a reduction of epithelial cell proliferation (Lilla and Werb 2010). These data indicate that hematopoietic cells of the innate immune system play an important role in the branching morphogenesis of the mammary gland. The precise location of the different types of cells suggests they have an important role in patterning as over-abundance can also lead to inhibited development. Indeed the migratory nature of these cells makes them perfectly equipped for precisely delivering growth factors or growth inhibitors as well as proteases in a temporal and spatial manner. However, the mechanisms whereby these innate immune cells potentiate mammary development are largely unknown.

TEB proliferation is accompanied by apoptosis so that the multilaminate epithelium is remodeled to give a duct consisting of a single layer of columnar epithelium overlaying a single layer of myoepithelial cells. Failure of this ductal clearance inhibits proper mammary development (Humphreys 1999). Macrophages are found in the TEBs engulfing apoptotic cells and in their absence, this process may be inefficient (Humphreys et al. 1996; Gouon-Evans et al. 2000). However, even in macrophage-deficient mice, the ductal structure is reduced to a single columnar layer (Gouon-Evans et al. 2000) and thus there are likely to be compensatory mechanisms through the action of nonprofessional phagocytes as is found for development of other organs (Dai et al. 2002).

TEBs are surrounded by a collagenous matrix (Hinck and Silberstein 2005; Schwertfeger et al. 2006b) and these structures grow

out through a funnel of organized fibers that are composed primarily of fibrillar collagen type 1 (Ingman et al. 2006). Intravital imaging of TEBs using multiphoton microscopy of mice expressing green fluorescent protein (GFP) regulated by a *Csf1r*-promoter (Sasmono et al. 2003) show that macrophages travel up and down these fibers at a fast rate and also "jump" between them (Ingman et al. 2006). Macrophage depletion using $Csf1^{op/op}$ mice results in a reduced number of these complex fibers compared to wild-type mice but without any effect on collagen 1 synthesis that is from the fibroblasts and epithelial cells. Furthermore the fibers that remain in the macrophage-depleted mice are also less orientated than the fibers in wild-type mammary glands (Ingman et al. 2006). Multiphoton imaging shows that in the absence of macrophages, the orientation of TEB outgrowth is perturbed with a failure to form the smooth fanlike pattern at the ductal front as observed in normal mice. In addition, macrophage deficiency results in TEBs that are rounder and more distorted than those in wild-type mice (Fig. 3). Correction of the macrophage defect by transgenic expression of CSF-1 in the mammary epithelium resulted in restoration of collagen bundling and a correction of the distortion of the TEBs. These data suggest a primary role for macrophages in bundling of these large collagen fibers and that these fibers in turn are required for the guiding the morphogenesis of the TEBS to generate a properly spaced ductal structure (Ingman et al. 2006). The mechanism for this action on collagen bundling is unknown but macrophages synthesize many proteases including matrix metalloprotease (MMP)-7 an enzyme that remodels collagen that might be necessary for the processing of the extracellular matrix (ECM) and in particular, the collagenous structures. Macrophages may also significantly affect other matrix molecules through expression of different proteases as well as molecules involved in matrix remodeling such as Lysyl oxidase and SPARC (Sangaletti et al. 2008). For example, macrophages are rich sources of MMP9 that is involved in ECM remodeling (Djonov et al. 2001; Egeblad and Werb 2002).

Mast cell degranulation is required for ductal outgrowth indicating a requirement for granule products. These products consist of proteases and growth factors. Indeed, depletion of serine proteases with a mutation in the di-peptidyl peptidase 1 (DPPI; cathepsin C) gene that activates many serine proteases results in inhibited mammary ductal development that includes a reduction in the numbers of TEBs and an inhibition in terminal duct formation (Lilla and Werb 2010). Cathepsin C degrades collagens 1 and IV and this protease is synthesized by macrophages as well as mast cells (Gocheva et al. 2010; Lilla and Werb 2010). Thus, cathepsin C alterations of the ECM might also be part of the mechanism behind the effects of depletion of these two cell types on ductal development. However, cell type-specific ablation studies have not been performed to definitively assign these functions to mast cells or macrophages.

PREGNANCY AND LACTATION

Ductal development is re-initiated on pregnancy under the influence of many systemic and local factors including the hormones progesterone and prolactin. Around mid-pregnancy in mice, there is a dramatic growth of lobuloalveolar structures that decorate these newly expanded branches with the mammary fat pad expanding to accommodate the outgrowths. At the end of pregnancy the adipocytes have largely been replaced by the lobuloalveolar structures and a milk-producing gland is fully formed that is 7–10-times heavier than the virgin gland (Atabai et al. 2007). After parturition with its associated loss of progesterone and on pup suckling, a lactational switch is effected and the alveoli secrete milk and appear as flattened cells surrounding large milk-filled lumens (Neville et al. 2002). There are relatively few studies of the pregnant human breast but it appears as in rodents that there is an expansion of lobular-alveolar structures with commensurate loss of adipocytes. The lactating gland has alveoli full of milk that is followed by involution postweaning with the alveolar cells dying of apoptosis (Howard and Gusterson 2000).

Mast cells are found adjacent to the expanding alveolar structures and their density increases according to lobuloalveolar development, but their density significantly declines on the lactational switch (Szewczyk et al. 2000). Macrophages also accumulate during pregnancy and they are found in close apposition to and aligned alongside the epithelial structures (Gouon-Evans et al. 2002). Although these somewhat diminish in numbers during lactation, macrophages are found stretched along the outside of the alveolar walls (Gouon-Evans et al. 2002). Both macrophages and neutrophils are found abundantly in milk of farm species and humans where they are thought to have an antimicrobial role. In addition, B cells are found in milk and this is in turn a rich source of antibodies (Atabai et al. 2007). These innate and acquired immune cells are recruited and undergo a transepithelial migration into the alveolar lumens although the mechanisms behind this biology are poorly understood. Nevertheless, it is clear that the lactating mammary gland is an extension of the mucosal immune system such that the milk confers immune protection to the suckling neonate (Brandtzaeg 2010).

Transcriptome analysis of progesterone responsive genes in mammary organoid cultures has also shown up-regulation of many inflammatory molecules several of which are chemoattractants for innate immune cells including serum amyloid proteins A1, 2, and 3 *(Saa1-3)*, suggesting that these molecules may be involved in leukocyte recruitment (Santos et al. 2009). In progesterone treated mice undergoing alveolar development, there was increased expression of SAA1 that is associated with leukocyte recruitment (Santos et al. 2009). CSF-1 is also synthesized in the epithelia of pregnant and lactating mammary glands and milk is a rich source of CSF-1 (Roth 1991; Sapi et al. 1998; Ryan et al. 2001). This epithelial CSF-1 synthesis is regulated by prolactin and progesterone (Sapi and Kacinski 1999). In the absence of CSF-1 in the $Csf1^{op}$ null mutant mouse, there is an almost complete absence of macrophages in the pregnant and lactational glands suggesting that CSF-1 is a major chemoattractant and survival factor for these cells during these phases of development (Gouon-Evans et al. 2002).

Transcriptome analysis of total RNA extracted from mammary gland isolated during pregnancy and lactation also revealed a surprising number of immune mediators expressed at these stages. These include T-cell regulatory cytokines and acute phase proteins (Watson 2009). Some of these molecules (such as IL-4 and IL-13), which under most circumstances act on immune cells, appear to play important roles by directly regulating mammary epithelial function (Khaled et al. 2007) whereas others synthesized by the mammary epithelium undoubtedly influence the immune cell population during pregnancy and lactation (Watson 2009). This plethora of immune mediators remains a fertile area both for discovery and for functional studies.

There are few functional studies on leukocytes during pregnancy and lactation except in their immune roles in response to infection, particularly in farm species (Wheeler et al. 1997a,b; Paape et al. 2002; Marshall et al. 2006; Atabai et al. 2007; Bleck et al. 2009). However, loss of CSF-1 in $Csf1^{op/op}$ mice results in a failure of ductal branching and premature differentiation of the lobuloalveolar structures with earlier expression of milk proteins than detected in wild-type mice (Pollard and Hennighausen 1994). This suggests a tradeoff between growth and differentiation during epithelial development. This effect can be partially rescued by recombinant CSF-1 administered subcutaneously (Pollard and Hennighausen 1994). These data showing that macrophage depletion results in a loss of ductal outgrowth during pregnancy reinforces the concept that macrophages have an important role in branching morphogenesis in the mammary gland. Ablation of immune cell types other than macrophages during pregnancy has not been reported and thus their roles in the processes of lobuloalveolar development and during lactation are unknown.

INVOLUTION

The cessation of suckling triggers involution in the mammary gland. Usually this occurs gradually and thus the process is not synchronized.

However, synchronization can be achieved by abrupt removal of pups. This forced weaning model revealed two stages of involution. The first within 24 hours of pup removal is characterized by epithelial cell apoptosis—a process that can be reversed by re-exposure to pups causing lactation to continue. The second phase of involution at around 48 hr postweaning involves massive apoptosis, matrix modeling and regression of the alveolar structure. With the loss of epithelial structures the gland is repopulated with adipocytes and eventually it returns to a virgin-like state (Atabai et al. 2007).

It was originally thought that the professional phagocytic macrophages would be recruited early in involution to ingest the apoptotic bodies. However, this does not appear to be the case (Monks and Henson 2009). Instead macrophage recruitment is only observed 3–4 days postweaning and instead of professional phagocytes, the removal of the early apoptotic bodies is effected through autophagy by adjacent epithelial cells (Monks and Henson 2009). In mice there is also evidence of neutrophils that are recruited before macrophages and, these cells together with macrophages are scattered through the interstitum and in the alveolar spaces (Monks et al. 2002; Atabai et al. 2007). In the human, cells bearing the pan-leukocytic marker CD45, are also found abundantly in the involuting gland (O'Brien and Schedin 2009). Similarly in farm species where it has been studied extensively, macrophages, neutrophils, and lymphocytes are found in the secretions from involuting glands. It is extremely likely that these macrophages scavenge debris and apoptotic cells (Atabai et al. 2007; Monks and Henson 2009) as well as bacteria (Tatarczuch et al. 2000, 2002). They may therefore be important in the prevention of mastitis in the vulnerable period of milk stasis (Atabai et al. 2007).

This sequential recruitment of neutrophils, macrophages, and lymphocytes at day 3–4 of involution is supported by the transcriptome analysis of involuting mammary glands. These data show expression of a wide range of inflammatory and acute phase molecules in the involuting mammary gland (Stein et al. 2004; Watson 2009). At 3–4 d postweaning these include markers for monocytes and macrophages such as F4/80, Ly6c, CD11b CSF-1R, and CD14, as well as markers for alternatively activated tissue-remodeling macrophages such as Arginase 1 (Stein et al. 2004; Monks and Henson 2009; O'Brien and Schedin 2009). This is preceded by the expression of leukocytic chemoattractants including CCL6, CCL7, CCL8, and CXCL14 that are expressed 24 h postweaning (Clarkson et al. 2004). However, the exact role of these chemoattractive molecules remains to be determined.

The other functions of these immune cells in involution, apart from garbage clearance and immunity, remains elusive because transient depletions of particular populations have not been performed. Interestingly *Csf1*$^{op/op}$ mice are unable to efficiently undergo a lactational switch and consequently there is a rapid involution in these mice (Pollard and Hennighausen 1994). However, this may be due less to the biology of the mammary gland and more to do with the failure of these mice to feed their young perhaps because of problems in olfaction required for pup finding (Erblich et al. unpubl.). Recent studies show that recruited macrophages are polarized to a tissue remodeling state and that their presence, just as in the developing gland, is associated with collagen deposition and matrix remodeling (O'Brien et al. 2010). Macrophages have also recently been shown to be important in adipogenesis at least during obesity, through their secretion of adipocyte growth factors and matrix remodeling molecules (Pollard 2009). However, whether they function as such during involution remains to be determined.

The intense remodeling of the mammary gland during involution associated with abundant macrophages may have negative consequences. This immediate postpartum period is associated with a higher risk of breast cancer that can manifest itself in a very aggressive form (O'Brien and Schedin 2009). Because macrophages play an important role in breast cancer progression and promotion of metastasis (see "Myeloid Cells and Breast Carcinogenesis") (Qian and Pollard 2010), it has been suggested

that these cells during involution may encourage the growth of stem cells carrying oncogenic mutations through their effects on matrix remodeling and production of growth and angiogenic factors (Schedin 2006; O'Brien and Schedin 2009). Indeed, the matrix formed during early involution is tumor promoting in models of breast cancer when compared with virgin and late-involution matrix (McDaniel et al. 2006; O'Brien et al. 2010). Interestingly, this increased risk of breast cancer is transient and in the longer term, parity is protective. This has been suggested to be because of sustained expression of transforming growth factor (TGF)-β3 (D'Cruz et al. 2002) that in many contexts is tumor inhibiting in part through effects on stem cell senescence (Boulanger and Smith 2001) and inhibition of mammary epithelial cell proliferation (Ewan et al. 2002). TGFβ3 is one of the earliest signaling molecules starting involution by stimulating apoptosis and it is expressed in two waves through involution (Nguyen and Pollard 2000). Coincidently macrophages are also important TGFβ producing and modifying cells suggesting that they may have dual roles in cancer risk dependent on context.

IMMUNE CELLS AND BREAST CANCER DEVELOPMENT

Breast cancer (BrCa) is the most frequent malignant tumor of women in North America (Society 2007). Although genetic and epigenetic changes in genes that regulate mammary epithelial cell (MEC) proliferation, survival, polarity, and/or differentiation are likely "initiators" of breast carcinogenesis, several lines of evidence indicate that stromal cell responses in premalignant mammary tissue may "promote" progression to cancer and/or metastatic capability of malignant MECs. Cellular components of tumor stroma include (myo)fibroblasts, vascular cells, infiltrating leukocytes, and specialized mesenchymal support cells unique to each tissue microenvironment.

Although BrCa has not historically been linked to underlying inflammation or infection, it shows tumor-associated inflammation as defined by infiltration of leukocytes into developing tumors where increases in some immune cell subsets in neoplastic stroma parallels disease progression (de Visser et al. 2006; DeNardo and Coussens 2007; DeNardo et al. 2009). Breast cancer development in woman is characterized by a significant increase in the presence of both innate and adaptive immune cells, with B and T lymphocytes as well as macrophages representing the most abundant leukocytes present in neoplastic stroma (DeNardo and Coussens 2007) (Fig. 5). Retrospective clinical studies examining identity of leukocytes in human breast cancer have revealed that high immunoglobulin (Ig) levels in tumor stoma (and serum), and increased presence of extrafollicular B cells, T regulatory (T_{reg}) cells, high ratios of CD4/CD8, or T_H2/T_H1 T lymphocytes in primary tumors or in draining lymph nodes correlates with tumor grade, stage, and overall patient survival (Shimokawara et al. 1982; Lee et al. 1985; Chin et al. 1992; Punt et al. 1994; Coronella et al. 2001; Coronella-Wood and Hersh 2003; Fernandez Madrid 2005; Kohrt et al. 2005; Bates et al. 2006); thus, some facets of adaptive immunity may indeed be involved in fostering cancer development in the breast. On the other hand, clinical (Leek and Harris 2002) studies show association with poor prognosis and experimental studies have shown that macrophages in primary mammary adenocarcinomas regulate early tumor development by activating angiogenic programs, as well as late-stage carcinogenesis by virtue of their secretion of paracrine factors required for stimulating proinvasive and prometastatic programs in malignant mammary epithelial cells (MECs) (Lin et al. 2001; Wyckoff et al. 2004, 2007; Goswami et al. 2005; Lin and Pollard 2007; Wang et al. 2007; Philippar et al. 2008; Yang et al. 2008; DeNardo et al. 2009; Gocheva et al. 2010).

MYELOID CELLS AND BREAST CARCINOGENESIS

Myeloid-lineage immune cells, such as mast cells, macrophages, and neutrophils, have been shown to promote tumor progression by exerting a number of protumoral activities, e.g., by stimulating angiogenesis (Coussens et al. 1999;

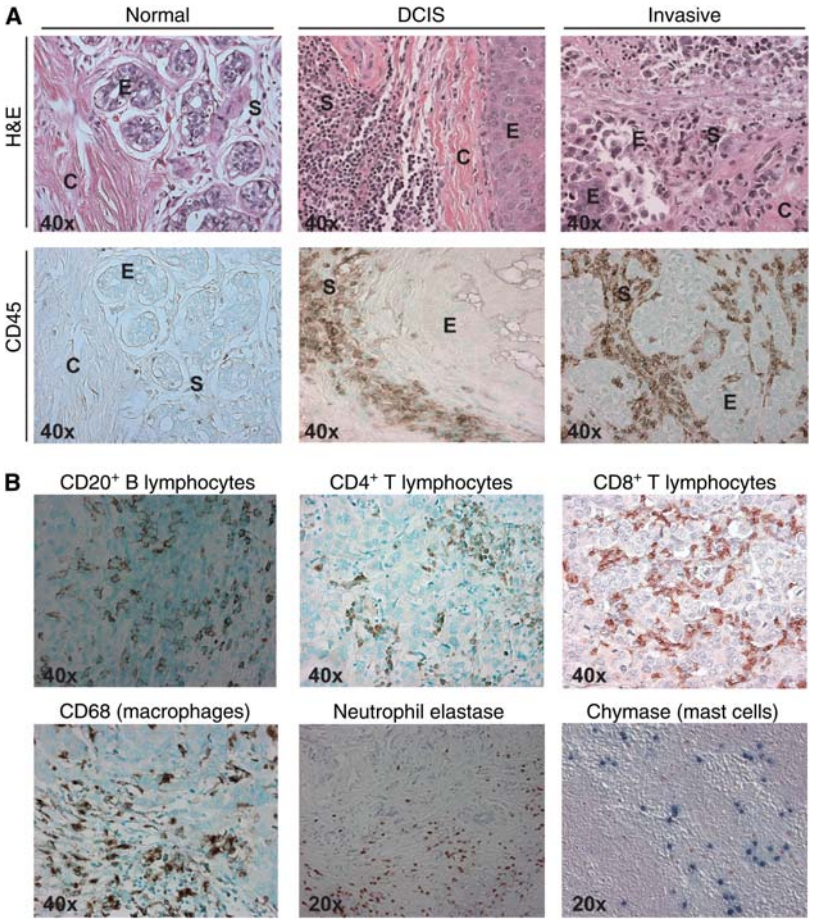

Figure 5. Leukocytes in human breast, ductal carcinoma in situ, and invasive carcinoma. (*A*) Hematoxylin and Eosin (H&E; *top* row) staining of *normal* human breast tissue, ductal carcinoma in situ (DCIS) and invasive breast cancer (invasive), showing ductal epithelial structures (E), collagenous stroma (C), and darkly stained immune cells infiltrating stroma (S). Immunodetection of infiltrating leukocytes by CD45 (leukocyte common antigen) immunoreactivity (brown staining; bottom row) reveals significant leukocyte infiltration in DCIS and invasive cancer, as compared to *normal* breast tissue. (*B*) Imunodetection of specific lineages of immune cells in invasive breast cancer, B lymphocytes ($CD20^+$; brown staining), $CD4^+$ T lymphocytes (brown staining), $CD8^+$ T lymphocytes (brown staining), macrophages ($CD68^+$; brown staining), neutrophils (neutrophils $elastase^+$; brown staining), and mast cells ($chymase^+$; blue staining). Original magnifications are shown for each panel.

De Palma et al. 2005; Okamoto et al. 2005; Lin et al. 2006; Nozawa et al. 2006; Takakura 2006; Lin and Pollard 2007), suppressing antitumor immunity (Blankenstein 2005; Zou 2005; Bronte et al. 2006), and enhancing tumor cell migration and metastasis (Wyckoff et al. 2004; Condeelis and Pollard 2006; DeNardo et al. 2009; Gocheva et al. 2010). Nucleated hematopoietic cells that have been directly implicated in tumor angiogenesis include mast cells (Coussens et al. 1999; Soucek et al. 2007), tumor-associated macrophages (Pollard 2004; Balkwill et al. 2005; Lewis and Pollard 2006), Tie2-expressing monocytes (De Palma et al. 2005; De Palma and Naldini 2006), neutrophils (Nozawa et al. 2006), dendritic cell precursors (Coukos et al. 2005), and myeloid immune suppressor cells (Yang et al. 2004; Serafini et al. 2006). Other hematopoietic cell types, such as platelets (Kisucka et al. 2006), eosinophils

(Puxeddu et al. 2005), and hematopoietic progenitors (Takakura et al. 2000), also participate in angiogenic processes, but it remains to be established whether they can directly promote tumor angiogenesis, rather than having a broader function in supporting tissue inflammation and remodeling.

The potential of neoplastic cells to spread locally (i.e., progress to malignancy) and systemically (i.e., metastasize) is linked to activation of angiogenic vasculature (Hanahan and Weinberg 2000). Tumor-associated blood vessels generated by angiogenesis support tumor growth and development, as well as provide escape routes for malignant cells to intravasate into the circulation (Hanahan and Weinberg 2000; Wyckoff et al. 2007). Although several studies have found that some highly metastatic breast cancer cell lines have up-regulated expression of genes encoding proangiogenic factors such as vascular endothelial growth factor (VEGF) that favor activation of angiogenic vessels (Lee et al. 2007), tumor angiogenesis in the mammary gland, as well as in other organs, is likely initially activated by activated myeloid cells attracted to neoplastic tissue (Coussens et al. 1999; Bergers et al. 2000; Condeelis and Pollard 2006).

In breast carcinomas, macrophages are one of the most abundant innate immune cell types in which they enhance angiogenic programming by production of proangiogenic factors such as VEGF and proteases, i.e., urokinase-type plasminogen activator (uPA) and MMP9 (Lin and Pollard 2007). In a mouse model of mammary adenocarcinoma development, e.g., MMTV-PyMT mice (Guy et al. 1992), increased macrophage infiltration in premalignant tissue occurs immediately before the angiogenic switch and the onset of malignancy (Lin et al. 2001, 2006). CSF-1 is broadly expressed by tumors of the reproductive system and its expression correlates with the extent of leukocyte infiltration and represent a poor prognostic indicator in these tumors (Lin and Pollard 2004). By using PyMT mice carrying a *Csf1* null mutation ($Csf1^{op/op}$), it was further shown that depletion of CSF-1 markedly decreased infiltration of macrophages at tumor sites, inhibited the angiogenic switch and significantly delayed tumor progression. The knockdown of CSF-1 in transplanted tumor cells (by using antisense oligonucleotides) also resulted in inhibition of tumor growth, with tumors showing extensive necrosis and poor vascularization, phenotypes that could be reversed by treatment of mice with CSF-1. The premature macrophage infiltration in the mammary gland of MMTV-LTR-CSF-1 transgenic mice induced robust angiogenesis even at early pre-malignant stages, providing evidence for a direct link between macrophage infiltration and angiogenesis, independent of tumor stage (Lin et al. 2006). These studies have provided evidence that CSF-1 is a major regulator of macrophage recruitment to tumors and shed light on the important roles of macrophages in tumor progression, and in particular with tumor-associated angiogenesis. Myeloid cell-delivery of VEGF to mammary tumors is clearly significant because myeloid-specific deletion of VEGF inhibited the angiogenic switch but surprisingly accelerated tumor development (Stockmann et al. 2008). In this regard, myeloid VEGF regulates formation of high-density vessel networks, attenuation of which blocks angiogenic programming of tumors while also inducing normalization of vasculature that thus results in accelerated tumor progression by decreasing tumor cell death and tumor hypoxia, together indicating that myeloid-derived VEGF-A is essential for tumor-associated alterations of vasculature. It is known that macrophages heavily infiltrate necrotic areas in tumors where they scavenge cellular debris and cooperate with tumor cells to promote angiogenesis (Murdoch et al. 2004; Lewis and Murdoch 2005). In fact, hypoxia stimulates expression of several proangiogenic molecules by activating hypoxia-inducible factors (HIFs) in macrophages. Expression of the monocyte chemoattractantss VEGF, endothelin 2, and endothelial monocyte-activating polypeptide II (EMAP II) by hypoxic tumor cells can attract macrophages into hypoxic areas within tumors. It is believed that macrophages are then retained in hypoxic tumor areas because of abrogation of chemotactic signal transduction and the down-regulation

of chemo-attractant receptors. Once in hypoxic areas, macrophages produce a wide array of proangiogenic molecules and matrix-remodeling factors, including IL-8/CXCL8, VEGF, FGF, platelet-derived growth factor (PDGF), MMPs, and uPA, but it remains to be clarified how crucial these macrophage-secreted factors are in the economy of tumor angiogenesis, because many proangiogenic molecules are also produced by other components of the tumor stroma and by the tumor cells themselves.

In addition to the aforementioned proangiogenic factors, macrophages release other molecules that can influence angiogenesis (Pollard 2004; Ojalvo et al. 2009). Macrophages are key producers of TNF-α, that can up-regulate expression of thymidine phosphorylase and MMP-9. Macrophages also produce IL-1 that may increase VEGF transcription by up-regulating expression of HIF-1α through cyclooxygenase (COX)-2. In addition, macrophages also release nitric oxyde (NO), a molecule that provokes vasodilation and increased vascular flow, through the activity of inducible NO synthase (iNOS). Macrophages can also produce FGF, hepatocyte growth factor (HGF), other EGFR ligands (HB-EGF), as well as platelet-derived growth factor (PDGF) and TGFα (Condeelis and Pollard 2006). In particular, leukocyte-derived tumor necrosis factor (TNF)α has been found to enhance invasive/migratory phenotypes of breast cancer cells in culture (Hagemann et al. 2005). In breast cancer cell lines, TNFα regulates epithelial invasion through activation of downstream signaling cascades including Jun amino-terminal kinase (JNK) and nuclear factor κB (NFκB) transcription factor. Activated JNK and NFκB in turn induce gene expression of proinvasive factors such as EMMPRIN (extracellular matrix metallo-protease inducer) and MIF (migration inhibitory factor), whose expression enhances MMP-2 and -9 secretion and activity (Hagemann et al. 2005). Production of TGFβ by alternatively activated macrophages in mammary tumors, mesenchymal support cells, and immature myeloid cells (IMCs) can also enhance the invasive and metastatic programming of malignant cells (Stover et al. 2007; Bierie et al. 2008; Yang et al. 2008) consistent with existence of a TGFβ-responsive gene signature that a predicts breast cancer pulmonary metastasis (Arribas et al. 1997).

Another myeloid population recently implicated in tumor angiogenesis are the so-called immature myeloid suppressor cells (Gallina et al. 2006; Serafini et al. 2006). Myeloid suppressor cells express low to undetectable levels of MHC-II and costimulatory molecules, thus cannot induce antitumor responses. Rather, these cells promote tumor development by exerting a profound inhibitory activity on both tumor-specific and nonspecific T lymphocytes and, as recently described, by providing factors essential for tumor growth and neovascularization such as TGFβ (Yang et al. 2004, 2008). The frequency of myeloid suppressor cells is significantly increased in the bone marrow (BM) and spleen of cancer patients and mice carrying large tumors. Moses and colleagues (Yang et al. 2004) found that $CD11b^{+}Gr1^{+}$-myeloid suppressor cells obtained from spleens of tumor-bearing mice promoted angiogenesis and tumor growth when co-injected with tumor cells, largely in response to their high level secretion of TGFβ (Yang et al. 2008). Myeloid suppressor cells also produce high levels of MMP-9, deletion of which minimizes their tumor-promoting activity. Similar to DC precursors, $CD11b^{+}Gr1^{+}$cells have also been found to occasionally incorporate into tumor endothelium as endothelial-like cells (Yang et al. 2004).

Concentration gradients of growth factors established by leukocytes present in neoplastic stroma coordinate tumor cell movements toward, and intravasation into, tumor-associated vasculature. For example, macrophages are the primary source of EGF in breast cancer microenvironments (Leek et al. 2000; Lewis and Pollard 2006). EGF promotes invasion/chemotaxis and intravasation of breast carcinoma cells through its interaction with EGFR as shown using an in vivo needle chemotaxis assay through engagement of cofilin-dependent actin polymerization (Wyckoff et al. 2000; Wang et al. 2007). Paracrine interactions between macrophages and breast carcinoma cells form positive feed-forward loops involving

macrophage-expressed EGF and CSF-1 expressed by neoplastic cells, that together result in breast carcinoma cells showing "high-velocity" polarized movement (chemotaxis) along collagen fibers toward blood vessels directed by perivascular macrophages (Condeelis and Segall 2003; Condeelis and Pollard 2006). These experimental data combined with the positive correlation between CSF-1 levels, macrophage recruitment and poor prognosis in human cancers (Scholl et al. 1994; Leek and Harris 2002), together support the notion that macrophages and/or their products play a major role in facilitating late-stage metastatic progression of tumors (Lin et al. 2001). Significantly a stromal CSF-1 gene expression signature in breast cancer correlated with poor outcomes and was observed to be similar between primary tumors and lymph node metastases (Webster et al. 2010).

Similar to CSF-1, several CC chemokines, particularly CCL2 and CCL5 (RANTES, or regulated on activation normal T cells expressed and secreted), have been implicated in recruitment of monocytes to tumors (Lin and Pollard 2004). CCL2/MCP-1 overexpression by genetically modified tumor cells implanted in mice promoted monocyte uptake by the tumor mass. In human tumors, CCL2/MCP-1 and CCL5/RANTES are produced predominately by neoplastic cells and fibroblasts correlating with macrophage infiltration. Furthermore, both CCL2/MCP-1 and CCL5/RANTES have been found to stimulate monocyte/macrophage-lineage cells to secrete MMP-9 and urokinase-type plasminogen activator (uPA), which through their ECM-remodeling functions are potent activators of angiogenesis (Murdoch et al. 2004). Thus, thanks to their ability to attract proangiogenic monocytes/macrophages to tumors, both CSF-1 and CCL2/MCP-1 can be regarded as major players in the orchestration of the angiogenic process in tumors.

T LYMPHOCYTES AND BREAST CANCER

Clinical evaluation of human breast adenocarcinomas reveals that presence of $CD4^+$ T_H2 T cells and T_{reg} cells increases with disease progression. High percentages of $CD4^+$ T cells in primary breast cancers positively correlate with markers of disease progression, including metastatic spread to sentinel lymph nodes and increased primary tumor size (Chin et al. 1992; Kohrt et al. 2005). The extent of T-cell infiltration into invasive breast carcinomas has been reported to range from 1%–45% of the total cellular mass (Chin et al. 1992). In rapidly proliferating tumors, presence of T lymphocytes (by histopathological determination) at tumor sites is a good prognostic indicator when compared to nonimmunogenic tumors, and correlates with auxillary lymph node negativity, smaller tumor diameter, lower histological grade, and recurrence-free survival (Aaltomaa et al. 1992); thus, supporting an overall role for T cells in immune surveillance. However, the exact composition of T-lymphocyte infiltration varies greatly and may profoundly affect disease progression and overall patient survival. Perhaps more significant, the ratio of $CD4^+$ to $CD8^+$ T cells or T_H2 to T_H1 cells present in primary tumors, where $CD4^+$ or T_H2 cells are more frequent than $CD8^+$ or T_H1 cells, correlates with lymph node metastasis and reduced overall patient survival (Chin et al. 1992; Kohrt et al. 2005). More recently, unsupervised expression profiling from breast cancer-associated stroma revealed a gene signature predictive of good prognostic outcome (>98%, 5-year survival) that was functionally enriched for elements of a T_H1-type immune response, including genes suggestive of cytotoxic T lymphocyte (CTL) and natural killer (NK) cell activity (Finak et al. 2008). Conversely, high levels of $FOXP3^+$ T_{reg} cells predict diminished relapse-free and overall patient survival (Bates et al. 2006, 2007). Although it is unclear if presence of $CD8^+$ CTLs alone provides any prognostic information for breast cancer, the presence of high percentages of $CD4^+$ T helper cells at primary tumor sites positively correlates with disease progression, e.g., metastatic spread to sentinel lymph node (LN) and primary tumor size (Chin et al. 1992; Kohrt et al. 2005). The interpretation based on these clinical studies is that the type of $CD4^+$ effector T-cell response elicited in an emergent breast cancer may in

part determine malignant and metastatic potential. Although presence of metastatic MECs in sentinel lymph nodes draining the primary tumor represents the strongest prognostic indicator for disease progression and overall patient outcome, combinatorial analysis evaluating presence and composition of leukocytes, together with other clinical markers indicative of stage, may provide utility for predicting outcome.

Why are $CD8^+$ CTL-mediated responses not more effective in eradicating or minimizing cancer occurrence and how might $CD4^+$ T cells be involved in enhancing breast cancer progression? One plausible mechanism may have to do with the "polarity" of the $CD4^+$ T_{helper} cell response at primary tumor sites and/or their distant metastases. We recently reported that interleukin (IL)-4-expressing T_H2 $CD4^+$ T lymphocytes promote invasion and metastasis of mammary adenocarcinomas by directly regulating T_H2 activity in macrophages, their bioeffector function and EGF expression, that in turn regulate invasive tumor growth, presence of circulating tumor cells (CTCs), and pulmonary metastasis (DeNardo et al. 2009). Using the PyMT mouse model of mammary carcinogenesis (Guy et al. 1992), we found that whereas primary tumor development was unchanged in PyMT/$CD4^{-/-}$ mice, both showed a significantly attenuated metastatic phenotype (DeNardo et al. 2009) similar to that of tissue macrophage-deficient/PyMT mice (Lin et al. 2001). We found that $CD11b^+Gr1^+$ monocytes and macrophages of CD4-deficient/PyMT tumors expressed significantly elevated levels of type 1 cytokines (e.g., TNFα, IL-6, IL-12p40, and IL-1β) and *Nos2* mRNA, indicative of a prevalent T_H1 immune microenvironment and M1 phenotype (DeNardo et al. 2009), whereas $CD11b^+Gr1^+$ monocytes and macrophages from CD4-proficient/PyMT mice were instead indicative of alternatively activated M2 cells that expressed higher levels of *arginase-1* (Arg-1) and *Tgfβ*, thus characterizing a T_H2 microenvironment (DeNardo et al. 2009). To determine if $CD4^+$ T-cell regulated myeloid cell phenotypes by a T_H2 pathway, we evaluated $CD4^+$ T cells from LNs and carcinomas of PyMT mice and found elevated expression of GATA3, IL-4, IL-13, and IL-10 (T_H2) and to a lesser extent T-bet (T_H1) and the T_H1 cytokine IFNγ, but neither FOXP3 (T_{reg}) nor IL-17a were expanded as compared to wild-type littermates indicating presence of both T_H1 and T_H2 effector lineages.

PyMT/IL4Rα-deficient mice and PyMT mice treated with neutralizing antibodies to IL-4 phenocopied PyMT/CD4-deficient mice with diminished pulmonary metastasis and presence of M1-$CD11b^+Gr1^+$ monocytes and macrophages in carcinomas (DeNardo et al. 2009). Moreover, Joyce and colleagues recently reported that IL-4 activation of tumor-associated macrophages was significant for cathepsin B and S production, that together also enhance invasion and metastatic progression (Gocheva et al. 2010). Together this data indicates that T_H2-$CD4^+$ T cells promote metastasis by enhancing the protumor bioactivities of macrophages, and engaging intracellular signaling cascades (EGF) required for dissemination and metastasis and indicating that blockade of T_H2-based and/or IL-4/IL-13-regulated pathways may provide a survival advantage by limiting late-stage disease progression and metastatic spread. By comparison, loss of $CD4^+$ T lymphocytes, similarly to loss of macrophage recruitment to mammary tumors, results in reduced presence of CTCs and diminished development of pulmonary metastasis, but without impacting microvessel density or the character of angiogenic vasculature in mammary carcinomas. These distinctions reflect the fact that $CD4^+$ T lymphocyte-derived factors, including IL-4, partially regulate the protumor properties of macrophages, in particular a unique population that promotes invasion and metastasis (Ojalvo et al. 2010), likely because of their impact on EGF expression. Therefore, the proangiogenic properties of macrophages are regulated independently of their T_H1- or T_H2-type cytokine expression characteristics, and instead are more likely dependent on other factors such as hypoxia (Lewis and Murdoch 2005; Zinkernagel et al. 2007).

In addition to indirectly potentiating cancer development by regulating protumor properties

of myeloid cells, work from several groups have reported that IL-4 and IL-13 also regulate tumor growth directly through activation of IL-4/13 receptors on epithelial cells. In some human breast carcinoma cell lines, particularly those that express the estrogen receptor α, IL-4, and IL-13 inhibit both basal and estrogen-induced cell proliferation in vitro and in xenograph tumor models in vivo (Toi et al. 1992; Gingras et al. 2000; Nagai and Toi 2000; Gooch et al. 2002). However, in other breast carcinoma cell lines, IL-4 regulates tumor cell survival by conferring resistance to apoptosis (in vitro) that translates to resistance to chemotherapy in xenograph models (Todaro et al. 2008). Palucka and colleagues reported that $CD4^+$ T cells directly enhance early breast tumor development in xenograph tumors using humanized NOD/SCID mice, and reported that the ability of $CD4^+$ T cells to produce T_H2 cytokines including IL-4 and IL-13 was key (Aspord et al. 2007). Taken together these data indicate that the effects of CD4-derived T_H2 cytokines on tumor development and progression is likely regulated by the organ microenvironment or IL-4/13 receptor status of malignant cells, and may also be subject to tumor cell etiology and the specific genetic programs altered during the initiation phase of cancer development. This realization, however, has profound implications for development of future therapeutics targeting $CD4^+$ T cells to either bolster antitumor immunity or neutralize protumor immunity by revealing the complexities of their bioeffector functions that are regulated by multiple factors not common to all tumor models.

B LYMPHOCYTES, HUMORAL IMMUNITY, AND BREAST CANCER

During breast carcinogenesis, mature B cells (including naive and activated) can be found in secondary lymphoid tissues as well as in tumor-associated stroma. As compared to healthy patients without evidence of cancer, the sentinel (draining) LNs of breast cancer patients contains enriched populations of proliferating and affinity matured (IgG^+) B lymphocytes. Moreover, data from retrospective studies examining percentages of B cells present in sentinel and auxiliary LNs of breast cancer patients reveals that their presence and/or maturation (IgG^+) correlates with increases in disease stage (stage I versus stage II) and total tumor burden (Wernicke 1975). Urdiales-Viedma and colleagues used immunohistochemical detection of IgA, IgG, and IgM in axillary LNs from 50 unselected ductal breast carcinomas and found that LNs with IgG^+ lymphoid follicles and/or metastatic LNs with IgM^+ lymphoid cells were statistically related to breast tumors of high histologic grade and more than three LN metastases (Urdiales-Viedma et al. 1986). These data correlate the presence of various populations of B cells in lymph nodes with malignancy.

During breast cancer development, Ig deposition in neoplastic mammary stroma is known to increase the bioavailability of VEGF by Ig binding to FcRs on tumor-associated macrophages and thus triggering release VEGF into the interstitium (Barbera-Guillem et al. 2002). Moreover, in breast carcinoma tissues, presence of endocytosed Ig in macrophages corresponds with local extracellular VEGF protein levels and local angiogenic vascular buds (Barbera-Guillem et al. 2002).

How might B lymphocytes regulate carcinoma development? A vast literature exists describing the occurrence of (auto)antibodies in either the serum of cancer patients, or interstitial antibody deposition in tumors (Tomer et al. 1998). Early presence of autoantibodies (in particular antinuclear and smooth muscle antibodies) in serum of cancer patients is well known to correlate with unfavorable prognosis (Tan and Shi 2003). Approximately 50% of breast cancer patients contain circulating Igs that specifically react with tumor-derived antigens—autoantibodies against ErbB2/HER2/neu are present in 20% of patients with ErbB2-positive breast cancer, making it the most common breast cancer "auto-antigen" (Disis et al. 1994). Paradoxically, presence of specific autoantibodies in serum and/or at tumor sites correlates with poor patient survival (Tomer et al. 1998; Tan and Shi 2003; Fernandez Madrid

2005); thus, perhaps indicating that Igs resulting from chronic B cell activation in response to tumor-specific antigens might promote disease progression. Despite the presence of antitumor antibodies in greater than half of all breast cancer patients, there are only few reports of spontaneous tumor regression (presumed to be immunologic) in the absence of therapy (Sheikh et al. 1979; Lee et al. 1985). Several factors may influence efficiency of antitumor antibodies in inducing tumor regression/destruction, including Ig concentration, HLA expression, tumor tolerance/immune suppression, and impaired cytotoxic T-cell activity. Thus, whether individuals with progressing tumors harbor a higher antigen load that thus triggers enhanced Ig production, or whether increased presence of serum or interstitial Igs predisposes patients to development of more advanced or recurrent cancers, requires further study. That said, B-cell-depletion in MMTV-PyMT mice was shown to be without consequence in regulating early or late-stage mammary carcinogenesis (DeNardo et al. 2009).

CONCLUSIONS

During the last decade, insights have been gained regarding mechanisms underlying the dynamic roles of immune cells as mediators of developmental processes, tissue homeostasis in adult tissues, and tumorigenesis. During the various phase of mammary development the immune system is engaged at every stage. This response appears to be restricted to the innate immune system except during lactation. Although these cells of the innate system are called immune cells this attribution appears to be a misnomer in these developmental contexts as there are no pathogens present. Instead these cells are trophic to developing tissue and act to enhance rates of epithelial growth and invasion through the fat pad and influence complexity of the ductal structures (Pollard 2009). During tumorigenesis a similar cast of innate immune cells are recruited. However in contrast to the developmental context there is a much greater engagement of the acquired immune response. This presence of many acquired immune cells in tumors suggests recognition of new "foreign" tumor antigens or of the extensive tissue damage caused by tumor growth. Indeed in some cases a tumor-directed immune response involving cytolytic $CD8^+$ T cells, T_H1 cells, and NK cells appears to protect against tumor development and progression. However, if the immune response involves B cells and activation of humoral immunity, and infiltration of T_H2 cells as well as innate inflammatory cells into an organ harboring initiated tumor cells, the likely outcome is promotion of tumor development and progression. Thus innate and acquired immune cells are in an evolving dynamic that result in the trophic functions of the innate system being conferred on the epithelial tumors in a fashion that mimics development. In mammary cancers of both mice and women macrophages appear to be the dominant innate immune cell type. Differing populations of macrophages confer on the tumor an ability to outgrow, invade, and become vascularized. Unfortunately unlike the developing mammary gland that has intrinsic programs to suppress the external trophic support once it is no longer required, the oncogenic and tumor suppressor mutations in cancer cells result in the loss of these "off" switches. Consequently the tumors obtain continuing support from these recruited immune cells and malignancy is enhanced. However, the balance between a protective cytotoxic response and a harmful humoral or T_H2 response can be regulated systemically by the general immune status of the individual, as well as locally by myeloid suppressor cells and T_{reg} cells, and thus offers clinicians with attractive targets for anticancer immune-based therapies.

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REFERENCES

Aaltomaa S, Lipponen P, Eskelinen M, Kosma VM, Marin S, Alhava E, Syrjanen K. 1992. Tumor size, nuclear morphometry, mitotic indices as prognostic factors in axillary-lymph-node-positive breast cancer. *Eur Surg Res* **24:** 160–168.

American Cancer Society. 2007. *Cancer facts and figures*. http://www.cancer.org/downloads/STT/caff2007PWSecured.pdf.

Anbazhagan R, Bartek J, Monaghan P, Gusterson BA. 1991. Growth and development of the human infant breast. *Am J Anat* **192:** 407–417.

Arribas J, Lopez-Casillas F, Massague J. 1997. Role of the juxtamembrane domains of the transforming growth factor-α precursor and the β-amyloid precursor protein in regulated ectodomain shedding. *J Biol Chem* **272:** 17160–17165.

Aspord C, Pedroza-Gonzalez A, Gallegos M, Tindle S, Burton EC, Su D, Marches F, Banchereau J, Palucka AK. 2007. Breast cancer instructs dendritic cells to prime interleukin 13-secreting CD4+ T cells that facilitate tumor development. *J Exp Med* **204:** 1037–1047.

Atabai K, Sheppard D, Werb Z. 2007. Roles of the innate immune system in mammary gland remodeling during involution. *J Mammary Gland Biol Neoplasia* **12:** 37–45.

Balkwill F, Charles KA, Mantovani A. 2005. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* **7:** 211–217.

Barbera-Guillem E, Nyhus JK, Wolford CC, Friece CR, Sampsel JW. 2002. Vascular endothelial growth factor secretion by tumor-infiltrating macrophages essentially supports tumor angiogenesis, and IgG immune complexes potentiate the process. *Cancer Res* **62:** 7042–7049.

Bates GJ, Fox SB, Han C, Launchbury R, Leek RD, Harris AL, Banham AH. 2007. Expression of the forkhead transcription factor FOXP1 is associated with that of estrogen receptorbeta in primary invasive breast carcinomas. *Breast Cancer Res Treat* **111:** 453–459.

Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, Banham AH. 2006. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* **24:** 5373–5380.

Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, et al. 2000. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol* **2:** 737–744.

Bierie B, Stover DG, Abel TW, Chytil A, Gorska AE, Aakre M, Forrester E, Yang L, Wagner KU, Moses HL. 2008. Transforming growth factor-beta regulates mammary carcinoma cell survival and interaction with the adjacent microenvironment. *Cancer Res* **68:** 1809–1819.

Blankenstein T. 2005. The role of tumor stroma in the interaction between tumor and immune system. *Curr Opin Immunol* **17:** 180–186.

Bleck GT, Wheeler MB, Hansen LB, Chester-Jones H, Miller DJ. 2009. Lactose synthase components in milk: Concentrations of α-lactalbumin and β1,4-galactosyltransferase in milk of cows from several breeds at various stages of lactation. *Reprod Domest Anim* **44:** 241–247.

Boulanger CA, Smith GH. 2001. Reducing mammary cancer risk through premature stem cell senescence. *Oncogene* **20:** 2264–2272.

Brandtzaeg P. 2010. Function of mucosa-associated lymphoid tissue in antibody formation. *Immunol Invest* **39:** 303–355.

Bronte V, Cingarlini S, Marigo I, De Santo C, Gallina G, Dolcetti L, Ugel S, Peranzoni E, Mandruzzato S, Zanovello P. 2006. Leukocyte infiltration in cancer creates an unfavorable environment for antitumor immune responses: A novel target for therapeutic intervention. *Immunol Invest* **35:** 327–357.

Chin Y, Janseens J, Vandepitte J, Vandenbrande J, Opdebeek L, Raus J. 1992. Phenotypic analysis of tumor-infiltrating lymphocytes from human breast cancer. *Anticancer Res* **12:** 1463–1466.

Clarkson RW, Wayland MT, Lee J, Freeman T, Watson CJ. 2004. Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. *Breast Cancer Res* **6:** R92–109.

Condeelis J, Pollard JW. 2006. Macrophages: Obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124:** 263–266.

Condeelis J, Segall JE. 2003. Intravital imaging of cell movement in tumours. *Nat Rev Cancer* **3:** 921–930.

Coronella JA, Telleman P, Kingsbury GA, Truong TD, Hays S, Junghans RP. 2001. Evidence for an antigen-driven humoral immune response in medullary ductal breast cancer. *Cancer Res* **61:** 7889–7899.

Coronella-Wood JA, Hersh EM. 2003. Naturally occurring B-cell responses to breast cancer. *Cancer Immunol Immunother* **52:** 715–738.

Coukos G, Benencia F, Buckanovich RJ, Conejo-Garcia JR. 2005. The role of dendritic cell precursors in tumour vasculogenesis. *Br J Cancer* **92:** 1182–1187.

Coussens LM, Raymond WW, Bergers G, Laig-Webster M, Behrendtsen O, Werb Z, Caughey GH, Hanahan D. 1999. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes Dev* **13:** 1382–1397.

D'Cruz CM, Moody SE, Master SR, Hartman JL, Keiper EA, Imielinski MB, Cox JD, Wang JY, Ha SI, Keister BA, et al. 2002. Persistent parity-induced changes in growth factors, TGF-β3, and differentiation in the rodent mammary gland. *Mol Endocrinol* **16:** 2034–2051.

Dai X, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, Sylvestre V, Stanley ER. 2002. Targeted disruption of the mouse CSF-1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies and reproductive defects. *Blood* **99:** 111–120.

Daniel CW, Silberstein GB. 1987. Postnatal development of the rodent mammary gland. In *The mammary gland, development, regulation, and function* (ed. MC Neville, CW Danile), pp. 1–35. Plenum, New York.

De Palma M, Naldini L. 2006. Role of haematopoietic cells and endothelial progenitors in tumour angiogenesis. *Biochim Biophys Acta* **1766:** 159–166.

De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M, Naldini L. 2005. Tie2 identifies a

hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* **8:** 211–226.

de Visser KE, Eichten A, Coussens LM. 2006. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* **6:** 24–37.

DeNardo DG, Coussens LM. 2007. Inflammation and breast cancer. Balancing immune response: Crosstalk between adaptive and innate immune cells during breast cancer progression. *Breast Cancer Res* **9:** 212.

DeNardo DG, Baretto JB, Andreu P, Vasquez L, Kolhatkar N, Tawfik D, Coussens LM. 2009. CD4+ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell* **16:** 91–102.

Disis ML, Calenoff E, McLaughlin G, Murphy AE, Chen W, Groner B, Jeschke M, Lydon N, McGlynn E, Livingston RB, et al. 1994. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. *Cancer Res* **54:** 16–20.

Djonov V, Andres AC, Ziemiecki A. 2001. Vascular remodelling during the normal and malignant life cycle of the mammary gland. *Microsc Res Tech* **52:** 182–189.

Egeblad M, Werb Z. 2002. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* **2:** 161–174.

Ewan KB, Shyamala G, Ravani SA, Tang Y, Akhurst R, Wakefield L, Barcellos-Hoff MH. 2002. Latent transforming growth factor-βactivation in mammary gland: Regulation by ovarian hormones affects ductal and alveolar proliferation. *Am J Pathol* **160:** 2081–2093.

Fernandez Madrid F. 2005. Autoantibodies in breast cancer sera: Candidate biomarkers and reporters of tumorigenesis. *Cancer Lett* **230:** 187–198.

Finak G, Bertos N, Pepin F, Sadekova S, Souleimanova M, Zhao H, Chen H, Omeroglu G, Meterissian S, Omeroglu A, et al. 2008. Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med* **14:** 518–527.

Gallina G, Dolcetti L, Serafini P, De Santo C, Marigo I, Colombo MP, Basso G, Brombacher F, Borrello I, Zanovello P, et al. 2006. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *J Clin Invest* **116:** 2777–2790.

Gingras D, Renaud A, Mousseau N, Beliveau R. 2000. Shark cartilage extracts as antiangiogenic agents: Smart drinks or bitter pills? *Cancer Metastasis Rev* **19:** 83–86.

Gocheva V, Wang HW, Gadea BB, Shree T, Hunter KE, Garfall AL, Berman T, Joyce A. 2010. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev* **24:** 241–255.

Gooch JL, Christy B, Yee D. 2002. STAT6 mediates interleukin-4 growth inhibition in human breast cancer cells. *Neoplasia* **4:** 324–331.

Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, Pixley FJ, Stanley ER, Segall JE, Condeelis JS. 2005. Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. *Cancer Res* **65:** 5278–5283.

Gouon-Evans V, Lin EY, Pollard JW. 2002. Requirement of macrophages and eosinophils and their cytokines/chemokines for mammary gland development. *Breast Cancer Res* **4:** 155–164.

Gouon-Evans V, Rothenberg ME, Pollard JW. 2000. Postnatal mammary gland development requires macrophages and eosinophils. *Development* **127:** 2269–2282.

Guy CT, Cardiff RD, Muller WJ. 1992. Induction of mammary tumors by expression of polyomavirus middle T oncogene: A transgenic mouse model for metastatic disease. *Mol Cell Biol* **12:** 954–961.

Gyorki DE, Asselin-Labat ML, van Rooijen N, Lindeman GJ, Visvader JE. 2009. Resident macrophages influence stem cell activity in the mammary gland. *Breast Cancer Res* **11:** R62.

Hagemann T, Wilson J, Kulbe H, Li NF, Leinster DA, Charles K, Klemm F, Pukrop T, Binder C, Balkwill FR. 2005. Macrophages induce invasiveness of epithelial cancer cells via NF-κ B and JNK. *J Immunol* **175:** 1197–1205.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. *Cell* **100:** 57–70.

Hinck L, Silberstein GB. 2005. Key stages in mammary development. The mammary end bud as a motile organ. *Breast Cancer Res* **7:** 245–251.

Howard BA, Gusterson BA. 2000. Human breast development. *J Mammary Gland Biol Neoplasia* **5:** 119–137.

Humphreys R. 1999. Programmed cell death in the terminal endbud. *J Mamm Gland Biol Neoplasia* **4:** 213–220.

Humphreys RC, Krajewska M, Krnacik S, Jaeger R, Weiher H, Krajewski S, Reed JC, Rosen JM. 1996. Apoptosis in the terminal endbud of the murine mammary gland: A mechanism of ductal morphogenesis. *Development* **122:** 4013–4022.

Ingman WV, Wyckoff J, Gouon-Evans V, Condeelis J, Pollard JW. 2006. Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Dev Dyn* **235:** 3222–3229.

Khaled WT, Read EK, Nicholson SE, Baxter FO, Brennan AJ, Came PJ, Sprigg N, McKenzie AN, Watson CJ. 2007. The IL-4/IL-13/Stat6 signalling pathway promotes luminal mammary epithelial cell development. *Development* **134:** 2739–2750.

King BL, Crisi GM, Tsai SC, Haffty BG, Phillips RF, Rimm DL. 2002. Immunocytochemical analysis of breast cells obtained by ductal lavage. *Cancer* **96:** 244–249.

Kisucka J, Butterfield CE, Duda DG, Eichenberger SC, Saffaripour S, Ware J, Ruggeri ZM, Jain RK, Folkman J, Wagner DD. 2006. Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage. *Proc Natl Acad Sci* **103:** 855–860.

Kohrt HE, Nouri N, Nowels K, Johnson D, Holmes S, Lee PP. 2005. Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer. *PLoS Med* **2:** e284.

Landskroner-Eiger S, Park J, Israel D, Pollard JW, Scherer PE. 2010. Morphogenesis of the developing mammary gland: Stage-dependent impact of adipocytes. *Dev Biol* **344:** 968–978.

Lee TH, Seng S, Sekine M, Hinton C, Fu Y, Avraham HK, Avraham S. 2007. Vascular endothelial growth factor mediates intracrine survival in human breast carcinoma

cells through internally expressed VEGFR1/FLT1. *PLoS Med* **4:** e186.

Lee YT, Sheikh KM, Quismorio FP Jr, Friou GJ. 1985. Circulating anti-tumor and autoantibodies in breast carcinoma: Relationship to stage and prognosis. *Breast Cancer Res Treat* **6:** 57–65.

Leek RD, Harris AL. 2002. Tumor-associated macrophages in breast cancer. *J Mammary Gland Biol Neoplasia* **7:** 177–189.

Leek RD, Hunt NC, Landers RJ, Lewis CE, Royds JA, Harris AL. 2000. Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. *J Pathol* **190:** 430–436.

Lewis C, Murdoch C. 2005. Macrophage responses to hypoxia: Implications for tumor progression and anti-cancer therapies. *Am J Pathol* **167:** 627–635.

Lewis CE, Pollard JW. 2006. Distinct role of macrophages in different tumor microenvironments. *Cancer Res* **66:** 605–612.

Lilla JN, Werb Z. 2010. Mast cells contribute to the stromal microenvironment in mammary gland branching morphogenesis. *Dev Biol* **337:** 124–133.

Lin EY, Pollard JW. 2004. Macrophages: Modulators of breast cancer progression. *Novartis Found Symp* **256:** 158–168; discussion 168–172, 259–269.

Lin EY, Pollard JW. 2007. Tumor-associated macrophages press the angiogenic switch in breast cancer. *Cancer Res* **67:** 5064–5066.

Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, Qian H, Xue XN, Pollard JW. 2006. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res* **66:** 11238–11246.

Lin EY, Nguyen AV, Russell RG, Pollard JW. 2001. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* **193:** 727–740.

Marshall KM, Hurley WL, Shanks RD, Wheeler MB. 2006. Effects of suckling intensity on milk yield and piglet growth from lactation-enhanced gilts. *J Anim Sci* **84:** 2346–2351.

McDaniel SM, Rumer KK, Biroc SL, Metz RP, Singh M, Porter W, Schedin P. 2006. Remodeling of the mammary microenvironment after lactation promotes breast tumor cell metastasis. *Am J Pathol* **168:** 608–620.

Monks J, Henson PM. 2009. Differentiation of the mammary epithelial cell during involution: Implications for breast cancer. *J Mammary Gland Biol Neoplasia* **14:** 159–170.

Monks J, Geske FJ, Lehman L, Fadok VA. 2002. Do inflammatory cells participate in mammary gland involution? *J Mammary Gland Biol Neoplasia* **7:** 163–176.

Mor G, Yue W, Santen RJ, Gutierrez L, Eliza M, Berstein LM, Harada N, Wang J, Lysiak J, Diano S, et al. 1998. Macrophages, estrogen and the microenvironment of breast cancer. *J Steroid Biochem Mol Biol* **67:** 403–411.

Murdoch C, Giannoudis A, Lewis CE. 2004. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood* **104:** 2224–2234.

Nagai S, Toi M. 2000. Interleukin-4 and breast cancer. *Breast Cancer* **7:** 181–186.

Neville MC, McFadden TB, Forsyth I. 2002. Hormonal regulation of mammary differentiation and milk secretion. *J Mammary Gland Biol Neoplasia* **7:** 49–66.

Neville MC, Medina D, Monks J, Hovey RC. 1998. The mammary fat pad. *J Mammary Gland Biol Neoplasia* **3:** 109–116.

Nguyen AV, Pollard JW. 2000. Transforming growth factor β3 induces cell death during the first stage of mammary gland involution. *Development* **127:** 3107–3118.

Nozawa H, Chiu, Hanahan D. 2006. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proc Natl Acad Sci* **103:** 12493–12498.

O'Brien J, Schedin P. 2009. Macrophages in breast cancer: Do involution macrophages account for the poor prognosis of pregnancy-associated breast cancer? *J Mammary Gland Biol Neoplasia* **14:** 145–157.

O'Brien J, Lyons T, Monks J, Lucia MS, Wilson RS, Hines L, Man YG, Borges V, Schedin P. 2010. Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species. *Am J Pathol* **176:** 1241–1255.

Ojalvo LS, King W, Cox D, Pollard JW. 2009. High-density gene expression analysis of tumor-associated macrophages from mouse mammary tumors. *Am J Pathol* **174:** 1048–1064.

Ojalvo LS, Whittaker CA, Condeelis JS, Pollard JW. 2010. Gene expression analysis of macrophages that facilitate tumor invasion supports a role for Wnt-signaling in mediating their activity in primary mammary tumors. *J Immunol* **184:** 702–712.

Okamoto R, Ueno M, Yamada Y, Takahashi N, Sano H, Suda T, Takakura N. 2005. Hematopoietic cells regulate the angiogenic switch during tumorigenesis. *Blood* **105:** 2757–2763.

Paape M, Mehrzad J, Zhao X, Detilleux J, Burvenich C. 2002. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. *J Mammary Gland Biol Neoplasia* **7:** 109–121.

Philippar U, Roussos ET, Oser M, Yamaguchi H, Kim HD, Giampieri S, Wang Y, Goswami S, Wyckoff JB, Lauffenburger DA, et al. 2008. A Mena invasion isoform potentiates EGF-induced carcinoma cell invasion and metastasis. *Dev Cell* **15:** 813–828.

Pollard JW. 2004. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* **4:** 71–78.

Pollard JW. 2009. Trophic macrophages in development and disease. *Nat Rev Immunol* **9:** 259–270.

Pollard JW, Hennighausen L. 1994. Colony stimulating factor 1 is required for mammary gland development during pregnancy. *Proc Natl Acad Sci* **91:** 9312–9316.

Pollard JW, Stanley ER. 1996. Pleiotropic roles for CSF-1 in development defined by the mouse mutation osteopetrotic (*op*). *Adv Dev Biochem* **4:** 153–193.

Punt CJ, Barbuto JA, Zhang H, Grimes WJ, Hatch KD, Hersh EM. 1994. Anti-tumor antibody produced by human tumor-infiltrating and peripheral blood B lymphocytes. *Cancer Immunol Immunother* **38:** 225–232.

Puxeddu I, Alian A, Piliponsky AM, Ribatti D, Panet A, Levi-Schaffer F. 2005. Human peripheral blood eosinophils

induce angiogenesis. *Int J Biochem Cell Biol* **37:** 628–636.

Qian BZ, Pollard JW. 2010. Macrophage diversity enhances tumor progression and metastasis. *Cell* **141:** 39–51.

Richert MM, Schwertfeger KL, Ryder JW, Anderson SM. 2000. An atlas of mouse mammary gland development. *J Mammary Gland Biol Neoplasia* **5:** 227–241.

Roth P. 1991. Colony-stimulating factor 1 levels in the human newborn infant. *J Pediatr* **119:** 113–116.

Ryan GR, Dai XM, Dominguez MG, Tong W, Chuan F, Chisholm O, Russell RG, Pollard JW, Stanley ER. 2001. Rescue of the colony-stimulating factor 1 (CSF-1)-nullizygous mouse ($Csf1^{op}/Csf1^{op}$) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. *Blood* **98:** 74–84.

Sakakura T. 1987. Mammary embryogenesis. In *The Mammary gland: Development, regulation and function* (ed. MC Neville, CW Danile), pp. 37–66. Plenum Press, New York.

Sangaletti S, Di Carlo E, Gariboldi S, Miotti S, Cappetti B, Parenza M, Rumio C, Brekken RA, Chiodoni C, Colombo MP. 2008. Macrophage-derived SPARC bridges tumor cell-extracellular matrix interactions toward metastasis. *Cancer Res* **68:** 9050–9059.

Santos SJ, Aupperlee MD, Xie J, Durairaj S, Miksicek R, Conrad SE, Leipprandt JR, Tan YS, Schwartz RC, Haslam SZ. 2009. Progesterone receptor A-regulated gene expression in mammary organoid cultures. *J Steroid Biochem Mol Biol* **115:** 161–172.

Sapi E, Kacinski BM. 1999. The role of CSF-1 in normal and neoplastic breast physiology. *Proc Soc Exp Biol Med* **220:** 1–8.

Sapi E, Flick MB, Rodov S, Carter D, Kacinski BM. 1998. Expression of CSF-1 and CSF-1 receptor by normal lactating mammary epithelial cells. *J Soc Gynecol Investig* **5:** 94–101.

Sasmono RT, Oceandy D, Pollard JW, Tong W, Pavli P, Wainwright BJ, Ostrowski MC, Himes SR, Hume DA. 2003. A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood* **101:** 1155–1163.

Schedin P. 2006. Pregnancy-associated breast cancer and metastasis. *Nat Rev Cancer* **6:** 281–291.

Scholl SM, Pallud C, Beuvon F, Hacene K, Stanley ER, Rohrschneider LR, Tang R, Pouillart P, Lidereau R. 1994. Anti-colony-stimulating factor-1 antibody staining in primary breast adenocarcinomas correlates with marked inflammatory cell infiltrates and prognosis. *J Natl Cancer Inst* **86:** 120–126.

Schwertfeger KL, Rosen JM, Cohen DA. 2006a. Mammary gland macrophages: Pleiotropic functions in mammary development. *J Mammary Gland Biol Neoplasia* **11:** 229–238.

Schwertfeger KL, Xian W, Kaplan AM, Burnet SH, Cohen DA, Rosen JM. 2006b. A critical role for the inflammatory response in a mouse model of preneoplastic progression. *Cancer Res* **66:** 5676–5685.

Serafini P, Borrello I, Bronte V. 2006. Myeloid suppressor cells in cancer: Recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol* **16:** 53–65.

Sferruzzi-Perri AN, Robertson SA, Dent LA. 2003. Interleukin-5 transgene expression and eosinophilia are associated with retarded mammary gland development in mice. *Biol Reprod* **69:** 224–233.

Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE. 2006. Generation of a functional mammary gland from a single stem cell. *Nature* **439:** 84–88.

Sheikh KM, Quismorio FP, Friou GJ, Lee YT. 1979. Ductular carcinoma of the breast: Serum antibodies to tumor-associated antigens. *Cancer* **44:** 2083–2089.

Shimokawara I, Imamura M, Yamanaka N, Ishii Y, Kikuchi K. 1982. Identification of lymphocyte subpopulations in human breast cancer tissue and its significance: an immunoperoxidase study with anti-human T- and B-cell sera. *Cancer* **49:** 1456–1464.

Soucek L, Lawlor ER, Soto D, Shchors K, Swigart LB, Evan GI. 2007. Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. *Nat Med* **13:** 1211–1218.

Stein T, Morris JS, Davies CR, Weber-Hall SJ, Duffy MA, Heath VJ, Bell AK, Ferrier RK, Sandilands GP, Gusterson BA. 2004. Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. *Breast Cancer Res* **6:** R75–R91.

Stockmann C, Doedens A, Weidemann A, Zhang N, Takeda N, Greenberg JI, Cheresh DA, Johnson RS. 2008. Deletion of vascular endothelial growth factor in myeloid cells accelerates tumorigenesis. *Nature* **456:** 814–818.

Stover DG, Bierie B, Moses HL. 2007. A delicate balance: TGF-β and the tumor microenvironment. *J Cell Biochem* **101:** 851–861.

Szewczyk G, Szukiewicz D, Zaczek R, Maslinska D. 2000. Mast cells in the mouse mammary gland–correlation with the development of lactiferous structures. *Folia Biol (Krakow)* **48:** 13–17.

Takakura N. 2006. Role of hematopoietic lineage cells as accessory components in blood vessel formation. *Cancer Sci* **97:** 568–574.

Takakura N, Watanabe T, Suenobu S, Yamada Y, Noda T, Ito Y, Satake M, Suda T. 2000. A role for hematopoietic stem cells in promoting angiogenesis. *Cell* **102:** 199–209.

Tan EM, Shi FD. 2003. Relative paradigms between autoantibodies in lupus and autoantibodies in cancer. *Clin Exp Immunol* **134:** 169–177.

Tatarczuch L, Bischof RJ, Philip CJ, Lee CS. 2002. Phagocytic capacity of leucocytes in sheep mammary secretions following weaning. *J Anat* **201:** 351–361.

Tatarczuch L, Philip C, Bischof R, Lee CS. 2000. Leucocyte phenotypes in involuting and fully involuted mammary glandular tissues and secretions of sheep. *J Anat* **196:** 313–326.

Todaro M, Lombardo Y, Francipane MG, Alea MP, Cammareri P, Iovino F, Di Stefano AB, Di Bernardo C, Agrusa A, Condorelli G, et al. 2008. Apoptosis resistance in epithelial tumors is mediated by tumor-cell-derived interleukin-4. *Cell Death Differ* **15:** 762–772.

Toi M, Bicknell R, Harris AL. 1992. Inhibition of colon and breast carcinoma cell growth by interleukin-4. *Cancer Res* **52:** 275–279.

Tomer Y, Sherer Y, Shoenfeld Y. 1998. Autoantibodies, autoimmunity and cancer. *Oncol Rep* **5:** 753–761.

Urdiales-Viedma M, Nogales-Fernandez F, Martos-Padilla S, Sanchez-Cantalejo E. 1986. Correlation of histologic grade and lymph node status with some histopathologic discriminants in breast cancer. *Tumori* **72:** 43–51.

Van Nguyen A, Pollard JW. 2002. Colony stimulating factor-1 is required to recruit macrophages into the mammary gland to facilitate mammary ductal outgrowth. *Dev Biol* **247:** 11–25.

Wang W, Wyckoff JB, Goswami S, Wang Y, Sidani M, Segall JE, Condeelis JS. 2007. Coordinated regulation of pathways for enhanced cell motility and chemotaxis is conserved in rat and mouse mammary tumors. *Cancer Res* **67:** 3505–3511.

Watson CJ. 2009. Immune cell regulators in mouse mammary development and involution. *J Anim Sci* **87:** 35–42.

Webster JA, Beck AH, Sharma M, Esphinosa IBW, Schreuder M, Montgomery KD, Jensen KC, van de Rijn M, West R. 2010. Variations in stromal signature in breast and colorectal cancer metastases. *J Pathol* **222:** 158–165.

Wernicke M. 1975. Quantitative morphologic assessment of immunoreactivity in regional lymph nodes of patients with carcinoma of the breast. *Surg Gynecol Obstet* **140:** 919–924.

Wheeler TT, Broadhurst MK, Rajan GH, Wilkins RJ. 1997a. Differences in the abundance of nuclear proteins in the bovine mammary gland throughout the lactation and gestation cycles. *J Dairy Sci* **80:** 2011–2019.

Wheeler TT, Kuys YM, Broadhurst MM, Molenaar AJ. 1997b. Mammary Stat5 abundance and activity are not altered with lactation state in cows. *Mol Cell Endocrinol* **133:** 141–149.

Wyckoff JB, Segall JE, Condeelis JS. 2000. The collection of the motile population of cells from a living tumor. *Cancer Res* **60:** 5401–5404.

Wyckoff JB, Wang Y, Lin EY, Li JF, Goswami S, Stanley ER, Segall JE, Pollard JW, Condeelis J. 2007. Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res* **67:** 2649–2656.

Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Graf T, Pollard JW, Segall J, Condeelis J. 2004. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res* **64:** 7022–7029.

Yang L, Debusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian LM, Carbone DP, Lin PC. 2004. Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* **6:** 409–421.

Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, Carbone DP, Matrisian LM, Richmond A, Lin PC, et al. 2008. Abrogation of TGF β signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer Cell* **13:** 23–35.

Zinkernagel AS, Johnson RS, Nizet V. 2007. Hypoxia inducible factor (HIF) function in innate immunity and infection. *J Mol Med* **85:** 1339–1346.

Zou W. 2005. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* **5:** 263–274.

RESEARCH ARTICLE

Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy

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ABSTRACT

Immune-regulated pathways influence multiple aspects of cancer development. In this article we demonstrate that both macrophage abundance and T-cell abundance in breast cancer represent prognostic indicators for recurrence-free and overall survival. We provide evidence that response to chemotherapy is in part regulated by these leukocytes; cytotoxic therapies induce mammary epithelial cells to produce monocyte/macrophage recruitment factors, including colony stimulating factor 1 (CSF1) and interleukin-34, which together enhance CSF1 receptor (CSF1R)–dependent macrophage infiltration. Blockade of macrophage recruitment with CSF1R-signaling antagonists, in combination with paclitaxel, improved survival of mammary tumor–bearing mice by slowing primary tumor development and reducing pulmonary metastasis. These improved aspects of mammary carcinogenesis were accompanied by decreased vessel density and appearance of antitumor immune programs fostering tumor suppression in a $CD8^+$ T-cell–dependent manner. These data provide a rationale for targeting macrophage recruitment/response pathways, notably CSF1R, in combination with cytotoxic therapy, and identification of a breast cancer population likely to benefit from this novel therapeutic approach.

SIGNIFICANCE: These findings reveal that response to chemotherapy is in part regulated by the tumor immune microenvironment and that common cytotoxic drugs induce neoplastic cells to produce monocyte/macrophage recruitment factors, which in turn enhance macrophage infiltration into mammary adenocarcinomas. Blockade of pathways mediating macrophage recruitment, in combination with chemotherapy, significantly decreases primary tumor progression, reduces metastasis, and improves survival by $CD8^+$ T-cell–dependent mechanisms, thus indicating that the immune microenvironment of tumors can be reprogrammed to instead foster antitumor immunity and improve response to cytotoxic therapy. *Cancer Discovery; 1(1); 54–67. ©2011 AACR.*

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INTRODUCTION

Tumor-associated macrophages (TAM) have been identified as regulators of solid tumor development based on their capacity to enhance angiogenic, invasive, and metastatic programming of neoplastic tissue (1, 2). Colony stimulating factor-1 (CSF1) is a key cytokine involved in recruitment and activation of tissue macrophages, exerting these effects through binding to a high-affinity receptor tyrosine kinase, the cFMS/CSF1 receptor (CSF1R; ref. 3). A second CSF1R ligand, interleukin 34 (IL-34), possesses similar binding affinities and regulates macrophage recruitment to tissues, but exhibits distinct tissue distribution characteristics (4).

Macrophage presence in several types of human cancer, including breast, ovarian, non–small cell lung cancer, and Hodgkin's lymphoma, correlates not only with increased vascular density but also a worse clinical outcome (1, 5, 6). Accordingly, a CSF1-response gene signature was identified in human breast cancer that predicts risk of recurrence and metastasis, and is similarly predictive for clinical outcome in colon cancer and leiomyosarcoma (7–9). On the basis of these findings, it seems reasonable to postulate that blockade of the molecular programs enhancing macrophage recruitment or protumor bioactivity in tumors may represent tractable targets for anticancer therapy. Accordingly, genetic or pharmacologic blockade of CSF1 or its receptor has been reported to decrease macrophage presence in tissues and in some experimental solid tumors, correlating with diminished tumor angiogenesis, reduced primary tumor growth, and pulmonary metastasis (1, 2).

Experimental studies have recently revealed that B and T lymphocytes can exert protumor activity indirectly by regulating the bioactivity of myeloid cells, including macrophages, monocytes, and mast cells, resulting in resistance to endocrine therapies and development of metastasis (10–13). We reported that in the absence of a significant $CD8^+$ CTL response, $CD4^+$ T-effector lymphocytes potentiate mammary adenocarcinoma metastasis by directly enhancing the protumor bioactivity of TAMs (11). On the basis of these results, we hypothesized that human breast cancers containing leukocytic infiltrates dominated by $CD4^+$ T lymphocytes and $CD68^+$ macrophages, without significant $CD8^+$ T-cell infiltration, would have a higher relative risk for metastasis and therefore reduced overall survival (OS) of patients. In this article, we report on an immune signature consisting of $CD68^{high}/CD4^{high}/CD8^{low}$ that significantly correlates with reduced OS for patients with breast cancer. Moreover, to demonstrate the biologic significance of this signature, we investigated a combination of standard-of-care chemotherapy and agents that block TAM infiltration of mammary tumors in an aggressive transgenic mouse model of mammary adenocarcinoma development [MMTV–polyoma middle T (PyMT) mice; ref. 14], in which late-stage carcinogenesis and pulmonary metastasis are regulated by CSF1 and tissue macrophages (15). We found that when TAM presence in mammary adenocarcinomas was minimized, antitumor immunity and $CD8^+$ CTL infiltration were enhanced; together, this improved chemosensitivity and resulted in reduced primary tumor development, significant decrease in pulmonary metastases, and improved OS, when compared with treatment using standard chemotherapy alone.

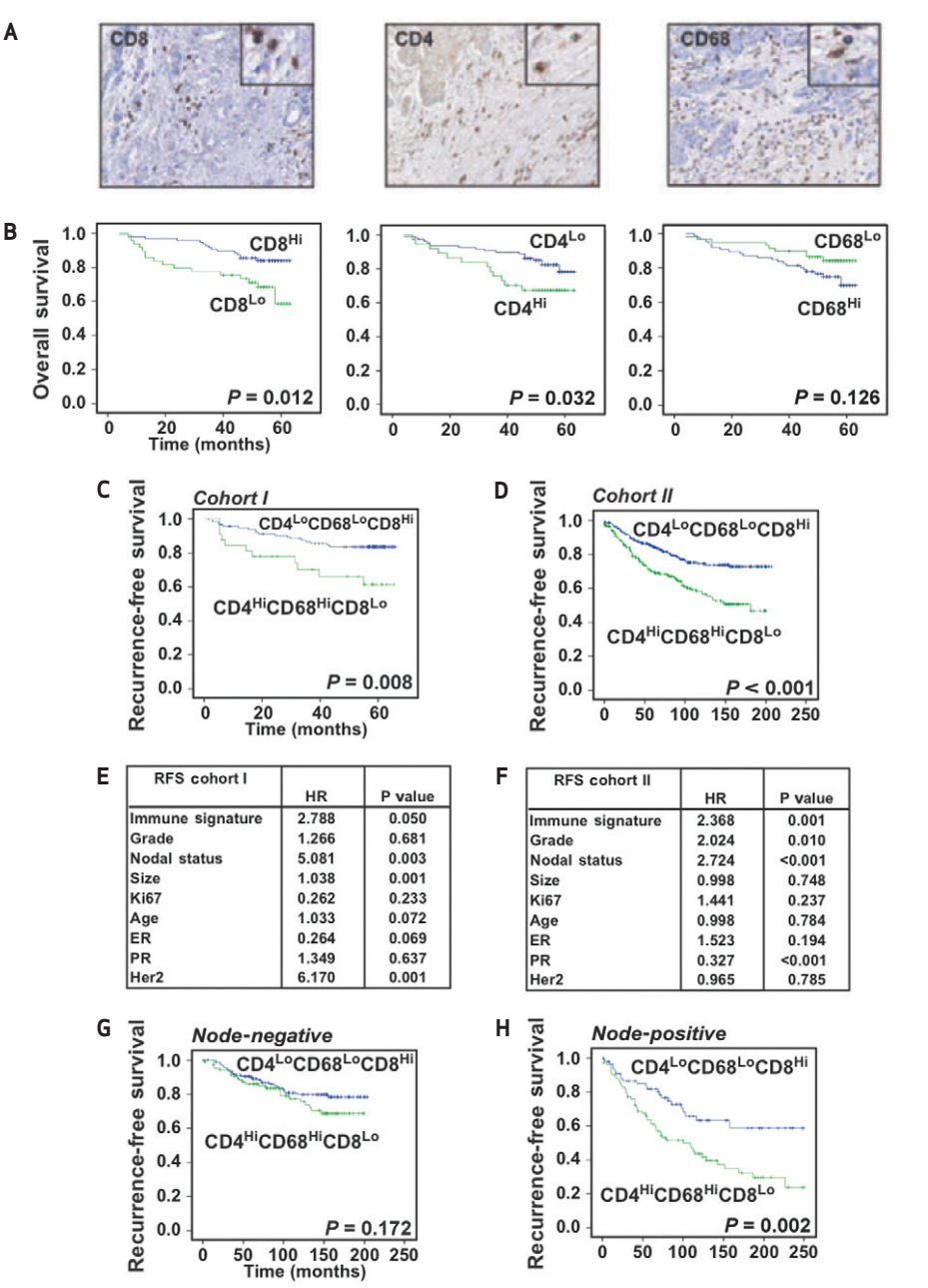
RESULTS

Single Immune Marker Analysis of Leukocyte Infiltration Predicts Breast Cancer Survival

In previous studies, we demonstrated that in the absence of a significant $CD8^+$ CTL response, $CD4^+$ T lymphocytes indirectly promoted invasion and metastasis of mammary adenocarcinomas by directly regulating the protumor bioactivities of TAMs (11). From these data, we predicted that infiltration of primary human breast cancer by $CD4^+$ and $CD8^+$ T lymphocytes and $CD68^+$ macrophages would correlate with aspects of breast cancer regulating OS. To address this issue, we analyzed $CD4^+$, $CD8^+$, and $CD68^+$ leukocyte density in tissue microarrays consisting of tumor tissue obtained at the time of primary surgery from 179 treatment-naïve breast cancer patients (Fig. 1A). We employed a fully automated nuclear algorithm to quantify $CD4^+$, $CD8^+$, and $CD68^+$ cells following immunohistochemical (IHC) detection. For survival analyses, high and low thresholds for each marker were established using a classification and regression decision tree analysis with 10-fold cross-validation (16). Kaplan-Meier analyses indicated that, as single variables, "high" $CD4^+$ T-cell density ($P = 0.032$) and "low" $CD8^+$ T-cell density ($P = 0.012$) correlated with reduced OS, whereas $CD68^+$ cell density alone showed no statistical significance (Fig. 1B). However, analysis of $CD68^+$ and $CD8^+$ immune cell infiltration demonstrated an inverse association between stromal infiltration by $CD68^+$ macrophages and $CD8^+$ T lymphocytes in human breast cancer tissues (Spearman's rho, −0.38; $P < 0.001$; Supplementary Table S1).

Three-Marker Immune Signature Is Prognostic for Breast Cancer Survival

Heterotypic interactions between diverse leukocyte populations often determine the outcome of immune responses in tissues (17). As such, we proposed that combined analysis of CD4, CD8, and CD68 would allow for improved prognostic stratification of breast cancer patients by assessing both antitumor immunity (i.e., $CD8^+$ density) and protumor immunity (i.e., high $CD4^+$ and $CD68^+$ leukocyte density). Thus, we predicted that an immune profile characterized by $CD68^{low}/CD4^{low}/CD8^{high}$ would represent primary breast cancer controlled by local resection, with improved OS and relapse-free survival (RFS). In contrast, an immune response characterized by $CD68^{high}/CD4^{high}/CD8^{low}$ would instead represent a population of patients at risk for distant metastasis and thus reduced OS. A classification and regression tree algorithm was used to define the signature in the screening cohort (Cohort I, $n = 179$; Supplementary Fig. S1). High and low thresholds for each marker were established by decision tree analysis with 10-fold cross-validation



RFS cohort I	HR	P value
Immune signature	2.788	0.050
Grade	1.266	0.681
Nodal status	5.081	0.003
Size	1.038	0.001
Ki67	0.262	0.233
Age	1.033	0.072
ER	0.264	0.069
PR	1.349	0.637
Her2	6.170	0.001

RFS cohort II	HR	P value
Immune signature	2.368	0.001
Grade	2.024	0.010
Nodal status	2.724	<0.001
Size	0.998	0.748
Ki67	1.441	0.237
Age	0.998	0.784
ER	1.523	0.194
PR	0.327	<0.001
Her2	0.965	0.785

Figure 1. CD68/CD4/CD8 immune signature is an independent prognostic indicator of breast cancer survival. **A,** high-magnification images (40×; 80× for inlays) of human breast cancer tissue sections showing immunoreactivity for representative $CD68^+$, $CD4^+$, and $CD8^+$ leukocyte infiltration. **B,** automated analysis of $CD68^+$, $CD4^+$, and $CD8^+$ immune cell detection, revealing relationship between leukocyte density and OS. Kaplan-Meier estimate of OS comparing autoscore leukocyte high- and low-infiltration groups is shown; 179 samples from Cohort I were used for analyses, and log-rank (Mantel-Cox) *P* values are denoted for difference in OS. **C** and **D,** Kaplan-Meier estimate of RFS, comparing $CD68^{high}/CD4^{high}/CD8^{low}$ and $CD68^{low}/CD4^{low}/CD8^{high}$ immune profiles as assigned by random forest clustering to identify optimal thresholds using Cohort I (**C**). Identified $CD68^{high}/CD4^{high}/CD8^{low}$ and $CD68^{low}/CD4^{low}/CD8^{high}$ immune profiles were used to stratify a second independent cohort, Cohort II (**D**). Cohort I ($n = 179$) and Cohort II ($n = 498$) samples were assessed, and the log-rank (Mantel-Cox) *P* value is denoted for difference in RFS. **E** and **F,** results from multivariate Cox regression analysis of RFS for the CD68/CD4/CD8 signature in Cohort I (**E**) and Cohort II (**F**). Hazard ratios (HR) and *P* values are shown for all characteristics. **G** and **H,** RFS in node-positive breast cancer predicted by CD68/CD4/CD8 immune signature. Kaplan-Meier estimates of RFS comparing $CD68^{high}/CD4^{high}/CD8^{low}$ and $CD68^{low}/CD4^{low}/CD8^{high}$ immune profiles as assigned by random forest clustering of breast cancer tissues. breast cancers were stratified into node-negative (**G**) and node-positive (**H**) patients and analyzed for RFS. The log-rank (Mantel-Cox) *P* value is denoted for difference in RFS.

of each tree model (16). All patients were categorized as having either a $CD68^{high}/CD4^{high}/CD8^{low}$ or a $CD68^{low}/CD4^{low}/CD8^{high}$ immune signature, and the same thresholds were then applied to a validation cohort (Cohort II, $n = 498$ patients, primary tumor samples). Kaplan-Meier analysis in the 2 independent cohorts (totaling 677 patients) showed significantly reduced OS and RFS in patients whose tumors harbored the $CD68^{high}/CD4^{high}/CD8^{low}$ signature (Fig. 1C and D; Supplementary Fig. S2A and B). Multivariate Cox regression analysis revealed that the $CD68^{high}/CD4^{high}/CD8^{low}$ signature was an independent predictor of decreased OS and RFS after controlling for grade, nodal status, tumor size, estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki-67 status in both cohorts (Fig. 1E and F; Supplementary Tables S2–S5), indicating that the immune signature predicted OS independently of established histopathologic parameters.

Three-Marker Immune Signature Is an Independent Predictor of RFS in Node-Positive Patients

The OS of breast cancer patients is greatly reduced if metastasis to regional or draining lymph nodes is present at the time of primary tumor detection. Therefore, node-positive patients require aggressive treatment with neoadjuvant or adjuvant systemic chemotherapy, or targeted therapies such as anti-estrogens or trastuzumab. To assess whether immune infiltration by macrophages and T lymphocytes affected the survival of this high-risk group, we examined the impact of the CD68/CD4/CD8 signature following stratification for nodal status. Whereas the CD68/CD4/CD8 signature was not predictive in node-negative patients (Fig. 1G), Kaplan-Meier analysis of Cohort II demonstrated significantly reduced RFS in node-positive patients whose tumors harbored the $CD68^{high}/CD4^{high}/CD8^{low}$ signature

(Fig. 1H). Multivariate Cox regression analysis revealed that the $CD68^{high}/CD4^{high}/CD8^{low}$ signature was an independent predictor of decreased RFS after controlling for grade, tumor size, ER, PR, HER2, and Ki-67 status (Supplementary Table S6), indicating that the immune signature predicts RFS independently of established histopathologic parameters. Thus, tumor infiltration by macrophages and T lymphocytes may influence breast cancer recurrence in lymph node–positive patients, a group often aggressively treated with neoadjuvant and adjuvant chemotherapy.

Cytotoxic Therapies Induce TAM Recruitment and CSF1 and IL-34 Cytokine Expression

These findings led us to hypothesize that blocking TAM infiltration in breast cancer patients bearing the $CD68^{high}/CD4^{high}/CD8^{low}$ signature might enhance antitumor T-cell responses and facilitate $CD8^+$ CTL infiltration and/or repolarization of $CD4^+$ T-cell responses toward T-helper 1 cell (T_H1). Consistent with this hypothesis, we found an inverse association between stromal infiltration by $CD68^+$ macrophages and $CD8^+$ T lymphocytes in human breast cancer tissues (Spearman's rho, –0.38; $P < 0.001$; Supplementary Table S1). On the basis of this observation, we postulated that chemosensitivity might in part be regulated by TAM and/or $CD8^+$ T-cell presence in breast cancer tissue. To address this, we initially evaluated leukocyte infiltration in a small cohort of freshly isolated breast tumors from women who had received neoadjuvant chemotherapy, compared with those undergoing primary surgery without preoperative treatment (Supplementary Table S7). We found a higher percentage of $CD45^+CD11b^+CD14^+$ macrophages in breast cancer from women who had received neoadjuvant chemotherapy than in tumors from women treated with surgery alone (Fig. 2A). In contrast, we observed no difference in tumor-infiltrating $CD45^+CD3^+CD8^+$ T lymphocytes between the 2 groups (Supplementary Fig. S3A).

To determine whether TAM presence in breast cancers was directly enhanced by chemotherapy, we evaluated leukocyte responses in MMTV-PyMT mice, a transgenic mouse model of mammary carcinogenesis, following treatment with paclitaxel (PTX; Supplementary Fig. S4). We found that infiltration of mammary tumors by $CD45^+CD11b^+Ly6C^{low}Ly6G^-F4/80^+$ TAMs was significantly increased following PTX treatment, with no significant change in the presence of $CD3^+CD8^+$ T lymphocytes (Fig. 2B; Supplementary Fig. S3B). Similar PTX-induced TAM recruitment was observed in syngeneic orthotopic PyMT-derived tumors (Supplementary Fig. S3C). Consistent with our hypothesis, PTX treatment of MMTV-PyMT mice only modestly slowed primary tumor growth (Fig. 2B). For studies herein, we defined TAMs as $CD45^+CD11b^+Ly6G^-Ly6C^{low}F4/80^+$, monocytic immature myeloid cells (iMC) as $CD45^+CD11b^+Ly6G^-Ly6C^{high}$, and granulocytic iMCs/neutrophils as $CD45^+CD11b^+Ly6G^{high}Ly6C^+$ (Supplementary Fig. S5A and B), in agreement with previously published studies (18, 19).

To reveal molecular mediators involved in regulating PTX-induced TAM recruitment, we examined mRNA expression of several monocyte/macrophage cytokines and chemokines in murine mammary epithelial carcinoma cells (MEC) following exposure to several forms of cytotoxic therapy *in vitro*. *CSF1, CCL8/MCP2,* and *IL34* mRNAs were increased in MMTV-PyMT–derived MECs following exposure to PTX (Fig. 2C). Of these, *CSF1* and *IL34* mRNAs were also increased following exposure to either CDDP or ionizing radiation (Fig. 2D; Supplementary Fig. S3D). Increased mRNA expression was not merely a response of malignant PyMT-derived MECs (pMEC), because a similar induction was also observed in nontransgenic MECs exposed to PTX in culture (Fig. S3E). Similarly, *CSF1* mRNA expression was also induced by PTX and CDDP in 5 of 6 human breast cancer cell lines reflecting the major subtypes of breast cancer (Fig. 2E). *In vivo, CSF1* mRNA was increased in mammary tissue of MMTV-PyMT mice following treatment with either PTX or ionizing radiation (Fig. 2F; Supplementary Fig. S3F). Together, these data indicate that induction of *CSF1* (and *IL34*) mRNA and subsequent TAM recruitment into mammary tissue represent a common response of MECs to cytotoxic agents.

Blockade of Chemotherapy-Induced TAM Recruitment

To determine whether tumor-infiltrating TAMs also regulate sensitivity of MECs to cytotoxic therapy, we blocked TAM infiltration *in vivo* with immunologic and pharmacologic agents (Supplementary Fig. S4) and evaluated myeloid cell infiltration of tumors from treated mice (Supplementary Fig. S5). Mice bearing orthotopic mammary tumors were treated with neutralizing monoclonal antibodies (mAb) CSF1 (clone 5S1) or CD11b (clone M1/70), or a competitive ATP inhibitor with potent (nM) specificity for CSF1 and cKIT receptor tyrosine kinases (PLX3397), either as a monotherapy or in combination with PTX. CD11b is an integrin cell adhesion molecule expressed on granulocytes, macrophages, monocytes, dendritic cells (DC), and natural killer cells that in part regulates transendothelial migration of cells into tissue and tumor parenchyma. PLX3397 has 10- to 100-fold selectivity for cKIT and CSF1R, as opposed to other related kinases, such as KDR (see Supplementary Fig. S6A and Methods; ref. 20).

Fluorescence-activated cell sorting analysis of the predominant myeloid subtypes infiltrating mammary tumors revealed that either as monotherapy, or in combination with PTX, $CD45^+CD11b^+Ly6C^-Ly6G^-F4/80^+$ TAM recruitment was significantly diminished following treatment with either αCSF1 mAb or PLX3397, with no effect on infiltration of $CD45^+CD11b^+LY6G^{high}$ iMCs or $CD45^+CD11b^{low/-}Ly6C^-CD22^-Ly6G^-CD11c^{high}MHCII^{high}$ DCs (Fig. 3A and B; Supplementary Fig. S5A and B). Treatment with aCD11b mAb decreased both TAM and iMC infiltration (Fig. 3A). Analysis of the maturation and differentiation status of TAMs remaining in mammary tumor tissue following αCSF1 or PLX3397 treatment revealed no significant change in CD11b, CD11c, F4/80, CD45, or MHCII expression (Supplementary Fig. S5B). However, examination of mammary tumor sections revealed a population of perivascular CSF1-independent $F4/80^+$ TAMs remaining (Fig. 3C). Blockade of TAM recruitment was a direct effect of CSF1/CSF1R blockade: *In vitro* CSF1R inhibition efficiently blocked $CD11b^+$ monocyte chemotaxis in response to control or PTX-treated pMEC–conditioned medium, with no effect on chemotaxis of $CD3^+$ T lymphocytes (Fig. 3D; Supplementary Fig. S3G). These results were mirrored *in vivo*; treatment of late-stage MMTV-PyMT mice with PLX3397 significantly inhibited both steady-state and PTX-induced tumor infiltration by $CD45^+CD11b^+Ly6C^-Ly6G^-F4/80^+$ TAMs (Fig. 3E; Supplementary Fig. S5) without altering TAM maturation/differentiation (Supplementary Fig. S6B).

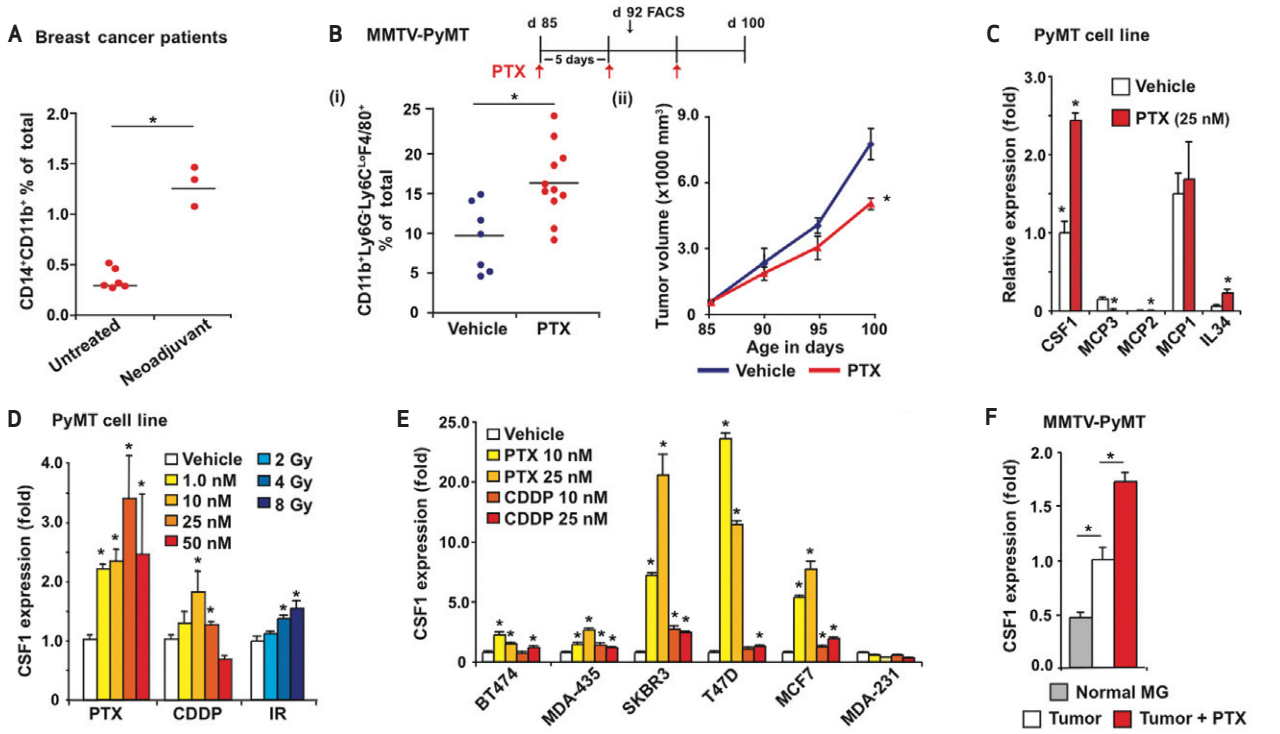


Figure 2. Cytotoxic therapy induces macrophage recruitment, as well as *CSF1* and *IL-34* mRNA expression. **A,** macrophage percentage in fresh human primary breast cancer tissues, depicted as mean of $CD45^+CD11b^+CD14^+$ macrophages as a percentage of total cells (analyzed by flow cytometry). "Neo-adjuvant" denotes patients who received chemotherapy prior to surgical resection of their primary breast cancer, as opposed to those who did not, denoted as "untreated"; *, statistically significant differences ($P = 0.004$) between the 2 groups. **B,** PTX-induced *CSF1* mRNA expression regulates tumor infiltration of macrophages and limits PTX response. (i), TAM percentage in mammary tumors of MMTV-PyMT mice following PTX treatment with mean number of $CD45^+Ly6G^-Ly6C^-CD11b^+F4/80^+$ TAMs as a percentage of total cells shown (analyzed by flow cytometry); (ii), primary tumor growth reduced by treatment with PTX. The 85-day-old MMTV-PyMT mice were treated with PTX and total tumor burden per animal was assessed every 5 days until endpoint. Treatment schematic is depicted at top, and data are displayed as mean tumor burden ±SEM; *, statistically significant differences between controls and PTX-treated mice (>8 mice/group). **C,** expression of monocyte/macrophage chemoattractants following chemotherapy. Quantitative reverse transcriptase PCR (qRT-PCR) analyses of *CSF1*, *MCP1*, *MCP2*, *MCP3*, and *IL34* expression in MMTV-pMECs–derived MECs treated with PTX for 24 hours ex vivo, expressed as mean fold change, compared with controls. Samples were assayed in triplicate for each tested condition; *, statistically significant differences between control and PTX-treated groups. **D,** Dose-dependent expression of *CSF1* following chemotherapy or radiation therapy. qRT-PCR analysis of mRNA expression in MMTV-PyMT–derived pMECs 24 hours after treatment with either cisplatin (CDDP), PTX, or a single dose of ionizing radiation, expressed as mean fold change, compared with control ±SEM. Drug and radiation doses are shown. Samples were assayed in triplicate for each tested condition; *, statistically significant differences between control and the indicated treatment. **E,** *CSF1* expression induced by chemotherapy in human breast carcinoma cell lines. qRT-PCR analysis of mRNA expression in BT474, MDA-MB-435, SKBR3, T47D, MCF7, and MDA-MB-231 at 24 hours after treatment with either CDDP or PTX, expressed as mean fold change, compared with vehicle-treated cells ±SEM. Chemotherapeutic doses are denoted. Samples were assayed in triplicate for each condition; *, statistically significant differences between control and indicated treatment. **F,** *CSF1* expression induced by cytotoxic therapy in MMTV-PyMT mammary tumors. qRT-PCR analysis of mRNA expression isolated from normal mammary tissue or MMTV-PyMT mammary tumors from mice treated with either PTX (10 mg/kg) every 5 days, or ionizing radiation (single dose of 8 Gy), expressed as mean fold change, compared with vehicle-treated tumors (4 mice/group). SE is depicted; *, statistically significant differences ($P < 0.05$, Mann-Whitney) for all gene expression analyses (**C–F**).

We next treated 80-day-old MMTV-PyMT mice, or mice bearing syngeneic orthotopic PyMT-derived tumors (~1.0 cm) with αCSF1, αCD11b, or PLX3397 (vs controls) for 5 days, followed by 4 cycles of PTX (10 mg/kg, i.v.; Supplementary Fig. S4). Primary tumor burden at study endpoints (2.0 cm primary tumors or 100 days of age) was significantly reduced in mice treated with combined αCSF1/PTX, αCD11b/PTX, or PLX3397/PTX therapy, compared to mice treated with these as single agents (Fig. 4A and B; Supplementary Fig. S7A). Similar results were observed in syngeneic mice bearing orthotopic PyMT-derived mammary tumors receiving combined PLX3397/carboplatin (CBDCA) therapy (Fig. 4B).

Mammary tumors in MMTV-PyMT mice progress through well-defined stages of cancer development, similar to progression of breast cancer in women, including tissue with florid ductal hyperplasia, ductal carcinoma *in situ* with early stromal invasion, and poorly differentiated invasive ductal carcinoma (15, 21). Using this staging criterion, we observed that mammary tumors arising in MMTV-PyMT mice treated with combined PLX3397/PTX therapy exhibited decreased development of late-stage carcinoma, compared with tumors in age-matched mice treated with either PTX or PLX3397 as monotherapy (Fig. 4C; Supplementary Fig. S7B). Moreover, the late-stage carcinomas that did develop in PLX3397/PTX-treated mice contained large areas of necrosis (Supplementary Fig. S7C) characterized by increased presence of apoptotic cells, as measured by cleaved caspase 3-positivity (Fig. 4D) with no accompanying change in epithelial proliferation (Supplementary Fig. S7D).

Decreased Vascular Density Accompanies Improved Chemosensitivity

It is known that TAMs provide VEGF to developing mammary tumors and thereby regulate angiogenic programming of tissue (22–24). Chemosensitivity to CDDP in MMTV-PyMT mice is in part regulated by myeloid-derived VEGF (25); thus, we sought to determine if TAM depletion altered VEGF expression and/

or density of CD31$^+$ vessels in MMTV-PyMT mice treated with PTX. Whereas total *VEGF* mRNA expression was significantly reduced by PLX3397 (Fig. 4E), this 70% reduction did not correlate with a change in vascular density (Fig. 4F). In contrast, combined PLX3397/PTX therapy resulted in a significant reduction in CD31$^+$ vessel density within mammary tumors, paralleling induction of apoptosis and necrosis (Fig. 4F).

CSF1-Signaling Blockade Enhances Antitumor Immunity and CTL Infiltration in Response to Chemotherapy

Because analysis of human breast cancer tissues revealed that high stromal TAM density inversely correlated with CD8$^+$ T-cell infiltration (Supplementary Table S1), we predicted that depletion of TAMs would enhance CD8$^+$ CTL infiltration and thereby foster an antitumor immune microenvironment. Analyses of tumor-infiltrating T lymphocytes in mice treated with αCSF1/PTX or PLX3397/PTX by flow cytometry or IHC revealed significantly increased presence of CD4$^+$ and CD8$^+$ T cells in mammary tumors (Fig. 5A and B; Supplementary Fig. S8A). Consistent with these findings, cytokine mRNA expression in mammary tissue derived from PLX3397/PTX-treated MMTV-PyMT mice revealed increased mRNA expression of cytotoxic effector molecules, including *IFN-γ*, *granzyme A*, *granzyme B*, *perforin-1*, and the type 1 DC effector molecules *IL12p35* and *IFN-α* (Fig. 5C). In contrast, expression of the immunosuppressive molecule *arginase-1* was

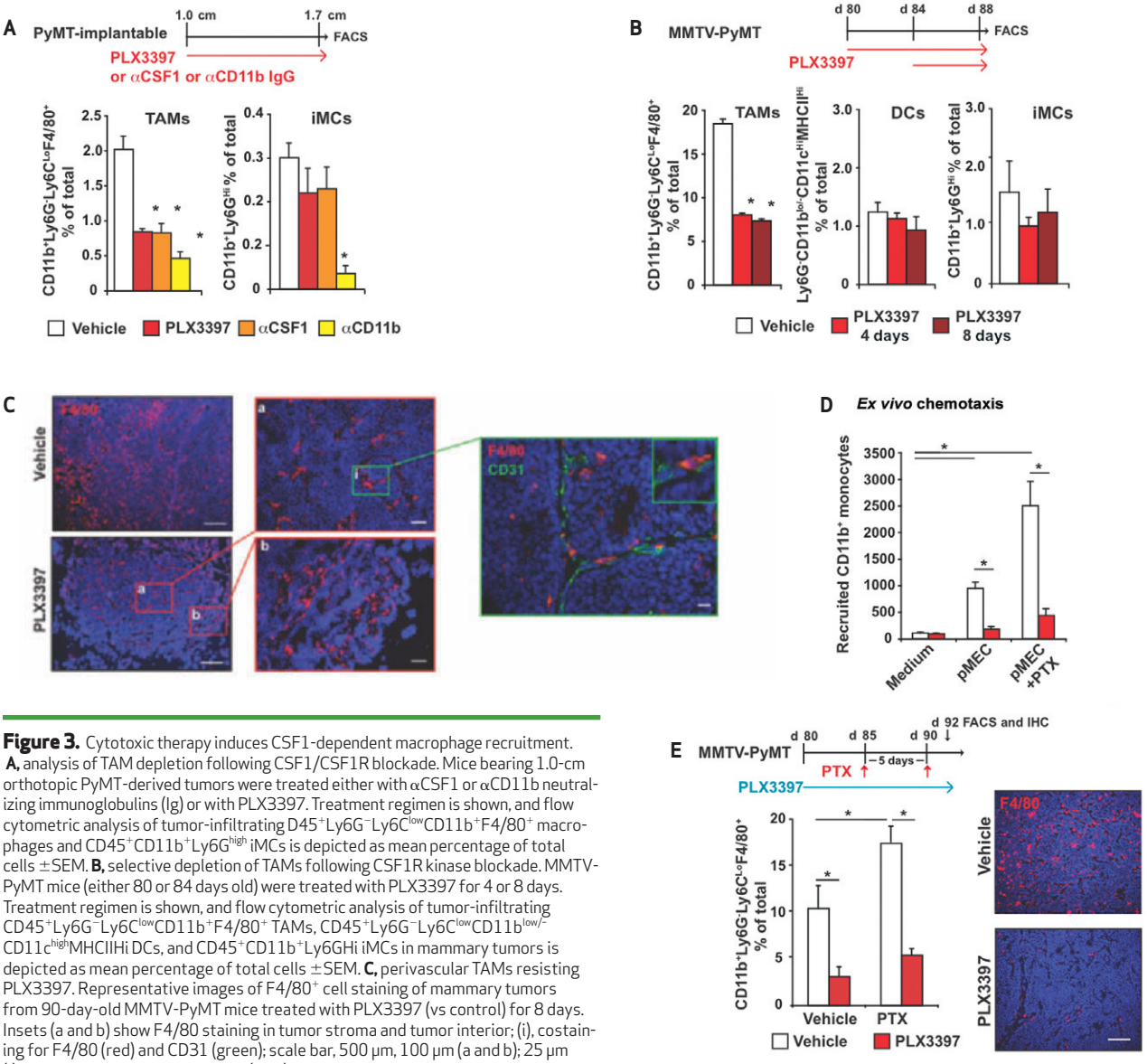


Figure 3. Cytotoxic therapy induces CSF1-dependent macrophage recruitment. **A,** analysis of TAM depletion following CSF1/CSF1R blockade. Mice bearing 1.0-cm orthotopic PyMT-derived tumors were treated either with αCSF1 or αCD11b neutralizing immunoglobulins (Ig) or with PLX3397. Treatment regimen is shown, and flow cytometric analysis of tumor-infiltrating D45$^+$Ly6G$^-$Ly6C^{low}CD11b$^+$F4/80$^+$ macrophages and CD45$^+$CD11b$^+$Ly6G^{high} iMCs is depicted as mean percentage of total cells ±SEM. **B,** selective depletion of TAMs following CSF1R kinase blockade. MMTV-PyMT mice (either 80 or 84 days old) were treated with PLX3397 for 4 or 8 days. Treatment regimen is shown, and flow cytometric analysis of tumor-infiltrating CD45$^+$Ly6G$^-$Ly6C^{low}CD11b$^+$F4/80$^+$ TAMs, CD45$^+$Ly6G$^-$Ly6C^{low}CD11b$^{low/-}$CD11c^{high}MHCIIHi DCs, and CD45$^+$CD11b$^+$Ly6GHi iMCs in mammary tumors is depicted as mean percentage of total cells ±SEM. **C,** perivascular TAMs resisting PLX3397. Representative images of F4/80$^+$ cell staining of mammary tumors from 90-day-old MMTV-PyMT mice treated with PLX3397 (vs control) for 8 days. Insets (a and b) show F4/80 staining in tumor stroma and tumor interior; (i), costaining for F4/80 (red) and CD31 (green); scale bar, 500 μm, 100 μm (a and b); 25 μm (i). **D,** peripheral blood lymphocyte (PBL) migration in response to conditioned medium from MMTV-PyMT MECs treated with either vehicle or PTX (25 nM for 24 hours), evaluated by Boyden chamber assay. CD45$^+$CD11b$^+$ peripheral blood monocytes after migration to the lower chamber, in the presence or absence of PLX3397 (50 nM), as quantified by flow cytometry. Data are depicted as mean cell number assayed in triplicate. **E,** PTX-induced TAM recruitment inhibited by PLX3397. TAM density in mammary tumors removed from MMTV-PyMT mice following treatment with PTX ± PLX3397. Treatment regimen is shown with mouse age, and data are depicted as mean number of CD45$^+$Ly6G$^-$Ly6C$^-$CD11b$^+$F4/80$^+$ TAMs as a percentage of total cells ±SEM (analyzed by flow cytometry, >5 mice/group). Representative IHC staining for F4/80 (red) and nuclear DNA (blue) from the same cohort of animals is shown. Scale bar, 500 μm; *, statistically significant differences ($P < 0.05$, Mann-Whitney) in **A**–**E**.

decreased by PLX3397/PTX therapy (Fig. 5C). This reprogramming of the immune microenvironment was accompanied by increased tumor infiltration of $CD45^+CD11b^{low/-}CD19^-Ly6G^-Ly6C^{low}CD11c^{high}MHCII^{high}$ DCs (Fig. 5D), indicating that combined treatment of MMTV-PyMT mice with PLX3397/PTX fostered an antitumor immune response by T lymphocytes expressing high levels of cytotoxic effector molecules.

Given these findings, we assessed the capacity of TAMs (isolated from mammary tumors of MMTV-PyMT mice) to directly repress $CD8^+$ T-cell activation *in vitro*. Using carboxyfluorescein succinimidyl ester (CFSE) dilution as a marker for T-cell proliferation, we found that $CD45^+CD11b^+F4/80^+Ly6G^-Ly6C^{low}$ TAMs significantly repressed $CD8^+$ T-cell activation and proliferation in a dose-dependent manner (Fig. 5E) that was reflective of the altered ratio of TAMs to $CD8^+$ T cells in mammary tumors of untreated versus PLX3397/PTX-treated MMTV-PyMT mice (Fig. 5F).

One mechanism by which TAMs may suppress $CD8^+$ T cells involves expression of inhibitory B7 family members that interact with "checkpoint" receptors expressed on infiltrating CD8 T cells. In particular, growing interest has been expressed in the PD1- PDL1 ligand system, in which PDL1/B7-H1 expression by TAMs represents a major source of the inhibitory PD1 ligand. Thus, we evaluated TAMs isolated from mammary tumors for expression of PDL1/B7-H1 and PDL2/B7-DC, as well as co-stimulatory molecules CD80 and CD86, and MHCII. TAMs expressed high levels of MHCII and B7-H1, but relatively lower levels of CD80 and CD86, indicating a possible role in inducing tolerance/anergy in tumor antigen–specific $CD4^+$ and $CD8^+$ T lymphocytes (Supplementary Fig. S5C).

Macrophage Depletion Enhances Chemotherapeutic Response in a $CD8^+$ CTL-Dependent Manner

To determine whether increased chemosensitivity of mammary tumors in PLX3397/PTX-treated mice was dependent on enhanced $CD8^+$ T-cell response, we depleted $CD8^+$ T cells from late-stage MMTV-PyMT mice treated with PTX or PLX3397 or both. Findings from this study revealed that the improved outcome with enhanced chemosensitivity resulting

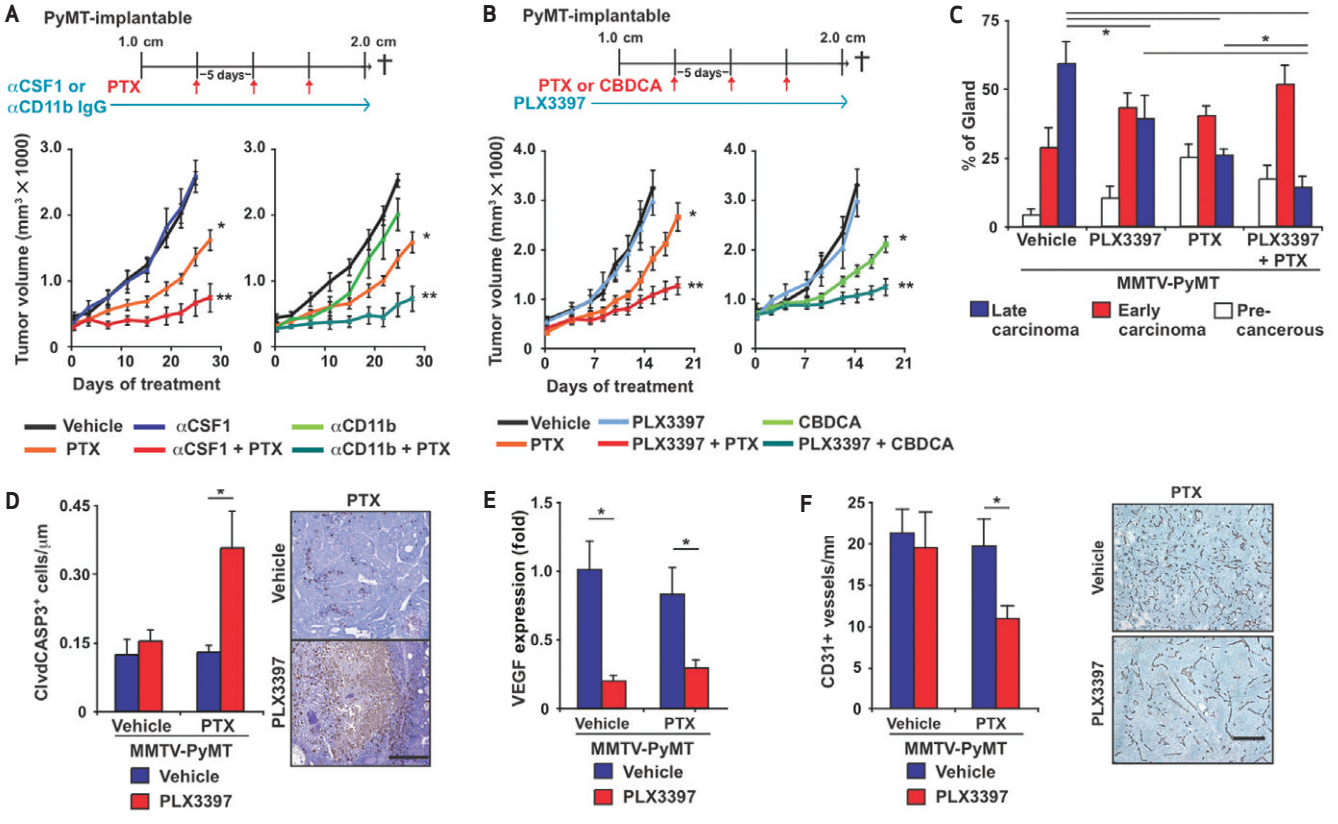


Figure 4. Macrophage depletion improves response to chemotherapy. **A** and **B,** primary tumor growth reduced by treatment with macrophage-depleting agents in combination with chemotherapy. Orthotopic PyMT-derived tumors were grown to a median diameter of 1.0 cm, and mice were then treated with PTX, CBDCA, and/or αCSF1, αCD11b neutralizing Igs ± PLX3397 for 21 or 28 days, with total tumor burden per animal assessed every 2 to 3 days. Treatment regimens are depicted for all cohorts, and data displayed as mean tumor burden ±SEM; *, statistically significant differences between vehicle- and PTX-treated mice. **, significant differences between mice treated with PTX alone and mice treated with PTX/PLX3397 or αCSF1 or αCD11b in combination. **C,** histologic stage analysis of MMTV-PyMT tumors. Tumors from 100-day-old MMTV-PyMT mice treated with PTX or PLX3397 or both were assessed for the presence of premalignant tissue and early- and late-stage carcinoma; data expressed as mean percentage of total gland area ±SEM. **D,** quantification of cleaved caspase-3–positive cells in mammary tumors of MMTV-PyMT mice treated with PTX or PLX3397 or both versus control (vehicle). Graph depicts mean positive cells per μm^2 of tumor tissue. Representative images show cleaved caspase-3–positive cells (brown staining) in tumors of MMTV-PyMT mice; scale bar, 500 μm. **E,** *VEGF* mRNA expression assessed by qRT-PCR of tumor tissue from MMTV-PyMT mice treated with vehicle or PTX or PLX3397 or both. Graph depicts mean fold change in gene expression compared with vehicle-treated control group. **F,** quantification of $CD31^-$ positive vessels in mammary tumors from MMTV-PyMT mice treated with PTX or PLX3397 or both, versus control (vehicle). Data represent the mean number of $CD31^+$ positive vessels per mm^2 of carcinoma tissue. Representative photomicrographs show CD31-positive vessels (brown staining); scale bar, 400 μm; *, statistically significant differences ($P < 0.05$, Mann-Whitney) in **C–F**.

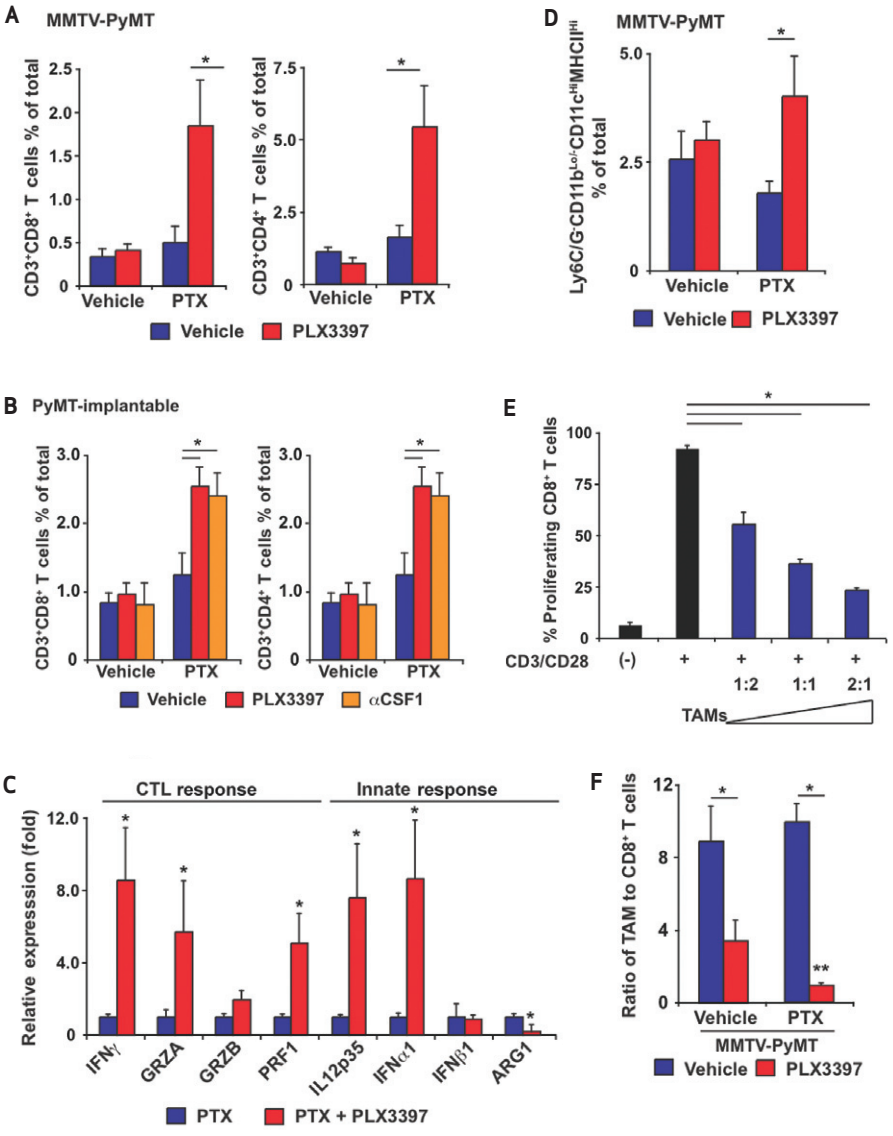


Figure 5. PTX in combination with PLX397 induces antitumor T-cell response. **A** and **B,** tumor infiltration by T lymphocytes enhanced by combined PTX and CSF1 or CSF1R blockade. Flow cytometric analyses of tumor infiltrating $CD3^+CD8^+$ and $CD3^+CD4^+$ T lymphocytes depicted as the mean number of positive cells, assessed as a percentage of total cells following treatment of MMTV-PyMT mice with PTX or PLX3397 or both (**A**), or treatment of mice having orthotopic PyMT-derived tumors with combined PTX/αCSF1 or PTX/PLX3397 (**B**), compared with controls. Mean values ±SEM are depicted. **C,** cytokine mRNA expression assessed in orthotopic PyMT-derived tumors from mice treated with PTX alone or in combination with PLX3397. Graph depicts mean fold change in mRNA expression compared with PTX treatment group (5 animals/group). SEM is depicted. **D,** tumor infiltration by DCs enhanced by combined PTX/PLX3397. Flow cytometric analysis of tumor-infiltrating $CD45^+Ly6G^-Ly6C^{low}CD11b^{low/-}CD11c^{high}MHCII^{high}$ DCs depicted as mean percentage of positive cells as a percentage of total cells from MMTV-PyMT mice treated with PTX or PLX3397 or both, versus controls. **E**, $CD8^+$ T-lymphocyte activation repressed by TAMs. Purified T cells were loaded with CFSE and activated *in vitro* by plate-bound CD3/28 and cocultured with the indicated ratio of $CD45^+Ly6G^-Ly6C^{Low}$ $CD11b^+F4/80^+$ TAMs isolated from late-stage mammary tumors of MMTV-PyMT mice. Data are depicted as the percentage of live $CD8^+$ T lymphocytes exhibiting CFSE dilution after 60 hours. Data are representative of 2 independent experiments run in triplicate. Error bars represent SEM. **F,** analysis of the ratio of tumor-infiltrating $CD45^+Ly6G^-Ly6C^{low}CD11b^+F4/80^+$ TAMs to $CD3^+CD8^+$ T lymphocytes depicted as mean ratio (TAM/CD8 CTL) ±SEM from MMTV-PyMT mice treated with vehicle or with PTX and/or PLX3397. *, statistically significant differences ($P < 0.05$, Mann-Whitney) in **A**–**F**. **, statistically significant differences ($P < 0.05$, Mann-Whitney) between PLX3397-treated groups in **F**.

from combined PLX3397/PTX therapy was indeed a $CD8^+$ T-cell–dependent response (Fig. 6A and B; Supplementary Fig. S8B). We found that CD8 depletion also resulted in increased tumor grade and decreased presence of cleaved caspase-3–positive cells in mice that had received combined PLX3397/PTX therapy (Fig. 6C and D). Taken together, these data indicate that the enhanced cytotoxic response elicited by CSF1R-signaling blockade was $CD8^+$ T-cell–dependent.

Combined Macrophage Depletion and Chemotherapy Blocks Metastasis in a CD8-Dependent Manner

Long-term survival of breast cancer patients is often limited by disseminated metastases following surgical resection of primary tumors. Analysis of leukocyte profiles in human breast cancers demonstrated that OS, and thus presumably metastatic spread, were regulated by the spectrum of tumor-infiltrating T lymphocytes and macrophages present. In MMTV-PyMT mice, although neither CSF1R-signaling blockade nor PTX therapy alone inhibited development of pulmonary metastasis, mice receiving combined PLX3397/PTX exhibited >85% reduction in pulmonary metastases that was in part $CD8^+$ T-cell–dependent (Fig. 6E).

Macrophages and CD8 Infiltration Predicts Survival and Chemotherapeutic Response

Overall, our data indicated that in the absence of TAMs, antitumor $CD8^+$ CTLs bolster response to chemotherapy and thereby influence outcome; thus, we predicted that TAM and CD8 T-cell ratios would correlate with pathologic responses in patients with breast cancer. Accordingly, we analyzed *CD68* and *CD8a* mRNA expression in a cohort of 311 patients constructed from 2 independent datasets (26, 27). All patients provided fine-needle aspirates (FNA) prior to neoadjuvant chemotherapy, and pathologic response was assessed at the time of definitive surgery. *CD8* mRNA expression in FNA samples correlated with pathologic complete response (pCR; $R = 0.216$; $P < 0.001$); however, CD68 did not. With median expression as a threshold, examination of both *CD8* and *CD68* mRNA revealed 3 groups—CD68 > CD8, CD68 < CD8, and CD68 = CD8 (denoted $CD68^{high}/CD8^{low}$, $CD68^{low}/CD8^{high}$, and CD68/

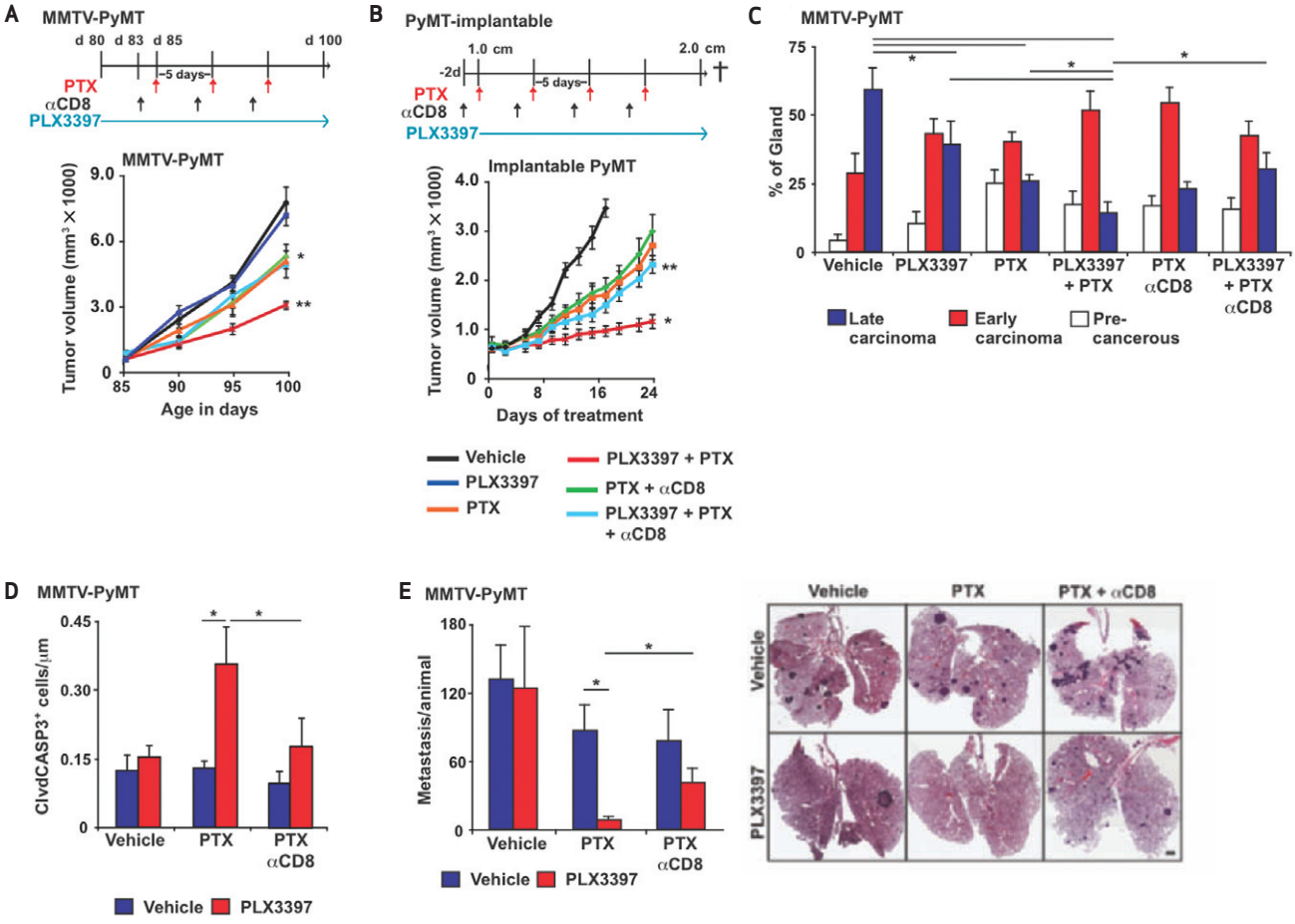


Figure 6. Combined PLX3397 and PTX treatment inhibits metastasis in a CD8-dependent manner. **A** and **B,** improved outcome following PLX3397/PTX treatment dependent on $CD8^+$ T cells. **A**, 85-day-old MMTV-PyMT mice were treated with PTX or PLX3397 or both, as well as anti-CD8 IgG. Total tumor burden per animal was assessed every 5 days. **B**, orthotopic PyMT-derived tumors were grown to a median diameter of 1.0 cm, at which time mice were treated with PTX or PLX3397 or both in combination with anti-CD8 or control IgG for 21 days, and total tumor burden per animal was assessed every 2 to 3 days. Treatment regimens are depicted along with SEM; *, statistically significant differences between mice treated with PTX alone and those treated with PLX3397/PTX. **, significant differences between mice treated with PLX3397/PTX and those treated with anti-CD8 and PTX/PLX3397/control IgG. **C,** histologic stage analysis of MMTV-PyMT tumors. Tumors from 100-day-old MMTV-PyMT mice treated with anti-CD8 IgG or with PTX and/or PLX3397 were assessed for presence of premalignant tissue and early- and late-stage carcinoma; data expressed as mean percentage of total gland area ±SEM. **D,** quantification of cleaved caspase-3–positive cells in mammary tumors of MMTV-PyMT mice treated with anti-CD8 IgG or with PTX and/or PLX3397 versus control (vehicle). Graph depicts mean positive cells per μm^2 of tumor tissue. **E,** quantification of metastatic foci per lung section per mouse from 100-day-old MMTV-PyMT mice treated with PTX and/or PLX3397 and/or anti-CD8 IgG, versus controls. Each lung was serially sectioned, 6 sections 100 μm apart were stained with hematoxylin and eosin (H&E), and the total number of metastatic foci (>8 cells) was quantified per mouse ($n \geq 10$ mice per cohort). SEM is depicted. *, Statistically significant differences ($P < 0.05$, Mann-Whitney). Representative photomicrographs of lung tissue sections reveal metastatic foci from 100-day-old MMTV-PyMT mice treated with vehicle or with PTX and/or PLX3397. Scale bar, 500 μm.

$CD8^{equal}$, respectively)—with the $CD68^{high}/CD8^{low}$ group correlating with a significantly lower rate of pCR (7%) and the $CD68^{low}/CD8^{high}$ exhibiting the highest rate of pCR at 27% (Fig. 7A). Thus, the ratio of *CD68/CD8a* expression represents a predictive response biomarker for neoadjuvant chemotherapy.

We next evaluated *CD68* and *CD8a* mRNA expression in breast cancers representing ~4000 patients assembled from 22 retrospective gene expression datasets (Supplementary Table S8). Median expression for both *CD8* and *CD68* was used to determine high and low groups. All patients were categorized as either $CD68^{high}/CD8^{low}$ or $CD68^{low}/CD8^{high}$. Kaplan-Meier estimates of survival demonstrated significantly reduced OS in the $CD68^{high}/CD8^{low}$ group (Fig. 7B). Not surprisingly, these gene expression results were validated using Kaplan-Meier analysis of OS on IHC data from Cohort I and II stratified for $CD68^{high}/CD8^{low}$ or $CD68^{low}/CD8^{high}$ (Supplementary Fig. S9A and B).

Because breast cancer represents a spectrum of distinct molecular subtypes (luminal A, luminal B, HER2-positive, basal type/triple negative), possessing distinct histopathologic and molecular features and correlating with differential responses to therapy and outcome (28–30), we evaluated *CD68/CD8* expression within breast cancer subtypes. $CD68^{high}/CD8^{low}$ expression correlated with reduced OS for breast cancer patients whose tumors were classified as either basal or HER2-positive (Fig. 7C; Supplementary Fig. S10).

DISCUSSION

The immune microenvironment in which a tumor evolves influences multiple parameters of the tumorigenic process. In this article we demonstrate that the immune microenvironment in breast cancer is a predictor of RFS and OS. Moreover, we provide evidence that response to chemotherapy is in part regulated by the immune microenvironment and that cytotoxic therapies induce neoplastic cells to produce monocyte/macrophage recruitment factors, including CSF1 and IL-34, which in turn enhance CSF1R-dependent macrophage infiltration into mammary adenocarcinomas. This is significant in light of our finding that blockade of the CSF1-signaling pathway mediating TAM recruitment, in combination with chemotherapy, decreases primary tumor progression, reduces metastasis, and improves survival—a $CD8^+$ T-cell–dependent outcome resulting from a reprogrammed immune microenvironment that fosters antitumor immunity. These data provide a compelling rationale for combinatorial therapies targeting TAM recruitment, notably CSF1R-mediated signaling pathways, in combination with cytotoxic therapy for breast cancer.

The Immune Microenvironment in Breast Cancer Predicts Outcome

In a previous study, we reported that T-helper 2 (T_H2)–$CD4^+$ T-effector cells regulate TAM bioactivity and thereby promote late-stage mammary carcinogenesis and development of pulmonary metastasis (11). We now extend these findings and demonstrate that the complexity of $CD8^+$ T lymphocytes, $CD4^+$ T lymphocytes (presumably T_H2 or T regulatory cells or both), and $CD68^+$ TAMs is a predictive biomarker for OS and RFS in node-positive breast cancer (Fig. 1). Retrospective clinical studies have previously revealed that the ratio of $CD4^+$ to $CD8^+$ T lymphocytes, or T_H2 to T_H1 $CD4^+$ T cells, infiltrating breast cancer correlates with increased tumor grade, lymph node metastasis, and reduced OS (31). Unsupervised expression profiling of breast cancer-associated stroma revealed a gene signature predictive of good prognostic outcome (>98% 5-year survival) that was functionally enriched for genes suggestive of CTL and natural killer cell activity (32). Moreover, elucidation of a CSF1-response gene expression signature in breast cancer demonstrated that CSF1 signaling correlates with response to therapy and OS (7–9). In this article, we revealed that not only does increased macrophage density correlate with poor outcome (Fig. 1), as reported by others, as well (5, 31, 33), but also the ratio of macrophages/*CD68* to $CD8^+$ T lymphocytes/*CD8a* in breast cancer is inversely correlated, an important finding when considering that TAMs can also suppress antitumor immunity. These findings indicate that our immune-based signature may be a useful predictor of recurrence and poor OS for multiple breast cancer subtypes, and, as such, may improve existing gene expression-based prognostic profiling to evaluate risk.

Breast Cancer CSF1 and CSF1R Expression

Our findings demonstrate that macrophage CSF1R signaling is necessary for their recruitment following induction of *CSF1* mRNA and interaction with ligand in carcinoma cells treated with chemotherapy (Fig. 3). Recent studies by Patsialou and colleagues (34) demonstrated that in some human breast carcinoma cell lines, specifically MDA-MB231, steady-state *CSF1R* mRNA is expressed at high levels and autocrine CSF1-CSF1R signaling enhances invasion (44). Notably, MDA-MB231 cells in our studies did not respond to CTX with increased *CSF1* mRNA expression (Fig. 3), likely because these cells already express 10- to 50-fold higher levels of *CSF1* mRNA than do other breast cancer cell lines evaluated (data not shown). Carcinoma cells isolated from MMTV-PyMT mice do not express significant levels of *CSF1R* mRNA (15, 22), indicating that CSF1R blockade in MMTV-PyMT mice influences myeloid biology, as opposed to MECs.

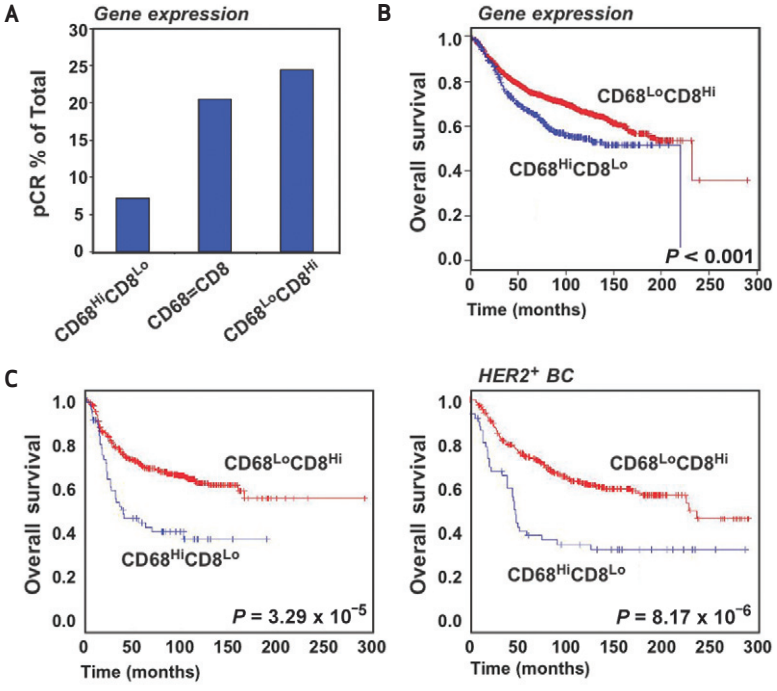


Figure 7. Ratio of *CD68* to *CD8* predicts patient survival and response to neoadjuvant chemotherapy. **A,** frequency of pCR in a cohort of 311 patients constructed from 2 independent datasets. All patients received FNAs prior to neoadjuvant chemotherapy and pathologic response was assessed at definitive surgery. With median expression as a threshold, examination of *CD8*a and *CD68* mRNA in FNA samples revealed 3 separate groups: CD68 > CD8, CD68 < CD8, and CD68 = CD8 (denoted $CD68^{high}/CD8^{low}$, $CD68^{low}/CD8^{high}$, and $CD68/CD8^{equal}$, respectively). Analysis of the rate of pCR in the groups is shown. **B,** Kaplan-Meier estimate of survival, comparing $CD68^{high}/CD8^{low}$ and $CD68^{low}/CD8^{high}$ immune profiles as assessed by mRNA expression from 3,872 patient samples assembled from 14 different platforms. Median expression for both *CD8* and *CD68* was used to determine high and low groups within each of the 22 individual datasets. Once a sample was assigned to a particular group, the 22 datasets were combined and a global survival analysis was performed. The log-rank (Mantel-Cox) *P* value is shown for difference in survival. **C,** Kaplan-Meier estimate of survival, comparing $CD68^{high}/CD8^{low}$ and $CD68^{low}/CD8^{high}$ immune profiles as assessed by mRNA expression from 3872 patient samples for tumors stratified into basal and $HER2^+$ breast cancer. The log-rank (Mantel-Cox) *P* value is shown for difference in survival.

Immunosuppressive Macrophages and Chemosensitivity

Development and progression of pulmonary metastases in mammary carcinoma is impaired in mice containing a recessive null mutation in the *CSF1* gene (15, 35). Similarly, blockade of CSF1R signaling impairs aspects of mammary carcinogenesis (36) and metastases (37). We used αCSF1 mAb and PLX3397, a novel small-molecular-weight tyrosine kinase inhibitor, to efficiently deplete $CD11b^+Ly6G^-Ly6C^{low}F4/80^+$ TAMs (70%) without altering the presence of $CD11b^+Ly6G^{high}F4/80^-$ iMCs or perivascular $F4/80^+$ macrophages in mammary tumor stroma (Fig. 3).

Malignant mammary epithelial cells from MMTV-PyMT mice express high levels of CSF1, which directly regulates TAM recruitment (and EGF expression) and induction of macrophage HIF1α (11, 38, 39). We previously reported that IFN-γ^+ $CD8^+$ CTL activity is impaired by myeloid-derived ARG1 and nitric oxide synthase that is HIF1α-dependent (38). Thus, on the basis of inverse correlation between TAMs and $CD8^+$ T cells in human breast cancer, and the fact that TAMs infiltrating mammary carcinomas directly suppress CTL activity in a HIF1α-dependent manner (38), we postulated that TAM depletion would foster antitumor immunity by relieving TAM-mediated CTL suppression and thereby enhance response to cytotoxic therapy. Accordingly, combined treatment of MMTV-PyMT mice with PTX, and either αCSF1 mAb or PLX3397, slowed primary tumor development, reduced development of high-grade carcinomas (Fig. 4), and decreased pulmonary metastasis by 85% (Fig. 6), features of mammary carcinogenesis accompanied by decreased vascular density (Fig. 4), and increased $CD4^+$ and $CD8^+$ T-cell infiltration in primary tumors (Fig. 5). Increased presence of $CD8^+$ T cells was significant in this regard, as when specifically depleted, the added benefit of combined PLX3397/PTX therapy was lost (Fig. 6).

Immunosuppressive myeloid cells encompass a diverse population of $CD11b^+Gr1^+Ly6G^+$ cells, including myeloid-derived suppressor cells, inflammatory monocytes, neutrophils, and iMCs. Human equivalents have been identified as $LIN^{-/low}$ human leukocyte antigen (HLA)–$DR^-CD33^+CD11b^+$ and $CD14^+HLA\text{-}DR^{-/low}$ cells (40). CSF1R blockade by PLX3397 depleted $CD11b^+Ly6G^-Ly6C^{low}F4/80^+$ TAMs, but not $CD11b^+Ly6G^+$ cells (Fig. 3C), which are 12-fold less abundant in MMTV-PyMT carcinomas. In contrast, immunosuppressive $CD11b^+Ly6G^+$ cells are more abundant in other mammary tumor models, such as 4T1 (41). This may be an important distinction between tumor types considering the fact that monocyte mobilization from bone marrow is impaired by genetic loss of CSF1, but unaltered following pharmacologic or immunologic inhibition of CSF1R. $Gr1^+CCR2^+CX3CR1^{low}$ iMCs are highly responsive to CCL2 (42), and CCL2 (MCP1) is expressed at high levels in MMTV-PyMT mammary tumors (Fig. 2C). Therefore, in extrapolating to the clinical scenario, it will be important to stratify human breast cancers containing predominantly high levels of mature tissue TAMs, compared with those containing $LIN^{-/low}HLA\text{-}DR^-CD33^+CD11b^+$ or $CD14^+HLA\text{-}DR^{-/low}$ iMCs, because these breast cancers would likely be less responsive to combinatorial therapy involving CSF1R-targeted agents.

Tissue Specificity and Clinical Implications

Stromal infiltration of TAMs is a poor prognostic indicator for some solid tumor types (43); however, infiltration of TAMs inside tumor nests, particularly when $CD8^+$ CTLs are also present, can correlate with improved survival outcome (44). These differences might be explained in part by the fact that TAMs produce either protumor or antitumor bioactivities depending on the types of cytokines to which they are exposed (43). TAMs regulated by T_H1 cytokines including IFN-γ, TNF-α, and granulocyte monocyte colony stimulating factor enhance TAM cytotoxic activity, production of proinflammatory cytokines, and antigen presentation (45). In contrast, tissue macrophages exposed to T_H2 cytokines, immune complexes, or immunosuppressive cytokines instead block CTL activity and promote angiogenesis and tissue remodeling (43, 45). In non–small cell lung cancer, TAMs that localize to tumor nests and correlate with favorable clinical outcomes exhibit an $M1/T_H1$ cytokine profile and express high levels of HLA-DR (46, 47). In contrast, CD163 and CD204 expressing TAMs ($M2/T_H2$ markers) correlate with poor clinical outcomes in melanoma, non–small cell lung cancer, and pancreatic cancer (48, 49). We found that tumor tissue from PLX3397/PTX-treated mice had increased *IL12p35* and *IFNα1* mRNA expression, indicative of bolstered antitumor immunity, indicating that PLX3397/PTX therapy fostered a general reprogramming of the immune microenvironment, in addition to blocking TAM infiltration that together favored $CD8^+$ T-cell–mediated tumor suppression. Data presented herein do not reveal whether the improved outcome for tumor-bearing mice or the antitumor immune microenvironment fostered under these conditions resulted directly from reduced presence of alternatively activated TAMs, decreased vessel density, or a combination of the two. Given the fact that PLX3397 as monotherapy efficiently reduced TAM presence but had no effect on vessel density, primary carcinoma (Fig. 4), or pulmonary metastasis development (Fig. 6), it seems reasonable to speculate that TAM depletion resulted in loss of an important epithelial cell survival pathway (possibly mediated by EGF) that resists chemotherapy-induced cell death; certainly, however, effects on vascular pathways may also play a role, as has been recently reported by Rolny and colleagues (50). It will be interesting to determine whether directly reprogramming TAMs—for example, by neutralization of IL-4—to favor the presence of classically activated (M1) TAMs, as we have previously reported (11), also similarly enhances antitumor immune programs and chemosensitivity and, if so, whether those responses are also $CD8^+$ T-cell–dependent.

Microtubule inhibitors constitute one of the most effective classes of cytotoxic agents available for treating both early- and late-stage breast cancer, and are considered the standard of care for treatment of metastatic disease. Several agents that affect microtubule dynamics are active antitumor agents and induce polymerization or cause nonfunctional tubulin aggregates. These compounds block cell division by interfering with function of the mitotic spindle and consequently result in cell-cycle arrest and cell death. PTX is among the most widely used agents in this class. Despite the clinical activity of taxanes, median time to progression in patients treated with PTX is only 6 to 9 months in the first- and second-line setting, and 3 to 4 months in patients with previous exposure to taxanes (51). Although addition of the antiangiogenic agent bevacizumab to PTX improved response and time to progression, it was without impact on OS (52). Glucocorticoid premedication is required for PTX to prevent increased bone marrow and peripheral nerve toxicity as well as allergic reactions and anaphylaxis due to the Cremaphor solvent base. For other cytotoxic agents, glucocorticoids are also commonly used to prevent

toxicities such as nausea, vomiting, and fluid retention. Although these agents are standard additions to many chemotherapies, they suppress production of proinflammatory cytokines and chemokines, severely impair differentiation of antigen presentation by DCs, suppress development of T_H1 cells, and bias immune responses toward T_H2 cell types (53). Our neoadjuvant studies in MMTV-PyMT and orthotopic tumor–bearing mice were performed without dexamethasone, an H_2 antagonist, or diphenhydramine. Thus, it is possible that the $CD8^+$ T-cell–dependent antitumor program fostered by combined PLX3397/PTX would have been dampened in the presence of dexamethasone. An understanding of the mechanisms that lead to inadequate or poor response to taxanes is urgently needed, as are prognostic biomarkers that predict which patients will respond favorably. That said, it seems reasonable that administration of cytotoxic agents not requiring steroids, in combination with novel strategies such as TAM ablation (or TAM reprogramming), which together bolster natural antitumor immunity, would improve outcomes and extend long-term survival for patients with breast cancer, as well as other cancer types. The clinical outcome of pharmacologically (or immunologically) targeting TAMs directly or the pathways that regulate their recruitment must be considered carefully because all cancer types may not respond in a similar fashion. This study provides a compelling rationale for clinical evaluation of combinatorial therapies inhibiting TAM recruitment, in combination with standard-of-care chemotherapy for treatment of breast cancer, and underscores the importance of identifying a population of patients who, by virtue of their immune profile and CSF1R status, may benefit most from such therapies.

METHODS

Patients and Tumor Samples

Tissue microarray studies were conducted on 2 separate patient cohorts. The screening cohort, Cohort I, described elsewhere in detail (54), was constructed from 179 cases of invasive breast cancer diagnosed at the Department of Pathology, Malmö University Hospital, Malmö, Sweden, between 2001 and 2002. The median age at diagnosis was 65 years of age, and the median follow-up time for OS was 52 months. Patients had not received neoadjuvant therapy and were treated with either modified radical mastectomy or wide local excision. The median tumor size was 2.2 cm; 62% of the tumors were PR-positive and 72% were ER-positive. Complete endocrine treatment data were available for 143 patients, 67 of whom received adjuvant tamoxifen, 3 an aromatase inhibitor, and 25 a combination of tamoxifen and an aromatase inhibitor. Information on adjuvant chemotherapy was available for 143 patients, of whom 30 received treatment. The second (validation) cohort, Cohort II, included 498 patients with primary invasive breast cancer diagnosed at the Malmö University Hospital between 1988 and 1992. These cases belonged to an original cohort of 512 patients, as previously described in detail (55). The median age at diagnosis was 65 years, and median follow-up time to first breast cancer event was 128 months. Information regarding the date of death was obtained from regional cause-of-death registries for all patients in both cohorts. Complete endocrine treatment data were available for 379 patients, 160 of whom received adjuvant tamoxifen. Information on adjuvant chemotherapy was available for 382 patients, of whom 23 received treatment. To assemble tissue microarrays, clearly defined areas of tumor tissue were indicated on a slide with a fresh tissue section from the paraffin block. Two biopsy samples, 1.0 mm in diameter, were taken from each donor paraffin block corresponding to the marked area. Recipient blocks were limited to ~200 cores each. In general, cores were taken from the peripheral aspect of the tumor. Necrotic tissue was avoided. For IHC analyses, 4.0-μm paraffin sections were used. The ethical committee at Lund University (Malmö, Sweden) approved this study.

Automated Image Acquisition, Management, and Analysis

Fully automated image acquisition was used for the results presented in this article. The Aperio ScanScope XT Slide Scanner (Aperio Technologies) was used to capture whole-slide digital images with a 20× objective. Slides were dearrayed to visualize individual cores, using Spectrum software (Aperio). A tumor-specific nuclear algorithm (IHC-MARK) developed in house was modified to quantify CD4, CD8, and CD68 expression. IHC-MARK was initially designed to identify tumor cells on the basis of nuclear morphologic features (56); however, this was modified for evaluating leukocyte infiltration based on specific nuclear morphologic features.

Image and Statistical Analysis

A decision tree–supervised algorithm was used to group patients based on immune cell IHC density. For decision tree analysis, all patients were randomly divided into 10 subsets. A decision tree model was selected using a 10-fold cross-validation approach (16). Ten consecutive decision tree models were independently constructed with the immune cell infiltration density continuous output from 9 subsets. Survival outcome predictive power of each decision tree model was tested on the remaining set of patients, and the model with the higher accuracy was selected as optimal for the dataset. Kaplan-Meier analysis and the log-rank test were used to illustrate differences between OS and RFS according to individual CD68, CD4, and CD8 expression. A Cox regression proportional hazards model was used to estimate the relationship to OS of the CD68/CD4/CD8 immune profile; lymph node status; tumor grade; and HER2, PR, and ER status in the patient cohorts. A P value of <0.05 was considered statistically significant, and calculations were assessed using Statistical Package for the Social Sciences (SPSS, Inc.).

Neoadjuvant Cohort

The neoadjuvant cohort consisted of 2 gene expression cohorts representing 311 patients treated with neoadjuvant chemotherapy (26, 27), with complete pathologic response in 60 (19%) patients. The majority of patients received PTX and fluorouracil-doxorubicin-cyclophosphamide. Both datasets were examined on the same array platform (Affymetrix U133A), using a standard operating procedure and normalization method (dCHIP) as previously reported (57, 58). Data were downloaded from the Gene Expression Omnibus (59) and an institutional website (60). Normalized expression values for both CD8 and CD68 were established as previously described (54).

Preclinical Mouse Models and Animal Husbandry

Mice harboring the PyMT transgene under the control of the MMTV promoter in the FVB/n strain were obtained from Dr. Zena Werb [University of California, San Francisco (UCSF), San Francisco, California] and have been previously described (14). Two murine models of mammary tumor development were used to analyze response to chemotherapy (Supplementary Fig. S3). The first model used MMTV-PyMT mice (Supplementary Fig. S3A). The 80-day-old MMTV-PyMT female littermates were randomized by initial tumor volume and fed either PLX3397 (20, 61, 62) formulated in mouse chow or control chow (provided by Plexxikon Inc). PLX3397 was formulated in mouse chow so that the average dose per animal per day was 40 mg/kg. When PLX3397-treated MMTV-PyMT mice reached 85 days of age, they were then administered PTX (Hospira) every 5 days by i.v. injection into the retroorbital plexus. PTX was given at 10 mg/kg of the animal per injection, diluted in PBS. Tumor burden was evaluated by caliper measurement every 5 days following the start of PLX3397 treatment. Prior to tissue collection, mice were cardiac-perfused with PBS to clear peripheral blood. Mammary tumor tissue from PBS-perfused MMTV-PyMT mice was analyzed by flow cytometry and qRT-PCR 2 days after the second dose of PTX, when metastatic burden and tumor grade were determined. Primary tumor burden was determined by caliper measurements on live sedated mice. Metastatic burden was assessed by serial sectioning of formalin-fixed paraffin-embedded lung tissue whereby the entire lung was sectioned and the number of metastatic foci (>5 cells) was determined on 6 sections taken every 100 μm following H&E staining. Lungs from >10 mice/group were analyzed.

To assess tumor grade, the stage characterization technique classified tumor tissue into 3 levels of histologic progression by quantifying the area of transformed glands occupied by each stage (15, 38). Progression follows from a "precancerous stage" characterized by premalignant hyperplasia and adenoma/mouse intestinal epithelium but with the retention of some normal ductal and acinar mammary gland morphology, to a more epithelial cell–dense "early carcinoma" with some stromal invasion, and finally to an invasive, high–mitotic index "late-stage carcinoma."

The IHC analysis was conducted on tissue sections following the end of studies on 100-day-old MMTV-PyMT mice (detailed in Supplementary Fig. S4A). Vehicle-treated mice received PBS-only injections. We also used a syngeneic orthotopic implantable tumor model (referred to as PyMT-implantable in all figures and detailed in Supplementary Fig. S4B). For this model, single-cell suspensions of tumor cell pools isolated from mammary tumors of 3 or 4 100-day-old MMTV-PyMT mice were generated following collagenase A digestion (see discussion of flow cytometry analysis earlier). A total of 1.0 million tumor cells from pools were diluted in medium and basement membrane extract (Matrigel, BD Pharmingen) and injected orthotopically into uncleared mammary fat pads (4th gland) of 10-week-old virgin FVB/n female mice. Following implantation, tumors were allowed to grow to a mean diameter of 1.0 cm before enrollment into studies. Mice were randomized into treatment groups based on tumor size and treated with PLX3397 and PTX, as described above. For some studies, CBDCA (Hospira) was used and administered at 10 mg/kg of mouse per injection, in a similar manner to administration of PTX (see above). For mice with implantable tumors, tumor burden was evaluated by caliper measurement every 2 to 3 days following the start of PLX3397 treatment, and mammary tissue was analyzed by flow cytometry, IHC, and qRT-PCR at the end of the study (Supplementary Fig. S3B). Immune-depleted mice were injected i.p. every 5 days with either 1.0 mg anti-CD8 immunoglobulin G (YTS169.4) or isotype control rat immunoglobulin on day 1 followed by 500 μg every 5 days. All mice were maintained within the UCSF Laboratory for Animal Care barrier facility, and the UCSF Institutional Animal Care and Use Committee approved all experiments involving animals.

Additional information on methods and cohorts is available in the Supplementary Data.

Disclosure of Potential Conflicts of Interest

E. Rexhepaj, D.J. Brennan, and W.M. Gallagher are inventors of a pending patent application in relation to the development of novel automated image analysis approaches in histopathology, and D.G. DeNardo, D.J. Brennan and L.M. Coussens are inventors of a pending patent application in relation to immune-based signatures for predicting breast cancer risk. B.L. West is an employee of Plexxikon Inc. but had no involvement in data collection, analysis, or interpretation.

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REFERENCES

1. Pollard JW. Trophic macrophages in development and disease. Nat Rev Immunol 2009;9:259–70.
2. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell 2010;141:39–51.
3. Tang R, Beuvon F, Ojeda M, Mosseri V, Pouillart P, Scholl S. M-CSF (monocyte colony stimulating factor) and M-CSF receptor expression by breast tumour cells: M-CSF mediated recruitment of tumour infiltrating monocytes? J Cell Biochem 1992;50:350–6.
4. Wei S, Nandi S, Chitu V, Yeung YG, Yu W, Huang M, et al. Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. J Leukoc Biol 2010;88:495–505.
5. Campbell MJ, Tonlaar NY, Garwood ER, Huo D, Moore DH, Khramtsov AI, et al. Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. Breast Cancer Res Treat 2010 [Epub ahead of print].
6. Steidl C, Lee T, Shah SP, Farinha P, Han G, Nayar T, et al. Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. N Engl J Med 2010;362:875–85.
7. Sharma M, Beck AH, Webster JA, Espinosa I, Montgomery K, Varma S, et al. Analysis of stromal signatures in the tumor microenvironment of ductal carcinoma in situ. Breast Cancer Res Treat 2010;123:397–404.
8. Espinosa I, Beck AH, Lee CH, Zhu S, Montgomery KD, Marinelli RJ, et al. Coordinate expression of colony-stimulating factor-1 and colony-stimulating factor-1-related proteins is associated with poor prognosis in gynecological and nongynecological leiomyosarcoma. Am J Pathol 2009;174:2347–56.
9. Beck AH, Espinosa I, Edris B, Li R, Montgomery K, Zhu S, et al. The macrophage colony-stimulating factor 1 response signature in breast carcinoma. Clin Cancer Res 2009;15:778–87.
10. de Visser KE, Korets LV, Coussens LM. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. Cancer Cell 2005;7:411–23.
11. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, et al. CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. Cancer Cell 2009;16:91–102.
12. Andreu P, Johansson M, Affara NI, Pucci F, Tan T, Junankar S, et al. FcRgamma activation regulates inflammation-associated squamous carcinogenesis. Cancer Cell 2010;17:121–34.
13. Ammirante M, Luo JL, Grivennikov S, Dedospasov S, Karin M. B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. Nature 2010;464:302–6.
14. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol 1992;12:954–61.
15. Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 2001;193:727–40.
16. Breiman L, Friedman JH, Olshen R, Stone CJ. Classification and regression trees. Pacific Grove, (CA): Wadsworth and Brooks/Cole Advanced Books and Software; 1984.
17. DeNardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. Cancer Metastasis Rev 2010;29:309–16.
18. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;9:162–74.
19. Haile LA, Gamrekelashvili J, Manns MP, Korangy F, Greten TF. CD49d is a new marker for distinct myeloid-derived suppressor cell subpopulations in mice. J Immunol 2010;185:203–10.
20. Artis DR, Bremer R, Gillette S, Hurt CR, Ibrahim PL, Zuckerman RL, Inventors; Plexxikon Inc., assignee. Molecular scaffolds for kinase ligand development. United States patent US-20050164300. July 28, 2005.

21. Lin EY, Jones JG, Li P, Zhu L, Whitney KD, Muller WJ, et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. Am J Pathol 2003;163:2113–26.
22. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res 2006;66:11238–46.
23. Lin EY, Pollard JW. Tumor-associated macrophages press the angiogenic switch in breast cancer. Cancer Res 2007;67:5064–6.
24. Lin EY, Li JF, Bricard G, Wang W, Deng Y, Sellers R, et al. VEGF restores delayed tumor progression in tumors depleted of macrophages. Mol Oncol 2007;1:288–302.
25. Stockmann C, Doedens A, Weidemann A, Zhang N, Takeda N, Greenberg JI, et al. Deletion of vascular endothelial growth factor in myeloid cells accelerates tumorigenesis. Nature 2008;456:814–8.
26. Hess KR, Anderson K, Symmans WF, Valero V, Ibrahim N, Mejia JA, et al. Pharmacogenomic predictor of sensitivity to preoperative chemotherapy with paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide in breast cancer. J Clin Oncol 2006;24:4236–44.
27. Tabchy A, Valero V, Vidaurre T, Lluch A, Gomez H, Martin M, et al. Evaluation of a 30-gene paclitaxel, fluorouracil, doxorubicin, and cyclophosphamide chemotherapy response predictor in a multicenter randomized trial in breast cancer. Clin Cancer Res 2010;16:5351–61.
28. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature 2000;406:747–52.
29. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98:10869–74.
30. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 2003;100:8418–23.
31. Kohrt HE, Nouri N, Nowels K, Johnson D, Holmes S, Lee PP. Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer. PLoS Med 2005;2:e284.
32. Finak G, Bertos N, Pepin F, Sadekova S, Souleimanova M, Zhao H, et al. Stromal gene expression predicts clinical outcome in breast cancer. Nat Med 2008;14:518–27.
33. Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res 1996;56: 4625–9.
34. Patsialou A, Wyckoff J, Wang Y, Goswami S, Stanley ER, Condeelis JS. Invasion of human breast cancer cells in vivo requires both paracrine and autocrine loops involving the colony-stimulating factor-1 receptor. Cancer Res 2009;69:9498–506.
35. Nowicki A, Szenajch J, Ostrowska G, Wojtowicz A, Wojtowicz K, Kruszewski AA, et al. Impaired tumor growth in colony-stimulating factor 1 (CSF-1)–deficient, macrophage-deficient op/op mouse: evidence for a role of CSF-1–dependent macrophages in formation of tumor stroma. Int J Cancer 1996;65:112–9.
36. Qian B, Deng Y, Im JH, Muschel RJ, Zou Y, Li J, et al. A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. PLoS One 2009;4:e6562.
37. Manthey CL, Johnson DL, Illig CR, Tuman RW, Zhou Z, Baker JF, et al. JNJ-28312141, a novel orally active colony-stimulating factor-1 receptor/FMS-related receptor tyrosine kinase-3 receptor tyrosine kinase inhibitor with potential utility in solid tumors, bone metastases, and acute myeloid leukemia. Mol Cancer Ther 2009;8:3151–61.
38. Doedens AL, Stockmann C, Rubinstein MP, Liao D, Zhang N, Denardo DG, et al. Macrophage expression of hypoxia-inducible factor-1α suppresses T-cell function and promotes tumor progression. Cancer Res 2010;70:7465–75.
39. Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res 2004;64:7022–9.
40. Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. Semin Cancer Biol 2006;16:53–65.
41. Bunt SK, Yang L, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. Cancer Res 2007;67:10019–26.
42. Zhang J, Patel L, Pienta KJ. CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. Cytokine Growth Factor Rev 2010;21:41–8.
43. Ruffell B, Denardo DG, Affara NI, Coussens LM. Lymphocytes in cancer development: polarization towards pro-tumor immunity. Cytokine Growth Factor Rev 2010;21:3–10.
44. Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, et al. Predominant infiltration of macrophages and CD8(+) T cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer. Cancer 2008;113:1387–95.
45. Mantovani A, Locati M. Orchestration of macrophage polarization. Blood 2009;114:3135–6.
46. Ma J, Liu L, Che G, Yu N, Dai F, You Z. The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. BMC Cancer 2010;10:112.
47. Ohri CM, Shikotra A, Green RH, Waller DA, Bradding P. Macrophages within NSCLC tumour islets are predominantly of a cytotoxic M1 phenotype associated with extended survival. Eur Respir J 2009;33:118–26.
48. Bronkhorst IH, Ly LV, Jordanova ES, Vrolijk H, Versluis M, Luyten GP, et al. Detection of M2 macrophages in uveal melanoma and relation with survival. Invest Ophthalmol Vis Sci 2011;52:643–50.
49. Kurahara H, Shinchi H, Mataki Y, Maemura K, Noma H, Kubo F, et al. Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. J Surg Res 2009 [Epub ahead of print].
50. Rolny C, Mazzone M, Tugues S, Laoui D, Johansson I, Coulon C, et al. HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. Cancer Cell 2011;19:31–44.
51. Twelves C, Cortes J, Vahdat LT, Wanders J, Akerele C, Kaufman PA. Phase III trials of eribulin mesylate (E7389) in extensively pretreated patients with locally recurrent or metastatic breast cancer. Clin Breast Cancer 2010;10:160–3.
52. Miller K, Wang M, Gralow J, Dickler M, Cobleigh M, Perez EA, et al. Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. N Engl J Med 2007;357:2666–76.
53. Zitvogel L, Kroemer G. The dilemma of anticancer therapy: tumor-specific versus immune effects. Blood 2008;112:4364–5.
54. Brennan DJ, Rexhepaj E, O'Brien SL, McSherry E, O'Connor DP, Fagan A, et al. Altered cytoplasmic-to-nuclear ratio of survivin is a prognostic indicator in breast cancer. Clin Cancer Res 2008;14:2681–9.
55. Paulsson J, Sjoblom T, Micke P, Ponten F, Landberg G, Heldin CH, et al. Prognostic significance of stromal platelet-derived growth factor beta-receptor expression in human breast cancer. Am J Pathol 2009;175:334–41.
56. Rexhepaj E, Brennan DJ, Holloway P, Kay EW, McCann AH, Landberg G, et al. Novel image analysis approach for quantifying expression of nuclear proteins assessed by immunohistochemistry: application to measurement of oestrogen and progesterone receptor levels in breast cancer. Breast Cancer Res 2008;10:R89.
57. Mazouni C, Peintinger F, Wan-Kau S, Andre F, Gonzalez-Angulo AM, Symmans WF, et al. Residual ductal carcinoma in situ in patients with complete eradication of invasive breast cancer after neoadjuvant chemotherapy does not adversely affect patient outcome. J Clin Oncol 2007;25:2650–5.
58. Moody SE, Perez D, Pan TC, Sarkisian CJ, Portocarrero CP, Sterner CJ, et al. The transcriptional repressor Snail promotes mammary tumor recurrence. Cancer Cell 2005;8:197–209.
59. Gene Expression Omnibus [database on the Internet]. Bethesda (MD): National Center for Biotechnology Information/National Library of Medicine/NIH. 2010 [cited 2011]. Available from http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20271
60. Department of Bioinformatics and Computational Biology [database on the Internet]. Houston (TX): The University of Texas MD Anderson Cancer Center. 2003 [cited 2011]. Available from http://bioinformatics.mdanderson.org/pubdata.html
61. Tsai J, Lee JT, Wang W, Zhang J, Cho H, Mamo S, et al. Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. Proc Natl Acad Sci U S A 2008;105:3041–6.
62. Louvet C, Szot GL, Lang J, Lee MR, Martinier N, Bollag G, et al. Tyrosine kinase inhibitors reverse type 1 diabetes in nonobese diabetic mice. Proc Natl Acad Sci U S A 2008;105:18895–900.

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Special Section Paper

Leukocytes as paracrine regulators of metastasis and determinants of organ-specific colonization

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It is now well recognized that tumor cell–host interactions regulate all aspects of cancer development. Amongst the various host response programs that facilitate primary cancer development, an emerging body of literature points to a critical role for leukocytes and their soluble mediators as regulating discrete events during primary tumor development and metastasis. This review focuses on the multiple aspects of leukocytes and their effector molecules as regulators of the metastatic process.

Dissemination of malignant cancer cells to distant organs is a multistage process requiring detachment and escape from primary tumor sites, extravasation through multiple basement membranes and matrix, survival in peripheral blood or lymphatics and ability to survive and proliferate in foreign tissue locales. As, for many tumor types, there is a temporal lag (months to decades) between when malignant cells arrive in ectopic locations and when proliferative capabilities are acquired,[1,2] this implies that in addition to activation of survival programs at the metastatic site, disseminated malignant cells must acquire additional capabilities enabling their survival that likely rely on harnessing embedded regulatory programs at secondary sites. Thus, although cell-intrinsic programs are necessary for successful progression through each of these hurdles,[2,3] cell-extrinsic programs regulated by non-neoplastic cells of mesenchymal, vascular and immune origins are also critical determinants for successful metastatic progression.[4]

Chronic infiltration of tissue by leukocytes, *i.e.*, chronic inflammation, is associated with predisposition to cancer.[5] Chronic inflammation triggered by bacterial and viral infections or by autoimmune disease is estimated to be linked with 20% of all deaths from cancer worldwide.[6] Indeed, epidemiological studies reporting that nonsteroidal anti-inflammatory drugs reduce the risk of some cancers provide evidence for a causal link between inflammation and cancer.[7–9] Thus, chronic inflammation that precedes neoplasia provides a fertile microenvironment whereby secreted growth factors, reactive oxygen species and cytokines support epithelial proliferation and create a permissive microenvironment to foster ongoing genetic instability and accumulation of genetic alterations that predispose to malignancy.[5,6] Alternatively, inflammation can also be a physiological response to aberrant proliferation and tissue remodeling initiated by mutational activation of intrinsic programs that sustain proliferation and/or block cell death, and thus represent a secondary event enabling progression of neoplastic cells.[10] In any event, hallmarks of inflammation such as the infiltration of neoplastic tissue by innate and adaptive leukocytes, activated angiogenic vasculature, tissue remodeling, and high levels of chemokines and cytokines that regulate these processes typify most solid tumors.[5,6,11] This review discusses how key components and pathways of the immune microenvironment are associated with adult solid tumors and thereby promote the multistep cascade of tumor metastasis to distant organs.

Key words: leukocytes, inflammation, cancer, metastasis
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Leukocytes Implicated in Mediating Solid Tumor Metastasis

It has been generally accepted that the chronic presence of activated leukocytes in primary tumors is a "hallmark" of the tumorigenic process and also represents a predictor of aggressive disease. Tumor-associated macrophages (TAMs) are one of the most abundant innate immune cells present in several types of human cancer (DeNardo *et al.*, manuscript submitted),[12–15] regulated in part by colony-stimulating factor (CSF)-1, a key cytokine involved in macrophage maturation, tissue recruitment and activation mediated by the CSF-1 receptor (CSF-1R/cFMS).[16] A second CSF-1R ligand, interleukin (IL)-34, possesses similar binding affinities and also regulates TAM recruitment to tissues, but exhibits distinct tissue

distribution.[17–19] TAM presence in several types of human cancer correlates with increased vascular density and worse clinical outcomes.[20–25] Studies in transgenic mouse models of mammary carcinogenesis revealed that TAMs promote tumor growth and enhance pulmonary metastasis by high-level expression of epidermal growth factor (EGF) and by activation of EGF-regulated signaling in mammary epithelial cells critical for invasive tumor growth and metastatic dissemination.[14] In mouse models of mammary carcinogenesis, TAMs activated by IL-4 and CSF-1 have been identified as essential determinants of pulmonary metastasis because of the prometastatic mediators they secrete.[26–29] The transcription factor *Ets2* was recently implicated in regulating some aspects of these activities as selective deletion of *Ets2* in TAMs decreased the frequency and size of pulmonary metastases in mouse models.[30]

T lymphocytes were classically studied in the context of their tumor surveillance and antitumor capabilities. However, recent investigations have revealed that $CD4^+$ T cells and T-regulatory cells instead promote pulmonary metastasis in part by regulating protumor *versus* antitumor bioactivity of innate leukocytes. We reported that IL-4-expressing $CD4^+$ T cells promote invasion and metastasis of mammary adenocarcinomas by directly regulating TAM phenotype, bioeffector function and EGF expression, which in turn regulates invasion, presence of circulating tumor cells (CTCs) and pulmonary metastasis.[28] Other mediators found to be significant with regards to T-cell enhancement of pulmonary metastasis mammary carcinomas are S100A4 and receptor activator of nuclear factor-B ligand (RANKL).[31,32] S100A4 protein mediates T cell attraction to developing neoplasms and premetastatic lungs of tumor-bearing mice, and in turn, it stimulates T-cell production of cytokines, particularly granulocyte CSF and eotaxin-2.[32] RANTES stimulates externalization of S100A4 via microparticle shedding from plasma membranes and induces upregulation of fibronectin (FN) from fibroblasts and a number of other cytokines, including RANTES in tumor cells that together enhance tumor cell motility.[31] During prostate carcinogenesis, T lymphocyte and macrophage-derived RANKL induces metastasis through activation and nuclear localization of inhibitor of nuclear factor kappa-B kinase subunit alpha leading to repression of *maspin*, a critical suppressor of metastasis.[33,34] Lung metastasis of mammary carcinomas is also regulated by $CCR4^+$ T regulatory cells that can directly kill natural killer (NK) cells.[35]

Other myeloid cell types implicated in regulating metastasis include neutrophils, mast cells and monocytes harboring T-cell suppressive activity[36–39] that are potent suppressors of antitumor adaptive immunity and directly facilitate metastasis by regulating angiogenic programs via enhanced metalloproteinase (MMP) activity.[11,40–42] Cancer-associated fibroblasts (CAFs) are implicated in regulating the activities of these myeloid cell types through their secretion of proinflammatory chemokines that recruit immune cells to sites of developing neoplasms.[32,43,44]

Proinflammatory Signals that Impact Exit from Primary Tumor Sites

Regulators of the invasive phenotype

For malignant cells to detach from primary tumors and move through their substratum basement membrane, they must transiently acquire a motile and migratory phenotype, sometimes also referred to as epithelial to mesenchymal transition (EMT).[45,46] This motile state is characterized by loss of homotypic cellular adhesions and apical–basal polarity and increased migratory capabilities. At the molecular level, this transition is largely driven by intrinsic alterations in gene expression, including suppression of *E-Cadherin*, mediated by activation of transcriptional repressors Snail, Slug, Twist and Zeb.[47]

Extrinsic regulation is also important. Specifically, the chronic inflammatory microenvironment, provided by leukocytes and CAFs, plays an important role in regulating the invasive and motile phenotype of potential metastatic cells. Several leukocyte-regulated mediators have been identified as key to these processes. Notably, tumor necrosis factor (TNF)-α secreted by TAMs activates the NF-κB transcription factor in neoplastic (and other) cells, directly leading to expression of Snail1 and Zeb.[44,48,49] Other leukocyte-derived cytokines (including IL-6 and IL-23) induce activation of intracellular STAT3 that in turn leads to induced expression of Twist.[50–52]

Local hypoxia in neoplastic tissue also contributes to induction of motility programs[53,54] in part by activation of transcription factors hypoxia inducible factor-1α and NF-κB, both implicated in EMT via transcription of Snail.[48] Moreover, the chemokine receptor CXCR4 is upregulated in mammary carcinomas by hypoxia and is associated with invasive behavior in response to its ligand stromal-derived factor-1 (SDF-1/CXCL12).[55,56] Thus, hypoxic conditions select for a more metastatic phenotype partially through activation of proinflammatory signaling cascades.

Invasion

Movement of malignant cells through basement membrane and stromal matrix requires remodeling of matrix proteins. This process is coordinated by proteolytic enzymes spanning several catalytic classes and includes matrix MMPs, cysteine cathepsins and serine proteases.[4] Indeed, the increased expression and activity of various proteases has been observed in multiple human and murine tumor types and can be used as a prognostic indicator of shorter survival rates in patients with breast, ovarian, colorectal and head and neck cancers.[57,58] Although many proteolytic enzymes are produced by motile neoplastic cells, the majority of tumor-promoting proteases are produced by activated stromal cells in the local tumor microenvironment,[40,59–62] *e.g.*, fibroblasts[63] or tumor-associated immune cells. Mast cells, neutrophils and macrophages secrete matrix remodeling proteases[64–66] implicated in prometastatic activity, as well as serine proteases that are associated with higher tumor grades and lymph node metastasis in breast cancer.[37]

In particular, murine macrophages are known to express elevated levels of the cysteine protease cathepsin B following exposure to IL-4.[67] Macrophages at the invasive edge of pancreatic islet cancers express cathepsin B, and this is associated with loss of epithelial E-cadherin on neighboring malignant cells.[4] Secretion of proteases by cells within the tumor microenvironment may not only foster metastatic activity and motility of neoplastic cells through matrix and into vasculature but also enhance and/or regulate the presence and activity of leukocytes: overexpression of cathepsin B in a transgenic mouse model of mammary carcinoma regulates pulmonary metastases, accompanied by increased numbers of B cells, Ig deposition and degranulation of mast cells in the primary tumor site.[68]

Protease secretion by TAMs is in part regulated by IL-6 emanating from neoplastic cells. Tumor cell-derived IL-6 induces secretion and activation of the cysteine protease cathepsin B and secretion of matrix MMPs by monocytes.[69] Similarly, neoplastic cell and T-cell-derived IL-4 induces cathepsin expression and activity in TAMs in several cancer types.[29] TAMs in turn regulate neoplastic cell motility by secreting factors such as migration-stimulating factor (MSF).[70] MSF is an oncofetal isoform of FN and is induced in TAMs by macrophage-CSF, IL-4 and transforming growth factor beta (TGF-β). Notably, some immune cell-derived proteases also harbor tumor-suppressive activity: the aspartic proteinase cathepsin E, expressed predominantly by immune cells, including lymphocytes, macrophages and dendritic cells, mediates neoplastic cell apoptosis by catalyzing the proteolytic release of soluble TNF-related apoptosis-inducing ligand from the cell surface. Tumor growth is subsequently enhanced by this cascade, and survival in tumor-bearing mice is impaired.[71]

Leukocytes and Survival of CTCs

Before productive metastatic colonization is possible, CTCs must develop mechanisms enabling their survival within the circulation. Mechanical shear stress, detachment-induced cell death (anoikis) and cell-mediated cytotoxicity within the microcirculation effectively clear most CTCs.[72] It has been estimated that only 0.01% of CTCs survive and eventually extravasate at distal locales.[4] Mechanisms that enhance the probability for CTC survival rely on physical interaction with leukocytes. Activated platelets aggregate around CTC and thereby protect them from NK cell-mediated lysis[72] by both thrombin-dependent and -independent mechanisms.[73]

Adhesion to capillary walls is largely regulated by the availability of adhesion molecules on CTCs, the endothelium, and the composition of the underlying extracellular matrix (ECM). Platelets[73] and neutrophils facilitate these interactions via their production of matrix attachment molecules such as beta(2)-integrin/intracellular adhesion molecule-1 (Refs. 74 and 75) and selectins.[76] Once CTCs attach to capillary lumens, another obstacle to surmount is inhibition of detachment-induced cell death, or anoikis, which is thought to be a major impediment for productive metastatic spread. Chemokine receptors CXCR4 and CCR7 and their ligands reduce the sensitivity of CTCs to not only arrest on capillary lumens but also on CTC anoikis by selective regulation of proapoptotic Bmf and antiapoptotic Bcl-xL proteins; thus, in the absence of appropriate cell–ECM interactions, selectins and chemokine receptors regulate CTC survival by mediating attachment and blocking cell death.[76,77]

Site-specific colonization

Organ-specific migration. The development of productive metastasis is a highly regulated process that is also subject to organ-specific mechanisms. Some solid tumors metastasize to preferred organ sites, for example, breast cancers metastasize to lung, liver, bone and brain; melanoma to liver, brain and skin; prostate cancer to bone; colorectal cancer to liver and lung; and lung adenocarcinoma metastasizes to bone, liver and brain.[2,4] Several studies have reported that tropism of CTCs to specific organ locales is regulated by the complexity of genetic alterations intrinsic to neoplastic cells,[2] while also recognizing that altered expression of important genes can also regulate tropism. Cyclooxygenase-2 (also known as PTGS2), the EGF receptor (EGFR) ligand heparin-bound EGF and the α-2,6-sialyltransferase (ST6GALNAC5) all act as mediators of malignant cell passage through the blood–brain barrier when breast cancers metastasize to brain.[78,79] In contrast, when breast cancer metastasizes to bone, IL-11 and connective tissue growth factor regulated by TGF-β are important.[80]

However, a growing body of literature has also identified cell-extrinsic mechanisms, in addition to intrinsic, that dictate organ specificity of metastases, including differential expression of chemokines and their receptors. Chemokines expressed by specific organs promote tumor cell adhesion to microvessel walls, facilitate extravasation into target tissues and induce tumor cell migration. CXCL12-CXCR4, CCR7 and its corresponding chemokine ligands, CCL21 and CCL19, significantly regulate lymph node metastasis, whereas CCR10-CCL27 and CCR4-CCL22 regulate melanoma metastasis.[81,82] Many malignant cells upregulate expression of chemokine receptors during premalignancy, partially as a result of autocrine and paracrine signaling mediated by TNF-α, IL-1 and IL-6 at the primary tumor site and subsequent chemokine gradients and then in part regulate migration toward specific organs.[70] One such functional chemokine-signaling axis involves CXCR4 and its ligand CXCL12. Expression of CXCL12 by mesenchymal bone marrow-derived cells directs migration of metastatic breast cancer cells to bone. These cells constitutively secrete the chemokine SDF-1 (SDF-1/CXCL12) and thereby attract $CXCR4^{+}$ malignant cells. Activation of CXCR4 promotes tumor progression by enabling survival and growth programs in malignant cells in ectopic tissues, regulates survival and growth of neoplastic cells in a paracrine manner and promotes tumor angiogenesis by attracting endothelial cells.[83] This important axis has been implicated for metastasis of multiple solid tumors including

pancreatic, hepatocellular, melanoma, lung and renal cancers.[84–91] Other functional chemokine receptors include CCR7, implicated in lymphatic metastasis, and CCR9 that is associated with metastasis to small intestine where its ligand is expressed.[92] Other chemokine receptors including CCR10, CXCR1, CXCR2, CXCR3, CXCR5 and CXCR7 expressed by malignant cells by a variety of solid tumors have also been implicated in organ-specific metastasis.[11]

Chemokine signaling is also an important feature of site-directed metastasis where neoplastic cell-secreted cytokines and chemokines signal to receptors expressed by various subtypes of myeloid cells, particularly significant with regards to colon carcinoma metastasis to liver.[93] Both murine and human colon cancer cells secrete the CC-chemokine ligands CCL9 and CCL15, and thereby induce recruitment of $CD34^{+}Gr1^{-}$ immature myeloid cells that express the CCL9/15 receptor CCR1, activation of which directly induces MMP-2 and MMP-9 expression. Lack of the *Ccr1*, *Mmp2* or *Mmp9* genes in myeloid cells suppresses disseminated tumor growth in the liver and significantly prolongs the survival of tumor-bearing mice.[93]

Autocrine signaling loops by malignant cells have also been implicated in organ-specific metastasis. Malignant cell-derived TGF-β induces expression of the cytokine angiopoietin-like 4 (ANGPTL4) in mammary carcinoma cells, which is critical for carcinoma dissemination and colonization in lungs. TGF-β induces expression of ANGPTL4 through Smad-signaling cascades in carcinoma cells just prior to their entry into circulation. This subsequently enhances their retention in lungs, but not in bone, by disruption of vascular endothelial cell–cell junctions, which increases permeability of lung capillaries and facilitates transendothelial passage of tumor cells.[94] These results indicate that a cytokine in the primary tumor microenvironment can induce expression of another cytokine in exiting tumor cells, thus enabling those cells to disrupt lung capillary walls and seed pulmonary metastases.

Immune cell support for colonization and organ-specific metastasis is also mediated by nonchemokine mechanisms: The two NF-κB targets, S100A8 and S100A9, are inflammatory mediators with chemotactic activity expressed and secreted by neoplastic cells, as well as by tumor-associated myeloid cells, and are associated with metastasis and a poor outcome in a variety of human tumors.[95] S100A8 and S100A9 act through the RAGE receptor (receptor for advanced glycation end products). They induce migration of myeloid cells with T-cell suppressive activity into tumors, in murine models of mammary and squamous carcinogenesis. Recruited suppressive myeloid cells facilitate tumor progression by inhibiting T cell and NK cell activation and by polarizing immunity toward a tumor-promoting type 2 phenotype.[96,97] Recently, S100A8/S100A9 were also implicated in site-specific colonization of melanoma to lungs: lack of the endogenous anti-inflammatory protein uteroglobin in mice leads to overexpression of S100A8/S100A9. Overexpression results in induction of MMP expression by neoplastic cells, and chemoattraction of melanoma cells according to the S100A8/S100A9 gradient, thus enhancing colonization of B16 melanoma in lungs.[98] Another mechanism by which tumor cell-secreted S100A8/S100A9 facilitate metastasis is through serum amyloid A-3 (SAA). Secretion of S100A8/S100A9 into premetastatic lungs induces local expression of SAA, that acts through Toll-like receptor (TLR) 4 to recruit additional myeloid cells, thus creating an inflammatory environment that accelerates migration of primary tumor cells to lung parenchyma.[99]

Leukocyte support for tumor cell survival and colonization at the metastatic organ. The temporal gap between infiltration to distant organs and the ability to colonize and form macrometastases, a process sometimes requiring decades, depending on tumor type, suggests that to grow at the metastatic site, disseminated tumor cells must acquire an ability to "educate" their new microenvironment to support their own survival. Although changes in the metastatic microenvironment that enable growth of disseminated cells are poorly defined, emerging data indicate that immune-mediated signaling plays an important role. During the earliest stages of liver metastasis, microvascular arrest of neoplastic cells triggers a local inflammatory response: tumor-secreted vascular endothelial growth factor (VEGF) induces expression of proinflammatory cytokines by sinusoidal endothelial cells resulting in upregulation of adhesion molecules such as VCAM-1, allowing arrest of metastatic melanoma cells.[100] Another prometastatic mechanism in liver supporting survival of CTCs is mediated by tumor-activated proinflammatory cytokine signaling by liver stellate cells, hepatocytes and myofibroblasts. These are recruited into sites of avascular micrometastases and create a microenvironment that supports metastatic growth through specific release of both proangiogenic factors and tumor cell invasion- and proliferation-stimulating factors provided by tumor-activated hepatocytes and myofibroblasts.[101]

An intriguing mechanism by which tumor cells take advantage of immune pathways to increase their metastatic potential is the ectopic expression of FcγRIIB by metastatic melanoma cells. FcγRIIB is an inhibitory low-affinity receptor for IgG that terminates activation signals initiated by antigen crosslinking of the B-cell receptor through its inhibitory immunoreceptor tyrosine-based inhibiting motif.[102] Forty percent of human metastatic melanomas gain expression of FcγRIIB, in particular, in liver metastases (69%), suggesting that gain of expression supports their survival in liver by escaping humoral immunity.[103,104] Experimental studies with B16 melanoma cells in immunocompetent mice indicate that tumor-expressed FcγRIIB operates as a decoy receptor inhibiting antibody-dependent cell cytotoxicity mediated by tissue macrophages, neutrophils and NK cells, which are abundant in liver: antitumor antibodies bind tumor cells via Fab domains, whereas the Fc portion is "caught" by the tumor FcγRIIB and cannot be recognized by FcγR of the effector

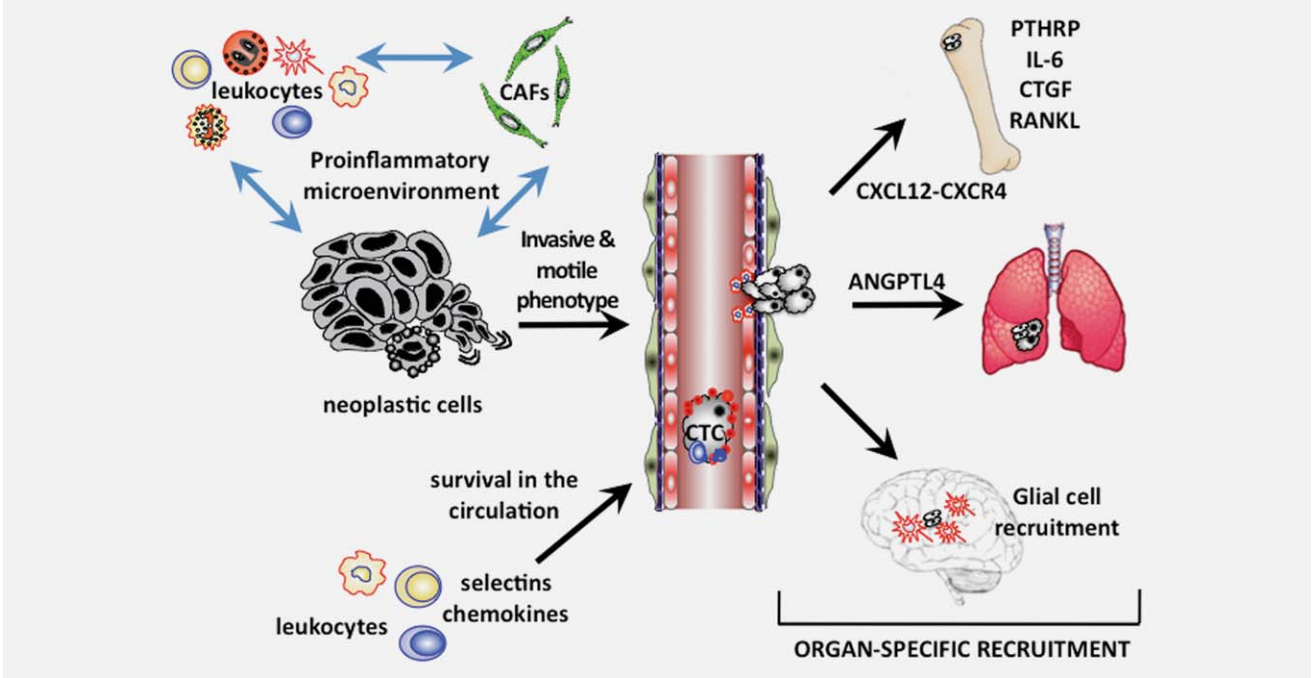

Figure 1. Immune signaling in tumor microenvironments facilitates all stages of tumorigenesis. Soluble mediators secreted by infiltrating and resident leukocytes and by carcinoma-associated fibroblasts (CAFs) within primary tumor sites support signaling programs within neoplastic cells that enable motile and invasive growth. Survival of circulating tumor cells (CTCs) in peripheral blood is facilitated by platelets, neutrophils and production of selectins and chemokine receptors. Organ-specific metastasis is directed by differential expression of chemokines and their receptors that together promote extravasation and retention of CTCs in distal organs. Colonization of distal organs is accomplished by mobilization of leukocytes and other stromal cells in ectopic organs, such as activation of osteoclasts in bone and recruitment of glial cells in brain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cells.[105] Thus, liver offers a prometastatic microenvironment that supports metastasis of cancer cells able to resist antitumor hepatic defenses and takes advantage of hepatic cell-derived factors that are key phenotypic properties of liver-metastasizing cancer cells.

The bone and bone marrow are also among the most frequent sites of cancer metastasis. During bone metastasis, breast carcinoma cells, through secretion of IL-6, IL-11, TNF-α and parathyroid hormone-related peptide, are able to activate osteoclasts through RANKL, which are critical for the formation of osteolytic metastases.[106] In other cancer types, such as neuroblastomas, IL-6 is secreted by bone marrow stromal cells and promotes osteolysis through the induction of RANKL in osteoblasts as well as in tumor cells.[107] NF-κB-regulated signaling in breast carcinoma cells promotes osteolytic bone metastasis by induction of osteoclastogenesis via granulocyte macrophage colony-stimulating factor.[108] Late-stage breast cancers also metastasize to brain, where recruitment of glial cells and a brain inflammatory response correlates with tumor cell proliferation and growth in both experimental metastasis in mice and in human brain metastases (Figure 1).[109]

A Premetastatic Niche

Several studies suggest that primary tumors can "prepare" the distant target organ of metastasis by creating a premetastatic niche,[110] whereas other studies indicate that a small number of metastatic cells activate their new microenvironment on arrival.[111] Regardless, neoplastic cells secrete factors that mobilize bone marrow-derived VEGF receptor (VEGFR)-1-expressing hematopoietic progenitor cells to sites of metastasis that induce expression of FN by resident fibroblasts, thus creating favorable conditions for arrival of would-be metastatic cells.[112]

$Gr1^+CD11b^+$ myeloid cells have also been identified to play a potential role in mediating changes that activate premetastatic lung into a permissive haven by diminishing immune-protective programs.[113] Mammary tumor cells growing in mammary pads remotely activate expression of TARC/CCL17 and MDC/CCL22 in lungs. These chemokines acting through CCR4 attract both tumor and immune cells.[35] Distant primary tumor-derived factors induce the expression of the inflammatory chemoattractants, S100A8 and S100A9, which in turn attract $Mac1^+$ myeloid cells to premetastatic lungs mediated by TLR-4-expressing cells that accelerate migration of primary tumor cells to lung tissues.[99,114] Lysyl oxidase (*LOX*) is a tumor cell-derived factor often induced in primary tumors in response to hypoxia.[115] However, systemic secretion of LOX leads to its accumulation in the lung, where it has been found to act on ECM proteins establishing a permissive niche for infiltrating cancer cells by crosslinking collagen IV in basement membranes and by recruiting CD11b+ myeloid cells that adhere to

crosslinked collagen IV, produce MMP-2 and thereby enhance the invasion and recruitment of BMDCs and metastasizing tumor cells.[116] These data indicate that, through multiple mechanisms, creation of a proinflammatory microenvironment in metastatic organs, whether that be prior to or at the time of malignant cell arrival, enhances the survival and proliferative possibilities for metastatic cells.

Implications for Therapy and Perspectives

Elucidation of the changes in metastatic microenvironments is a significant clinical goal for eradicating cancer-associated death. Immune-based signaling pathways have emerged as central players in facilitating growth of micrometastases into clinically relevant macrometastases. Anticancer therapies that target these programs are gaining attention and in a few cases are being evaluated in clinical trials.[117] Denosumab, an anti-RANKL antibody, originally developed for treatment of osteoporosis, has been found effective for inhibiting bone metastasis in prostate cancer.[118] Disruption of tumor cell adhesion to protective stroma by targeting the CXCR4-CXCL12 axis using a small molecule of CXCR4 antagonist, such as Plerixafor (AMD3100), is a novel, attractive therapeutic approach being explored in ongoing clinical trials for metastatic multiple myeloma, leukemia and other types of cancer.[119,120]

Several therapeutic agents that limit IL-1 activity are approved for treating chronic inflammatory diseases, *e.g.*, recombinant IL-1Rα (anakinra), and neutralizing monoclonal antibodies to IL-1β and a soluble receptor to IL-1, which have also been found to exert benefits in animal models of metastasis and tumor-associated angiogenesis. A goal for the future would be to evaluate this activity in clinical trials of IL-1 blockade.[121] Despite their critical involvement in invasion and metastasis, there has been conflicting results with antiproteases, possibly due to antitumorigenic activity of some enzymes.[122]

As cancer research and clinical oncology progress increasingly toward a new era of integrative cancer therapy based on combinatorial drug regimens that act synergistically by targeting intrinsic pathways in neoplastic cells, as well as extrinsic pro-oncogenic pathways in the tumor microenvironment, the intensive research in deciphering the role of the metastatic microenvironment and of tumor-promoting inflammation will hopefully result in innovative therapeutic strategies in the future.

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References

1. Folkman J, Kalluri R. Cancer without disease. *Nature* 2004;427:787.
2. Nguyen DX, Bos PD, Massague J. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* 2009;9:274–84.
3. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer* 2002;2:563–72.
4. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer* 2009;9:239–52.
5. Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;420:860–7.
6. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001;357:539–45.
7. Koehne CH, Dubois RN. COX-2 inhibition and colorectal cancer. *Semin Oncol* 2004;31:12–21.
8. Howe LR, Dannenberg AJ. COX-2 inhibitors for the prevention of breast cancer. *J Mammary Gland Biol Neoplasia* 2003;8:31–43.
9. Flossmann E, Rothwell PM. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. *Lancet* 2007;369:1603–13.
10. Feng Y, Santoriello C, Mione M, Hurlstone A, Martin P. Live imaging of innate immune cell sensing of transformed cells in zebrafish larvae: parallels between tumor initiation and wound inflammation. *PLoS Biol* 2010;8: e1000562.
11. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008;454:436–44.
12. DeNardo DG, Johansson M, Coussens LM. Immune cells as mediators of solid tumor metastasis. *Cancer Metastasis Rev* 2008;27:11–18.
13. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol* 2002;196: 254–65.
14. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* 2004;4:71–8.
15. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell* 2010;141:39–51.
16. Tang R, Beuvon F, Ojeda M, Mosseri V, Pouillart P, Scholl S. M-CSF (monocyte colony stimulating factor) and M-CSF receptor expression by breast tumour cells: M-CSF mediated recruitment of tumour infiltrating monocytes? *J Cell Biochem* 1992;50: 350–6.
17. Wei S, Nandi S, Chitu V, Yeung YG, Yu W, Huang M, Williams LT, Lin H, Stanley ER. Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. *J Leukoc Biol* 2010;88:495–505.
18. Chihara T, Suzu S, Hassan R, Chutiwitoonchai N, Hiyoshi M, Motoyoshi K, Kimura F, Okada S. IL-34 and M-CSF share the receptor Fms but are not identical in biological activity and signal activation. *Cell Death Differ* 2010; 17:1917–27.
19. Lin H, Lee E, Hestir K, Leo C, Huang M, Bosch E, Halenbeck R, Wu G, Zhou A, Behrens D, Hollenbaugh D, Linnemann T, et al. Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. *Science* 2008;320: 807–11.
20. Tsutsui S, Yasuda K, Suzuki K, Tahara K, Higashi H, Era S. Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. *Oncol Rep* 2005;14:425–31.
21. Steidl C, Lee T, Shah SP, Farinha P, Han G, Nayar T, Delaney A, Jones SJ, Iqbal J, Weisenburger DD, Bast MA, Rosenwald A, et al. Tumor-associated macrophages

and survival in classic Hodgkin's lymphoma. *N Engl J Med* 2010;362: 875–85.
22. Bolat F, Kayaselcuk F, Nursal TZ, Yagmurdur MC, Bal N, Demirhan B. Microvessel density, VEGF expression, and tumor-associated macrophages in breast tumors: correlations with prognostic parameters. *J Exp Clin Cancer Res* 2006;25:365–72.
23. Chen JJ, Lin YC, Yao PL, Yuan A, Chen HY, Shun CT, Tsai MF, Chen CH, Yang PC. Tumor-associated macrophages: the double-edged sword in cancer progression. *J Clin Oncol* 2005;23:953–64.
24. Zhang J, Patel L, Pienta KJ. CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. *Cytokine Growth Factor Rev* 2010;21:41–8.
25. Campbell MJ, Tonlaar NY, Garwood ER, Huo D, Moore DH, Khramtsov AI, Au A, Baehner F, Chen Y, Malaka DO, Lin A, Adeyanju OO, et al. Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. *Breast Cancer Res Treat* September 15, 2010.
26. Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* 2001;193:727–40.
27. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 2006;124: 263–6.
28. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, Coussens LM. CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell* 2009;16:91–102.
29. Gocheva V, Wang HW, Gadea BB, Shree T, Hunter KE, Garfall AL, Berman T, Joyce JA. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev* 2010;24:241–55.
30. Zabuawala T, Taffany DA, Sharma SM, Merchant A, Adair B, Srinivasan R, Rosol TJ, Fernandez S, Huang K, Leone G, Ostrowski MC. An ets2-driven transcriptional program in tumor-associated macrophages promotes tumor metastasis. *Cancer Res* 2010;70:1323–33.
31. Forst B, Hansen MT, Klingelhofer J, Moller HD, Nielsen GH, Grum-Schwensen B, Ambartsumian N, Lukanidin E, Grigorian M. Metastasis-inducing S100A4 and RANTES cooperate in promoting tumor progression in mice. *PLoS One* 2010;5:e10374.
32. Grum-Schwensen B, Klingelhofer J, Grigorian M, Almholt K, Nielsen BS, Lukanidin E, Ambartsumian N. Lung metastasis fails in MMTV-PyMT oncomice lacking S100A4 due to a T-cell deficiency in primary tumors. *Cancer Res* 2010;70:936–47.
33. Luo JL, Tan W, Ricono JM, Korchynskyi O, Zhang M, Gonias SL, Cheresh DA, Karin M. Nuclear cytokine-activated IKKalpha controls prostate cancer metastasis by repressing Maspin. *Nature* 2007;446:690–4.
34. Affara NI, Coussens LM. IKKalpha at the crossroads of inflammation and metastasis. *Cell* 2007;129:25–6.
35. Olkhanud PB, Baatar D, Bodogai M, Hakim F, Gress R, Anderson RL, Deng J, Xu M, Briest S, Biragyn A. Breast cancer lung metastasis requires expression of chemokine receptor CCR4 and regulatory T cells. *Cancer Res* 2009; 69:5996–6004.
36. Nozawa F, Hirota M, Okabe A, Shibata M, Iwamura T, Haga Y, Ogawa M. Elastase activity enhances the adhesion of neutrophil and cancer cells to vascular endothelial cells. *J Surg Res* 2000;94: 153–8.
37. Xiang M, Gu Y, Zhao F, Lu H, Chen S, Yin L. Mast cell tryptase promotes breast cancer migration and invasion. *Oncol Rep* 2010;23:615–19.
38. Lazennec G, Richmond A. Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends Mol Med* 2010;16:133–44.
39. Ostrand-Rosenberg S. Immune surveillance: a balance between protumor and antitumor immunity. *Curr Opin Genet Dev* 2008;18:11–18.
40. Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, Carbone DP, Matrisian LM, Richmond A, Lin PC, Moses HL. Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer Cell* 2008;13: 23–35.
41. Bunt SK, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. *J Immunol* 2006;176:284–90.
42. Bunt SK, Yang L, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. *Cancer Res* 2007;67: 10019–26.
43. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. *Cancer Cell* 2010;17: 135–47.
44. Wu Y, Deng J, Rychahou PG, Qiu S, Evers BM, Zhou BP. Stabilization of snail by NF-kappaB is required for inflammation-induced cell migration and invasion. *Cancer Cell* 2009;15:416–28.
45. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* 2009;9:265–73.
46. Kalluri R. EMT: when epithelial cells decide to become mesenchymal-like cells. *J Clin Invest* 2009;119:1417–19.
47. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; 139:871–90.
48. Lopez-Novoa JM, Nieto MA. Inflammation and EMT: an alliance towards organ fibrosis and cancer progression. *EMBO Mol Med* 2009;1: 303–14.
49. Maier HJ, Schmidt-Strassburger U, Huber MA, Wiedemann EM, Beug H, Wirth T. NF-kappaB promotes epithelial-mesenchymal transition, migration and invasion of pancreatic carcinoma cells. *Cancer Lett* 2010;295:214–28.
50. Cheng GZ, Zhang WZ, Sun M, Wang Q, Coppola D, Mansour M, Xu LM, Costanzo C, Cheng JQ, Wang LH. Twist is transcriptionally induced by activation of STAT3 and mediates STAT3 oncogenic function. *J Biol Chem* 2008;283:14665–73.
51. Sullivan NJ, Sasser AK, Axel AE, Vesuna F, Raman V, Ramirez N, Oberyszyn TM, Hall BM. Interleukin-6 induces an epithelial-mesenchymal transition phenotype in human breast cancer cells. *Oncogene* 2009;28:2940–7.
52. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer* 2009;9: 798–809.
53. Rundqvist H, Johnson RS. Hypoxia and metastasis in breast cancer. *Curr Top Microbiol Immunol* 2010;810:121–39.
54. Liao D, Johnson RS. Hypoxia: a key regulator of angiogenesis in cancer. *Cancer Metastasis Rev* 2007;26: 281–90.
55. Cronin PA, Wang JH, Redmond HP. Hypoxia increases the metastatic ability of breast cancer cells via upregulation of CXCR4. *BMC Cancer* 2010;10:225.
56. Tafani M, Russo A, Di Vito M, Sale P, Pellegrini L, Schito L, Gentileschi S, Bracaglia R, Marandino F, Garaci E, Russo MA. Up-regulation of pro-inflammatory genes as adaptation to hypoxia in MCF-7 cells and in human mammary invasive carcinoma microenvironment. *Cancer Sci* 2010;101: 1014–23.

57. Berdowska I. Cysteine proteases as disease markers. *Clin Chim Acta* 2004;342:41–69.
58. Gocheva V, Joyce JA. Cysteine cathepsins and the cutting edge of cancer invasion. *Cell Cycle* 2007;6:60–4.
59. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* 2010;141: 52–67.
60. Littlepage LE, Sternlicht MD, Rougier N, Phillips J, Gallo E, Yu Y, Williams K, Brenot A, Gordon JI, Werb Z. Matrix metalloproteinases contribute distinct roles in neuroendocrine prostate carcinogenesis, metastasis, and angiogenesis progression. *Cancer Res* 2010;70:2224–34.
61. Gocheva V, Zeng W, Ke D, Klimstra D, Reinheckel T, Peters C, Hanahan D, Joyce JA. Distinct roles for cysteine cathepsin genes in multistage tumorigenesis. *Genes Dev* 2006;20:543–56.
62. Affara NI, Andreu P, Coussens LM. Delineating protease functions during cancer development. *Methods Mol Biol* 2009;539:1–32.
63. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 2005;121: 335–48.
64. Coussens LM, Raymond WW, Bergers G, Laig-Webster M, Behrendtsen O, Werb Z, Caughey GH, Hanahan D. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes Dev* 1999;13:1382–97.
65. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 2000;103:481–90.
66. Pahler JC, Tazzyman S, Erez N, Chen YY, Murdoch C, Nozawa H, Lewis CE, Hanahan D. Plasticity in tumor-promoting inflammation: impairment of macrophage recruitment evokes a compensatory neutrophil response. *Neoplasia* 2008;10:329–40.
67. Vasiljeva O, Papazoglou A, Kruger A, Brodoefel H, Korovin M, Deussing J, Augustin N, Nielsen BS, Almholt K, Bogyo M, Peters C, Reinheckel T. Tumor cell-derived and macrophage-derived cathepsin B promotes progression and lung metastasis of mammary cancer. *Cancer Res* 2006;66:5242–50.
68. Sevenich L, Werner F, Gajda M, Schurigt U, Sieber C, Muller S, Follo M, Peters C, Reinheckel T. Transgenic expression of human cathepsin B promotes progression and metastasis of polyoma-middle-T-induced breast cancer in mice. *Oncogene* 2011;30:54–64.
69. Mohamed MM, Cavallo-Medved D, Rudy D, Anbalagan A, Moin K, Sloane BF. Interleukin-6 increases expression and secretion of cathepsin B by breast tumor-associated monocytes. *Cell Physiol Biochem* 2010;25:315–24.
70. Solinas G, Schiarea S, Liguori M, Fabbri M, Pesce S, Zammataro L, Pasqualini F, Nebuloni M, Chiabrando C, Mantovani A, Allavena P. Tumor-conditioned macrophages secrete migration-stimulating factor: a new marker for M2-polarization, influencing tumor cell motility. *J Immunol* 2010;185:642–52.
71. Kawakubo T, Okamoto K, Iwata J, Shin M, Okamoto Y, Yasukochi A, Nakayama KI, Kadowaki T, Tsukuba T, Yamamoto K. Cathepsin E prevents tumor growth and metastasis by catalyzing the proteolytic release of soluble TRAIL from tumor cell surface. *Cancer Res* 2007;67: 10869–78.
72. Gassmann P, Haier J. The tumor cell-host organ interface in the early onset of metastatic organ colonisation. *Clin Exp Metastasis* 2008;25:171–81.
73. Camerer E, Qazi AA, Duong DN, Cornelissen I, Advincula R, Coughlin SR. Platelets, protease-activated receptors, and fibrinogen in hematogenous metastasis. *Blood* 2004;104:397–401.
74. Slattery MJ, Liang S, Dong C. Distinct role of hydrodynamic shear in leukocyte-facilitated tumor cell extravasation. *Am J Physiol Cell Physiol* 2005;288:C831–C839.
75. Slattery MJ, Dong C. Neutrophils influence melanoma adhesion and migration under flow conditions. *Int J Cancer* 2003;106:713–22.
76. McDonald B, Spicer J, Giannais B, Fallavollita L, Brodt P, Ferri LE. Systemic inflammation increases cancer cell adhesion to hepatic sinusoids by neutrophil mediated mechanisms. *Int J Cancer* 2009;125:1298–305.
77. Kochetkova M, Kumar S, McColl SR. Chemokine receptors CXCR4 and CCR7 promote metastasis by preventing anoikis in cancer cells. *Cell Death Differ* 2009;16: 664–73.
78. Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, Minn AJ, van de Vijver MJ, Gerald WL, Foekens JA, Massague J. Genes that mediate breast cancer metastasis to the brain. *Nature* 2009;459:1005–9.
79. Chiang AC, Massague J. Molecular basis of metastasis. *N Engl J Med* 2008;359: 2814–23.
80. Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA, Massague J. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 2003;3:537–49.
81. Ben-Baruch A. Organ selectivity in metastasis: regulation by chemokines and their receptors. *Clin Exp Metastasis* 2008; 25:345–56.
82. Izraely S, Klein A, Sagi-Assif O, Meshel T, Tsarfaty G, Hoon DS, Witz IP. Chemokine-chemokine receptor axes in melanoma brain metastasis. *Immunol Lett* 2010;130:107–14.
83. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001;410:50–6.
84. Marchesi F, Monti P, Leone BE, Zerbi A, Vecchi A, Piemonti L, Mantovani A, Allavena P. Increased survival, proliferation, and migration in metastatic human pancreatic tumor cells expressing functional CXCR4. *Cancer Res* 2004;64: 8420–7.
85. Monti P, Marchesi F, Reni M, Mercalli A, Sordi V, Zerbi A, Balzano G, Di Carlo V, Allavena P, Piemonti L. A comprehensive in vitro characterization of pancreatic ductal carcinoma cell line biological behavior and its correlation with the structural and genetic profile. *Virchows Arch* 2004;445:236–47.
86. Zhao FL, Guo W. Expression of stromal derived factor-1 (SDF-1) and chemokine receptor (CXCR4) in bone metastasis of renal carcinoma. *Mol Biol Rep* 2011;38: 1039–45.
87. Yoshitake N, Fukui H, Yamagishi H, Sekikawa A, Fujii S, Tomita S, Ichikawa K, Imura J, Hiraishi H, Fujimori T. Expression of SDF-1 alpha and nuclear CXCR4 predicts lymph node metastasis in colorectal cancer. *Br J Cancer* 2008;98: 1682–9.
88. Na IK, Scheibenbogen C, Adam C, Stroux A, Ghadjar P, Thiel E, Keilholz U, Coupland SE. Nuclear expression of CXCR4 in tumor cells of non-small cell lung cancer is correlated with lymph node metastasis. *Hum Pathol* 2008;39:1751–5.
89. Bartolome RA, Ferreiro S, Miquilena-Colina ME, Martinez-Prats L, Soto-Montenegro ML, Garcia-Bernal D, Vaquero JJ, Agami R, Delgado R, Desco M, Sanchez-Mateos P, Teixido J. The chemokine receptor CXCR4 and the metalloproteinase MT1-MMP are mutually required during melanoma metastasis to lungs. *Am J Pathol* 2009;174:602–12.
90. Wagner PL, Hyjek E, Vazquez MF, Meherally D, Liu YF, Chadwick PA, Rengifo T, Sica GL, Port JL, Lee PC, Paul S, Altorki NK, et al. CXCL12 and CXCR4 in adenocarcinoma of the lung:

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association with metastasis and survival. *J Thorac Cardiovasc Surg* 2009;137:615–21.
91. Xiang Z, Zeng Z, Tang Z, Fan J, Sun H, Wu W, Tan Y. Increased expression of vascular endothelial growth factor-C and nuclear CXCR4 in hepatocellular carcinoma is correlated with lymph node metastasis and poor outcome. *Cancer J* 2009;15:519–25.
92. Richmond A. CCR9 homes metastatic melanoma cells to the small bowel. *Clin Cancer Res* 2008;14:621–3.
93. Kitamura T, Fujishita T, Loetscher P, Revesz L, Hashida H, Kizaka-Kondoh S, Aoki M, Taketo MM. Inactivation of chemokine (C-C motif) receptor 1 (CCR1) suppresses colon cancer liver metastasis by blocking accumulation of immature myeloid cells in a mouse model. *Proc Natl Acad Sci USA* 2010;107: 13063–8.
94. Padua D, Zhang XH, Wang Q, Nadal C, Gerald WL, Gomis RR, Massague J. TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* 2008;133:66–77.
95. Gebhardt C, Nemeth J, Angel P, Hess J. S100A8 and S100A9 in inflammation and cancer. *Biochem Pharmacol* 2006;72: 1622–31.
96. Sinha P, Okoro C, Foell D, Freeze HH, Ostrand-Rosenberg S, Srikrishna G. Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells. *J Immunol* 2008;181: 4666–75.
97. Gebhardt C, Riehl A, Durchdewald M, Nemeth J, Furstenberger G, Muller-Decker K, Enk A, Arnold B, Bierhaus A, Nawroth PP, Hess J, Angel P. RAGE signaling sustains inflammation and promotes tumor development. *J Exp Med* 2008;205:275–85.
98. Saha A, Lee YC, Zhang Z, Chandra G, Su SB, Mukherjee AB. Lack of an endogenous anti-inflammatory protein in mice enhances colonization of B16F10 melanoma cells in the lungs. *J Biol Chem* 2010;285:10822–31.
99. Hiratsuka S, Watanabe A, Sakurai Y, Akashi-Takamura S, Ishibashi S, Miyake K, Shibuya M, Akira S, Aburatani H, Maru Y. The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. *Nat Cell Biol* 2008;10: 1349–55.
100. Mendoza L, Valcarcel M, Carrascal T, Egilegor E, Salado C, Sim BK, Vidal-Vanaclocha F. Inhibition of cytokine-induced microvascular arrest of tumor cells by recombinant endostatin prevents experimental hepatic melanoma metastasis. *Cancer Res* 2004;64:304–10.
101. Vidal-Vanaclocha F. The prometastatic microenvironment of the liver. *Cancer Microenviron* 2008;1:113–29.
102. Brown KS, Blair D, Reid SD, Nicholson EK, Harnett MM. FcgammaRIIb-mediated negative regulation of BCR signalling is associated with the recruitment of the MAPkinase-phosphatase, Pac-1, and the 3′-inositol phosphatase, PTEN. *Cell Signal* 2004;16:71–80.
103. Cohen-Solal JF, Cassard L, Fournier EM, Loncar SM, Fridman WH, Sautes-Fridman C. Metastatic melanomas express inhibitory low affinity fc gamma receptor and escape humoral immunity. *Dermatol Res Pract* 2010;2010:657406.
104. Ran M, Katz B, Kimchi N, Halachmi E, Teillaud JL, Even J, Berko-Flint Y, Atlas E, Fridman WH, Witz IP. In vivo acquisition of Fc gamma RII expression on polyoma virus-transformed cells derived from tumors of long latency. *Cancer Res* 1991;51:612–18.
105. Cassard L, Cohen-Solal JF, Fournier EM, Camilleri-Broet S, Spatz A, Chouaib S, Badoual C, Varin A, Fisson S, Duvillard P, Boix C, Loncar SM, et al. Selective expression of inhibitory Fcgamma receptor by metastatic melanoma impairs tumor susceptibility to IgG-dependent cellular response. *Int J Cancer* 2008;123:2832–9.
106. Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer* 2002;2: 584–93.
107. Ara T, Declerck YA. Interleukin-6 in bone metastasis and cancer progression. *Eur J Cancer* 2010;46:1223–31.
108. Park BK, Zhang H, Zeng Q, Dai J, Keller ET, Giordano T, Gu K, Shah V, Pei L, Zarbo RJ, McCauley L, Shi S, et al. NF-kappaB in breast cancer cells promotes osteolytic bone metastasis by inducing osteoclastogenesis via GM-CSF. *Nat Med* 2007;13:62–9.
109. Fitzgerald DP, Palmieri D, Hua E, Hargrave E, Herring JM, Qian Y, Vega-Valle E, Weil RJ, Stark AM, Vortmeyer AO, Steeg PS. Reactive glia are recruited by highly proliferative brain metastases of breast cancer and promote tumor cell colonization. *Clin Exp Metastasis* 2008;25: 799–810.
110. Kaplan RN, Psaila B, Lyden D. Bone marrow cells in the 'pre-metastatic niche': within bone and beyond. *Cancer Metastasis Rev* 2006;25:521–9.
111. Grivennikov SI, Karin M. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. *Cytokine Growth Factor Rev* 2009;21: 11–19.
112. Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA, Zhu Z, Hicklin D, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 2005;438:820–7.
113. Yan HH, Pickup M, Pang Y, Gorska AE, Li Z, Chytil A, Geng Y, Gray JW, Moses HL, Yang L. Gr-1+CD11b+ myeloid cells tip the balance of immune protection to tumor promotion in the premetastatic lung. *Cancer Res* 2010;70:6139–49.
114. Hiratsuka S, Watanabe A, Aburatani H, Maru Y. Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nat Cell Biol* 2006;8:1369–75.
115. Erler JT, Bennewith KL, Nicolau M, Dornhofer N, Kong C, Le QT, Chi JT, Jeffrey SS, Giaccia AJ. Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* 2006;440:1222–6.
116. Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A, Le QT, Giaccia AJ. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell* 2009;15:35–44.
117. Balkwill F. Tumour necrosis factor and cancer. *Nat Rev Cancer* 2009;9:361–71.
118. Vallet S, Smith MR, Raje N. Novel bone-targeted strategies in oncology. *Clin Cancer Res* 2010;16:4084–93.
119. Burger JA, Peled A. CXCR4 antagonists: targeting the microenvironment in leukemia and other cancers. *Leukemia* 2009;23:43–52.
120. Azab AK, Runnels JM, Pitsillides C, Moreau AS, Azab F, Leleu X, Jia X, Wright R, Ospina B, Carlson AL, Alt C, Burwick N, et al. CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. *Blood* 2009; 113:4341–51.
121. Dinarello CA. Why not treat human cancer with interleukin-1 blockade? *Cancer Metastasis Rev* 2010;29:317–29.
122. Overall CM, Kleifeld O. Tumour microenvironment—opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat Rev Cancer* 2006;6:227–39.

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Technical Report

Rapid Generation of *In Vitro* Multicellular Spheroids for the Study of Monoclonal Antibody Therapy

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Abstract

Tumor microenvironments present significant barriers to penetration by antibodies and immunoconjugates and are difficult to study *in vitro*. Cells cultured as monolayers typically exhibit less resistance to therapy than those grown *in vivo*. Therefore, it is important to develop an alternative research model that better represents *in vivo* tumors. We have developed a protocol to produce multicellular spheroids, a simple and more relevant model of *in vivo* tumors that allows for further investigations of the microenvironmental effects on drug penetration and tumor cell killing. The protocol is used to produce *in vitro* three-dimensional tumor spheroids from established human cancer cell lines and primary cancer cells isolated from patients without the use of any extracellular components. To study the ability of tumor-targeting immunoconjugates to penetrate these tumor spheroids *in vitro*, we have used an immunotoxin targeting mesothelin, a surface protein expressed in malignant mesotheliomas. This method for producing consistent, reproducible 3D spheroids may allow for improved testing of novel monoclonal antibodies and other agents for their ability to penetrate solid tumors for cancer therapy.

Key words: multicellular spheroids, protocol, monoclonal antibody therapy

INTRODUCTION

Treatment of solid tumors remains a major challenge despite the availability of a vast number of anti-cancer agents. Antibody-based therapies represent a promising new approach for treating solid tumors. However, a major challenge involves delivering sufficient amounts of antibodies and immunoconjugates within tumor masses. For an anti-cancer antibody agent to be successful, it must (*a*) be effective in the tumor microenvironment, and (*b*) reach the tumor cells in optimal quantities to exert a therapeutic effect[5]. Tumors are more resistant to therapy than cancer cells cultured as monolayers. This can be explained by "multicellular resistance," a mechanism for drug resistance attributed to cell-cell contacts, cell-matrix contacts, and the three-dimensional (3D) shape found in tissue[5]. The majority of the ~10^{13} cells in the human body are only within a few cell diameters of a blood vessel. This feature facilitates the delivery of oxygen and nutrients to the cells that form the tissues of the body (Fig. 1)[5] and also enables the efficient delivery of most medicines.

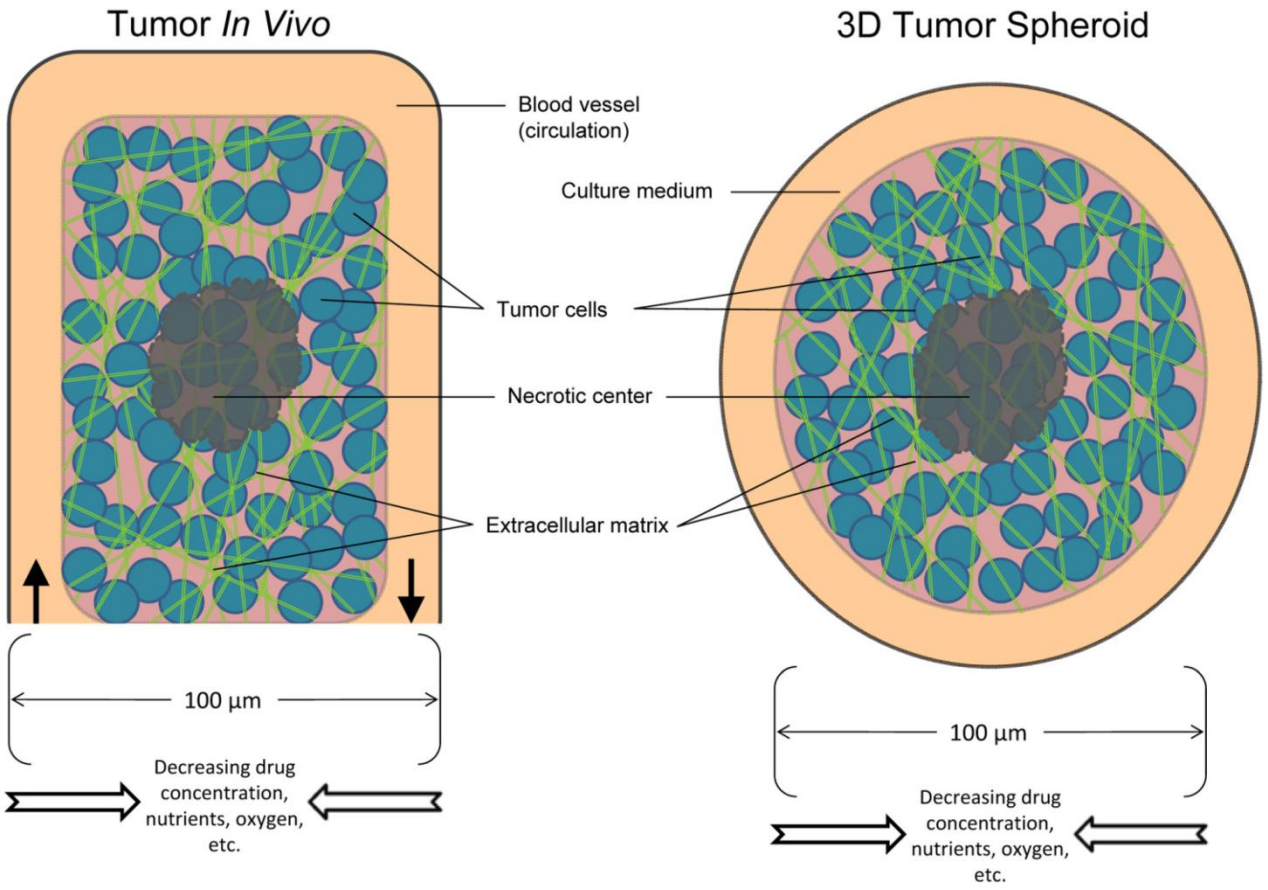

Figure 1. Schematic overview of the *in vivo* tumor and the *in vitro* tumor spheroid. Comparison of the components between a tumor and spheroid model.

In light of this, the solid tumor microenvironment has several major characteristics, including hypoxia, large distances between blood vessels, high interstitial fluid pressure, structure and composition of the extracellular matrix, and cell–cell adhesion. Overall, these features of the tumor microenvironment limit the delivery of anticancer drugs to cancer cells that are situated far from blood vessels. Multicellular resistance acquired by tumor cells may contribute to difficulties in translating promising findings from *in vitro* studies into clinical therapy. *In vitro* multicellular cancer spheroids have begun to bridge the complexity gap between monolayer cell culture and *in vivo* tumors and have become valuable models in the study of drug resistance. Spheroids exhibit many features of the tumor microenvironment and model the avascular region of tumors that is dependent on diffusion (Fig. 1)[4]. A simple, reliable, high-throughput and less expensive *in vitro* tumor model would be useful for characterizing and screening antibodies and immunoconjugates for cancer therapy.

Here, we describe a detailed protocol to establish an *in vitro* 3D tumor spheroid model. This model can be used to identify potential new therapeutic targets that are highly expressed in mesothelioma cells in 3D spheroids, but not in monolayers, and therefore be relevant in the 3D tumor. Furthermore, this protocol may be easily applied to *in vitro* studies of other tumor-targeting antibodies and immunoconjugates *in vitro*. It may also be a useful system for screening tumor penetrating monoclonal antibodies by antibody engineering technologies such as phage display [7].

Development of the protocol

Most studies of anticancer drugs consider only cellular and/or genetic mechanisms at the level of the single cell. However, drug penetration is a highly important additional mechanism and requires a more complex cellular environment to study. Indeed, *in vitro* spheroid models have become the most commonly used tools to assess drug penetration. Although animal studies, when feasible, hold the advantage of mimicking the clinical environment most closely, *in vitro* spheroids offer the benefit of being

able to examine the distribution of drugs in the absence of complicating factors such as pharmacokinetics, which often differ between mice and humans. Not only are tumor spheroids an excellent model to evaluate drug penetration, they play an increasingly meaningful role in drug discovery and development.

In 2006, Ivascu and Kubbies at Roche Pharmaceutical Research Oncology in Germany first reported a simple method to generate tumor spheroids for potential high-throughput functionality and toxicity analysis.[4] Briefly, a defined number of cancer cells ranging from 1,000 to 20,000 were seeded into wells of poly(2-hydroxyethylmethacrylate)–coated, 96-well, round- or conical-bottom plates in standard growth medium and centrifuged for 10 minutes at 1000 x g. Within 24 hours of culturing, this procedure generated individual spheroids in each well with homogeneous sizes, morphologies, and stratification of proliferating cells found in the rim that also include dying cells in the core region.[4] In addition, by adding basement membrane extract Matrigel to some cell lines, they were able to improve the structure from an aggregate to spheroid morphology. In 2008, after evaluating several techniques, V. Courtney Broaddus' group at the University of California San Francisco (USA) first established mesothelioma spheroids for the study of apoptotic resistance using multicellular spheroids[1], modifying the method originally reported by Ivascu and Kubbies.[1] Interestingly, although Broaddus' study did not use any basement membrane extract, they found the formation of spheroids to be stably intact.

Our laboratory at the National Cancer Institute (NCI) focuses on producing human monoclonal antibodies (mAbs) for the development of cancer therapy. Although leukemia treatments involving mAbs have been in clinical use for years, this approach has not been as successful for solid tumors. The proliferation of tumor cells forces blood vessels apart, reducing vascular density and creating a population of cells distant (>100μm) from vessels.[5] Drugs generally do not penetrate further than three to five cell diameters from blood vessels, thereby depriving more distantly located tumor cells of any drugs. Penetrating antibody technology is increasingly seen by many to be the holy grail of antibody therapy. A limitation in our ability to identify and evaluate effective penetrating antibody reagents has been the lack of an *in vitro* model that recapitulates the features of a solid tumor. In the last two years, in collaboration with our group, the Takayama laboratory at the University of Michigan has produced two publications related to 3D spheroid cultures [10, 11]. One study established co-cultures by introducing different types of cells on the membrane or in the bottom chamber of commercially available Transwells. They found that cancer cells formed 3D spheroids while maintaining their viability and used this procedure to evaluate mouse embryonic stem (mES) cell differentiation based on heterogeneous cell-cell interactions[11]. Co-culture of mES cells and HepG2 human hepatocellular cancinoma (HCC) cells decreased SOX17 expression of mES cells, and direct cell-cell contact further decreased SOX17 expression, indicating that co-culture with HepG2 cells inhibits endoderm differentiation. Another study described high-throughput 3D spheroid culture and drug testing using a 384-well format hanging drop culture plate[10].

In collaboration with V. Courtney Broaddus, we made a 3D spheroid model culture using a human mesothelioma cell line and primary cell lines isolated from the ascites of malignant mesothelioma patients. Using this model, we treated spheroids with SS1P, a recombinant immunotoxin targeting mesothelin[6], and evaluated how the microenvironment affected the ability of the drug to penetrate a 3D cancer cell mass and kill cancer cells.[8] Mesothelin is highly expressed in mesothelioma and several other human cancers, including pancreatic adenocarcinomas, ovarian cancers, lung adenocarcinomas and cholangiocarcinoma.[2, 3, 9] Although mesothelioma cells grown as monolayers or as spheroids express comparable levels of mesothelin, we observed that spheroids were at least 100 times less sensitive to SS1P and that penetration within spheroids was limited after four hours. The core area of spheroids revealed a substantial number of tight junctions, along with a significant increase in expression of E-cadherin, a protein involved in the assembly and sealing of tight junctions and highly expressed in malignant mesothelioma. Moreover, siRNA silencing and antibody inhibition targeting E-cadherin enhanced SS1P immunotoxin therapy *in vitro*.[8]

Advantages and limitations of the present protocol

3D spheroid culture is often complicated to follow and varies frequently according to individual practices. It can be cumbersome for labs with limited resources and expensive to manage. However, it is worthwhile to mention that although not all features of solid cancers, such as the influence of stroma and immune cells, are modeled by multicellular spheroids, spheroids do reflect many important properties of solid tumors, including the development of an extracellular matrix, tight junctions between epithelial cells, and gradients of nutrient concentration and cell proliferation from the exterior to the center. Spheroids

can develop central necrosis and regions of hypoxia. By incubating spheroids with an anticancer drug, it then becomes possible to examine the kinetics of drug penetration. Unlike a previous method[12], the protocol described here is relatively simple and straightforward to learn, rapid in producing samples, and economically feasible.

Experimental Design

NCI-H226 (American Type Culture Collection or ATCC, Rockville, MD) is a human lung squamous carcinoma cell line. HepG2 and Hep3B (ATCC) are two human liver cancer (HCC) cell lines. NCI-M-03 and NCI-M-13 (Raffit Hassan, NCI, Bethesda, MD) are two human mesothelioma primary cell lines isolated from the ascites of malignant mesothelioma patients treated at the NCI.[8]

MATERIALS

REAGENTS FOR FORMATION OF SPHEROIDS

- Poly-HEMA (Sigma; Cat. #P3932)
- 95% ethanol (in milliQ water)
- U-bottomed 96-well plates (Greiner; Cat. #650185)
- SS1P (Ira Pastan, Laboratory of Molecular Biology, NCI, Bethesda, MD)
- RPMI 1640 Media (Invitrogen; Cat. #21870-076) containing 10% fetal bovine serum (FBS), 1% L-glutamine (Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA)
- FreeStyle 293 Media (Invitrogen; Cat. #12338-018)
- Accutase (BD Biosciences; Cat. #561527)
- T-75cm^2 tissue culture flasks (Sarstedt; Cat. #83.1813.002)

ADDITIONAL REAGENTS FOR CHARCTERIZATION OF SPHEROIDS

- Coomassie Plus Protein Assay Reagent (Thermo Scientific; Cat. #1856210)
- RNeasy Mini Kit (Qiagen; Cat. #74104)
- Cell Counting Kit-8 (Dojindo Laboratories; Cat. #CK04)
- Costar white opaque-walled 96-well plates adequate for cell culture (Corning; Cat. #3922)
- CellTiter-Glo Luminescent Cell Viability Assay (Promega; Cat. #G7572)

REAGENT SETUP

Lysis buffer

To make a stock solution of lysis buffer, combine one "Complete Mini-EDTA free" protease inhibitor tablet (Roche; Cat. #1873580), 10 mL of RIPA buffer, and 1 mL of 20% SDS.

Poly-HEMA

Prepare a 120 mg/mL stock solution of poly-HEMA in 95% ethanol. Incubate while stirring with a sterile magnetic bar at room temperature (15–20°C) overnight. To make a working solution of Poly-HEMA, pipette 1 mL of Poly-HEMA stock solution into 23 mL of 95% ethanol to obtain a final concentration of 5 mg/mL (this volume is enough to coat four plates). Vortex solution and leave at room temperature (15–20°C) until ready to coat. Prepare fresh working solution every time new plates are made.

EQUIPMENT

Device (plate shaker) for mixing multiwell plates

Luminometer or charge-coupled device (CCD) camera imaging device capable of reading multiwell plates

PROCEDURE

Preparation of non-adsorbent poly-HEMA plates

1 Pipette 60 µL of poly-HEMA stock solution into each well of a 96-well U-bottomed plate.

SEE TROUBLESHOOTING (1)

2 Evaporate with lids on at room temperature (15–20°C) inside a sterile hood for 72 hours.

SEE TROUBLESHOOTING (2)

3 After plates are completely dried, seal with parafilm, and store at 4°C.

4 Before use, bring to room temperature (15-20°C) and sterilize in the hood with lids off for 30 minutes.

Preparation of Spheroids

5 Maintain cell line as adherent monolayer cultures in RPMI 1640 medium supplemented with 10% FBS, L-glutamine, and penicillin-streptomycin.

6 Incubate at 5% CO_2 with balance of air at 37°C.

7 Seed at 2x10^{5}cells/mL in T-75cm^2 tissue culture flasks.

8 Change cell media twice a week.

9 Rinse the cells with PBS and add 5 mL of Accutase to detach cells and incubate at 37°C for 3-5 minutes.

10 Neutralize with 5 mL of growth media and perform a cell count.

11 Centrifuge at 216 x g at room temperature (15–20°C) for 5 minutes.

12 Dilute cell concentration to $5x10^4$ cells/mL with growth media in order to form 10,000-cells per well spheroids (approximately 1 million cells are needed per plate).

13 Pipette 200 µL of the cell suspension into each well of the 96-well plate pre-coated with poly-HEMA.

14 Centrifuge the plate at 216 x g at room temperature (15–20°C) for 10 minutes.

15 Incubate for 24-48 hours at 37°C, 5% CO_2.

SEE TROUBLESHOOTING (3)

Preparation of Cell Lysate from Spheroids and Monolayers

Spheroids

16 Pipette growth media and spheroid completely from each well and collect in a 50 mL tube.

17 Centrifuge at 216 x g at room temperature (15–20°C) for 5 minutes, aspirate the supernatant, and resuspend the cells with 1.5 mL of PBS.

18 Centrifuge at 216 x g at room temperature (15–20°C) for 5 minutes, aspirate the supernatant, and resuspend the cells in 50 µL of lysis buffer (for approximately 1 million cells).

19 Vortex thoroughly.

20 Repeat four cycles of freezing at 80°C for approximately 10 minutes and thawing at 37°C for approximately 20 minutes.

21 Centrifuge at 216 x g at room temperature (15–20°C) for 1 minute, and collect all of the supernatant.

22 Measure concentration by Coomassie stain, a reliable standard for protein quantification. Load approximately 50-100 µg of cell protein lysate per lane for western blot analysis.

Monolayers

23 Aspirate growth media of monolayer cells and rinse three times with PBS.

Δ CRITICAL STEP Allow monolayer cells to grow for two to three days after seeding until 50-60% confluent.

24 Add 2 mL of PBS and scrape monolayer cells on ice, and collect in a 15 mL tube.

25 Add another 2 mL of PBS to the plate, scrape, and collect the remaining cells.

26 Centrifuge at 216 x g at room temperature (15–20°C) for 5 minutes, aspirate the supernatant, and resuspend the cells in 50 µL of lysis buffer (for approximately 1 million cells)

27 Vortex thoroughly.

28 Repeat four cycles of freezing and thawing (-80°/37°C).

29 Centrifuge at 216 x g at room temperature (15–20°C) for 5 minutes, and collect all supernatant.

30 Measure concentration by Coomassie stain. Load approximately 50-100 µg of cell protein lysate per lane for western blot analysis.

SEE TROUBLESHOOTING (4)

Purification of RNA for Microarray Analysis

31 Collect monolayer and spheroid cell lysate on three separate days.

Δ CRITICAL STEP Save a sufficient amount of cell lysate for RNA quality validation.

32 Follow manufacturer's kit instructions to purify RNA.

SEE TROUBLESHOOTING (5)

WST Assay

33 Form 3D spheroids on a 96-well poly-HEMA-coated plate.

34 Allow monolayers and spheroids to grow for 24 hours.

35 Add 20 µL of SS1P/BL22 (negative control)/Cycloheximide (CMH) (positive control) to 200 µL of RPMI growth media per well for a 1:10 dilution.

SEE TROUBLESHOOTING (6)

36 Allow monolayers and spheroids to incubate for 72 hours with immunotoxin.

37 Prepare a sufficient volume of WST reagent in serum-free FreeStyle media (e.g. 1 mL of reagent in 9 mL of media).

38 Remove growth media and replace with 100 µL/well of a working solution of WST reagent-FreeStyle media mentioned in the previous step.

39 Transfer all 100 µL containing each spheroid from each well to a flat-bottom 96-well plate.

40 Allow to incubate for 2-4 hours at 37°C until brown-yellow color develops.

41 Measure absorbance at 450 nm with reference wavelength of 650 nm.

ATP Assay

42 Form 3D spheroids on a 96-well poly-HEMA coated plate.

43 Allow monolayers and spheroids to grow for 24 hours.

44 Seed 10,000 cells/well for monolayer cells and one 10,000-cell spheroid/well containing 200 µL of RPMI growth media in a 96-well flat bottom plate.

45 Add 20 µL of SS1P and BL22 (negative control) from 1000 ng/mL to 0.0001 ng/mL to each well.

SEE TROUBLESHOOTING (7)

46 Incubate for 72 hours at 37°C.

47 Transfer monolayer cells and spheroids via a pipette to opaque-walled multiwell plates in culture medium (100 µL/well for 96-well plates).

48 Prepare control wells containing medium without cells to obtain background luminescence value.

49 Equilibrate the plate and its contents at room temperature (15-20°C) for approximately 30 minutes.

50 Add a volume of CellTiter-Glo Reagent equal to the volume of cell culture medium present in each well (e.g., add 100 µL of reagent to 100 µL of growth media containing cells for a 96-well plate).

51 Mix contents for 2 minutes on an orbital shaker to induce cell lysis.

52 Allow plate to incubate at room temperature (15-20°C) for 10 minutes to stabilize luminescent signal.

53 Measure luminescence.

SEE TROUBLESHOOTING (8)

TROUBLESHOOTING

1. For proper storage, the stock solution of poly-HEMA can be stored at room temperature (15-20°C) indefinitely.

2. It is optional to evaporate with the lids on at room temperature (15–20°C) on the bench for 1 to 2 weeks for slower drying, or to evaporate at 37°C inside a humidity-free bacterial incubator for 48 hours.

3. Within six hours, mesothelioma cells begin to form as an aggregate and should be left alone as they may be easily disturbed by pipetting. By 24 hours, most mesothelioma cells form into a disk-like shape called a multicellular spheroid and can be transferred gently by a pipette without dissociating.

4. For proteins that may be more difficult to visualize or detect (e.g. E-Cadherin), use at least 100 µg of cell protein lysate per lane for western blot analysis.

5. Selected GeneChip Human Genome U133 Plus 2.0 Array was performed by NCI Affymetrix Group (Frederick, MD) for reliability. For optimal results, the recommended final concentration of RNA for array analysis is 100 ng/mL in a 6 µL volume per sample.

6. To properly prepare SS1P at the original concentration (0.25 mg/mL): For 100 ng/mL final concentration, add 20 µL of 1 µg/mL SS1P into 100 µL growth media per well. For 1000 ng/mL final concentration, add 20µl of 10µg/ml SS1P into 200µl growth media per well. Original concentration of BL22 (1 µg/mL): For 100 ng/mL final concentration, add 20 µL of 1 µg/mL BL22 into 200 µL growth media per well. Original concentration of CHM (100 µg/mL): For working solution, dilute 1 µL in 99 µL of growth media. For 1:1000 final concentration, add 20 µL of CHM working solution into 200 µL growth media per well.

7. To properly prepare SS1P at the original concentration (0.25 mg/mL): Dilute to 10,000 ng/mL working solution (adding 20 µL of this in 200 µL of cell suspension results in a final concentration of 1000 ng/mL). For a 1000 ng/mL working solution, add 100 µL of 10,000 ng/mL SS1P in 900 µL of growth media (adding 20 µL of this in 200 µL of cell suspension results in a final concentration of 100 ng/mL). Continue to serially dilute 100 µL of SS1P in 900 µL of growth media. Original concentration of BL22 (684 µg/mL): Dilute to 10,000 ng/mL working solution (adding 20 µL of this in 200 µL of cell suspension results in a final concentration of 1000 ng/mL). For a 1000 ng/mL working solution, add 100 µL of 10,000 ng/mL BL22 in 900 µL of growth media (adding 20 µL of this in 200 µL of cell suspension results in a final concentration of 100 ng/mL). Continue to serially dilute 100 µL of BL22 in 900 µL of growth media.

8. It is highly recommended to do more than one type of cell proliferation or viability assay (e.g. WST and ATP).

TIMING

Step 1-4, Preparation of non-adsorbent Poly-HEMA plates: 3 days

Step 5-15, Preparation of Spheroids: 2 days

Step 16-30, Preparation of Cell Lysate: 3 hours

Step 31-32, Purification of RNA for Microarray Analysis: 1 day

Step 33-41, WST Assay for SS1P-Treated Monolayers and Spheroids: 4 days

Step 42-53, ATP Assay for SS1P-Treated Monolayers and Spheroids: 4 days

ANTICIPATED RESULTS

Using the experimental conditions described in this protocol, almost all of the established cancer cell lines and primary cells isolated from patients are able to form 3D spheroids *in vitro* (Fig. 2). We found that the mesothelioma and HCC cells we used formed spheroids well.

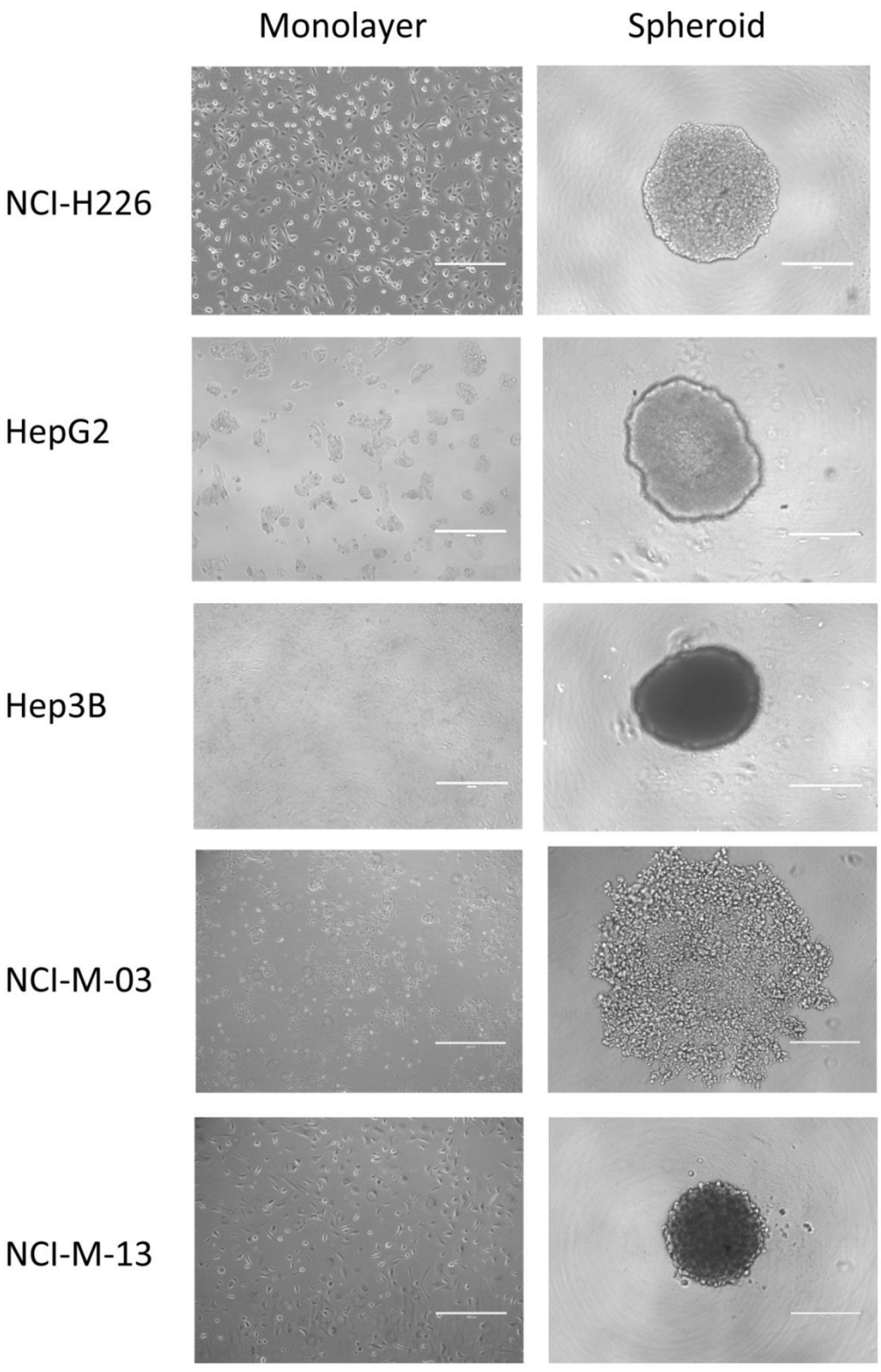

Figure 2. Establishment of *in vitro* tumor spheroids. Microscopic images of monolayers and spheroids of human cancer cell lines, NCI-H226 (mesothelioma), HepG2 (hepatocellular carcinoma or HCC), Hep3B (HCC), and primary mesothelioma lines, NCI-M-03 and NCI-M-13, isolated from patients taken after 24 hours. Scale bars, 400 μm.

Within only 2 days after seeding cells, spheroids are ready for tumor penetration studies of antibodies or immunoconjugates, RNA extraction for microarray analysis, protein lysis for proteomics analysis or discovery of tumor penetration antibodies by phage display and other antibody technologies.

To investigate how tumor microenvironments affect the killing activity and penetration of an antibody agent, monolayers and spheroids were treated with SS1P and a negative control. Cell growth inhibition (WST) and cell viability (ATP) assays showed that the IC_{50} of SS1P for spheroids was >1000 ng/mL, at least 100 times the IC_{50} for monolayers, ~10 ng/mL, after 72 hours. Both assays revealed that greater than 50% of the cancer cells from spheroids could not be killed by SS1P concentrations as high as 1,000 ng/mL. Finally, we tested SS1P on primary lines isolated from malignant mesothelioma patients and confirmed that SS1P was far less effective on spheroids.

We explored two different experimental approaches to examine the roles of protein regulation in regard to immunotoxin penetration. The first involved silencing E-Cadherin using siRNA. A reduction in protein expression (greater than 80%) was observed followed by an increased sensitization to SS1P therapy. Finally, an inhibitory antibody against E-Cadherin also demonstrated enhanced anti-tumor activity only if added before, but not after, spheroid formation.

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Author Contributions

MH conceived and designed the experiments. YP performed the experiments. MH, VCB and YP analyzed the data. YP and MH wrote the manuscript. YP, MH, VCB and DB revised the manuscript.

Conflict of Interest

There is no conflict of interest and the financial disclosure has been described in the Acknowledgements.

References

1. Barbone D, Yang T.M, Morgan J.R, Gaudino G, Broaddus V.C. Mammalian target of rapamycin contributes to the acquired apoptotic resistance of human mesothelioma multicellular spheroids. J. Biol. Chem. 2008; 283: 13021–13030.
2. Hassan R, Ho M. Mesothelin targeted cancer immunotherapy. Eur. J. Cancer. 2008; 44: 46-53.
3. Ho M, et al. Mesothelin expression in human lung cancer. Clin. Cancer Res. 2007; 13: 1571-1575.
4. Ivascu A, Kubbies M. Rapid generation of single-tumor spheroids for high-throughput cell function and toxicity analysis. J Biomol Screen. 2006 Dec;11(8):922-32.
5. Minchinton A.I, Tannock I.F. Drug penetration in solid tumours. Nat. Rev. Cancer. 2006; 6: 583-92.
6. Pastan I, Hassan R, Fitzgerald D.J, Kreitman R.J.. Immunotoxin therapy of cancer. Nat. Rev. Cancer. 2006; 6: 559-65.
7. Winter G, Griffiths A.D, Hawkins R.E, Hoogenboom H.R. Making antibodies by phage display technology. Annu. Rev. Immunol. 1994; 12: 433-55.
8. Xiang X, et al. The development and characterization of a human mesothelioma in vitro 3D model to investigate immunotoxin therapy. PLoS One. 2011; 6: e14640.
9. Yu L, et al. Mesothelin as a potential therapeutic target in human cholangiocarcinoma. J Cancer. 2010; 1: 141-9.
10. Tung YC, Hsiao AY, Allen SG, Torisawa YS, Ho M, Takayama S. High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. Analyst. 2011; 136: 473-8.
11. Torisawa YS, Mosadegh B, Cavnar SP, Ho M, Takayama S. Transwells with Microstamped Membranes Produce Micropatterned Two-Dimensional and Three-Dimensional Co-Cultures. Tissue Eng Part C Methods. 2011; 17: 61-7
12. Hjelstuen MH, Rasch-Halvorsen K, Brekken C, Bruland O, de L Davies C. Penetration and binding of monoclonal antibody in human osteosarcoma multicell spheroids. Comparison of confocal laser scanning microscopy and autoradiography. Acta Oncol. 1996; 35: 273-9.

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Clinical Cancer Research

Imaging, Diagnosis, Prognosis

MRI of Tumor-Associated Macrophages with Clinically Applicable Iron Oxide Nanoparticles

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Abstract

Purpose: The presence of tumor-associated macrophages (TAM) in breast cancer correlates strongly with poor outcome. The purpose of this study was to develop a clinically applicable, noninvasive diagnostic assay for selective targeting and visualization of TAMs in breast cancer, based on magnetic resonanceI and clinically applicable iron oxide nanoparticles.

Experimental Design: F4/80-negative mammary carcinoma cells and F4/80-positive TAMs were incubated with iron oxide nanoparticles and were compared with respect to magnetic resonance signal changes and iron uptake. MMTV-PyMT transgenic mice harboring mammary carcinomas underwent nanoparticle-enhanced magnetic resonance imaging (MRI) up to 1 hour and 24 hours after injection. The tumor enhancement on MRIs was correlated with the presence and location of TAMs and nanoparticles by confocal microscopy.

Results: *In vitro* studies revealed that iron oxide nanoparticles are preferentially phagocytosed by TAMs but not by malignant tumor cells. *In vivo*, all tumors showed an initial contrast agent perfusion on immediate postcontrast MRIs with gradual transendothelial leakage into the tumor interstitium. Twenty-four hours after injection, all tumors showed a persistent signal decline on MRIs. TAM depletion via αCSF1 monoclonal antibodies led to significant inhibition of tumor nanoparticle enhancement. Detection of iron using 3,3′-diaminobenzidine-enhanced Prussian Blue staining, combined with immunodetection of CD68, localized iron oxide nanoparticles to TAMs, showing that the signal effects on delayed MRIs were largely due to TAM-mediated uptake of contrast agent.

Conclusion: These data indicate that tumor enhancement with clinically applicable iron oxide nanoparticles may serve as a new biomarker for long-term prognosis, related treatment decisions, and the evaluation of new immune-targeted therapies. *Clin Cancer Res; 17(17); 5695–704. ©2011 AACR.*

Introduction

Although breast cancer has not historically been linked to underlying inflammation or infection, it exhibits tumor-associated inflammation marked by infiltration of leukocytes into developing tumors where increases in some leukocyte subsets parallels disease progression (1–3). In the majority of cases, however, the natural immunity to cancer that is present is not protective, but instead fosters progression. Studies in transgenic mouse models of mammary carcinogenesis revealed that tumor-associated macrophages (TAM) promote tumor growth and enhance pulmonary metastasis by high-level expression of epidermal growth factor (EGF) and activation of EGF-regulated signaling in mammary epithelial cells (MEC) critical for invasive tumor growth and metastatic dissemination (4). Histopathologic and flow cytometric evaluations have revealed that TAMs are the most abundant innate immune cell present in murine mammary carcinomas and in human breast cancers (2, 5). TAM presence in several types of human cancer, including breast, correlates with increased vascular density and worse clinical outcome (6–11). A clinically reliable noninvasive *in vivo* imaging test that could reliably detect and quantify TAMs could be employed as a novel, widely applicable prognostic assay for stratifying individual patients to more aggressive and/or TAM-targeted therapies.

Intravenously injected superparamagnetic iron oxide (SPIO) nanoparticles are effective contrast agents for magnetic resonance imaging (MRI). SPIO are phagocytosed by macrophages in various target tissues depending on their particle size and composition. Relatively large SPIO with

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Translational Relevance

The presence of tumor-associated macrophages (TAM) in adenocarcinomas correlates strongly with poor outcome in patients with breast cancer. Our data indicate that the Food and Drug Administration (FDA)-approved iron oxide nanoparticle compound ferumoxytol (Feraheme) is preferentially phagocytosed by TAMs, but not by neoplastic tumor cells. *In vivo*, ferumoxytol administration was associated with an initial tumor perfusion, followed by tumor retention and persistent magnetic resonance-enhancement at 24 hours after intravenous administration, which correlated with phagocytosed nanoparticles in TAMs. Together, these data indicate that ferumoxytol-enhancement may serve as a new biomarker for long-term prognosis and related treatment decisions that will support ongoing development of new immune-targeted therapies. Since ferumoxytol is FDA-approved as an iron supplement, this application is immediately clinically applicable as an imaging approach via an "off label" use.

hydrodynamic diameters in the order of 80 to 150 nm are rapidly phagocytosed by macrophages of the reticuloendothelial system (RES), such as liver, spleen, and bone marrow, whereas ultra small SPIO (USPIO) with diameters of less than 50 nm escape RES phagocytosis to some extent, leading to a prolonged blood pool circulation and accumulation in inflamed tissues and tumors due to transendothelial leak and macrophage phagocytosis (12–14).

The goal of this study was to utilize novel USPIO to develop an immediately clinically applicable molecular imaging approach for enhanced imaging of TAMs in breast cancer. Our imaging technique relies on the iron supplement ferumoxytol (Feraheme), recently Food and Drug Administration–approved for intravenous treatment of iron deficiency in patients (15–17). Ferumoxytol is also a USPIO compound, providing a strong signal effect on MRIs and thus exerting properties of an magnetic resonance contrast agent (18–21). On the basis of these properties, we postulated that ferumoxytol would be phagocytosed by TAMs in breast cancer, thereby enabling selective detection of TAMs on delayed, postperfusion MRIs.

Materials and Methods

Contrast agents

Three USPIO nanoparticle compounds were investigated: (i) Ferumoxytol (Feraheme, AMAG Pharmaceuticals Inc.) is a USPIO nanoparticle applied for intravenous treatment of iron deficiency in patients with impaired renal function (15, 16, 20, 22, 23). Ferumoxytol consists of an iron oxide core and a carboxydextran coating. Ferumoxytol has a mean hydrodynamic diameter of 30 nm, an r_1 relaxivity of 38 $s^{-1}mM^{-1}$, and an r_2 relaxivity of 83 $s^{-1}mM^{-1}$ at 0.94T and at 37°C. Ferumoxytol was conjugated to fluorescein isothiocyanate (FITC; Ferumoxytol-FITC) for detection by immunofluorescent microscopy. (ii) P904 (Guerbet Group, Paris, France) is a USPIO compound currently in phase I clinical trials in Europe with plans for global distribution (24–27). P904 consists of an iron oxide core and a hydrophilic coating by a monomeric organic molecule with 20 hydroxylic groups. P904 has a mean hydrodynamic diameter of 21 nm, an r_1 of 14 $s^{-1}mM^{-1}$, and an r_2 relaxivity of 92 $s^{-1}mM^{-1}$ at 1.5 T and 37°C. (iii) P1133 (Guerbet) is a preclinical USPIO with potential future clinical development (24). P1133 is based on P904 but also incorporates 8 to 10 folate moieties per nanoparticle in its coating, added via an amino PEG derivative of folic acid coupled on its *g*-carboxylic moiety to the carboxylate-bearing iron core. P1133 has a mean hydrodynamic diameter of 26 nm, an r_1 relaxivity of 12 $s^{-1}mM^{-1}$, and an r_2 relaxivity of 95 $s^{-1}mM^{-1}$ at 1.5 T and 37°C.

Animal model

This study was approved by the animal care and use committees at the respective institutions. MMTV-PyMT mice that spontaneously develop multifocal, multiclonal mammary adenocarcinomas were used at 12 to 14 weeks of life (28). Seven animals each received intravenous injections of ferumoxytol, P904, or P1133. Six additional animals received injections of P1133 + free folic acid. Animal age, weight, and tumor size were not significantly different between experimental groups that received different contrast agents ($P > 0.05$). Additional experiments were carried out in 7 postpubertal female FVB/n mice (10–12 weeks), which received injections of 50,000 PyMT-derived tumor cells into the right lower mammary fat pad for induction of orthotopic tumors. Three of these animals were treated with anti-colony stimulating factor (CSF)–1 monoclonal antibody (mAb), clone 5A1, purified by the UCSF Hybridoma core using the ATCC hybridoma (#CRL-2702). The animals received an intraperitoneal injection of 2 mg of anti-CSF1 mAb, consisting of a 1 mg starting dose followed by 0.5 mg chaser doses on day 5 and 8, and ferumoxytol-enhanced MRI on day 9. Three additional animals served as controls and received intraperitoneal injections of PBS at the corresponding time points above, followed by ferumoxytol-enhanced MRI. One additional mouse received 3 subsequent MRIs at 0, 1, and 24 hours without any contrast agent injection to confirm that tumors did not show any changes in magnetic resonance signal over a 2 day observation period. For all animals, MRI experiments were carried out when mammary tumors reached an approximate size of 1.0 cm.

Macrophage isolation and *in vitro* labeling

Tumors from MMTV-PyMT mice at day 90 to 95, or PyMT-orthotopic tumors, were isolated and digested in collagenase and DNAse (Roche Applied Sciences), strained over a cell strainer (BD Falcon, BD Biosciences), and incubated with phycoerythrin (PE)-conjugated rat anti-mouse F4/80 antibody (clone CI:A3-1, Caltag). Cells were then incubated with anti-PE magnetic beads and isolated

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over a magnetic column to provide $F4/80^+$ cells (macrophages and monocytes) and $F4/80^-$ cell fractions (malignant mammary epithelial cells and other stromal populations). In a previous study, we reported that $F4/80^+$ cells represent $Ly6G^-Ly6C^-CD11b^+F4/80^+$ TAMs (2). A total of 4×10^6 of both $F4/80^+$ and $F4/80^-$ cells were plated onto cell culture dishes in DMEM supplemented with 10% fetal calf serum. A total of 200 μg [Fe]/mL of either Ferumoxytol, P1133, or P904 were added to the cell culture medium. Additional samples were incubated with P1133 + 1.67 μmol/mL of free folic acid, a dose that corresponds to 10 times the dose of folic acid engrafted onto P1133. Cells were incubated overnight at standard cell culture conditions (37°C, 5% CO_2). The next day, nonadherent cells were discarded and adherent cells were removed via a cell lifter. Removed cells were washed 3 times in PBS and resuspended in 400 μL of ficoll at a density of 1.07 g/mL and placed into 2.0 mL conical tubes for imaging. Experiments were done in duplicates.

In vitro cell imaging and data analysis

For *in vitro* imaging of nanoparticle-loaded cells, a clinical 3T scanner was used (Signa Excite HD, GE Medical Systems) with a standard wrist coil (USA Instruments). Test tubes were immersed in a water bath and a multiecho spin echo sequence was obtained with the following parameters: TE 15, 30, 45, 60 ms, TR 2000 ms, FOV 8 × 8 cm, matrix 256 × 196 pixels, slice thickness 2 mm, and 2 acquisitions. Image processing was done by using MRVision software (MR Vision Co.). T2 relaxation times were calculated assuming a monoexponential signal decay and using nonlinear least square curve fitting on a pixel by pixel basis.

Determination of cell iron content

After imaging, cell samples were digested overnight in trypsin and placed in 10% HNO_3. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was done to quantify the iron content per sample (Perkin-Elmer).

In vivo imaging

Animals were randomly assigned to MRI when their tumor reached a size of approximately 1.0 cm. Imaging of MMTV-PyMT mice before and after injection of different nanoparticles was done with a 2 T Omega CSI-II magnetic resonance scanner (Bruker Instruments) and imaging of mice before and after anti-CSF1 mAb treatment was done with a 1 T desktop magnetic resonance scanner (Aspect M2 Compact High Performance MR System). Animals were anesthetized with isofluorane and placed on a recirculating water warming pad in a dedicated radiofrequency coil for high resolution MRI. A butterfly cannula filled with heparinized saline solution was introduced into the tail vein and left in place. T1, T2, and T2* weighted imaging sequences were obtained with the following parameters: T1 Spinecho (SE): TR 500 ms, TE 12 ms; T2 SE: TR 2000-2500 ms, TE 15, 30, 45, 60 ms (2T), and TE 20, 40, 60, 80 ms (1T); T2* Gradient echo (GE): TR 240 ms, TE 10 ms, flip angle 30 degrees (2T). MRIs were obtained with a field of view (FOV) of 3 × 3 cm (2T) or 6 × 6 cm (1T), a matrix of 128 × 128 or 200 × 200 pixels, and a slice thickness of 1 to 2 mm.

Following precontrast T1, T2, and T2* weighted imaging, 24 PyMT animals received intravenous injections of 0.5 mmol [Fe]/kg ferumoxytol ($n = 7$), P904 ($n = 7$), P1133 ($n = 7$), P1133 + 2.35 mmol/kg free folic acid (100 times the dose of folate engrafted onto P1133; $n = 3$), or P1133 + 0.235 mmol/kg free folic acid (10 times the dose of folate engrafted onto P1133; $n = 3$). Additional tumor-bearing mice after anti-CSF1 mAb treatment ($n = 3$) or controls ($n = 3$) were injected with 0.5 mmol [Fe]/kg ferumoxytol. After contrast media injection, without repositioning the mouse, 6 subsequent multiecho T2 SE sequences were obtained over the course of an hour, followed by T1- and T2*-weighted images. Mice were removed from the scanner, allowed to wake up, and imaged 24 hours later with T1, T2, and T2* weighted sequences. T2-relaxation times of the tumor were calculated based on multiecho SE sequences and converted to R2-relaxation rates (R2 = 1/T2), which is proportional to contrast agent concentration. The relative change in R2 data between pre- and postcontrast MRIs, ΔR2 (%), was determined as a quantitative measure of tumor contrast enhancement.

Histology

After the last MRI, at 24 hours postcontrast media injection, mice were sacrificed, and mammary tumors explanted and placed in optimal cutting temperature (OCT) compound on dry ice for histologic processing. Samples were cut onto slides and warmed to room temperature, followed by fixation in 100% ice-cold acetone. Some samples were then washed in H_2O, and iron deposits in the tissue were detected using the Accustain Iron Stain Kit (Sigma-Aldrich) according to the manufacturer's instructions, followed by signal enhancement with Fast 3,3′-diaminobenzidine (DAB, Vector Laboratories) for 2 minutes. After blocking sections with PBS containing 5% goat serum and 2.5% bovine serum albumin (blocking buffer), sections were incubated overnight at 4°C with 0.5× blocking buffer containing either rabbit anti-mouse folate receptor α (1:100, Abcam) or rat anti-mouse folate receptor β (1:8, kind gift from Prof. Matsuyama, Kagoshima University, Japan; ref. 29). Staining for folate receptor β was enhanced using a biotinylated anti-rat secondary antibody (1:200, Vector Laboratories) and the Tyramide Signal Amplification kit (Perkin-Elmer). After extensive washing, sections were incubated overnight with FITC-conjugated rat anti-mouse CD68 (1:50, Serotec), followed by Alexa 488 conjugated goat anti-FITC and either Alexa 546-conjugated donkey anti-rabbit or Alexa 546-conjugated Streptavidin (1:500, Invitrogen). For detection of Ferumoxytol-FITC, sections were stained with rat anti-mouse CD68 (1:100, Serotec), washed, and then incubated with a combination of Alexa 546-conjugated donkey anti-rat and Alexa 488-conjugated goat anti-FITC. All slides were mounted using ProLong Gold with DAPI (Invitrogen) and analyzed using an LSM510 confocal microscope (Zeiss).

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Statistics

Statistical analysis comparing the differences of tumor relaxation rates between mice receiving different contrast agents was done with a Wilcoxon rank sum test. A *t* test was used to determine the significance of differences between different cell samples and differences between age and tumor size of mice. A *P* value of less than 0.05 was considered significant.

Results

F4/80-positive TAMs phagocytose USPIO *in vitro*

Following incubation with the iron oxide nanoparticle ferumoxytol, F4/80$^+$ TAMs showed a markedly decreased signal on T2-weighted MRIs, whereas F4/80$^-$ cells showed minimal signal changes compared with untreated controls (Fig. 1A). Calculation of changes in relaxation rates (ΔR) as quantitative measures of the magnetic resonance signal enhancement (Fig. 1B) corroborated the qualitative findings with significantly higher ΔR2 data for ferumoxytol-exposed F4/80$^+$ TAMs compared with ferumoxytol-exposed F4/80$^-$ cells consisting primarily of carcinoma cells ($P > 0.05$). Determination of iron content in the samples revealed that increased iron uptake was responsible for the observed relaxation rate changes (Fig. 1C).

Since both TAMs and malignant epithelial cells highly express the folate receptor, folate-linked USPIO have been recently developed for "tumor-targeted imaging" (24, 30, 31). F4/80$^+$ TAMs incubated with folate-engrafted P1133 nanoparticles showed significantly stronger visual and quantitative magnetic resonance signal enhancement as compared with ferumoxytol and P904 (Fig. 1). However, folate-engraftment also leads to significantly increased nanoparticle uptake and magnetic resonance enhancement of F4/80$^-$ populations. The P1133-induced signal effect was inhibited by coincubation with free folic acid to P904 levels (Fig. 1), thus indicating that folate-targeting mediates increased USPIO uptake *in vitro*.

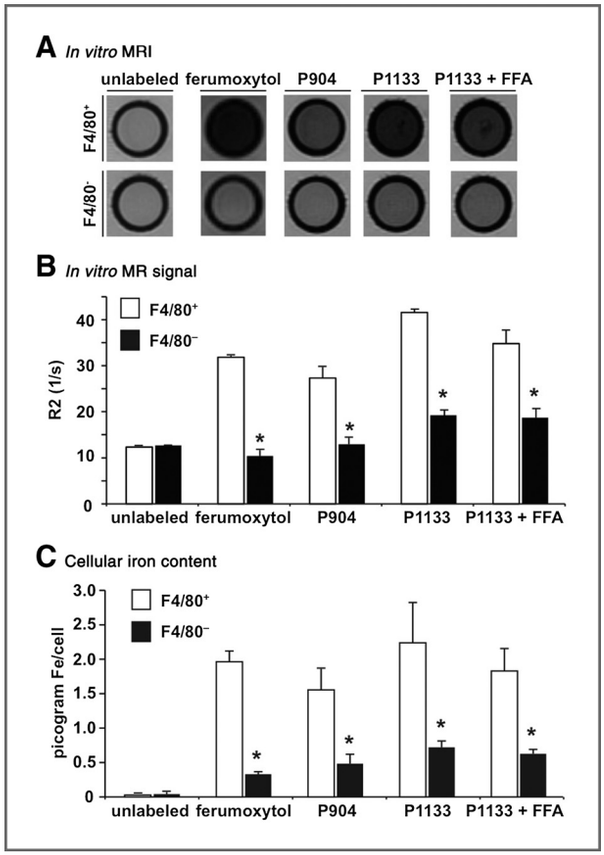

Figure 1. *In vitro* MRIs of iron oxide nanoparticle-labeled cells with corresponding quantitative magnetic resonance signal enhancement and spectrometry data. A, axial T2-weighted MRIs through test tubes containing F4/80$^+$ versus F4/80$^-$ cells labeled overnight with Ferumoxytol, P904, P1133 alone, or P1133 with free folic acid (FFA). Cells were kept in suspension in ficoll solution and test tubes were placed in a water bath to avoid artifacts by surrounding air (which would cause a dark magnetic resonance signal). Image parameters: 3 Tesla, SE 2000/60 (TR/TE in ms). B, corresponding R2 relaxation rates that quantitatively measure the magnetic resonance signal effect of iron oxide nanoparticle labeled F4/80$^+$ versus F4/80$^-$ cells, displayed as mean ± SD from duplicate experiments. C, iron content of the same cell samples as shown in B, as determined by ICP-OES.

Ferumoxytol leads to persistent tumor enhancement on delayed, postperfusion MRIs and corresponds to specific nanoparticle retention in TAMs

We investigated 90-day-old MMTV-PyMT mice bearing late-stage mammary adenocarcinomas before and after intravenous injection of ferumoxytol, as well as syngeneic mice with PyMT-derived orthotopic mammary tumors. All tumors showed an initial negative (dark) enhancement on immediate postcontrast T2-weighted MRIs, which was most pronounced in the tumor periphery and increased slowly and gradually up to 1.0-hour postinjection (p.i.). This corresponds to an initial blood pool perfusion of USPIO with slow, gradual transendothelial leakage of the nanoparticles into the tumor interstitium (32–35). At 24-hour p.i. of ferumoxytol, all tumors showed a persistent signal decline, which was most pronounced in tumor centers (Fig. 2). We used DAB-enhanced Prussian Blue staining for detection of iron, and immunodetection of CD68$^+$ TAMs in tissue sections of mammary tumors localized ferumoxytol to CD68$^+$ TAMs (Fig. 3A). As it was difficult to show selective uptake using DAB-generated contrast due to high background, we also generated ferumoxytol-FITC to show colocalization by immunofluorescence using an Alexa 488-conjugated anti-FITC antibody. As shown in Figure 3B, ferumoxytol was specifically found within CD68$^+$ TAMs, but not keratin 18-expressing malignant epithelial cells. Although ferumoxytol was not found within all TAMs, these results indicate that the magnetic resonance signal effects on delayed MRIs were largely due to TAM-mediated uptake of contrast agent.

USPIO-mediated TAM enhancement on delayed MRIs can be increased by folate receptor targeting of nanoparticles

To determine whether folate receptor targeting could enhance the MRIs, we obtained additional MRIs of

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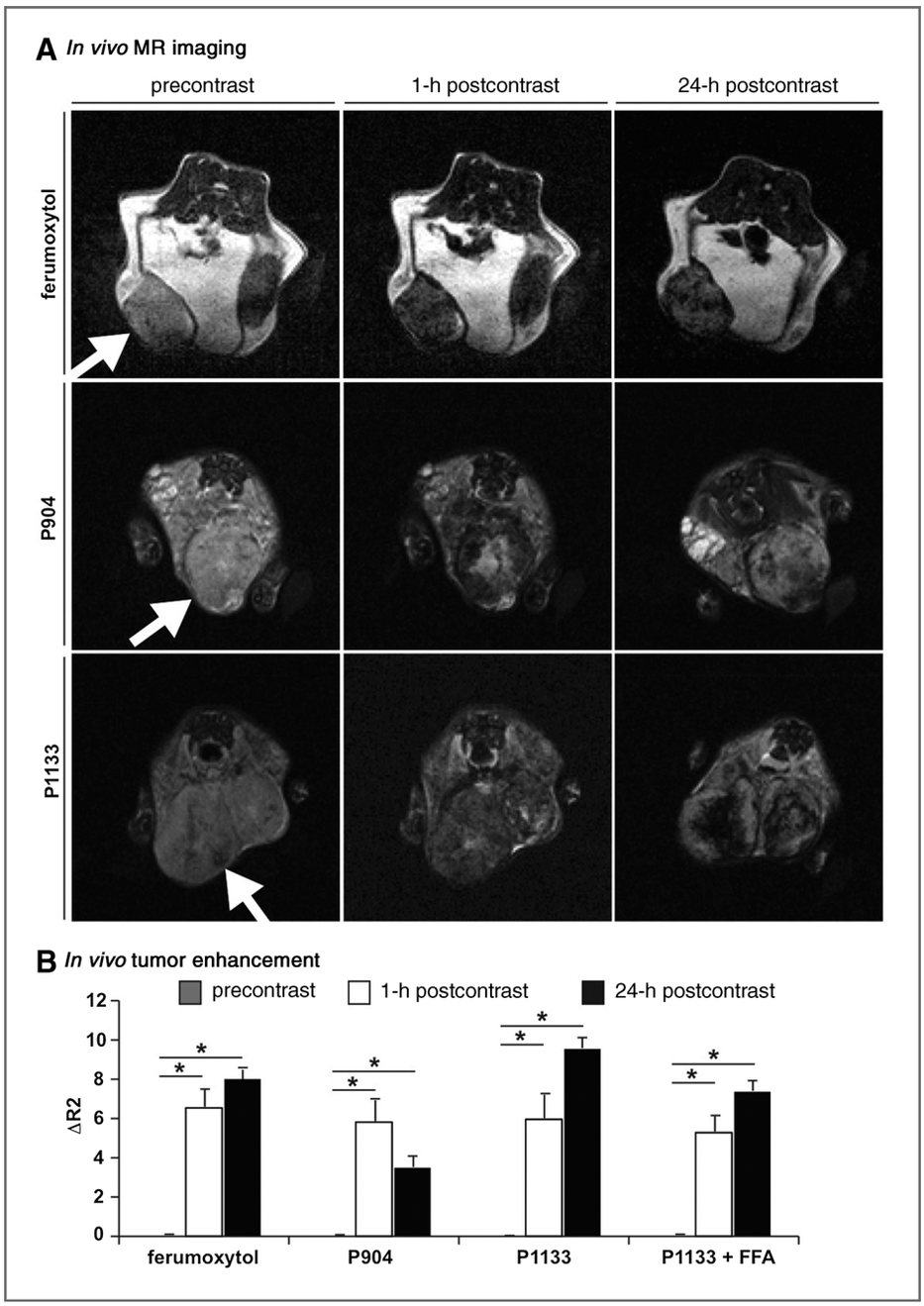

Figure 2. *In vivo* MRI of iron oxide nanoparticles. A, T2-weighted SE images of representative mammary tumors in MMTV-PyMT mice prior to (precontrast) 1 and 24 hours after administration of 0.5 mmol [Fe]/kg of ferumoxytol, P904 or P1133. The iron oxide nanoparticle-based contrast agents cause a negative (dark) signal effect in the tumor tissue on these scans (arrows point to tumors). B, quantitation of magnetic resonance signal enhancement (delta R2 measurement) of mammary tumors in MMTV-PyMT mice before and after iron oxide-nanoparticle administration, displayed as means ± SD ($n = 7$ mice/group, except P1133 + FFA which contained 3 mice). Note that all tumors show a nanoparticle retention at 24 hours, which is most pronounced for the folate-linked nanoparticle P1133.

MMTV-PyMT mice with late-stage mammary adenocarcinomas injected with the folate-engrafted USPIO P1133, the nontargeted analogue P904, or P1133 plus free folic acid. P1133 and P904 caused a nonspecific tumor-perfusion effect on T2-weighted images during the first hour p.i., which was not significantly different as compared with tumor-bearing mice injected with ferumoxytol (Fig. 2). Delayed MRIs showed a significantly stronger persistent tumor signal decline at 24-hour p.i. of P1133 compared with ferumoxytol ($P < 0.05$).

In vivo inhibition experiments with free folic acid are limited due to rapid liver uptake and renal elimination of free folic acid (36). Inhibition experiments with free folic acid at a 10 times increased dose as compared with the folate dose delivered with P1133 resulted in a minor, albeit not significant inhibition of the P1133-induced tumor enhancement ($P > 0.05$). Inhibition experiments with higher folic acid doses proved toxic in tumor-bearing mice, similar to previous reports (37). However, delayed MRIs showed significantly less tumor enhancement at 24-hour

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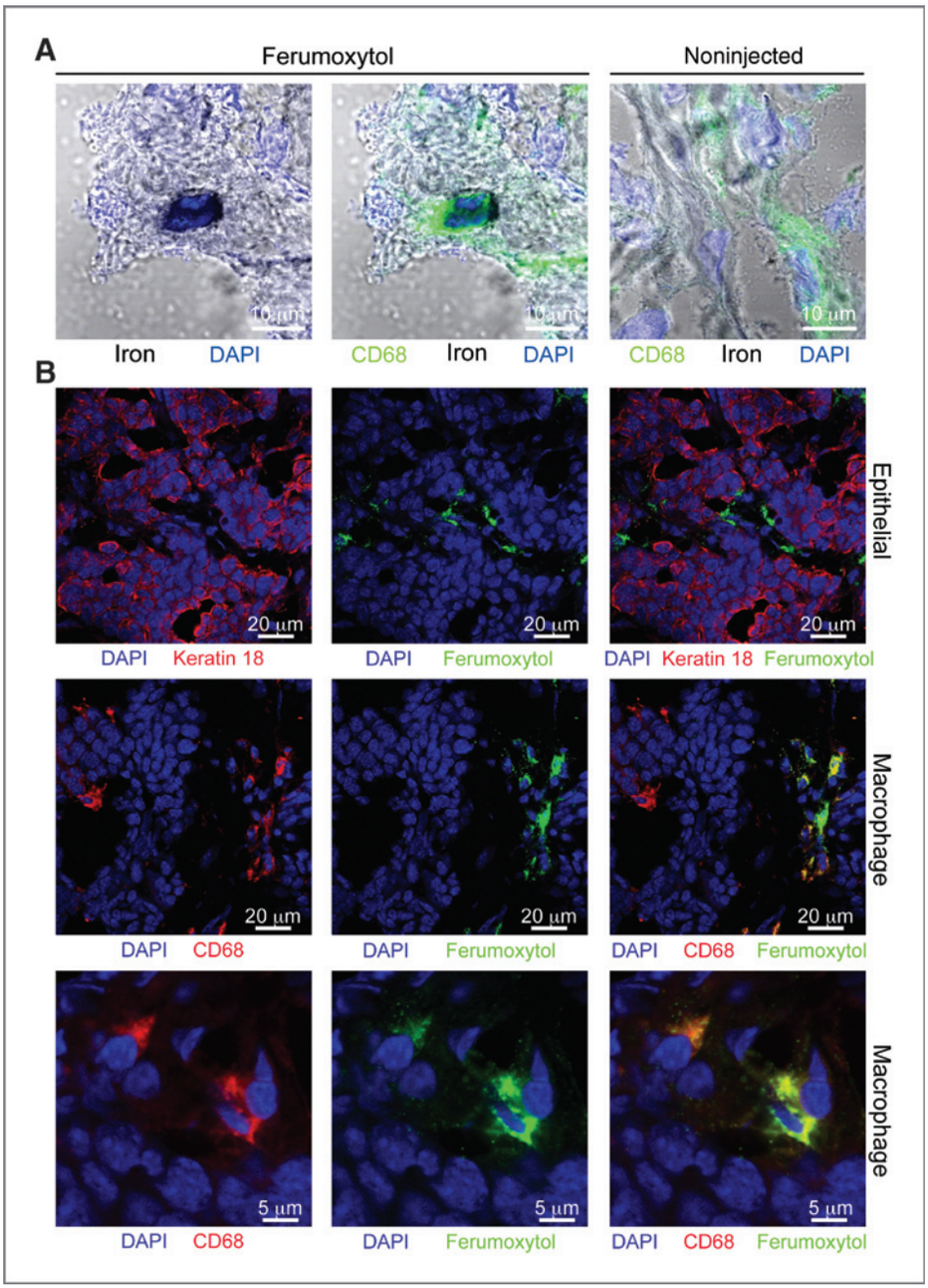

Figure 3. Uptake of ferumoxytol by TAMs *in vivo*. A, localization within OCT-embedded mammary tumors of ferumoxytol (iron; black contrast) to CD68$^+$ macrophages (green) using phase contrast of DAB staining and confocal microscopy. B, localization of ferumoxytol-FITC (green) to CD68$^+$ macrophages (red) but not Keratin 18$^+$ carcinoma cells (red) within mammary tumors. Scale bars are shown in images.

p.i. of folate-free P904 compared with folate-linked P1133 ($P < 0.05$; Fig. 2). Because P1133 and P904 are chemically identical except for folate engraftment on P1133, this data indicates increased nanoparticle uptake via folate receptor targeting.

We next evaluated TAMs versus epithelial cells for expression of α and β folate receptor in mammary tumors and revealed folate receptor α staining throughout epithelium, with no expression detectable on CD68$^+$ TAMs (Fig. 4A). In contrast, expression of folate receptor β was observed exclusively on CD68$^+$ cells, although these represented only a portion of the total CD68$^+$ TAMs found within tumors (Fig. 4B) and seemed to be primarily associated with vascular and peripheral regions of mammary tumors. Consistent with the MRIs, Prussian Blue staining for iron was more prominent within tumors from mice injected with P1133 compared with P904 or ferumoxytol (Fig. 4C). Furthermore, although some iron staining was observed in areas that did not seem occupied by TAMs (blue arrow), this was minor compared with staining within stromal areas likely enriched with TAMs (red arrow). Thus, while folate engraftment did increase uptake of USPIOs by cells

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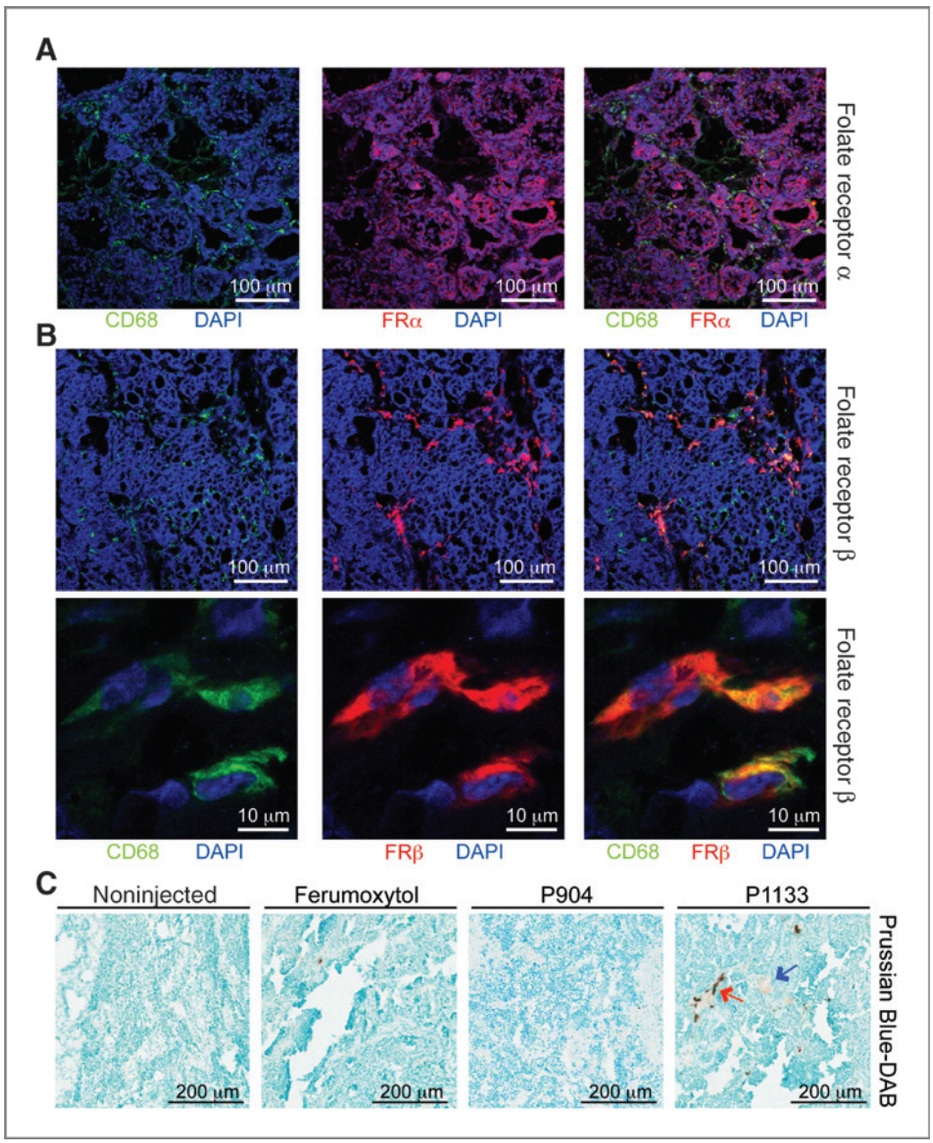

Figure 4. Folate receptor expression and folate-targeted uptake of nanoparticles. A, staining for folate receptor α (FRα; red) and CD68$^+$ macrophages (green) shows that expression of FRα is localized to carcinoma cells within mammary tumors. B, a subpopulation of CD68$^+$ macrophages (green) expresses folate receptor β (FRβ; red staining). C, Prussian Blue staining for iron with DAB enhancement within mammary tumors from mice injected with ferumoxytol, P904, or P1133. Scale bars are shown in images.

other than TAMs, these results indicate that folate modification of USPIOs may still improve their clinical use as evaluators of TAM presence within tumors.

Ferumoxytol-enhanced MRI detects TAM-depletion after anti-CSF1-mAb treatment

Imaging data from a control mouse that underwent 3 subsequent MRIs at 0, 1, and 24 hours without any contrast agent injection confirmed that MMTV-PyMT tumors do not show any intrinsic changes in magnetic resonance signal within a 2-day observation period. Mice treated with anti-CSF1 mAb showed a similar ferumoxytol-tumor perfusion effect compared with untreated controls during the first hour after intravenous ferumoxytol-injection. However, at 24-hour p.i., anti-CSF1 mAb-treated tumors showed less magnetic resonance contrast effects and significantly smaller ΔR2 enhancement data compared with untreated controls (Fig. 5A). Corresponding confocal microscopy evaluations confirmed TAM-depletion of anti-CSF1 mAb-treated tumors (Fig. 5B), indicating that ferumoxytol-enhanced MRI is related to TAM density.

Discussion

Results from this study show that ferumoxytol can be used as a reliable tool to quantitatively monitor macrophage presence in tumors, suggesting that this imaging technique can be readily investigated as a surrogate measure to predict outcomes for patients with breast cancer, and applied to monitor TAM-targeted therapies now in clinical trials.

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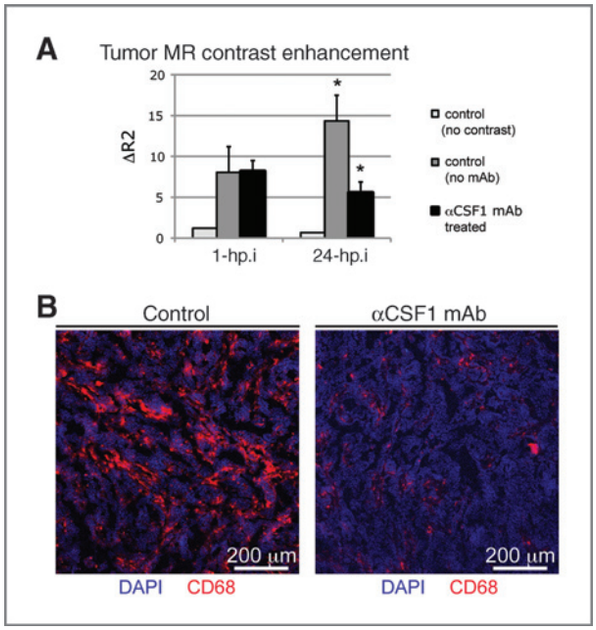

Figure 5. Ferumoxytol-enhanced MRI detects TAM-depletion noninvasively *in vivo*. A, quantitative magnetic resonance signal enhancement (delta R2 measurement) of MMTV-PyMT mammary tumors before and after iron oxide-nanoparticle administration, displayed as mean ± SD of 3 mice treated with anti-CSF1 mAb or PBS control. An additional control mouse underwent serial magnetic resonance without any contrast agent injection to confirm that MMTV-PyMT tumors do not show any intrinsic changes in magnetic resonance signal within a 2-day observation period. Note that mice treated with anti-CSF1 mAb showed significantly smaller ΔR2 enhancement data compared with untreated controls. B, corresponding confocal microscopy evaluations confirmed TAM-depletion within anti-CSF1 mAb-treated tumors.

To the best of our knowledge, this is the first report of utilizing a clinically applicable nanoparticle for TAM-detection by MRI. Other investigators have reported TAM-detection with nanoparticles that are not clinically applicable, for either MRI (38) or optical imaging (39). In addition, there have been reports of radiotracer-based approaches for TAM detection by positron emission tomography (PET; ref. 39). The latter is associated with radiation exposure and therefore not used routinely for breast imaging. MRI, on the other hand, is radiation free, established for breast cancer detection, and integrates near-microscopic anatomic resolution, high sensitivity, and excellent soft tissue contrast. Although histologic methods for quantifying TAMs are more precise, they are invasive, limited to one or few observations, and not representative of the whole tumor in the case of biopsies.

Preclinical and clinical evidence indicates that chronic presence of diverse leukocyte subsets within the stroma of breast cancers promotes tumor growth and metastasis (3, 40, 41). TAMs play a significant protumorigenic role in this context by augmenting neoplastic cell survival and motility via elaboration of cytokines, chemokines, proteases, and reactive oxygen species (3, 4, 42, 43). TAMs also potentiate pulmonary metastasis of mammary adenocarcinomas through enhanced angiogenesis via regulation of VEGF bioavailability and supplying epidermal growth factor (EGF) to mammary epithelium (5, 44), in addition to suppression of protective adaptive immune responses (3, 42, 43, 45, 46). Exuberant macrophage recruitment to breast cancer has been reported to be strongly associated with poor prognosis, both in animal models and in patients (2–4, 42–44). Although phagocytotic capacity can be altered by *in vitro* polarization of macrophages, we have no evidence that ferumoxytol uptake corresponds to a particular TAM phenotype. Regardless, aggressive human breast cancers have been reported to contain few (if any) T_H1-polarized macrophages (47), and in the MMTV-PyMT transgenic model in particular, TAMs are strongly T_H2-polarized by interleukin 4 (2).

Ferumoxytol enhancement is a new, noninvasive indicator for TAM-tumor infiltration, which may serve as a novel biomarker for breast cancers with poor outcome and may be utilized to stratify tumors with high TAM infiltration for immune-targeted therapies. There have been multiple approaches for specific targeting and/or blockade of TAMs for therapeutic purposes (29, 48, 49), some of which are currently in clinical trials based on experimental data showing that genetic, immunologic, or pharmacologic blockade of CSF1, or its receptor (CSF1R), decreases TAM presence in tissues and in experimental solid tumors, correlating with diminished tumor angiogenesis, and reduced primary tumor growth and pulmonary metastasis (50–54). Because these therapies are not cytotoxic, biomarkers of their efficiency at inducing macrophage depletion would be of great clinical benefit. Moreover, since clinical trials of new therapeutic drugs and new combination therapies are expensive and take years to complete, the immediate value and impact of imaging TAMs and/or TAM-depletion via MRI would be immense.

We recognize several limitations with this approach. Studies reported herein were done with ferumoxytol doses of 0.5 mmol/kg. Previously described ferumoxytol doses in humans were in the order of 0.035–0.072 mmol/kg (18, 19). Iron oxide nanoparticles are generally applied in higher doses in rodents as opposed to humans to compensate for the relatively shorter blood half life in rodents. However, future clinical applications must show if the currently applied dose in patients is sufficient for TAM detection, or if the dose can be safely increased. Of note, ferumoxytol showed an excellent safety profile in more than 700 patients (17). Larger superparamagnetic iron oxide nanoparticles (SPIO, diameter >50 nm) provide higher cellular uptake via *ex vivo* labeling. However, SPIOs are rapidly phagocytosed by macrophages in liver, spleen, and bone marrow and do not reach TAMs *in vivo*. USPIOs, on the other hand, are not as quickly recognized by the RES and have a longer circulation time, and can therefore leak into tumor interstitium, where they can be phagocytosed by TAMs. Thus, for "*in vivo* TAM labeling," USPIOs are advantageous (32, 33).

As shown by our data, an alternative approach to increase the sensitivity of MRI would be to utilize folate-engrafted nanoparticles. Although such particles are currently not available for clinical use, precursors of such compounds are currently entering clinical trials and thus folate-engrafted derivatives may become clinically available in the future. Folate-engraftment enhances nanoparticle uptake via the folate receptor β, which is highly expressed on TAMs (31). Several investigators including us have reported uptake of USPIO and folate-engrafted USPIO by neoplastic cells, which may be a confounding variable when aiming for TAM detection (24, 55, 56). However, data presented here reveal that the ferumoxytol and P1133 uptake in malignant epithelial cells is significantly lower as compared with macrophage uptake, leading to negligible interferences of our imaging approach.

In conclusion, we have shown that iron oxide nanoparticle-enhanced MRI can be utilized to detect TAMs in a mouse model of mammary carcinogenesis. Ferumoxytol is a clinically available nanoparticle that can be readily applied for TAM imaging in patients with breast cancer via an "off label" use. Macrophage detection may be enhanced by using folate-engrafted nanoparticles that may become available for clinical use in the near future. Clinical studies are underway to evaluate these findings in patients.

Disclosure of Potential Conflicts of Interest

C. Corot works for Guerbet Research and provided contrast agents for this study. She was not involved in data analyses. The other authors disclosed no potential conflicts of interest.

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References

1. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nature Reviews Cancer 2006;6:24–37.
2. DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, et al. CD4+ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. Cancer Cell 2009;16:91–102.
3. DeNardo DG, Coussens LM. Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. Breast Cancer Res 2007;9:212.
4. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 2004;4:71–8.
5. DeNardo DG, Johansson M, Coussens LM. Immune cells as mediators of solid tumor metastasis. Cancer Metastasis Rev 2008;27:11–8.
6. Bolat F, Kayaselcuk F, Nursal TZ, Yagmurdur MC, Bal N, Demirhan B, et al. Microvessel density, VEGF expression, and tumor-associated macrophages in breast tumors: correlations with prognostic parameters. J Exp Clin Cancer Res 2006;25:365–72.
7. Campbell MJ, Tonlaar NY, Garwood ER, Huo D, Moore DH, Khramtsov AI, et al. Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. Breast Cancer Res Treat 2011;128:703–11.
8. Chen JJ, Lin YC, Yao PL, Yuan A, Chen HY, Shun CT, et al. Tumor-associated macrophages: the double-edged sword in cancer progression. J Clin Oncol 2005;23:953–64.
9. Steidl C, Lee T, Shah SP, Farinha P, Han G, Nayar T, et al. Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. N Engl J Med 2010;362:875–85.
10. Tsutsui S, Yasuda K, Suzuki K, Tahara K, Higashi H, Era S, et al. Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. Oncol Rep 2005;14:425–31.
11. Zhang J, Patel L, Pienta KJ. CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. Cytokine Growth Factor Rev 2010;21:41–8.
12. Rosenblum LT, Kosaka N, Mitsunaga M, Choyke PL, Kobayashi H. *In vivo* molecular imaging using nanomaterials: General *in vivo* characteristics of nano-sized reagents and applications for cancer diagnosis (Review). Mol Membr Biol 2010;27:274–85.
13. Gossuin Y, Gillis P, Hocq A, Vuong QL, Roch A. Magnetic resonance relaxation properties of superparamagnetic particles. Wiley Interdiscip Rev Nanomed Nanobiotechnol 2009;1:299–310.
14. Corot C, Robert P, Idee JM, Port M. Recent advances in iron oxide nanocrystal technology for medical imaging. Adv Drug Deliv Rev 2006;58:1471–504.
15. Landry R, Jacobs PM, Davis R, Shenouda M, Bolton WK. Pharmacokinetic study of ferumoxytol: a new iron replacement therapy in normal subjects and hemodialysis patients. Am J Nephrol 2005; 25:400–10.
16. Lu M, Cohen MH, Rieves D, Pazdur R. FDA report: Ferumoxytol for intravenous iron therapy in adult patients with chronic kidney disease. Am J Hematol 2010;85:315–9.
17. Schwenk MH. Ferumoxytol: a new intravenous iron preparation for the treatment of iron deficiency anemia in patients with chronic kidney disease. Pharmacotherapy 2010;30:70–9.
18. Li W, Salanitri J, Tutton S, Dunkle EE, Schneider JR, Caprini JA, et al. Lower extremity deep venous thrombosis: evaluation with ferumoxytol-enhanced magnetic resonance imaging and dual-contrast mechanism–preliminary experience. Radiology 2007; 242:873–81.
19. Li W, Tutton S, Vu AT, Pierchala L, Li BS, Lewis JM, et al. First-pass contrast-enhanced MR angiography in humans using ferumoxytol, a novel ultrasmall superparamagnetic iron oxide (USPIO)-based blood pool agent. J Magn Reson Imaging 2005;21:46–52.
20. Neuwelt EA, Várallyay CG, Manninger S, Solymosi D, Haluska M, Hunt MA, et al. The potential of ferumoxytol nanoparticle magnetic resonance imaging, perfusion, and angiography in central nervous system malignancy: a pilot study. Neurosurgery 2007;60:601–11; discussion 611–602.

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21. Simon GH, von Vopelius-Feldt J, Fu Y, Schlegel J, Pinotek G, Wendland MF, et al. Ultrasmall supraparamagnetic iron oxide-enhanced magnetic resonance imaging of antigen-induced arthritis: a comparative study between SHU 555 C, ferumoxtran-10, and ferumoxytol. Invest Radiol 2006;41:45–51.
22. Balakrishnan VS, Rao M, Kausz AT, Brenner L, Pereira BJ, Frigo TB, et al. Physicochemical properties of ferumoxytol, a new intravenous iron preparation. Eur J Clin Invest 2009;39:489–96.
23. Pai AB, Nielsen JC, Kausz A, Miller P, Owen JS. Plasma pharmacokinetics of two consecutive doses of ferumoxytol in healthy subjects. Clin Pharmacol Ther 2010;88:237–42.
24. Meier R, Henning TD, Boddington S, Tavri S, Arora S, Piontek G, et al. Breast cancers: MR imaging of folate-receptor expression with the folate-specific nanoparticle P1133. Radiology 2010;255:527–35.
25. Sigovan M, Bessaad A, Alsaid H, Lancelot E, Corot C, Neyran B, et al. Assessment of Age Modulated Vascular Inflammation in ApoE-/- Mice by USPIO-Enhanced Magnetic Resonance Imaging. Invest Radiol 2010;45:702–7.
26. Sigovan M, Boussel L, Sulaiman A, Sappey-Marinier D, Alsaid H, Desbleds-Mansard C, et al. Rapid-clearance iron nanoparticles for inflammation imaging of atherosclerotic plaque: initial experience in animal model. Radiology 2009;252:401–9.
27. Wang ZJ, Boddington S, Wendland M, Meier R, Corot C, Daldrup-Link H, et al. MR imaging of ovarian tumors using folate-receptor-targeted contrast agents. Pediatr Radiol 2008;38:529–37.
28. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. Mol Cell Biol 1992;12:954–61.
29. Nagai T, Tanaka M, Tsuneyoshi Y, Xu B, Michie SA, Hasui K, et al. Targeting tumor-associated macrophages in an experimental glioma model with a recombinant immunotoxin to folate receptor beta. Cancer Immunol Immunother 2009;58:1577–86.
30. Kim S, Lee J. Folate-targeted drug-delivery systems prepared by nano-comminution. Drug Dev Ind Pharm 2011;37:131–8.
31. Puig-Kröger A, Sierra-Filardi E, Domínguez-Soto A, Samaniego R, Corcuera MT, Gómez-Aguado F, et al. Folate receptor beta is expressed by tumor-associated macrophages and constitutes a marker for M2 anti-inflammatory/regulatory macrophages. Cancer Res 2009;69:9395–403.
32. Daldrup-Link HE, Rydland J, Helbich TH, Bjørnerud A, Turetschek K, Kvistad KA, et al. Quantification of breast tumor microvascular permeability with feruglose-enhanced MR imaging: initial phase II multicenter trial. Radiology 2003;229:885–92.
33. Daldrup-Link HE, Simon GH, Brasch RC. Imaging of tumor angiogenesis: current approaches and future prospects. Curr Pharm Des 2006;12:2661–72.
34. Turetschek K, Huber S, Floyd E, Helbich T, Roberts TP, Shames DM, et al. MR imaging characterization of microvessels in experimental breast tumors by using a particulate contrast agent with histopathologic correlation. Radiology 2001;218:562–9.
35. Turetschek K, Roberts TP, Floyd E, Preda A, Novikov V, Shames DM, et al. Tumor microvascular characterization using ultrasmall superparamagnetic iron oxide particles (USPIO) in an experimental breast cancer model. J Magn Reson Imaging 2001;13:882–8.
36. Steinberg SE, Campbell CL, Hillman RS. Kinetics of the normal folate enterohepatic cycle. J Clin Invest 1979;64:83–8.
37. Parchure M, Ambaye RY, Lalitha VS, Gokhale SV. Acute toxicity of folic acid in mice. Experientia 1985;41:72–3.
38. Leimgruber A, Berger C, Cortez-Retamozo V, Etzrodt M, Newton AP, Waterman P, et al. Behavior of endogenous tumor-associated macrophages assessed *in vivo* using a functionalized nanoparticle. Neoplasia 2009;11:459–68, 452 p following 468.
39. Nahrendorf M, Keliher E, Marinelli B, Waterman P, Feruglio PF, Fexon L, et al. Hybrid PET-optical imaging using targeted probes. Proc Natl Acad Sci U S A 2010;107:7910–5.
40. Coussens LM, Werb Z. Inflammation and cancer. Nature 2002;420:860–7.
41. Ruffell B, DeNardo DG, Affara NI, Coussens LM. Lymphocytes in cancer development: polarization towards pro-tumor immunity. Cytokine Growth Factor Rev 2010;21:3–10.
42. Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL, et al. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res 1996;56:4625–9.
43. van Netten JP, Ashmed BJ, Cavers D, Fletcher C, Thornton IG, Antonsen BL, et al. 'Macrophages' and their putative significance in human breast cancer. Br J Cancer 1992;66:220–1.
44. Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 2001;193:727–40.
45. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, Qian H, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res 2006;66:11238–46.
46. Crowther M, Brown NJ, Bishop ET, Lewis CE. Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. J Leukoc Biol 2001;70:478–90.
47. Solinas G, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol 2009;86:1065–73.
48. Luo Y, Zhou H, Krueger J, Kaplan C, Lee SH, Dolman C, et al. Targeting tumor-associated macrophages as a novel strategy against breast cancer. J Clin Invest 2006;116:2132–41.
49. Zeisberger SM, Odermatt B, Marty C, Zehnder-Fjällman AH, Ballmer-Hofer K, Schwendener RA, et al. Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. Br J Cancer 2006;95:272–81.
50. Conway JG, McDonald B, Parham J, Keith B, Rusnak DW, Shaw E, et al. Inhibition of colony-stimulating-factor-1 signaling *in vivo* with the orally bioavailable cFMS kinase inhibitor GW2580. Proc Natl Acad Sci U S A 2005;102:16078–83.
51. Manthey CL, Johnson DL, Illig CR, Tuman RW, Zhou Z, Baker JF, et al. JNJ-28312141, a novel orally active colony-stimulating factor-1 receptor/FMS-related receptor tyrosine kinase-3 receptor tyrosine kinase inhibitor with potential utility in solid tumors, bone metastases, and acute myeloid leukemia. Mol Cancer Ther 2009;8:3151–61.
52. Nowicki A, Szenajch J, Ostrowska G, Wojtowicz A, Wojtowicz K, Kruszewski AA, et al. Impaired tumor growth in colony-stimulating factor 1 (CSF-1)-deficient, macrophage-deficient op/op mouse: evidence for a role of CSF-1-dependent macrophages in formation of tumor stroma. Int J Cancer 1996;65:112–9.
53. Priceman SJ, Sung JL, Shaposhnik Z, Burton JB, Torres-Collado AX, Moughon DL, et al. Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: combating tumor evasion of antiangiogenic therapy. Blood 2010;115:1461–71.
54. DeNardo D, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer Discovery 2011;1:54–67.
55. Choi H, Choi SR, Zhou R, Kung HF, Chen IW. Iron oxide nanoparticles as magnetic resonance contrast agent for tumor imaging via folate receptor-targeted delivery. Acad Radiol 2004;11:996–1004.
56. Sun C, Sze R, Zhang M. Folic acid-PEG conjugated superparamagnetic nanoparticles for targeted cellular uptake and detection by MRI. J Biomed Mater Res A 2006;78:550–7.

Immune microenvironments in solid tumors: new targets for therapy

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REVIEW

Immune microenvironments in solid tumors: new targets for therapy

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Leukocytes and their soluble mediators play important regulatory roles in all aspects of solid tumor development. While immunotherapeutic strategies have conceptually held clinical promise, with the exception of a small percentage of patients, they have failed to demonstrate effective, consistent, and durable anti-cancer responses. Several subtypes of leukocytes that commonly infiltrate solid tumors harbor immunosuppressive activity and undoubtedly restrict the effectiveness of these strategies. Several of these same immune cells also foster tumor development by expression of potent protumor mediators. Given recent evidence revealing that immune-based mechanisms regulate the response to conventional cytotoxic therapy, it seems reasonable to speculate that tumor progression could be effectively diminished by combining cytotoxic strategies with therapies that blunt protumor immune-based effectors and/or neutralize those that instead impede development of desired anti-tumor immunity, thus providing synergistic effects between traditional cytotoxic and immune-modulatory approaches.

Despite expanded appreciation for the diversity of cellular mechanisms fostering solid tumor development, anti-cancer therapy remains heavily reliant on cytotoxic modalities—including chemotherapy (CTX) and radiation therapy (RT)—that kill rapidly proliferating (neoplastic) cells within tumors. Emerging clinical and experimental data indicate that clinical responses to cytotoxic therapy can be improved if immunogenic cell death pathways are also concurrently activated (Ma et al. 2010). Evidence for simultaneous engagement of immunogenic cell death programs has been provided for some tumors following conventional cytotoxic therapy, based on the increased presence of molecules released by dying cells thought to be "sensed" by leukocytes (Kepp et al. 2011), the result of which leads to enhanced "killing" of target cells. While an obvious clinical strategy has been to bolster these anti-tumor mechanisms, achieving clinical success has been limited. Possible mechanisms underlying these clinical failures include the underappreciated properties of some immune cell types that can harbor both immunosuppressive activity—e.g., blunting malignant cell killing by $CD8^+$ cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells—simultaneously with protumor activities that promote survival, invasion, and dissemination of malignant cells (Ruffell et al. 2010). Experimental studies in immune-competent murine models of human cancer have provided support for this concept by revealing that blockade of some protumor immune-based pathways effectively bolsters anti-tumor immunity (neoplastic cell killing) when combined with cytotoxic therapy (DeNardo et al. 2011).

[*Keywords*: leukocyte; inflammation; chemotherapy; radiation therapy; cytotoxic therapy; cancer]
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Cancer and chronic inflammation

In homeostatic tissue, resident immune cells serve as sentinels that safeguard tissue and organ integrity. Following acute damage (e.g., infiltration/infection by pathogens or physical trauma), one activity of resident leukocytes is to limit tissue damage while engaging tissue repair programs (e.g., activation of stromal fibroblasts and vasculature for matrix resynthesis and angiogenesis, respectively, and recruitment of leukocytes from peripheral blood to remove damaged cells and debris) and facilitate re-epithelialization, all without inducing autoimmunity. Following resolution of wound responses, tissue damage is (hopefully) minimal and homeostatic maintenance programs return such that organ physiology is unperturbed.

In cancer, immune cells play dual roles with potential to either eliminate or promote malignancy. Premalignant tissues contain proliferating cells harboring genomic damage (e.g., "initiated" cells) that typically activate critical proliferation/survival pathways. In these tissues, chronic engagement/activation of immune cells, stromal fibroblasts, and vascular and mesenchymal support cells together fosters survival of "initiated" cells, culminating in tissue expansion and development of premalignant lesions via a process reminiscent of typical "inflammatory-type" responses observed in tissues responding to acute damage/trauma (Coussens and Werb 2002). When these chronic inflammatory-type events are sustained, neoplastic progression can ensue. Unresolved chronic immune responses thus resemble the resolution phase of wound healing, where the tumor microenvironment contains

significant infiltrations of cells with immunosuppressive activity akin to a wound failing to heal (Coussens and Werb 2002).

Consistent with this, studies evaluating leukocyte complexity by flow cytometry in human (and murine) tumors have identified multiple immune cell types that variably contain immunosuppressive activity (e.g., block anti-tumor CTL- or NK T-cell-mediated killing of malignant cells)—including regulatory T cells (T_{regs}), immature monocytes (iMCs), alternatively activated macrophages (AAMs), mast cells, neutrophils, Tie2$^+$ monocytes, dendritic cells (DCs), and T helper 2 (T_H2)-CD4$^+$ effector T cells (DeNardo et al. 2011; Rolny et al. 2011; Ruffell et al. 2011)—and thus afford developing malignancies a mechanism to escape killing by T cells. Mouse modeling studies indicate that the net effect of these assemblages results in favoring tumor expansion (Fig. 1; DeNardo et al. 2010; Grivennikov et al. 2010; Qian and Pollard 2010; Ruffell et al. 2010). Three types of leukocytes in particular have emerged as playing significant roles in suppressing anti-tumor immune responses: T_{reg} cells, iMCs, and AAMs.

Immune-based programs that blunt anti-tumor immunity

T_{reg} cells

T_{reg} cells, a subset of the CD4$^+$ T-cell population, constitutively express the high-affinity interleukin-2 (IL-2) receptor (CD25), CTL antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), and the lineage-specific transcription factor Foxp3 and play an important physiological role in suppressing responses to self-antigens, thereby preventing autoimmunity (Hori et al. 2003). As many malignant cell types express self-antigens (Kawakami and Rosenberg 1997), it follows that T_{reg} cells in their physiologic capacity

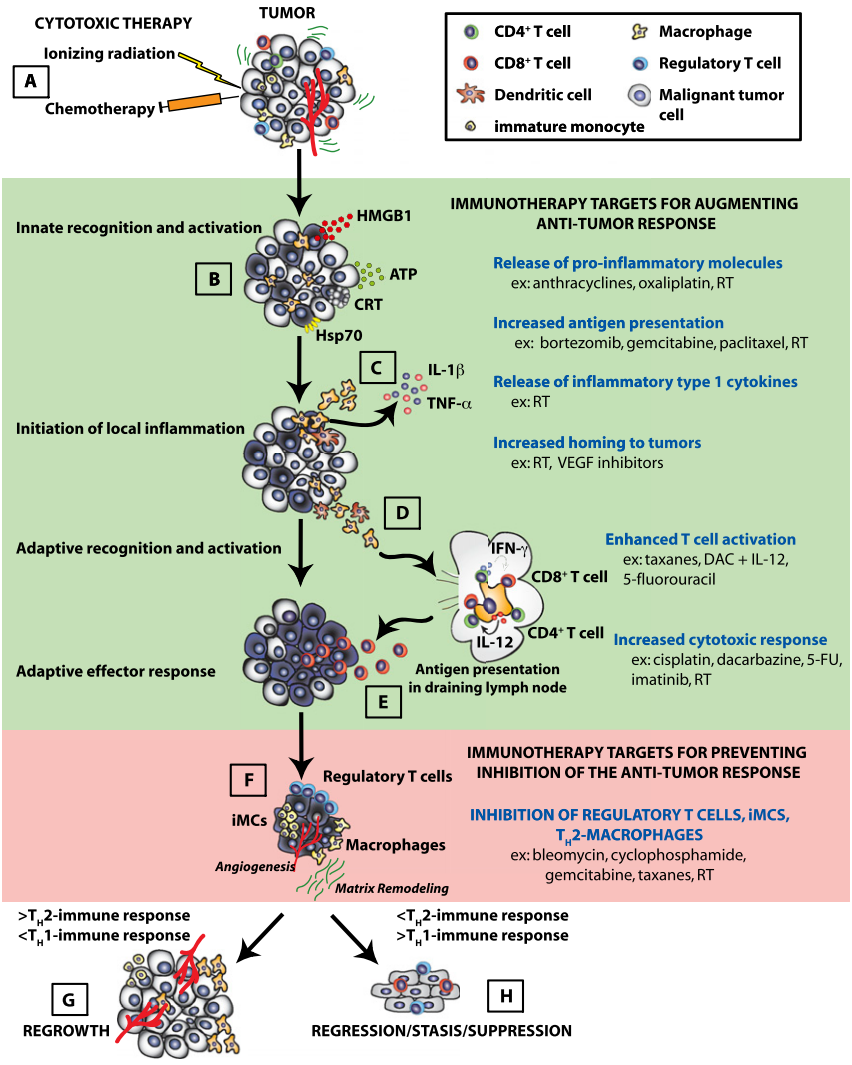

Figure 1. Schematic of immune response pathways induced following cytotoxic therapy. Traditional cytotoxic therapies (e.g., CTX and RT) trigger an immune response in tissues (*A*), leading to the release of inflammatory mediators (including HMGB1, calreticulin, ATP, and Hsp70) from tumor cells (*B*). (*C*) These molecules activate resident immune cells such as DCs and tissue macrophages through cognate receptors, including TLR4 and P2RX7, which triggers the release of TNF-α and IL-1, which further recruits peripheral blood leukocytes (PBLs) from the circulation. (*D*) Activation of resident DCs and tissue macrophages stimulates their migration to the lymphoid tissue bearing tumor antigens. In the lymphoid tissue, the DCs and macrophages present antigen to CD4$^+$ and CD8$^+$ T cells, leading to their activation. (*E*) Activated CD4$^+$ and CD8$^+$ T cells then reenter the circulation and return to the tumor to eliminate tumor cells. (*F*) Throughout this process, a portion of the PBLs recruited from the circulation into the tumor also possess suppressive function (e.g., T_{reg} cells and various subtypes of myeloid cells), and these become increasingly dominant as the tumor is cleared by a cytotoxic response (primarily CD8$^+$ T cells and NK cells) and function to reduce the anti-tumor cytotoxic response by a diverse array of mechanisms. (*H*) If the malignant cells are completely eradicated, the tissue can return to a normal, homeostatic state. (*G*) However, if incomplete eradication of malignant cells occurs, then, over time, tumor regrowth is evident in the form of recurrent disease at the primary site or metastases at distal sites. Events in the immune response that might serve as targets to enhance the immune response (shaded green) or prevent suppression of the immune response (shaded red) are outlined on the *right* with examples of cytotoxic agents that can mediate each of these events.

hamper anti-tumor immunity and that tumors may evade immune detection by engaging or activating T_{reg} cell-based pathways. This notion has been borne out by studies evaluating peripheral blood, tumor-draining lymph nodes, and tumors—e.g., breast (Bates et al. 2006) and gastrointestinal (Sasada et al. 2003)—where increased presence of T_{regs} is prominent. Importantly, increased frequency of T_{regs} also correlates with poor outcome for several cancer types (Sasada et al. 2003; Curiel et al. 2004; Bates et al. 2006). Further support for the notion of tumors activating development of T_{reg} cells comes from studies showing that stromal cells produce chemokines such as CCL22 (Curiel et al. 2004) and cytokines such as transforming growth factor-β (TGFβ) (Ghiringhelli et al. 2005) that enhance T_{reg} infiltration.

The ability of T_{regs} to block anti-tumor immunity has been confirmed in vivo, where adoptive transfer of $CD3^+CD25^-$ T cells from patients into NOD/SCID mice was found to retard tumor growth, while simultaneous transfer of T_{regs} abrogated the protective effect (Curiel et al. 2004). Mechanistically, in vitro studies have revealed that leukocytes isolated from melanoma and ovarian cancer patients depleted of T_{reg} cells ex vivo enabled remaining leukocytes to respond to selective tumor antigens (Nishikawa et al. 2005). Also significant is the observation that T_{regs} directly promote malignant cell proliferation and dissemination via soluble mediators they express (Tan et al. 2011). Given evidence demonstrating that T_{regs} block anti-tumor immunity (Dunn et al. 2004), it stands to reason that, in order to augment anti-tumor immunity therapeutically, neutralizing pathways that bolster the presence or activity of T_{regs} would likely provide a survival advantage.

AAMs

Unlike T_{regs}, macrophages derived from circulating immature myeloid precursors play a more complex role in regulating immune responses owing to their ability to possess both pro- and anti-tumor bioactivity, depending on the cytokine milieu they encounter once within tissue (Qian and Pollard 2010). Classically activated macrophages (CAMs) regulated by T_H1 cytokines—e.g., interferon γ (IFNγ), tumor necrosis factor α (TNFα), or granulocyte/monocyte colony-stimulating factor (GM-CSF)—possess enhanced cytotoxic activity, produce proinflammatory (T_H1) cytokines, and have antigen presentation capability (Mosser and Edwards 2008). In contrast, macrophages exposed to T_H2 cytokines (IL-4, IL-13, etc.), immune complexes, or immunosuppressive cytokines become alternatively activated (AAMs) (Qian and Pollard 2010) and instead typically lack cytotoxic activity, block $CD8^+$ T-cell proliferation or infiltration, and express a diverse assortment of proliferative, proangiogenic, and tissue remodeling mediators (DeNardo et al. 2009, 2011; Andreu et al. 2010; Qian and Pollard 2010; Ruffell et al. 2010). Experimental data from murine models indicate that AAMs become T_H2-skewed due to high levels of type 2 cytokines (IL-4 and IL-13) released by infiltrating $CD4^+$ T cells and neoplastic epithelial cells (DeNardo et al. 2009; Gocheva et al. 2010) or TSLP (thymic stromal lymphopoietin) also produced by neoplastic epithelial cells (Pedroza-Gonzalez et al. 2011). While AAMs are typical constituents of tissue repair processes, in solid tumors, rather than aiding in "healing," they instead foster neoplasia (Qian and Pollard 2010). AAMs produce a multitude of factors—including epidermal growth factor (EGF), TGFβ, and cathepsin proteases—that together provide a survival advantage to malignant epithelia and regulate their response to cytotoxic therapies (DeNardo et al. 2011; Shree et al. 2011). Data from human tumors support this hypothesis, since the presence of AAMs that are $CD163^+CD204^+$ correlate with reduced survival for patients with breast cancer, non-small-cell lung cancer, and Hodgkin's lymphoma (Kawai et al. 2008; Steidl et al. 2010; DeNardo et al. 2011). Owing to lack of specificity for CD68 as a macrophage-specific marker, however, some of these findings may need to be revisited (Ruffell et al. 2011).

The importance of macrophages in tumor progression is further underscored by mouse modeling data revealing that genetic loss of *CSF1/CSF1 receptors* (Lin et al. 2001) or blockade of M-CSF-induced signaling cascades (DeNardo et al. 2011) reduces macrophage presence in tumors and correlates with reduced mammary tumor metastasis. Thus, AAMs, through their ability to differentially regulate immunity and express molecules that support angiogenesis/tissue remodeling and proliferation, profoundly affect the development, maintenance, and dissemination of malignant tumors.

Immunosuppressive monocytes

Sharing the same common myeloid progenitor as macrophages, immunosuppressive monocytes in rodent tumor models encompass a diverse population of cells characterized by expression of surface markers, including CD11b and Gr1 (Ostrand-Rosenberg 2008; Gabrilovich and Nagaraj 2009), and include monocytes variably referred to as myeloid-derived suppressor cells (MDSCs), iMCs, inflammatory monocytes, and neutrophils (Ostrand-Rosenberg 2008). Human equivalents have been identified as $LIN^{-/Lo}$ human leukocyte antigen (HLA)-DR^-CD33^+ $CD11b^+$ and $CD14^+$HLA-$DR^{-/Lo}$ cells (Serafini et al. 2006); however, as with mice, these share markers with multiple mature granulocytic subtypes and thus likely represent a mixed population in which some cells contain immunesuppressive properties. MDSCs and iMCs are functionally characterized by their T-cell-suppressive activity; e.g., the ability to suppress T- and NK cell proliferation via arginase I, inducible nitric oxide synthase expression, and perioxynitrite, and, at the same time, promote generation of T_{reg} cells (Mazzoni et al. 2002; Gabrilovich and Nagaraj 2009; Doedens et al. 2010; Lu et al. 2011).

In mice, systemic increases in the presence of MDSCs and iMCs have been observed when syngeneic mice are transplanted with or develop spontaneous tumors (Ostrand-Rosenberg 2008). Significant increases in MDSCs in peripheral blood are also a common feature for patients with several types of cancer (Almand et al. 2001). Moreover, in murine models of cancer, MDSCs/iMCs have also been found to mediate resistance to some forms of anti-angiogenic

therapy (Shojaei et al. 2007; Priceman et al. 2010). Thus, strategies aiming to eliminate MDSCs/iMCs may result in shifting the immune microenvironment to instead favor anti-tumor type responses that improve survival.

Cytotoxic therapy and immune cells

Cytotoxic therapy and immunogenic cell death

Cytotoxic therapy (CTX and RT), in combination with surgery, forms the cornerstone of systemic treatment for most clinically detectable solid tumors. Significantly, most cytotoxic therapies result in immune suppression due to a higher sensitivity of bone marrow-derived stem cells and many leukocyte subsets, especially lymphocytes, to their cytotoxic effects. Through specialized cell death pathways, including Fas–FasL, lymphocytes respond to DNA damage induced by CTX and RT by undergoing early apoptosis at doses significantly lower than other cell types, especially epithelial or neural cell types. Bone marrow-derived stem cells are also uniquely sensitive to CTX and RT (Apetoh et al. 2007; Ghiringhelli et al. 2009), and their early destruction is likely a dose-limiting toxicity for many of these modalities; thus, administration of cytotoxic agents can lead to systemic immune suppression. That said, there is increasing evidence that within tumors, cell death generated by these agents also triggers activation of other immune response pathways that serendipitously also regulate therapeutic efficacy of the particular cytotoxic agent/modality (Table 1).

Whereas neoplastic cells have long been thought to undergo an "immunologically silent" demise following cytotoxic therapy, whereby apoptotic machinery eliminates them (Albert et al. 1998), recent studies have challenged this notion (Ma et al. 2011) and revealed that nonapoptotic and biochemically distinct cell death pathways are also activated following RT and some forms of CTX (e.g., anthracyclines and oxaliplatin) (Fig. 1). Mechanistically, leukocytes detect cell death through immune-based receptors selective for molecules released by dying cells (often termed "danger signals"), including Toll-like receptor-4 (TLR-4) and its ligand, the high-mobility group box protein 1 (HMGB1) (Apetoh et al. 2007). Detection of danger signals by resident leukocytes results in subsequent activation of both innate (myeloid and NK cells) and adaptive (T and B) cell lineages. Molecular mechanisms underlying immunogenic cell death following cytotoxic therapy involve activation of several critical sequential checkpoints. These include (1) exposure of endoplasmic reticulum (ER)-resident protein complexes, comprised of calreticulin/ERp57 on plasma membranes of neoplastic cells that serve as "eat me" signals for DCs; (2) release of the chromatin-binding HMGB1 protein, which by a TLR4/MyD88-dependent mechanism inhibits degradation of DC phagosomes, thereby facilitating antigen presentation (Apetoh et al. 2007); (3) ATP release from dying neoplastic cells and subsequent engagement of DC P2RX7 purinergic receptors, leading to IL-1β release (Ghiringhelli et al. 2009); and (4) effective antigen cross-presentation by DCs with increased production of IFNγ/IFNγ receptors and $CD8^+$ CTL-dependent killing responses. Experimental evidence supporting these pathways emanates from in vivo evaluation in murine tumor models where the immune response induced by CTX or RT efficiently prevents tumor growth dependent on activation of these pathways (Apetoh et al. 2007; Ghiringhelli et al. 2009). Clinical evidence for the importance of these mechanisms is provided by human breast cancer patients harboring Asp299Gly *TLR4* polymorphisms or loss-of-function mutations in the *P2RX7* gene, both of which disrupt DC–T-cell functional interactions by impairing DCs' ability to sense HMGB1 and ATP release by dying cells, and correlates clinically with resistance to CTX (anthracyclines) and RT (Ghiringhelli et al. 2009).

Recognition that immune-based mechanisms modulate the response to cytotoxic therapy implies that the ultimate effectiveness of cytotoxic modalities could be improved by combinatorial approaches that also engage immunogenic death programs. Thus, strategies improving antigen presentation (to T cells) and/or increasing macrophage cytolytic activity would theoretically impede tumorigenesis if the protumorigenic properties of those leukocytes following cytotoxic therapy could also be effectively blunted. Requisite for success of this scenario is that T_H1-based immune programs would be fostered, and dominant T_H2-type programming would be blunted. T_H1 programming in response to increased expression of type 1 cytokines (TNFα, IFNγ, and IL-2) activates cell-mediated responses that are "anti-tumor" in nature. T_H2 programming, on the other hand, is mediated by expression of type 2 cytokines (IL-4, IL-10, and TGFβ) that instead initiate tissue remodeling, angiogenesis, and (sometimes) humoral immunity, and together foster a protumorigenic state (Yang et al. 2008; DeNardo et al. 2010; Ruffell et al. 2010).

Evidence that forced T_H1 polarization of tumor microenvironments can improve response to cytotoxic therapy has been observed. For example, immunization with plasmacytoma supernatant plus IL-1 resulted in decreased tissue/tumor levels of IL-10 and TGFβ and increased levels of IFNγ and IL-2, thus favoring T_H1 immunity and tumor regression (Li et al. 1998). Other studies reported that combined CTX or RT with DC vaccination, which augments the initial T_H1 response through enhanced antigen presentation, also resulted in tumor regression (Koike et al. 2008; Matsumura et al. 2008). A general conclusion from these studies is that cytotoxic therapy indeed fosters an anti-tumor immune microenvironment; however, this response tends not to be durable, likely due to protumor, immunosuppressive programs that become dominant, thereby fueling tumor recurrence and subsequent resistance to therapy.

Cytotoxic therapy and cancer immunotherapy—a different approach

Harnessing the body's own immune system to fight cancer has long been considered the ultimate treatment for cancer because of its potential to specifically and durably target antigen-positive neoplastic cells while limiting damage to

Table 1. *Immune effects of cytotoxic agents*

		Effect on tumor immune response							
Cytotoxic agent	Example	Release of proinflammatory molecules	Antigen presentation	Release of inflammatory cytokines	Increased homing of inflammatory cells	Enhanced T-cell activation	Enhanced cytotoxic immune response	Inhibition of immunosuppressive cells	References
Alkylating agents	Cyclophosphamide, ifosfamide, busulfan, melphalan, dacarbazine					•		•	Bracci et al. 2007; Robert et al. 2011
Anthracyclines	Daunorubicin, doxorubicin	•							Ma et al. 2010; Ramakrishnan et al. 2010
Anti-metabolites	Methotrexate, 5-fluorouracil, fludarabine, gemcitabine		•			•	•	•	Suzuki et al. 2005; Ma et al. 2010
DNA methytransferase inhibitors	2′-Deoxy-5-azacytidine (decitabine or DAC)						•		Ma et al. 2010
Platinum agents	Carboplatin, cisplatin, oxaliplatin	•							Ghiringhelli et al. 2009; Ma et al. 2010; Ramakrishnan et al. 2010
Spindle poisons	Paclitaxel, docetaxel, vincristine, vinblastine (*Vinca* alkaloids)		•			•		•	Apetoh et al. 2007; Ma et al. 2010; Ramakrishnan et al. 2010; DeNardo et al. 2011
Topoisomerase inhibitors	Etoposide, irinotecan								
Tyrosine kinase inhibitors	Imatinib, dasatinib, sunitinib						•	•	Kao et al. 2009; Ozao-Choy et al. 2009
Radiation	γ/X-rays, electrons, Protons, α particles	•	•	•	•		•	•	Matsumura et al. 2008; Ahn et al. 2010; Meng et al. 2010; DeNardo et al. 2011

Common classes of cytotoxic agents are listed, with their known effects on immune responses indicated.

normal tissue. Given the immunogenic potential of cytotoxic therapies alone, it follows that strategies augmenting the immune response to cancer would synergize with anti-tumor immunity generated by cytotoxic therapy. That said, current cancer immunotherapies use a variety of strategies, including therapeutic monoclonal antibodies (mAbs) and adoptive cell transfer (ACT) involving transfer of ex vivo expanded autologous or allogeneic tumor-reactive lymphocytes and cancer vaccines, and thus attempt to stimulate anti-tumor immunity (Table 2). A critical appraisal of these approaches reveals limited overall objective response rates (3.6%) across several early-phase trials (Klebanoff et al. 2011). Although positive results with surrogate immunological endpoints have been reported, the vast majority of phase III immunotherapy trials in patients with solid tumors have failed to demonstrate improved overall survival. Analysis of these reveals that the strategies do indeed initiate and/or prime anti-tumor immunity; however, their limited success lies in their failure to also inhibit the pathways that block CTL and NK T-cell-mediated killing. As new data emerge expanding on our understanding of these complex immune-based mechanisms, new approaches are sure to develop that not only enhance generation of anti-tumor immunity, but also prevent its suppression.

Cancer immunotherapy I: augmenting the anti-tumor immune response

ACT ACT has been reported to induce objective tumor regression and long-term responses for a small fraction of melanoma patients (Rosenberg et al. 2008). Although first described in the 1980s, therapeutic efficacy and increased patient survival were only reported following addition of immuno-depleting CTX prior to ACT, which was subsequently further improved by myeloablative lympho-depleting regimens (Dudley et al. 2008). Mechanistically, (limited) removal of endogenous lymphocytes that act as "sinks" for homeostatic cytokines and elimination of immunosuppressive T_{regs} and iMCs underlay these objective clinical responses (Gattinoni et al. 2005; Dudley et al. 2008).

Gabrilovich and colleagues (Ramakrishnan et al. 2010) evaluated several cancer vaccines with ACT in murine models in combination with several widely used chemotherapeutic drugs. These researchers reported that CTX rendered tumor cells more susceptible to the cytolytic effects of CTLs via increased perforin-independent permeability to granzyme B, mediated by up-regulation of mannose-6-phosphate receptors on malignant cells (Ramakrishnan et al. 2010). When combined with CTX, CTLs raised against specific tumor antigens induced apoptosis in neighboring tumor cells that did not express the antigens. Thus, small numbers of CTLs can mediate potent anti-tumor effects when combined with CTX and provide a rationale for combining these modalities for treatment of patients with advanced cancer.

Cancer vaccines Inspired by success with vaccination against bacterial and some viral pathogens, a variety of approaches have been explored in an attempt to immunize patients against their own cancers, some of which include use of whole (killed) tumor cells, proteins, peptides, or DNA vaccines (Giaccone et al. 2005; Testori et al. 2008; Dougan and Dranoff 2009; Amato et al. 2010). In spite of limited success with these, there is renewed interest following recent positive clinical results in prostate cancer and lymphoma. Sipuleucel-T (Provenge), a cellular vaccine comprising autologous antigen-presenting cells (APCs) cultured with a fusion protein of prostatic acid phosphatase with GM-CSF, extended median survival in two independent phase III trials, leading to FDA approval for treatment of advanced prostate cancer (Small et al. 2006; Kantoff et al. 2010). Other encouraging results have been reported, most notably idiotype vaccination for follicular lymphoma in a phase III trial that demonstrated a prolonged period of CTX-induced remission (Neelapu et al. 2005).

DCs link innate and adaptive immunity and can induce contrasting states, including immunity and tolerance to self. Multiple populations of DCs are recognized in vivo in both human and murine tumors, each with distinct properties that variably regulate humoral and cellular immunity (Hashimoto et al. 2011). While antibody responses are preferentially mediated by $CD14^+$ dermal DCs, CTL responses are instead preferentially mediated by Langerhans cells (Hashimoto et al. 2011), thus indicating that DC-mediated mechanisms inducing humoral and/or cellular immunity are fundamentally distinct. Early clinical trials testing vaccination with ex vivo generated DCs pulsed with tumor antigens provided proof-of-principle evidence that therapeutic immunity could be elicited; however, clinical benefit measured by regression of established tumors in patients with stage IV cancer was observed in only a small percentage of patients (Palucka et al. 2008). Patients with soft tissue sarcoma who received fractionated external beam radiation in combination with administration of intratumoral DCs demonstrated an increased T-cell infiltration, with tumoral $CD4^+$ T cells positively correlating with tumor-specific immune responses (Finkelstein et al. 2011). Thus, new-generation DC vaccines are needed that generate large numbers of high avidity effector anti-tumor T cells able to overcome suppressive mechanisms in the tumor microenvironment. These, combined with therapies blunting T_H2-based pro-tumor immunity and CTX or RT, would thus be anticipated to provide much more durable tumor repression.

Cancer immunotherapy II: targeting immunosuppressive pathways and cells

T_{reg} cells Evidence for the role of T_{regs} in anti-tumor immunity was first provided by Sakaguchi and colleagues (Shimizu et al. 1999; Sakaguchi 2005) using a syngeneic murine heterotopic transplant model. This was later reproduced in several murine tumor models, all of which demonstrated that depletion of T_{regs} via anti-CD25 mAb prior to tumor inoculation led to syngeneic tumor rejection (Casares et al. 2003). In conjunction with cytotoxic therapy, strategies targeting CD25, such as depleting mAbs or an IL-2-diphtheria toxin fusion protein, enhanced

Table 2. *Current immunotherapy*

Immunotherapy type	Species	Cancer type	Agent	Comment	References
ACT	Mouse	Colon, breast, lymphoma	CTL + paclitaxel or cisplatin or doxorubicin	Significant tumor growth delay seen with combination therapy due to increased sensitivity of tumor cells, Granzyme B via mannose-6 phosphate receptor	Ramakrishnan et al. 2010
	Human	Melanoma	Ex vivo expanded tumor-infiltrating lymphocytes + IL-2; cyclophosphamide + fludarabine + total body irradiation	Objective response rate of 50%–70% in patients with combined regimen (vs. 15% with historical standard of IL-2 + dacarbazine)	Dudley et al. 2008
	Human	Melanoma	Ex vivo expanded $CD8^+$ T cells against melanoma antigen Melan-A	Tumor-specific immune response detected in 27.2% (three of 11 patients)	Mackensen et al. 2006
Cancer vaccines	Human	Melanoma	Peptide vaccine (Hsp96)	No improvement in overall survival ($P = 0.32$)	Testori et al. 2008
	Human	Kidney	Poxvirus encoding tumor antigen 5T4	Specific antibody responses detected, but no improvement in overall survival ($P = 0.55$)	Amato et al. 2010
	Human	Lung	Antibody that mimics ganglioside GD3	Specific antibody responses detected, but no improvement in overall survival ($P = 0.28$)	van Meerbeeck et al. 2005
	Human	Follicular lymphoma	Idiotype vaccination	Induction of prolonged remission	Neelapu et al. 2005
	Human	Sarcoma	Intratumoral DCs + RT	Tumor-specific immune responses detected in 52.9% of patients (nine of 17 patients)	Finkelstein et al. 2011
	Human	Prostate	Tumor antigen (prostatic acid phosphatase) coupled to GM-CSF (Sipuleucel-T)	4-mo improvement in median survival compared with no treatment ($P = 0.02$)	Small et al. 2006; Kantoff et al. 2010
Inhibition or depletion of T_{reg} cells	Mouse	Lymphoma, colon	Anti-CD25 depleting mAb + attenuated Poxvirus + RT	Increased antigen-specific immune responses with combined modalities	Kudo-Saito et al. 2005
	Mouse	Melanoma	Agonistic anti-OX40 mAb + cyclophosphamide	Improved survival and significantly delayed tumor growth secondary to intratumoral T_{reg}-specific hyperactivation and apoptosis	Hirschhorn-Cymerman et al. 2009
	Human	Melanoma	IL-2 fusion protein with diphtheria toxin (denileukin difitox) + DC vaccine	Increased T-cell and antibody response to tumor antigen CEA	Morse et al. 2008
	Human	Melanoma	Anti-CTLA4 (Ipilimumab) mAbs + dacarbazine	2-mo improvement in median survival versus dacarbazine alone ($P < 0.001$)	Hodi et al. 2010; Robert et al. 2011

(continued)

Table 2. *continued*

Immunotherapy type	Species	Cancer type	Agent	Comment	References
Depletion or repolarization of macrophages	Mouse	Melanoma	Clodronate liposomes + RT	Delayed tumor growth following RT only when macrophages were depleted	Meng et al. 2010
	Mouse	Breast	CSF-1 receptor (CSF-1R) inhibitor + paclitaxel	Delayed tumor growth with combination of paclitaxel and macrophage deletion but not with macrophage depletion alone	DeNardo et al. 2011
	Human, mouse	Pancreas	Agonistic CD40 mAb + gemcitabine	Tumor response rate of 30% (vs. 5.4% historically for gemcitabine alone) in both humans and mice; mechanism depends on repolarization of tumor-associated macrophages	Beatty et al. 2011
Depletion of iMCs/MDSCs	Human, mouse	Lung (non-small cell)	ATRA + paclitaxel/cisplatin	2.9 mo increase in progression-free survival ($P = 0.008$); mouse model indicates that ATRA mechanism may be combination of differentiation of iMC into macrophages and increased sensitivity of tumor cells to CTX	Arrieta et al. 2010
	Human, mouse	Multiple	Sunitinib (tyrosine kinase inhibitor) + RT	59% (21 of 36) of lesions had complete or partial response; mouse model shows decreased circulating T_{reg} and iMC/MDSC and suppressive function with sunitinib	Kao et al. 2009; Ozao-Choy et al. 2009
	Mouse	Head and neck, breast	Anti-CD11b mAb	Delayed tumor growth with combination of mAb and RT or paclitaxel	Ahn et al. 2010; DeNardo et al. 2011

Results from selected immunotherapy studies in murine models of human cancer and in patients with various types of solid cancers.

anti-tumor immune responses in both murine models and humans (Kudo-Saito et al. 2005; Mackensen et al. 2006; Morse et al. 2008). However, strategies targeting CD25 lack specificity, in that activated T cells also express CD25; thus, these agents may also blunt formation of robust anti-tumor T-cell responses while also depleting T_{reg} cells (Curtin et al. 2008). Therefore, other strategies targeting the T_{regs}—such as the agonistic antibody against OX40, a TNR receptor (TNFR) family costimulatory molecule expressed on T cells and DCs—in combination with cyclophosphamide minimize this paradox by inducing T_{reg}-specific apoptosis (Hirschhorn-Cymerman et al. 2009).

Targeting T_{regs} with CTLA-4 antagonists has been perhaps the most successful of the strategies targeting an immunosuppressive pathway, although others such as B7-H3, PD-L1, and CD73 are currently under investigation (Yi and Chen 2009; Ascierto et al. 2010; Jin et al. 2011). CTLA-4 is a negative costimulatory molecule expressed on both activated T cells and T_{reg} cells that helps dampen ongoing immune response, is frequently up-regulated on chronically activated and exhausted T cells (Engelhardt et al. 2006; Wherry et al. 2007), and not only inhibits T-cell activation, but also promotes T_{reg} function (Teft et al. 2006). Results from a phase III trial evaluating the CTLA-4-blocking mAb ipilimumab, recently approved for the treatment of advanced malignant melanoma by the FDA, revealed extended overall survival of previously treated melanoma patients, correlating with increased $CD8^+$ T-cell activation and T_{reg} inhibition (Hodi et al. 2010). A subsequent landmark study demonstrated improved survival in patients with untreated advanced melanoma who received ipilimumab combined with the CTX agent dacarbazine, as compared with those receiving CTX alone (Robert et al. 2011). This study supports the hypothesis that the combination of CTX with a reduction in the suppressive environment—in this case, elimination of T_{regs}—is a strategy that can lead to more effective anti-tumor immunity.

AAMs CTX and RT stimulate recruitment of macrophages (and monocytes) into tissues through direct induction of myeloid cell chemoattractant molecules. Epithelial cells rapidly respond to CTX (paclitaxel, cisplatin, and carboplatin) and RT by direct mRNA induction of monocyte-promoting chemokines such as *csf-1*, *IL-34*, *ccl2/MCP-1*, *ccl5*, *cxcl10*, *cxcl11*, *cx3cl1*, and *HIF1* (Kioi et al. 2010; DeNardo et al. 2011; Ruffell et al. 2011). Cyclophosphamide, oxaliplatin, and RT induce T_H1 (Bracci et al. 2007; Ghiringhelli et al. 2009) as well as T_H2 cytokines (Gremy et al. 2008), indicating that CTX and RT have the potential to skew macrophage phenotypes to either anti- or protumorigenic states. Thus, while CTX and RT may initially mediate a cytotoxic/cytolytic macrophage response (Lambert and Paulnock 1987), enhanced presence of T_H2 cytokines may contribute to ongoing skewing and maintenance of AAMs in tumors and subsequent repulsion of $CD8^+$ T-cell-mediated anti-tumor immunity (Doedens et al. 2010).

The duality of macrophages as mediators of cytotoxic therapy responses has been demonstrated in experimental murine models showing that macrophage depletion significantly slows tumor growth, but only when provided in combination with either CTX or RT. Selective depletion of macrophages using clodronate liposomes in an orthotopic murine melanoma model given before RT increased latency and slowed tumor regrowth, whereas coimplantation of malignant cells along with bone marrow-derived macrophages increased tumor radioresistance mediated by TNFα signaling pathways (Meng et al. 2010). Macrophage depletion strategies in combination with CTX or RT slow tumor development in murine models of sarcoma and melanoma in part by altering, or perhaps "normalizing," tumor vasculature (Meng et al. 2010; Rolny et al. 2011). Vascular normalization in this context likely improves tumor hemodynamics, thereby increasing delivery of chemotherapeutic agents and oxygenation of tumor parenchyma, and thereby reducing hypoxia.

Given the evidence that cytotoxic agents are also potent immune adjuvants, it would not be surprising that strategies abolishing or reprogramming AAMs would enhance both cell killing by cytotoxics and immunogenic cell death. Thus, in order to overcome the immunosuppressive barriers established by tumors, it may be important to not only provide antigenic stimulus in the form of cytotoxic therapy, but also neutralize myeloid-based pathways established in the tumor that blunt effective anti-tumor immune responses. To address this possibility, we recently used a mouse model of mammary carcinogenesis (MMTV-PyMT mice) and reported that CSF1R blockade depleted $CD11b^+Ly6G^-Ly6C^{Lo}F4/80^+$ macrophages, but not the less abundant population of granulocytic $CD11b^+Ly6G^+$-expressing myeloid cells, and resulted in slowed primary tumor growth and diminished metastasis, but only when given in combination with CTX, by $CD8^+$ T-cell-dependent mechanisms (DeNardo et al. 2011). Cathepsin protease-expressing macrophages have been found to mediate many of these effects, and cathepsin B and S protect malignant mammary epithelial cells from Taxol-induced (as well as etoposide and doxorubicin) tumor cell death in coculture (Shree et al. 2011). Combining Taxol with cathepsin inhibition in vivo significantly enhanced efficacy against primary and metastatic mammary tumors, supporting the therapeutic relevance for this effect (Shree et al. 2011). These experimental studies provide a compelling rationale for clinical evaluation of combinatorial approaches inhibiting macrophage recruitment or altering macrophage response pathways and mediator expression/activity in combination with "standard of care" CTX for treatment of some solid tumors in order to overcome inherent resistance to CTX. These strategies are an active area of clinical research in the phase I and II setting, testing a variety of agents designed to either block macrophage recruitment or stimulate alternative macrophage programming (Anthony et al. 2011) with the hope that combinations will provide improved clinical outcomes.

In addition to targeting macrophage recruitment, it is also possible to target macrophage polarization in an attempt to elicit the presence of more favorable T_H1-polarized cytotoxic macrophages in tumors. One such strategy currently being explored is via targeting CD40, a member of the TNFR

superfamily and a costimulatory molecule expressed on a diverse assortment of cells, including DCs, B cells, and macrophages, as well as endothelial, mesenchymal, and epithelial cells. Binding of the CD40 ligand (CD40L) CD154 to CD40 mediates distinct effects on cells, depending on cell type and the tissue and microenvironment in which they reside. On immune cells, CD40 regulates humoral and cellular immunity, while apoptotic and antiproliferative pathways are regulated by CD40 on some neoplastic cells (Fonsatti et al. 2010). Activation of APCs requires binding of CD40L on T_H cells to CD40, whereas macrophage activation requires IFNγ produced by T_H1-$CD4^+$ T cells in addition to CD40L–CD40 interaction. This results in macrophage up-regulation of CD40 and TNFR and induction of cytotoxic activity, including increased expression of nitric oxide and reactive oxygen species (Fonsatti et al. 2010). To investigate whether agonist CD40 mAbs would thwart tumor-induced immune suppression and instead invoke productive T-cell-dependent anti-tumor immunity, Beatty et al. (2011) treated 21 patients with pancreatic ductal adenocarcinoma (PDA) with a fully humanized agonistic CD40 mAb in combination with gemcitabine and reported tumor regression in some patients. Using a mouse model of PDA to reveal the molecular/cellular mechanisms underlying the improved response, tumor regression was found to be dependent on CD40-activated MHC-$II^{hi}CD86^+$ tumoricidal macrophages as opposed to $CD8^+$ T cells (Beatty et al. 2011).

In addition to these approaches, others have investigated the efficacy of CD47 blockade to foster macrophage and DC phagocytic activity (Jaiswal et al. 2010). CD47, also known as integrin-associated protein (IAP), encodes a membrane protein mediating intracellular calcium levels following cell adhesion to extracellular matrix. CD47 binds to the SIRPα inhibitor receptor on macrophages and DCs and thereby inhibits phagocytosis; in autoimmune processes, these interactions limit tissue damage (Jaiswal et al. 2010). Expression of CD47 has been found to be significantly increased on some malignant tumor cells, especially in non-Hodgkin's lymphoma, thus rendering malignant cells resistant to macrophage and DC phagocytosis (Chao et al. 2010). Since agonistic CD40 mAb in combination with gemcitabine provides a survival advantage for PDA dependent on tumoricidal macrophages, it seems reasonable to speculate that combining similar approaches with therapies blocking CD47 may be efficacious in solid tumors where CD47 is up-regulated. Taken together, the experience with immunotherapy makes a compelling case for integrating strategies that restrain and/or reprogram tumor immune microenvironments, resulting in bolstering of diverse anti-tumor pathways to achieve meaningful therapeutic gains.

Immunosuppressive myeloid cells Minimizing suppressive iMCs/MDSCs in tumors has been investigated using all-*trans* retinoic acid (ATRA), which induces differentiation of iMCs into macrophages and correlates with enhanced anti-tumor immunity in murine models (Kusmartsev and Gabrilovich 2003). In human clinical trials, addition of retinoic acid to standard CTX improved outcome for patients with advanced non-small-cell lung cancer (Arrieta et al. 2010). While ATRA decreased accumulation of iMCs in both tumor-bearing mice and humans, exposure to this agent also increased sensitivity of malignant cells to CTX, likely accounting for at least some of its anti-tumor efficacy (Arrieta et al. 2010). Other strategies to eliminate iMCs have used c-KIT antagonists that decrease accumulation of iMCs in murine and human tumors but have only improved anti-tumor immunity when given in the presence of tumor vaccines (Ozao-Choy et al. 2009). A phase I/II clinical study revealed that concurrent administration of sunitinib—an oral, small-molecule, multi-targeted receptor tyrosine kinase inhibitor of the vascular endothelial growth factor receptor (VEGFR), c-KIT, and platelet-derived growth factor receptor approved by the FDA for treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor—with RT in patients with one to five distant oligometastases improved progression-free survival; responses correlated with decreased peripheral blood monocyte levels 7 d following start of therapy (Kao et al. 2009). While evidence supporting the use of c-KIT antagonists and cytotoxic therapy is encouraging, agents that target c-KIT can also have effects on many other cell types, including hematopoietic stem cells, mast cells, and melanocytes, due to activity also against other related kinases, thus posing a significant challenge for interpreting data in terms of effects on iMC subtypes. Although many of the agents used for targeting iMCs lack specificity, data from agents such ATRA and c-KIT antagonists provide suggestive evidence that immature myeloid populations may have important roles in regulating anti-tumor immune responses.

Another approach for depletion of immunosuppressive myeloid cells has been treatment of tumor-bearing mice with αCD11b mAbs. CD11b is an integrin cell adhesion molecule involved in transendothelial migration expressed predominantly by myeloid lineage cells, including neutrophils, macrophages, monocytes, and DCs. Bone marrow-derived $CD11b^+$ myeloid cells are recruited to tumors following RT, where they restore vascular programming via VEGF secretion, thus aiding subsequent tumor (re)growth. Neutralizing CD11b mAbs inhibit recruitment of $CD11b^+$ myeloid cells into RT-treated tumors, slowing tumor regrowth and thus improving RT response (Ahn et al. 2010). Similarly, mice bearing syngeneic 4T1 mammary tumors treated with CTX and αCD11b mAbs demonstrated significantly slowed primary tumor growth as well as reduced pulmonary metastases (DeNardo et al. 2011). $Gr1^+CCR2^+CX3CR1^{Lo}$ iMCs are highly responsive to CCL2 (Zhang et al. 2010), and CCL2/MCP1 is expressed at high levels in mammary tumors and is now mechanistically demonstrated to potentiate pulmonary metastasis (Qian et al. 2011).

A neutralizing antibody specific for human CD11b–CD18 integrin heterodimers, rovelizumab (LeukArrest), has previously been investigated and was found to have an excellent safety profile, but lacked therapeutic efficacy in inflammatory diseases such as multiple sclerosis. However, based on murine studies, it seems reasonable to speculate that a drug like rovelizumab could be

administered safely for transient blockade of myeloid cell infiltration following local RT or systemic CTX and thereby provide a window of opportunity when tumors could be prevented from efficient revascularization and anti-tumor immunity could be bolstered. Thus, extrapolating to the clinical scenario, it will be important to stratify human tumors containing predominately high levels of mature tissue macrophages, as compared with those containing iMCs/MDSCs, as these tumors would likely be less responsive to therapy directed at CSF1R-positive macrophages, but might be expected to instead respond to drugs like rovelizumab.

Conclusions

The relatively modest gains provided by immunotherapy despite intense investigation can be in part attributed to the presence of pathways that suppress anti-tumor immunity. These mechanisms likely evolved as part of tumor development where the local microenvironment contains an immune set point skewed favoring T_H2-type pathways, as compared with homeostatic tissue. Cell types including T_{regs}, AAMs, and iMCs form an inhibitory network that suppresses local immunity, thereby limiting the efficacy of many forms of anti-cancer therapy reliant on formation of productive anti-tumor immune responses.

Studies investigating the mechanism of action for CTX and RT have historically focused on cell-intrinsic molecules regulated by these cytotoxic agents; however, recent evidence indicates the importance of cell-extrinsic factors, particularly for induction of anti-tumor immunity. Given this, inhibitory mechanisms that stymied development of effective immunotherapy may also play an important role in regulating response to cytotoxic agents. Emerging data indicate that targeting immune inhibitory/stimulatory pathways, in conjunction with conventional cytotoxic therapy and current immunotherapy, significantly enhances the effectiveness of cytotoxic therapy by augmenting anti-tumor immunity and preventing its suppression. Further exploration to better characterize and understand inhibitory immune pathways will aid in identification of new targets that redefine our understanding of the anti-tumor mechanism of traditional cytotoxic therapies and direct us to new strategies that improve the efficacy of standard therapy.

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References

Ahn GO, Tseng D, Liao CH, Dorie MJ, Czechowicz A, Brown JM. 2010. Inhibition of Mac-1 (CD11b/CD18) enhances tumor response to radiation by reducing myeloid cell recruitment. *Proc Natl Acad Sci* **107:** 8363–8368.

Albert ML, Sauter B, Bhardwaj N. 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392:** 86–89.

Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, Carbone DP, Gabrilovich DI. 2001. Increased production of immature myeloid cells in cancer patients: A mechanism of immunosuppression in cancer. *J Immunol* **166:** 678–689.

Amato RJ, Hawkins RE, Kaufman HL, Thompson JA, Tomczak P, Szczylik C, McDonald M, Eastty S, Shingler WH, de Belin J, et al. 2010. Vaccination of metastatic renal cancer patients with MVA-5T4: A randomized, double-blind, placebo-controlled phase III study. *Clin Cancer Res* **16:** 5539–5547.

Andreu P, Johansson M, Affara NI, Pucci F, Tan T, Junankar S, Korets L, Lam J, Tawfik D, DeNardo DG, et al. 2010. FcRγ activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell* **17:** 121–134.

Anthony SP, Puzanov I, Lin PS, Nolop KB, West B, Von Hof DD 2011. Pharmacodynamic activity demonstrated in phase I for PLX3397, a selective inhibitor of FMS and Kit. *J Clin Oncol* **29:** (suppl; abstr 3093).

Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, et al. 2007. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* **13:** 1050–1059.

Arrieta O, Gonzalez-De la Rosa CH, Arechaga-Ocampo E, Villanueva-Rodriguez G, Ceron-Lizarraga TL, Martinez-Barrera L, Vazquez-Manriquez ME, Rios-Trejo MA, Alvarez-Avitia MA, Hernandez-Pedro N, et al. 2010. Randomized phase II trial of All-*trans*-retinoic acid with chemotherapy based on paclitaxel and cisplatin as first-line treatment in patients with advanced non-small-cell lung cancer. *J Clin Oncol* **28:** 3463–3471.

Ascierto PA, Simeone E, Sznol M, Fu YX, Melero I. 2010. Clinical experiences with anti-CD137 and anti-PD1 therapeutic antibodies. *Semin Oncol* **37:** 508–516.

Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, Banham AH. 2006. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* **24:** 5373–5380.

Beatty GL, Chiorean EG, Fishman MP, Saboury B, Teitelbaum UR, Sun W, Huhn RD, Song W, Li D, Sharp LL, et al. 2011. CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. *Science* **331:** 1612–1616.

Bracci L, Moschella F, Sestili P, La Sorsa V, Valentini M, Canini I, Baccarini S, Maccari S, Ramoni C, Belardelli F, et al. 2007. Cyclophosphamide enhances the antitumor efficacy of adoptively transferred immune cells through the induction of cytokine expression, B-cell and T-cell homeostatic proliferation, and specific tumor infiltration. *Clin Cancer Res* **13:** 644–653.

Casares N, Arribillaga L, Sarobe P, Dotor J, Lopez-Diaz de Cerio A, Melero I, Prieto J, Borras-Cuesta F, Lasarte JJ. 2003. $CD4^+$/$CD25^+$ regulatory cells inhibit activation of tumor-primed $CD4^+$ T cells with IFN-γ-dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. *J Immunol* **171:** 5931–5939.

Chao MP, Alizadeh AA, Tang C, Myklebust JH, Varghese B, Gill S, Jan M, Cha AC, Chan CK, Tan BT, et al. 2010. Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell* **142:** 699–713.

Coussens LM, Werb Z. 2002. Inflammation and cancer. *Nature* **420:** 860–867.

Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, et al. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* **10:** 942–949.

Curtin JF, Candolfi M, Fakhouri TM, Liu C, Alden A, Edwards M, Lowenstein PR, Castro MG. 2008. Treg depletion inhibits efficacy of cancer immunotherapy: Implications for clinical trials. *PLoS One* **3:** e1983. doi: 10.1371/journal.pone.0001983.

DeNardo DG, Barreto JB, Andreu P, Vasquez L, Tawfik D, Kolhatkar N, Coussens LM. 2009. $CD4^+$ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell* **16:** 91–102.

DeNardo DG, Andreu P, Coussens LM. 2010. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev* **29:** 309–316.

DeNardo DG, Brennan D, Rexhapaj E, Ruffel B, Shiao S, Gallagher WM, Wadhani N, Kial SD, Junaid SA, Rugo HS, et al. 2011. Leukocyte complexity in breast cancer predicts overall survival and functionally regulates response to chemotherapy. *Cancer Discovery* **1:** 54–67.

Doedens AL, Stockmann C, Rubinstein MP, Liao D, Zhang N, Denardo DG, Coussens LM, Karin M, Goldrath AW, Johnson RS. 2010. Macrophage expression of hypoxia-inducible factor-1α suppresses T-cell function and promotes tumor progression. *Cancer Res* **70:** 7465–7475.

Dougan M, Dranoff G. 2009. Immune therapy for cancer. *Annu Rev Immunol* **27:** 83–117.

Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U, Robbins PF, Huang J, Citrin DE, Leitman SF, et al. 2008. Adoptive cell therapy for patients with metastatic melanoma: Evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* **26:** 5233–5239.

Dunn GP, Old LJ, Schreiber RD. 2004. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* **21:** 137–148.

Engelhardt JJ, Sullivan TJ, Allison JP. 2006. CTLA-4 overexpression inhibits T cell responses through a CD28-B7-dependent mechanism. *J Immunol* **177:** 1052–1061.

Finkelstein SE, Iclozan C, Bui MM, Cotter MJ, Ramakrishnan R, Ahmed J, Noyes DR, Cheong D, Gonzalez RJ, Heysek RV, et al. 2011. Combination of external beam radiotherapy (EBRT) with intratumoral injection of dendritic cells as neoadjuvant treatment of high-risk soft tissue sarcoma patients. *Int J Radiat Oncol Biol Phys* doi: 10.1016/j.ijrobp.2010.12.068.

Fonsatti E, Maio M, Altomonte M, Hersey P. 2010. Biology and clinical applications of CD40 in cancer treatment. *Semin Oncol* **37:** 517–523.

Gabrilovich DI, Nagaraj S. 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* **9:** 162–174.

Gattinoni L, Finkelstein SE, Klebanoff CA, Antony PA, Palmer DC, Spiess PJ, Hwang LN, Yu Z, Wrzesinski C, Heimann DM, et al. 2005. Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific $CD8^+$ T cells. *J Exp Med* **202:** 907–912.

Ghiringhelli F, Puig PE, Roux S, Parcellier A, Schmitt E, Solary E, Kroemer G, Martin F, Chauffert B, Zitvogel L. 2005. Tumor cells convert immature myeloid dendritic cells into TGF-β-secreting cells inducing $CD4^+CD25^+$ regulatory T cell proliferation. *J Exp Med* **202:** 919–929.

Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, Vermaelen K, Panaretakis T, Mignot G, Ullrich E, et al. 2009. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1β-dependent adaptive immunity against tumors. *Nat Med* **15:** 1170–1178.

Giaccone G, Debruyne C, Felip E, Chapman PB, Grant SC, Millward M, Thiberville L, D'Addario G, Coens C, Rome LS, et al. 2005. Phase III study of adjuvant vaccination with Bec2/bacille Calmette-Guerin in responding patients with limited-disease small-cell lung cancer (European Organisation for Research and Treatment of Cancer 08971-08971B; Silva Study). *J Clin Oncol* **23:** 6854–6864.

Gocheva V, Wang HW, Gadea BB, Shree T, Hunter KE, Garfall AL, Berman T, Joyce JA. 2010. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev* **24:** 241–255.

Gremy O, Benderitter M, Linard C. 2008. Acute and persisting Th2-like immune response after fractionated colorectal γ-irradiation. *World J Gastroenterol* **14:** 7075–7085.

Grivennikov SI, Greten FR, Karin M. 2010. Immunity, inflammation, and cancer. *Cell* **140:** 883–899.

Hashimoto D, Miller J, Merad M. 2011. Dendritic cell and macrophage heterogeneity in vivo. *Immunity* **35:** 323–335.

Hirschhorn-Cymerman D, Rizzuto GA, Merghoub T, Cohen AD, Avogadri F, Lesokhin AM, Weinberg AD, Wolchok JD, Houghton AN. 2009. OX40 engagement and chemotherapy combination provides potent antitumor immunity with concomitant regulatory T cell apoptosis. *J Exp Med* **206:** 1103–1116.

Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, et al. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* **363:** 711–723.

Hori S, Nomura T, Sakaguchi S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299:** 1057–1061.

Jaiswal S, Chao MP, Majeti R, Weissman IL. 2010. Macrophages as mediators of tumor immunosurveillance. *Trends Immunol* **31:** 212–219.

Jin D, Fan J, Wang L, Thompson LF, Liu A, Daniel BJ, Shin T, Curiel TJ, Zhang B. 2011. CD73 on tumor cells impairs antitumor T-cell responses: A novel mechanism of tumor-induced immune suppression. *Cancer Res* **70:** 2245–2255.

Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, et al. 2010. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* **363:** 411–422.

Kao J, Packer S, Vu HL, Schwartz ME, Sung MW, Stock RG, Lo YC, Huang D, Chen SH, Cesaretti JA. 2009. Phase 1 study of concurrent sunitinib and image-guided radiotherapy followed by maintenance sunitinib for patients with oligometastases: Acute toxicity and preliminary response. *Cancer* **115:** 3571–3580.

Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, Aokage K, Saijo N, Nishiwaki Y, Gemma A, et al. 2008. Predominant infiltration of macrophages and $CD8^+$ T Cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer. *Cancer* **113:** 1387–1395.

Kawakami Y, Rosenberg SA. 1997. Human tumor antigens recognized by T-cells. *Immunol Res* **16:** 313–339.

Kepp O, Galluzzi L, Martins I, Schlemmer F, Adjemian S, Michaud M, Sukkurwala AQ, Menger L, Zitvogel L, Kroemer G. 2011. Molecular determinants of immunogenic cell death elicited by anticancer chemotherapy. *Cancer Metastasis Rev* **30:** 61–69.

Kioi M, Vogel H, Schultz G, Hoffman RM, Harsh GR, Brown JM. 2010. Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. *J Clin Invest* **120:** 694–705.

Klebanoff CA, Acquavella N, Yu Z, Restifo NP. 2011. Therapeutic cancer vaccines: Are we there yet?*Immunol Rev* **239:** 27–44.

Koike N, Pilon-Thomas S, Mule JJ. 2008. Nonmyeloablative chemotherapy followed by T-cell adoptive transfer and dendritic cell-based vaccination results in rejection of established melanoma. *J Immunother* **31:** 402–412.

Kudo-Saito C, Schlom J, Camphausen K, Coleman CN, Hodge JW. 2005. The requirement of multimodal therapy (vaccine, local tumor radiation, and reduction of suppressor cells) to eliminate established tumors. *Clin Cancer Res* **11:** 4533–4544.

Kusmartsev S, Gabrilovich DI. 2003. Inhibition of myeloid cell differentiation in cancer: The role of reactive oxygen species. *J Leukoc Biol* **74:** 186–196.

Lambert LE, Paulnock DM. 1987. Modulation of macrophage function by γ-irradiation. Acquisition of the primed cell intermediate stage of the macrophage tumoricidal activation pathway. *J Immunol* **139:** 2834–2841.

Li L, Okino T, Sugie T, Yamasaki S, Ichinose Y, Kanaoka S, Kan N, Imamura M. 1998. Cyclophosphamide given after active specific immunization augments antitumor immunity by modulation of Th1 commitment of $CD4^+$ T cells. *J Surg Oncol* **67:** 221–227.

Lin EY, Nguyen AV, Russell RG, Pollard JW. 2001. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* **193:** 727–740.

Lu T, Ramakrishnan R, Altiok S, Youn JI, Cheng P, Celis E, Pisarev V, Sherman S, Sporn MB, Gabrilovich D. 2011. Tumor-infiltrating myeloid cells induce tumor cell resistance to cytotoxic T cells in mice. *J Clin Invest* **121:** 4015–4029.

Ma Y, Kepp O, Ghiringhelli F, Apetoh L, Aymeric L, Locher C, Tesniere A, Martins I, Ly A, Haynes NM, et al. 2010. Chemotherapy and radiotherapy: Cryptic anticancer vaccines. *Semin Immunol* **22:** 113–124.

Ma Y, Conforti R, Aymeric L, Locher C, Kepp O, Kroemer G, Zitvogel L. 2011. How to improve the immunogenicity of chemotherapy and radiotherapy. *Cancer Metastasis Rev* **30:** 71–82.

Mackensen A, Meidenbauer N, Vogl S, Laumer M, Berger J, Andreesen R. 2006. Phase I study of adoptive T-cell therapy using antigen-specific $CD8^+$ T cells for the treatment of patients with metastatic melanoma. *J Clin Oncol* **24:** 5060–5069.

Matsumura S, Wang B, Kawashima N, Braunstein S, Badura M, Cameron TO, Babb JS, Schneider RJ, Formenti SC, Dustin ML, et al. 2008. Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells. *J Immunol* **181:** 3099–3107.

Mazzoni A, Bronte V, Visintin A, Spitzer JH, Apolloni E, Serafini P, Zanovello P, Segal DM. 2002. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J Immunol* **168:** 689–695.

Meng Y, Beckett MA, Liang H, Mauceri HJ, van Rooijen N, Cohen KS, Weichselbaum RR. 2010. Blockade of tumor necrosis factor α signaling in tumor-associated macrophages as a radiosensitizing strategy. *Cancer Res* **70:** 1534–1543.

Morse MA, Hobeika AC, Osada T, Serra D, Niedzwiecki D, Lyerly HK, Clay TM. 2008. Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines. *Blood* **112:** 610–618.

Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* **8:** 958–969.

Neelapu SS, Gause BL, Nikcevich DA, Schuster SJ, Winter J, Gockerman JP, Loughran T Jr, Takeshita K, Inghirami G, McGaughey D, et al. 2005. Phase III randomized trial of patient-specific vaccination for previously untreated patients with follicular lymphoma in first complete remission: Protocol summary and interim report. *Clin Lymphoma* **6:** 61–64.

Nishikawa H, Jager E, Ritter G, Old LJ, Gnjatic S. 2005. $CD4^+$ $CD25^+$ regulatory T cells control the induction of antigen-specific $CD4^+$ helper T cell responses in cancer patients. *Blood* **106:** 1008–1011.

Ostrand-Rosenberg S. 2008. Immune surveillance: A balance between protumor and antitumor immunity. *Curr Opin Genet Dev* **18:** 11–18.

Ozao-Choy J, Ma G, Kao J, Wang GX, Meseck M, Sung M, Schwartz M, Divino CM, Pan PY, Chen SH. 2009. The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies. *Cancer Res* **69:** 2514–2522.

Palucka AK, Ueno H, Fay J, Banchereau J. 2008. Dendritic cells: A critical player in cancer therapy?*J Immunother* **31:** 793–805.

Pedroza-Gonzalez A, Xu K, Wu TC, Aspord C, Tindle S, Marches F, Gallegos M, Burton EC, Savino D, Hori T, et al. 2011. Thymic stromal lymphopoietin fosters human breast tumor growth by promoting type 2 inflammation. *J Exp Med* **208:** 479–490.

Priceman SJ, Sung JL, Shaposhnik Z, Burton JB, Torres-Collado AX, Moughon DL, Johnson M, Lusis AJ, Cohen DA, Iruela-Arispe ML, et al. 2010. Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: Combating tumor evasion of antiangiogenic therapy. *Blood* **115:** 1461–1471.

Qian BZ, Pollard JW. 2010. Macrophage diversity enhances tumor progression and metastasis. *Cell* **141:** 39–51.

Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, Kaiser EA, Snyder LA, Pollard JW. 2011. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature* **475:** 222–225.

Ramakrishnan R, Assudani D, Nagaraj S, Hunter T, Cho HI, Antonia S, Altiok S, Celis E, Gabrilovich DI. 2010. Chemotherapy enhances tumor cell susceptibility to CTL-mediated killing during cancer immunotherapy in mice. *J Clin Invest* **120:** 1111–1124.

Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, Lebbe C, Baurain JF, Testori A, Grob JJ, et al. 2011. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* **364:** 2517–2526.

Rolny C, Mazzone M, Tugues S, Laoui D, Johansson I, Coulon C, Squadrito ML, Segura I, Li X, Knevels E, et al. 2011. HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through down-regulation of PlGF. *Cancer Cell* **19:** 31–44.

Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. 2008. Adoptive cell transfer: A clinical path to effective cancer immunotherapy. *Nat Rev Cancer* **8:** 299–308.

Ruffell B, Denardo DG, Affara NI, Coussens LM. 2010. Lymphocytes in cancer development: Polarization towards pro-tumor immunity. *Cytokine Growth Factor Rev* **21:** 3–10.

Ruffell B, Au A, Rugo HS, Esserman LJ, Hwang ES, Coussens LM. 2011. Leukocyte composition in human breast cancer. *Proc Natl Acad Sci* doi: 10.1073/pnas.1104303108.

Sakaguchi S. 2005. Naturally arising Foxp3-expressing $CD25^+CD4^+$ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* **6:** 345–352.

Sasada T, Kimura M, Yoshida Y, Kanai M, Takabayashi A. 2003. $CD4^+CD25^+$ regulatory T cells in patients with gastrointestinal malignancies: Possible involvement of regulatory T cells in disease progression. *Cancer* **98:** 1089–1099.

Serafini P, Borrello I, Bronte V. 2006. Myeloid suppressor cells in cancer: Recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol* **16:** 53–65.

Shimizu J, Yamazaki S, Sakaguchi S. 1999. Induction of tumor immunity by removing $CD25^+CD4^+$ T cells: A common basis between tumor immunity and autoimmunity. *J Immunol* **163:** 5211–5218.

Shojaei F, Wu X, Malik AK, Zhong C, Baldwin ME, Schanz S, Fuh G, Gerber HP, Ferrara N. 2007. Tumor refractoriness to anti-VEGF treatment is mediated by $CD11b^+Gr1^+$ myeloid cells. *Nat Biotechnol* **25:** 911–920.

Shree T, Olson OC, Elie BT, Kester JC, Garfall AL, Simpson K, Bell-McGuinn KM, Zabor EC, Brogi E, Joyce JA. 2011. Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer. *Genes Dev* **25:**2465–2479.

Small EJ, Schellhammer PF, Higano CS, Redfern CH, Nemunaitis JJ, Valone FH, Verjee SS, Jones LA, Hershberg RM. 2006. Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. *J Clin Oncol* **24:** 3089–3094.

Steidl C, Lee T, Shah SP, Farinha P, Han G, Nayar T, Delaney A, Jones SJ, Iqbal J, Weisenburger DD, et al. 2010. Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. *N Engl J Med* **362:** 875–885.

Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. 2005. Gemcitabine selectively eliminates splenic $Gr\text{-}1^+/CD11b^+$ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res* **11:** 6713–6721.

Tan W, Zhang W, Strasner A, Grivennikov S, Cheng JQ, Hoffman RM, Karin M. 2011. Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL-RANK signalling. *Nature* **470:** 548–553.

Teft WA, Kirchhof MG, Madrenas J. 2006. A molecular perspective of CTLA-4 function. *Annu Rev Immunol* **24:** 65–97.

Testori A, Richards J, Whitman E, Mann GB, Lutzky J, Camacho L, Parmiani G, Tosti G, Kirkwood JM, Hoos A, et al. 2008. Phase III comparison of vitespen, an autologous tumor-derived heat shock protein gp96 peptide complex vaccine, with physician's choice of treatment for stage IV melanoma: The C-100-21 Study Group. *J Clin Oncol* **26:** 955–962.

van Meerbeeck JP, Gaafar R, Manegold C, Van Klaveren RJ, Van Marck EA, Vincent M, Legrand C, Bottomley A, Debruyne C, Giaccone G. 2005. Randomized phase III study of cisplatin with or without raltitrexed in patients with malignant pleural mesothelioma: An intergroup study of the European Organisation for Research and Treatment of Cancer Lung Cancer Group and the National Cancer Institute of Canada. *J Clin Oncol* **23:** 6881–6889.

Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, Subramaniam S, Blattman JN, Barber DL, Ahmed R. 2007. Molecular signature of $CD8^+$ T cell exhaustion during chronic viral infection. *Immunity* **27:** 670–684.

Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, Carbone DP, Matrisian LM, Richmond A, Lin PC, et al. 2008. Abrogation of TGF β signaling in mammary carcinomas recruits $Gr\text{-}1^+CD11b^+$ myeloid cells that promote metastasis. *Cancer Cell* **13:** 23–35.

Yi KH, Chen L. 2009. Fine tuning the immune response through B7-H3 and B7-H4. *Immunol Rev* **229:** 145–151.

Zhang J, Patel L, Pienta KJ. 2010. CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. *Cytokine Growth Factor Rev* **21:** 41–48.

Leukocyte composition of human breast cancer

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Retrospective clinical studies have used immune-based biomarkers, alone or in combination, to predict survival outcomes for women with breast cancer (BC); however, the limitations inherent to immunohistochemical analyses prevent comprehensive descriptions of leukocytic infiltrates, as well as evaluation of the functional state of leukocytes in BC stroma. To more fully evaluate this complexity, and to gain insight into immune responses after chemotherapy (CTX), we prospectively evaluated tumor and nonadjacent normal breast tissue from women with BC, who either had or had not received neoadjuvant CTX before surgery. Tissues were evaluated by polychromatic flow cytometry in combination with confocal immunofluorescence and immunohistochemical analysis of tissue sections. These studies revealed that activated T lymphocytes predominate in tumor tissue, whereas myeloid lineage cells are more prominant in "normal" breast tissue. Notably, residual tumors from an unselected group of BC patients treated with neoadjuvant CTX contained increased percentages of infiltrating myeloid cells, accompanied by an increased CD8/CD4 T-cell ratio and higher numbers of granzyme B-expressing cells, compared with tumors removed from patients treated primarily by surgery alone. These data provide an initial evaluation of differences in the immune microenvironment of BC compared with nonadjacent normal tissue and reveal the degree to which CTX may alter the complexity and presence of selective subsets of immune cells in tumors previously treated in the neoadjuvant setting.

inflammation | macrophage

Several subtypes of CD45-expressing leukocytes infiltrate breast cancer (BC), including $CD4^+$ and $CD8^+$ T cells, $CD20^+$ B cells, and multiple myeloid-lineage cells including tumor-associated macrophages (TAMs) that are often identified by immunohistochemical (IHC) detection of CD68 (1). High lymphocyte infiltration is associated with increased survival in patients <40 y of age (2) and with a favorable prognosis in subsets of patients whose tumors are also heavily infiltrated by TAMs (3). More specifically, large cohort studies of patients with BC have revealed that the presence of $CD68^+$ cells in tumor tissue correlates with poor prognostic features (4–6), higher tumor grade (7–9), increased angiogenesis (10–13), decreased disease-free survival (6, 11, 14, 15), and increased risk for systemic metastasis when assessed in conjunction with endothelial and carcinoma cell markers (16).

The functional significance of specific leukocytes in BC development has been implied based on experimental studies using murine models of mammary carcinogenesis where mice harboring homozygous null mutations in genes specifying leukocyte development or recruitment have been evaluated. In transgenic mice expressing the polyoma virus middle T antigen regulated by the mouse mammary tumor virus promoter (MMTV-PyMT mice), progression of mammary carcinomas and metastases to lungs are reduced in mice lacking the *colony-stimulating factor-1* (*csf1*) gene, a cytokine critical for macrophage maturation and recruitment (17, 18). TAMs in mammary tumor tissue are often associated with vasculature (19), where their production of VEGFA fosters angiogenic programming of tissue (20, 21), and their production of EGF promotes invasive tumor growth and subsequent metastases (22, 23). Moreover, TAMs regulated by epithelial CSF1 express higher levels of several hypoxia-induced genes (*iNOS* and *arginase-1*) that, in turn, mediate suppression of anti-tumor immunity by blocking cytotoxic T-cell proliferation and activation (6, 24). Thus, TAM presence and bioactivity within mammary tumors correspond to their clinical activity, further indicating the importance of TAMs, not only in promoting tumor development, but also in suppression of anti-tumor immunity.

$CD4^+$ T cells isolated from human BC produce high levels of type II helper (T_H2) cytokines including IL-4 and IL-13 (25, 26), which are significant in light of studies demonstrating that several protumor activities of TAMs are regulated by IL-4 derived from $CD4^+$ T cells (1, 27). Based on these findings, we recently reported that infiltration by $CD68^+$, $CD4^+$, and $CD8^+$ immune cells in human BC is predictive of overall survival, and that the ratio of *CD68* to *CD8a* mRNA in tumor tissue correlates with complete pathologic response (pCR) in patients undergoing neoadjuvant chemotherapy (CTX) for early stage BC (6). Despite the clear correlation between these specific immune cell types and BC clinical outcome, leukocyte complexity within tumor tissue remains poorly described, with most studies relying on single-marker IHC detection. Furthermore, although some studies have examined the effects of CTX on the presence and function of circulating peripheral blood leukocytes (28), data regarding the effect of CTX on tumor-infiltrating immune cells are limited (29).

Herein, we evaluated leukocytic infiltrates in breast tissue from predominantly hormone receptor positive patients who had, or had not, received CTX before definitive surgery. In CTX-naïve patients, we found that activated T lymphocytes comprised the majority of immune cells within tumors, whereas myeloid-lineage cells predominate in nonadjacent normal breast tissue. In contrast, tumors from patients with residual disease after neoadjuvant CTX contained higher levels of infiltrating myeloid cells, with a simultaneous shift away from a T_H2 dominated lymphocyte response.

Results

Increased Presence of T Cells in Tumor Tissue. To evaluate the composition of tumor-infiltrating leukocytes in human BC, tumors from 20 patients were evaluated by polychromatic flow cytometry and IHC detection of leukocyte lineages in tissue sections as described in *Materials and Methods*. Nine invasive ductal carcinomas (IDC) and five invasive lobular carcinomas (ILC)—mostly histological grade two or three—were obtained from patients with no prior exposure to CTX (CTX-naïve) at the time of primary surgery for early stage BC, although one patient had received neoadjuvant tamoxifen. Six tumor samples were obtained from patients previously treated with neoadjuvant CTX before resection (CTX-treated), consisting entirely of grade two or three IDC. Notably, three of six CTX-treated tumors were HER2/neu-positive, compared with only 1 of 14 CTX-naïve tumors, whereas both groups contained roughly equivalent percentages of tumors negative for estrogen, progesterone, and HER2 receptors (triple negative). Details of tumor pathology are outlined in Table S1. Ipsilateral nonadjacent tissue was also obtained from seven CTX-naïve and four CTX-treated patients

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for use as "normal" tissue, in addition to tissues from two contralateral prophylactic mastectomies from patients with ipsilateral ductal carcinoma in situ (DCIS).

Immune infiltrates detected with the pan-leukocyte marker CD45 were present in both normal and tumor tissue, but with substantially increased density in BC (Fig. 1*A*). Leukocyte subsets were evaluated by using a combination of lineage markers to identify specific subpopulations (Figs. S1 and S2), with the complexity of these populations shown in Fig. 1*B* as a percentage of the total number of $CD45^+$ cells in each sample. BC tissues from CTX-naïve patients contained infiltrates dominated by T lymphocytes ($CD3\varepsilon^+$), with minor populations of natural killer cells ($CD3\varepsilon^-CD56^+NKG2D^+$) and B lymphocytes ($CD19/20^+HLA\text{-}DR^+CD3^-$). In comparison, myeloid-lineage cells including macrophages ($CD14^{hi}CD11b^+HLA\text{-}DR^+$), mast cells ($Fc\varepsilon R1\alpha^+CD117^+CD11b^-CD49d^+$) and neutrophils ($CD15^+CD11b^+CD49d^-$) were more evident in the normal tissue from these patients. A similar immune profile was observed in breast tissues obtained from the two prophylactic mastectomies (Fig. 1*B*).

Increased Presence of Myeloid-Lineage Cells in Residual Tumors from Patients Exposed to Neoadjuvant CTX. Comparative analysis of residual BC tissue removed from patients after neoadjuvant CTX revealed an obvious difference in the percentages of myeloid-lineage cells compared with the CTX-naïve group. With some exceptions, this difference included an increased presence of macrophages as a percent of total leukocytes (Fig. 2*A*), as well as by density evaluation of CSF1 receptor (CSF1R)-positive cells in tissue by IHC (Fig. 2*B*). Increased percentages of mast cells (Fig. 2*C*) and neutrophils (Fig. 2*D*) were also evident in most CTX-treated patients, with an ≈14-fold increase in CTX-treated versus CTX-naïve groups. Basophils ($Fc\varepsilon R1\alpha^+CD117^-CD11b^-CD49d^+$; Fig. 2*E*) were highly increased in only one of six CTX-treated samples, whereas the percentage of myeloid dendritic cells ($CD11c^+HLA\text{-}DR^+CD14^{lo/-}$; Fig. 2*F*) was unchanged. Evaluation of plasmacytoid dendritic cells expressing CD85g/ILT7 detected an insufficient number of events for analysis. Thus, with the exception of basophils, dendritic cells, and $CD15^+CD11b^+CD49d^+$ eosinophils—which were present just at a detectable level in the tissues examined—increased presence of myeloid-lineage cells typified residual tumors of women treated with neoadjuvant CTX.

CD68 Is Not a Macrophage-Specific Marker in Human BC. Macrophages are well established as regulators of murine mammary tumorigenesis (30), where they can represent up to 80% of leukocytes present within late stage mammary carcinomas (1). In human BC, immunoreactivity for CD68 has been used extensively for identification of macrophages, with $CD68^+$ cell density associated with reduced overall survival (6, 11, 14, 15).

The high number of $CD68^+$ cells reported in the literature, and shown in Fig. 3*A*, was in contrast to the limited number of $CD14^{hi}CD11b^+HLA\text{-}DR^+$ macrophages observed by flow cytometry in the BC suspensions examined (Figs. 1*B* and 2*A*). To understand this discrepancy, we first evaluated CD68 expression in BC tissue sections, compared with CD163 (a hemoglobin scavenger receptor also commonly used as a marker for macrophages) and CSF1R (Fig. 3*A*). This comparative analysis revealed a lack of correlation in cell density among the three markers. We next evaluated frozen BC tissue sections by confocal microscopy after immunofluorescent detection of CD68 in combination with CSF1R or CD45 (Fig. 3*B*). Although all cells expressing high levels of the CSF1R also expressed CD68, there was a distinct population of $CD68^+$ cells that expressed neither CSF1R nor CD45. CD68 did not significantly colocalize with keratin$^+$ epithelial cells, $CD31^+$ endothelial cells, or smooth muscle actin α-expressing mural cells surrounding vasculature (Fig. 3*C*). This expression contrasted with murine mammary tumors isolated from MMTV-PyMT transgenic mice (17), where $CD68^+$ cells coexpressed both CSF1R and the murine macrophage marker F4/80 (Fig. S3). In agreement with historic literature (31, 32), these results thus indicate that CD68 is not a macrophage-specific marker in human BC.

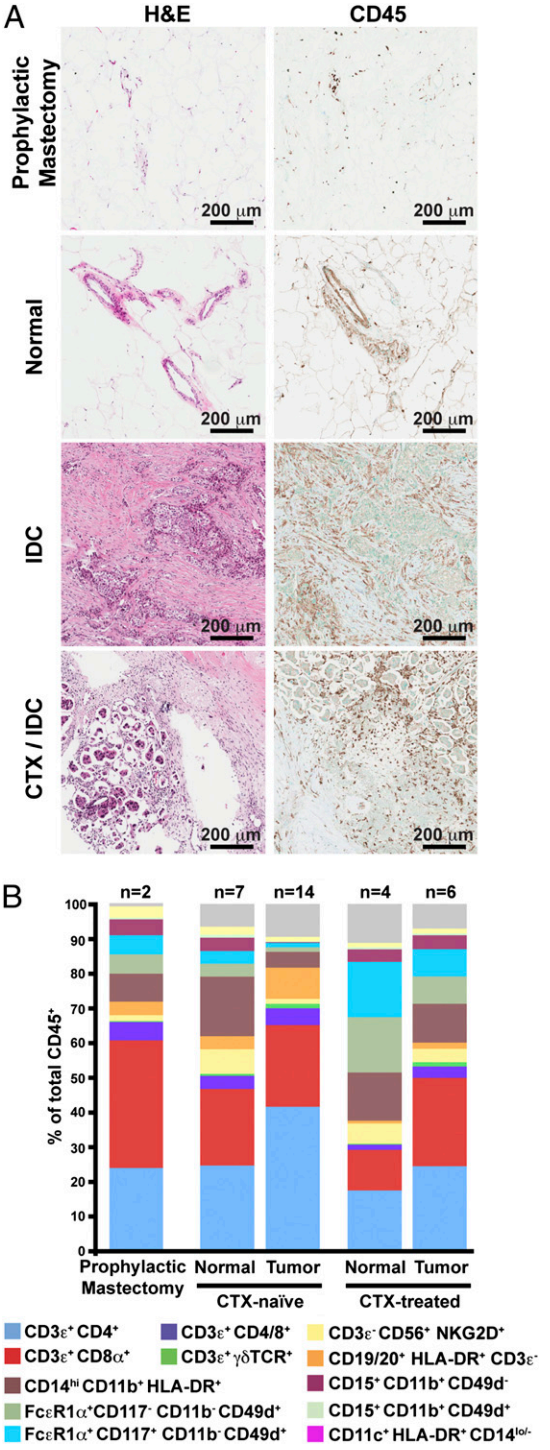

Fig. 1. Leukocyte infiltration of human breast tumors. (*A*) Hematoxylin and eosin (H&E) staining of tissue sections (*Left*) with representative immunohistochemistry for CD45 (*Right*) shown for each. (*B*) Flow cytometric analysis of leukocyte populations within human breast tumors. Results are shown as a percent of total $CD45^+$ cells with markers used to define specific lineages shown below.

Tumor-Infiltrating T Cells Display an Activated Phenotype. To reveal the phenotype of T cells infiltrating BCs, we examined surface marker and chemokine receptor expression of tissue-infiltrating $CD4^+$ and $CD8^+$ T cells (Fig. 4 *A* and *B*). Specifically, both $CD4^+$ and $CD8^+$ T cells displayed increased expression of activation markers CD69 and HLA-DR compared with peripheral

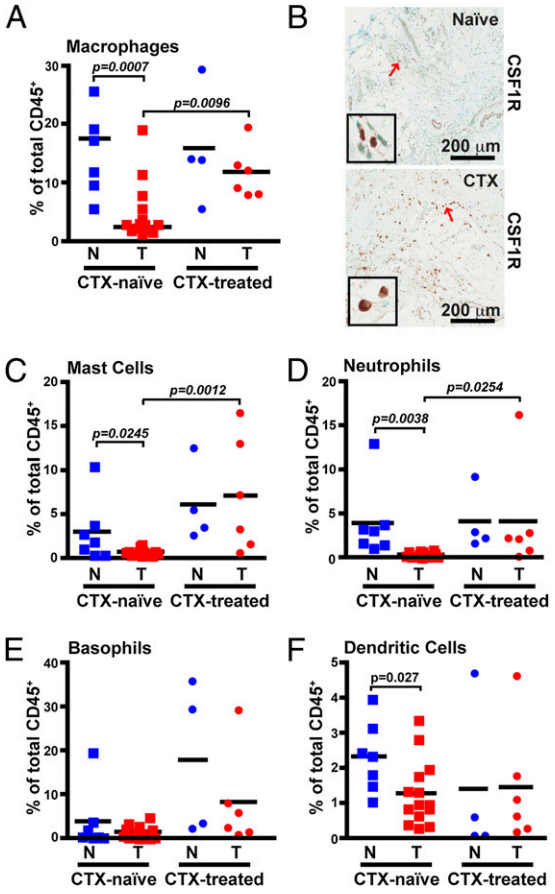

Fig. 2. Increased myeloid-lineage leukocyte infiltration within CTX-treated patients. (*A*) $CD14^{hi}CD11b^{+}HLA\text{-}DR^{+}$ macrophages shown as a percent of total $CD45^{+}$ cells as determined by flow cytometry. (*B*) Representative immunohistochemistry for CSF1R in tumors from either CTX-naïve (*Upper*) or CTX-treated (*Lower*) patients. Red arrows indicate cells displayed in enlarged insets. $Fc\varepsilon R1\alpha^{+}CD117^{+}CD11b^{-}CD49d^{+}$ mast cells (*C*), $CD15^{+}CD11b^{+}CD49d^{-}$ neutrophils (*D*), $Fc\varepsilon R1\alpha^{+}CD117^{-}CD11b^{-}CD49d^{+}$ basophils (*E*), and $CD11c^{+}HLA\text{-}DR^{+}CD14^{lo/-}$ (*F*) DCs shown as a percent of total $CD45^{+}$ cells. N, nonadjacent normal; T, tumor.

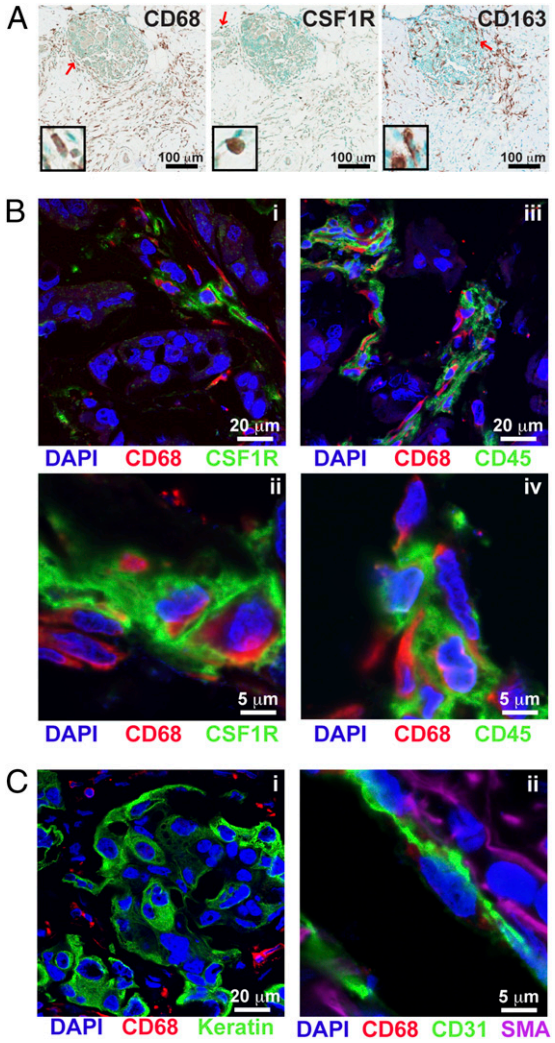

Fig. 3. CD68 is not a specific macrophage marker in human breast tumor tissue. (*A*) Representative immunohistochemistry within tumors for CD68 (*Left*), CSF1R (*Center*), and CD163 (*Right*) in serial sections from a CTX-treated patient. Red arrows indicate cells displayed in enlarged insets. (*B*) Immunofluorescent staining of human breast tumors for CD68 (red) in conjunction with CSF1R (*i* and *ii*) or CD45 (*iii* and *iv*). (C) Immunofluorescent staining for CD68 (red) in conjunction with pan-keratin (green; *i*), or CD31 (green) and smooth muscle actin-α (SMA; purple; *ii*).

blood T cells, with a corresponding loss of markers for naïve T cells, CD45RA, and CCR7. Furthermore, although all T cells constitutively expressed the costimulatory receptor CD28 (Fig. S4*A*), expression of CD27, another costimulatory receptor, was reduced in a large proportion of tissue-infiltrating cells, indicative of shedding after interaction with its ligand CD70 (33) and potential acquisition of effector functions (34, 35). $CD4^{+}$ and $CD8^{+}$ T cells also displayed substantially up-regulated expression of chemokine receptors CCR4 and CCR5 (Fig. 4*B*), and although $CD8^{+}$ T cells constitutively expressed CXCR3, tissue-infiltrating $CD4^{+}$ T cells exhibited higher CXCR3 expression than their counterparts in peripheral blood. Surface marker expression by tissue-infiltrating $CD4^{+}$ and $CD8^{+}$ T cells was subtly different between tumor and benign tissue in some samples; however, these changes were not consistent across patients, or between CTX-naïve and CTX-treated groups (Fig. S4*B*).

Altered Lymphocyte Balance in Residual Tumors After Neoadjuvant CTX. Although we observed no difference in the percent of $CD3e^{-}CD56^{+}NKG2D^{+}$ natural killer (NK) cells (Fig. 5*A*), higher levels of $CD19/CD20^{+}HLA\text{-}DR^{+}$ B cells were evident in several CTX-naïve tumors compared with both normal tissue and CTX-treated tumors. As has been reported (36), B cells were clustered together in association with T cells (Fig. 5*C*). Notably, $CD4^{+}$ T cells as a percent of the total $CD45^{+}$ population were also increased in CTX-naïve tumors compared with both normal tissue and residual postneoadjuvant tumors (Fig. 5*D*). As the percent of $CD8^{+}$ T cells was unchanged (Fig. 5*E*), the lower percentage of $CD4^{+}$ T cells within the CTX-treated group resulted in an increased CD8 to CD4 ratio (Fig. 5*F*). Although it was unclear whether the density of $CD8^{+}$ cells in CTX-treated residual tumors was increased (Fig. 5*G*), the number of cells expressing granzyme B was strikingly evident in two of six CTX-treated tumors (Fig. 5*H*), whereas minimal granzyme B staining was observed in CTX-naïve tumors, even in areas with high numbers of $CD8^{+}$ T cells (Fig. 5*I*).

Despite the reduced percentage of $CD4^{+}$ T cells in tumors from CTX-treated patients, there was no change in the density of IHC detected regulatory T cells expressing FoxP3 (Fig. S5*A*), which was specifically expressed by $CD3^{+}CD4^{+}$ cells in the tumor (Fig. S5*B*). Gating on $CD25^{hi}$ cells, consisting of >80% $FoxP3^{+}$ cells in all samples tested, also revealed that the relative percentage of these cells was invariant between groups (Fig. S5*C*). Phenotypically, $CD4^{+}FoxP3^{+}$ cells displayed an activated phenotype with equivalent surface levels of CD45RO and CD69

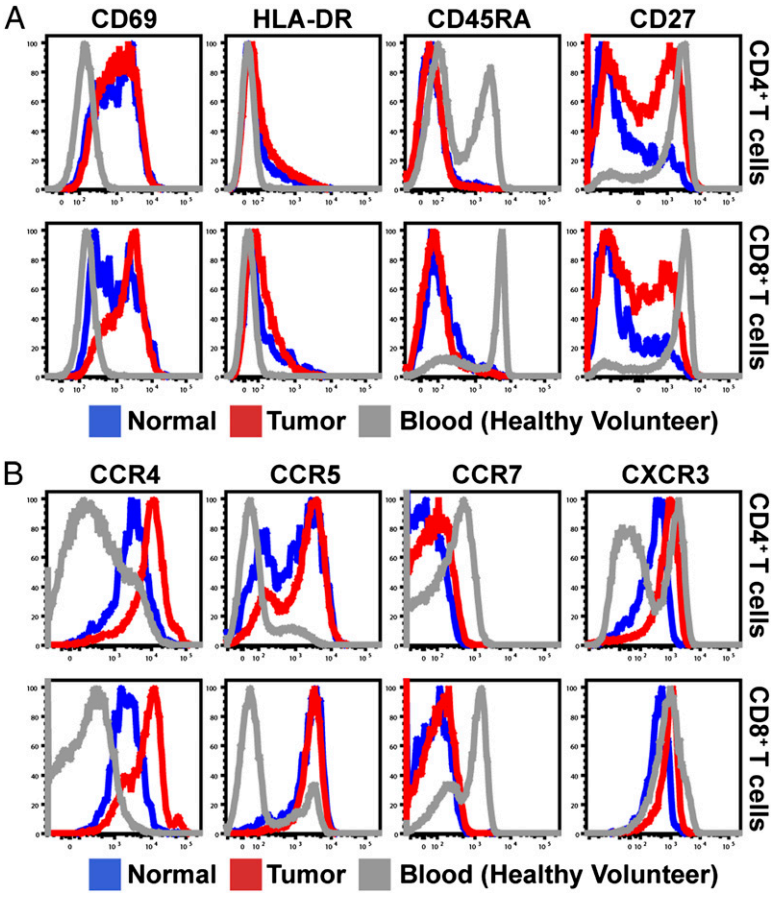

Fig. 4. Tissue-infiltrating T cells display an activated phenotype. (*A* and *B*) Representative histograms of $CD3^+CD4^+$ (*Upper*) or $CD3^+CD8^+$ (*Lower*) T cells isolated from a single CTX-treated patient with both normal (blue) and tumor (red) tissue. Expression of activation markers CD69 (*Left*), HLA-DR (*Center Left*), CD45RA (*Center Right*), and CD27 (*Right*) are shown in *A*, and expression of chemokine receptors CCR4 (*Left*), CCR5 (*Center Left*), CCR7 (*Center Right*) and CXCR3 (*Right*) are shown in *B*.

to $CD4^+FoxP3^-$ cells and, as has been reported for cells in peripheral blood (37), expressed lower levels of CD127 (Fig. S5*D*). Interestingly, although not all $FoxP3^+$ cells expressed HLA-DR, they did comprise the majority of HLA-DR–expressing $CD4^+$ T cells, in addition to coexpressing high levels of CD25.

These data collectively reveal a shift within tumors toward a T_H2-type response in BC characterized by increased presence of B cells and $CD4^+$ T cells, in comparison with nonadjacent normal breast tissue. This shift is reversed in tumors obtained from CTX-treated patients, with additional evidence of a cytotoxic T-cell response through a more favorable CD8/CD4 T-cell ratio and increased presence of granyzme B-expressing lymphocytes; thus, even residual tumors from patients with a poor response to CTX may contain immune microenvironments that are more favorably skewed towards an anti-tumor, T_H1-type immune response.

Discussion

Herein, we present a detailed description of leukocyte complexity in BC as evaluated in a cohort of CTX-naïve patients with stage 2/3 tumors, compared with patients with significant residual disease after neoadjuvant CTX. T lymphocytes were the major population within both CTX-naïve and CTX-treated tumors, found almost exclusively in an activated state as determined by increased expression of CD69 and chemokine receptors, with simultaneous loss of naïve markers CCR7 and CD45RA. The presence of activation markers, however, does not definitively demonstrate that intratumoral T cells are functionally active. In fact, granzyme B expression was minimal within tumors from CTX-naïve patients, suggesting negligible cytotoxic activity by infiltrating $CD8^+$ T cells. In comparison, granzyme B was highly expressed in one-third of the CTX-treated tumors, suggestive of a more cytotoxic T-cell response within some tumors after exposure to CTX.

Importantly, residual tumors from CTX-treated patients also contained reduced percentages of B cells and $CD4^+$ T cells. Tumor-infiltrating $CD4^+$ T cells in BC are known to express the T_H2 cytokines IL-4 and IL-13 concomitantly with the production of IFN-γ (25, 26), consistent with coexpression of CXCR3 and CCR4 (38, 39) as we observed herein. It remains to be determined whether cytokine production by $CD4^+$ T cells is altered by neoadjuvant CTX; however, the combined reduction in both $CD4^+$ T cells and B cells is indicative of a favorable shift away from a T_H2 microenvironment. This shift could be relevant for TAM function, as has been described in the MMTV-PyMT model where TAMs are programmed by IL-4 toward a T_H2 phenotype (1), and more recently in pancreatic ductal adenocarcinoma during treatment where an agonist CD40 monoclonal antibody fostered cytolytic macrophage activities (40).

Although the extent of lymphocyte infiltration has been associated with improved prognosis in subsets of patients (2, 3), and with pCR after CTX (41, 42), information regarding the relationship between individual lymphocyte subsets to survival is limited. High FoxP3 counts correlate with reduced overall and relapse-free survival in estrogen receptor (ER)-positive tumors (43), and pCR to neoadjuvant CTX is associated with reduced FoxP3 grading (44, 45). Although two studies examining T-cell infiltration by flow cytometry found conflicting results regarding the CD8:CD4 ratio and lymph node metastasis (46, 47), the number of $CD8^+$ T cells within tissue has been associated with improved patient survival (48). We have also reported a CD68/CD4/CD8 immune signature predicting overall and relapse-free survival, with inverse correlations evident for CD4 when used in conjunction with other markers (6). There is thus an urgent need for additional prospective investigations where multiple parameters of lymphocytic infiltration and functionality are evaluated to determine the most significant biomarker comparisons that predict outcome and guide specific therapy.

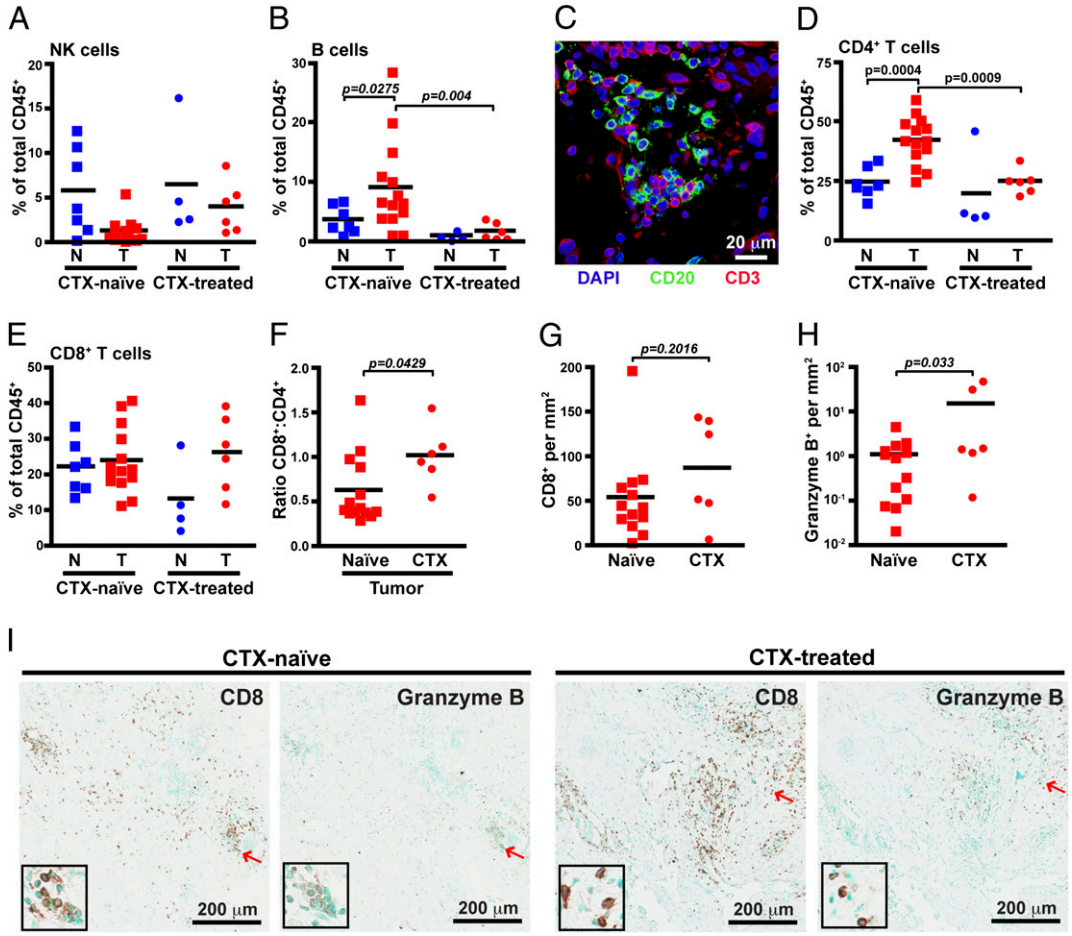

Fig. 5. Improved cytotoxic T-cell response in CTX-treated tumors. CD3ε$^-$CD56$^+$NKG2D$^+$ natural killer cells (*A*) and CD3ε$^-$CD19/20$^+$HLA-DR$^+$ B cells (*B*) shown as a percent of total CD45$^+$ cells as determined by flow cytometry. (*C*) Immunofluorescent staining of tumors for CD20 (green) and CD3 (red). CD3ε$^+$CD4$^+$ T cells (*D*) and CD3ε$^+$CD8$^+$ T cells (*E*) are shown as a percent of total CD45$^+$ cells. (*F*) Ratio of CD8$^+$ to CD4$^+$ T cells within CTX-naive versus CTX-treated tumors. Number of CD8-positive (*G*) and granzyme B-positive (*H*) cells per area as determined by automated counting. (*I*) Representative sections stained with CD8 or granzyme B from CTX-naive (*Left*) or CTX-treated (*Right*) tumors. Red arrows indicate cells displayed in enlarged insets. N, nonadjacent normal; T, tumor.

Although used successfully in multiple studies to relate TAM infiltration with clinically relevant outcomes, our results indicate that CD68 alone cannot accurately evaluate macrophage presence in human breast tissue given that multiple stromal cells express it and that a subset of these are CSF1R- and CD45-negative. We observed that the nonleukocytic CD68$^+$ cells were predominantly located within tumor stroma and, thus, based on this localization and morphology, we speculate that CSF1R$^-$CD68$^+$ cells likely reflect tumor-associated fibroblasts or monocyte-derived fibrocytes in agreement with other reports (31, 32, 49–52). Our findings do not invalidate CD68 as a clinically relevant marker and, importantly, CSF1-response gene signatures have been identified in breast adenocarcinomas that are predictive of recurrence risk and metastasis (53, 54). However, given the important role that fibroblasts (and perhaps fibrocytes) play in fostering aspects of tumorigenesis (55–57), differentiating among macrophages, fibroblasts, and other stromal populations within tumors has the potential to improve diagnostic information currently generated by immunodetection of CD68.

As we have reported for expression of *csf1* mRNA (6), multiple genes encoding myeloid cell chemoattractants are differentially expressed by human BC cell lines, with variable induction of these genes in response to CTX (Fig. S6). Although differential expression between cell lines corresponding to particular subtypes of BC is evident, it is doubtful these cell lines accurately represent the response of BC tumor tissue; thus, we are investigating whether differences in myeloid cell infiltrates reflect distinct molecular subtypes of BC and to what extent these differ in residual tumors from CTX-treated patients.

It is important to acknowledge that leukocyte composition within tumors responding to CTX likely differs substantially from residual or recurrent tumors from patients that have received CTX, given what is known regarding immune responses to CTX-induced cell death (28). However, we recently reported that in mammary carcinomas of MMTV-PyMT mice, blockade of the CSF1-CSF1R pathway critical for TAM recruitment improved response to CTX through a CD8$^+$ T-cell–dependent effect (6). Thus, even though the findings presented herein are based on a small dataset of heterogeneous tumor subtypes, and our results may be biased because of sample selection favoring large and/or less CTX-responsive tumors among the CTX-treated group, the clear distinctions in the myeloid profiles between CTX-naive and CTX-treated tumors is provocative and indicates that a CSF1-targeted strategy may be a promising approach to enhance therapeutic efficacy of cytotoxic CTX, particularly for treatment of refractory BC. Moreover, given the increase in granulocytic populations within tumors resistant to CTX, and the involvement of these cells in regulating immune responses in chronic inflammatory diseases (58–62), these populations may also be functionally relevant, and targeting common pathways of immune suppression within the tumor microenvironment may provide additional therapeutic opportunities to increase efficacy of neoadjuvant CTX.

Materials and Methods

Tissues were collected at the time of surgery from consenting patients at the University of California, San Francisco under approval from the institutional review board. Tumor and ipsilateral nonadjacent normal tissues were collected by a certified pathologist (A.A.) and were prepared for analysis on the day of resection. The percent of macrophages and $CD8^+$ T cells has been reported for a subset of the patients described here (6). Flow cytometry, immunohistochemistry, and immunofluorescence were performed as described (6), with detailed methods contained in *SI Materials and Methods*, and a list of antibodies available in Tables S2 and S3. Statistical differences between two independent groups were determined by using Student's *t* test via Prism 4.0 software (GraphPad Software).

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1. DeNardo DG, et al. (2009) CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell* 16:91–102.
2. Ménard S, et al. (1997) Lymphoid infiltration as a prognostic variable for early-onset breast carcinomas. *Clin Cancer Res* 3:817–819.
3. Pupa SM, et al. (1996) Macrophage infiltrate and prognosis in c-erbB-2-overexpressing breast carcinomas. *J Clin Oncol* 14:85–94.
4. Bingle L, Brown NJ, Lewis CE (2002) The role of tumour-associated macrophages in tumour progression: Implications for new anticancer therapies. *J Pathol* 196:254–265.
5. Mukhtar RA, Nseyo O, Campbell MJ, Esserman LJ (2011) Tumor-associated macrophages in breast cancer as potential biomarkers for new treatments and diagnostics. *Expert Rev Mol Diagn* 11:91–100.
6. DeNardo DG, et al. (2011) Leukocyte complexity in breast cancer predicts overall survival and functionally regulates response to chemotherapy. *Cancer Discovery* 1:54–67.
7. Esserman LJ, et al. (2006) Magnetic resonance imaging captures the biology of ductal carcinoma in situ. *J Clin Oncol* 24:4603–4610.
8. Volodko N, Reiner A, Rudas M, Jakesz R (1998) Tumour-associated macrophages in breast cancer and their prognostic correlations. *Breast* 7:99–105.
9. Lee AH, Happerfield LC, Bobrow LG, Millis RR (1997) Angiogenesis and inflammation in invasive carcinoma of the breast. *J Clin Pathol* 50:669–673.
10. Uzzan B, Nicolas P, Cucherat M, Perret GY (2004) Microvessel density as a prognostic factor in women with breast cancer: A systematic review of the literature and meta-analysis. *Cancer Res* 64:2941–2955.
11. Tsutsui S, et al. (2005) Macrophage infiltration and its prognostic implications in breast cancer: The relationship with VEGF expression and microvessel density. *Oncol Rep* 14:425–431.
12. Bolat F, et al. (2006) Microvessel density, VEGF expression, and tumor-associated macrophages in breast tumors: Correlations with prognostic parameters. *J Exp Clin Cancer Res* 25:365–372.
13. Chen JJ, et al. (2005) Tumor-associated macrophages: The double-edged sword in cancer progression. *J Clin Oncol* 23:953–964.
14. Leek RD, et al. (1996) Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res* 56:4625–4629.
15. Campbell MJ, et al. (2010) Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. *Breast Cancer Res Treat* 128:703–711.
16. Robinson BD, et al. (2009) Tumor microenvironment of metastasis in human breast carcinoma: A potential prognostic marker linked to hematogenous dissemination. *Clin Cancer Res* 15:2433–2441.
17. Guy CT, Cardiff RD, Muller WJ (1992) Induction of mammary tumors by expression of polyomavirus middle T oncogene: A transgenic mouse model for metastatic disease. *Mol Cell Biol* 12:954–961.
18. Lin EY, Nguyen AV, Russell RG, Pollard JW (2001) Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* 193:727–740.
19. Lin EY, et al. (2006) Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res* 66:11238–11246.
20. Stockmann C, et al. (2008) Deletion of vascular endothelial growth factor in myeloid cells accelerates tumorigenesis. *Nature* 456:814–818.
21. Lin EY, et al. (2007) Vascular endothelial growth factor restores delayed tumor progression in tumors depleted of macrophages. *Mol Oncol* 1:288–302.
22. Wyckoff J, et al. (2004) A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res* 64:7022–7029.
23. Wyckoff JB, et al. (2007) Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res* 67:2649–2656.
24. Doedens AL, et al. (2010) Macrophage expression of hypoxia-inducible factor-1 alpha suppresses T-cell function and promotes tumor progression. *Cancer Res* 70:7465–7475.
25. Aspord C, et al. (2007) Breast cancer instructs dendritic cells to prime interleukin 13-secreting CD4+ T cells that facilitate tumor development. *J Exp Med* 204:1037–1047.
26. Pedroza-Gonzalez A, et al. (2011) Thymic stromal lymphopoietin fosters human breast tumor growth by promoting type 2 inflammation. *J Exp Med* 208:479–490.
27. Gocheva V, et al. (2010) IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev* 24:241–255.
28. Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G (2008) Immunological aspects of cancer chemotherapy. *Nat Rev Immunol* 8:59–73.
29. Allan CP, Turtle CJ, Mainwaring PN, Pyke C, Hart DN (2004) The immune response to breast cancer, and the case for DC immunotherapy. *Cytotherapy* 6:154–163.
30. Qian BZ, Pollard JW (2010) Macrophage diversity enhances tumor progression and metastasis. *Cell* 141:39–51.
31. Pulford KA, Sipos A, Cordell JL, Stross WP, Mason DY (1990) Distribution of the CD68 macrophage/myeloid associated antigen. *Int Immunol* 2:973–980.
32. Kunz-Schughart LA, et al. (2003) [The "classical" macrophage marker CD68 is strongly expressed in primary human fibroblasts]. *Verh Dtsch Ges Pathol* 87:215–223.
33. Hintzen RQ, et al. (1994) Characterization of the human CD27 ligand, a novel member of the TNF gene family. *J Immunol* 152:1762–1773.
34. Hamann D, et al. (1997) Phenotypic and functional separation of memory and effector human CD8+ T cells. *J Exp Med* 186:1407–1418.
35. Okada R, Kondo T, Matsuki F, Takata H, Takiguchi M (2008) Phenotypic classification of human CD4+ T cell subsets and their differentiation. *Int Immunol* 20:1189–1199.
36. Nelson BH (2010) CD20+ B cells: The other tumor-infiltrating lymphocytes. *J Immunol* 185:4977–4982.
37. Liu W, et al. (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 203:1701–1711.
38. Kunkel EJ, et al. (2002) Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by human tissue-infiltrating lymphocytes. *Am J Pathol* 160:347–355.
39. Kim CH, et al. (2001) Rules of chemokine receptor association with T cell polarization in vivo. *J Clin Invest* 108:1331–1339.
40. Beatty GL, et al. (2011) CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. *Science* 331:1612–1616.
41. Demaria S, et al. (2001) Development of tumor-infiltrating lymphocytes in breast cancer after neoadjuvant paclitaxel chemotherapy. *Clin Cancer Res* 7:3025–3030.
42. Denkert C, et al. (2010) Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *J Clin Oncol* 28:105–113.
43. Bates GJ, et al. (2006) Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 24:5373–5380.
44. Ladoire S, et al. (2008) Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. *Clin Cancer Res* 14:2413–2420.
45. de Kruijf EM, et al. (2010) The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. *Clin Cancer Res* 16:1272–1280.
46. Macchetti AH, et al. (2006) Tumor-infiltrating CD4+ T lymphocytes in early breast cancer reflect lymph node involvement. *Clinics (Sao Paulo)* 61:203–208.
47. Sheu BC, et al. (2008) Clinical significance of tumor-infiltrating lymphocytes in neoplastic progression and lymph node metastasis of human breast cancer. *Breast* 17:604–610.
48. Mahmoud SM, et al. (2011) Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol* 29:1949–1955.
49. Gottfried E, et al. (2008) Expression of CD68 in non-myeloid cell types. *Scand J Immunol* 67:453–463.
50. Pilling D, Fan T, Huang D, Kaul B, Gomer RH (2009) Identification of markers that distinguish monocyte-derived fibrocytes from monocytes, macrophages, and fibroblasts. *PLoS ONE* 4:e7475.
51. Shao DD, Suresh R, Vakil V, Gomer RH, Pilling D (2008) Pivotal Advance: Th-1 cytokines inhibit, and Th-2 cytokines promote fibrocyte differentiation. *J Leukoc Biol* 83:1323–1333.
52. Azambuja D, et al. (May 2, 2011) Lack of association of tumor-associated macrophages with clinical outcome in patients with classical Hodgkin's lymphoma. *Ann Oncol*, 10.1093/annonc/mdr157.
53. Sharma M, et al. (2010) Analysis of stromal signatures in the tumor microenvironment of ductal carcinoma in situ. *Breast Cancer Res Treat* 123:397–404.
54. Beck AH, et al. (2009) The macrophage colony-stimulating factor 1 response signature in breast carcinoma. *Clin Cancer Res* 15:778–787.
55. Trimboli AJ, et al. (2009) Pten in stromal fibroblasts suppresses mammary epithelial tumours. *Nature* 461:1084–1091.
56. Erez N, Truitt M, Olson P, Arron ST, Hanahan D (2010) Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. *Cancer Cell* 17:135–147.
57. Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. *Nat Rev Cancer* 6:392–401.
58. Schmielau J, Finn OJ (2001) Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients. *Cancer Res* 61:4756–4760.
59. Fridlender ZG, et al. (2009) Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* 16:183–194.
60. Eller K, et al. (2011) IL-9 production by regulatory T cells recruits mast cells that are essential for regulatory T cell-induced immune suppression. *J Immunol* 186:83–91.
61. Galli SJ, Nakae S, Tsai M (2005) Mast cells in the development of adaptive immune responses. *Nat Immunol* 6:135–142.
62. Perrigoue JG, et al. (2009) MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat Immunol* 10:697–705.

LISA M. COUSSENS, PH.D.

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REGULATION OF PROTUMOR IMMUNITY AND CANCER DEVELOPMENT

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Clinical and experimental studies have established that chronic infiltration of neoplastic tissue by leukocytes, i.e., chronic inflammation, promotes development and/or progression of various solid tumors[1,2]; however, the organ-specific cellular and molecular programs that favor pro-tumor, as opposed to anti-tumor immunity by leukocytes are incompletely understood. While some leukocytes certainly exhibit anti-tumor activity, i.e., cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells[3], other leukocytes, most notably mast cells, $CD4^+$ T cells, B lymphocytes, dendritic cells (DCs), granulocytes, immature monocytes and macrophages exhibit more bipolar roles, by virtue of their capacity to either hinder or potentiate tumor progression[1,2]. A major question regarding these disparate leukocytes bioactivities is the degree to which their various pro- or anti-cancer activities are regulated by tissue-specificity and/or are responsive to individual oncogenic or tumor suppressive gene programming in early neoplastic tissue.

Leukocytes and Breast Carcinogenesis: In the breast, cancer development is in part characterized by a significant increase in both innate and adaptive immune cells, with B and T lymphocytes and macrophages representing the most abundant leukocytes present in neoplastic stroma[4]. Retrospective clinical studies examining identity of leukocytes in human breast cancer have revealed that high immunoglobulin (Ig) levels in tumor stoma (and serum), and increased presence of extra follicular B cells, T regulatory (T_{reg}) cells, high ratios of CD4/CD8 or T_H2/T_H1 T lymphocytes in primary tumors or in draining lymph nodes correlates with tumor grade, stage and overall patient survival[3]. On the other hand, experimental studies have demonstrated that macrophages in primary mammary adenocarcinomas regulate late-stage carcinogenesis by virtue of their pro-angiogenic properties[5,6], as well as fostering pulmonary metastasis by providing epidermal growth factor (EGF) and cathepsin proteases[7] to malignant mammary epithelial cells (MECs) and thereby enhancing their invasive (and metastatic) behavior[8,9]. Based on these seemingly disparate observations, we sought to determine if adaptive immunity also fostered malignancy in the breast by regulating the phenotype or effector functions of tumor-associated macrophages (TAMs) and either activated their pro-tumor properties or alternatively by suppressing their anti-tumor capabilities.

Utilizing the MMTV-PyMT mouse model of mammary carcinogenesis[10], we revealed a provocative role for $CD4^+$ T cells as potentiators of peripheral blood dissemination and pulmonary metastasis of malignant mammary adenocarcinomas through their ability to regulate pro-tumor properties of TAMs[11]. Specifically, T_H2-polarized $CD4^+$ T cells secrete high levels of interleukin (IL)-4 and thereby regulate M1 and M2-type TAM bioactivity by activation of IL4Rα-signaling cascades. M2-TAMs in turn promote invasive behavior of malignant MECs by high level production of cathepsin protease activity[7] and EGF that subsequently activates MEC invasion and EGF receptor signaling programs, activities that are essential for entry into peripheral blood, dissemination and outgrowth in the lung. These findings indicate that when $CD4^+$ T lymphocytes are present in a T_H2-type tumor microenvironment, they promote metastasis by regulating the pro-tumor properties of TAMs mediated by IL4Rα-signaling, as opposed to limiting or eradicating malignant cells by engaging cytotoxic mechanisms. These realizations provide rational for development of anti-cancer therapeutics that neutralize pro-tumor properties of IL4Rα-based signaling in both adaptive and innate immune cells in the tumor microenvironment and periphery, that when delivered in combination with cytotoxic drugs or therapeutics bolstering anti-tumor immunity, may extend survival of patients with advanced disease.

Leukocytes and Squamous Carcinogenesis: B lymphocytes constitute a central component of adaptive immunity and not only serve in antibody production but also as antigen-presenting cell; thus, B lymphocyte expression of major histocompatability complex (MHC) and co-stimulatory molecules as well as secretion of pro-inflammatory cytokines induces optimal $CD4^+$ and $CD8^+$ T cells activation,

expansion, memory T lymphocyte formation and antigenic spreading. As such, B cells have been historically associated with anti-tumor immunity. More recently, the heterogeneity of B lymphocyte responses has been recognized and diverse B cell subtypes with either pro-immune or regulatory properties have been identified *in vivo*. In particular, regulatory B lymphocytes, which include various flavors of IL-10 producing cells, have been identified in the context of autoimmune diseases that exert anti-inflammatory activities[12,13]. However, the role of these individual B lymphocyte subpopulations in malignant disease has yet to be fully elucidated.

Using a transgenic mouse model of multi-stage epithelial carcinogenesis, *i.e.*, K14-HPV16 mice[14], we previously revealed that adaptive immunity is an important regulator of inflammation-associated cancer development[15]. Combined B and T lymphocyte-deficiency in HPV16 mice, *e.g*. HPV16/RAG1$^{-/-}$ mice, resulted in a failure to initiate and/or sustain leukocyte infiltration during premalignancy[15]. As a consequence, tissue remodeling, angiogenesis and epithelial hyperproliferation were significantly reduced, culminating in attenuated premalignant progression and a 43% reduction in carcinoma incidence[15]. Importantly, adoptive transfer of B lymphocytes or serum from HPV16 mice into HPV16/RAG1$^{-/-}$ mice reinstated chronic inflammation in premalignant tissues, indicating that B cell-derived soluble mediators were necessary to potentiate malignant progression. More recently, we revealed that B cell-derived IgGs regulate neoplastic progression and subsequent carcinoma development by engagement of Fcγ receptors (FcγR) expressed on resident and recruited immune cells[16]. Specifically, we found that immune complex (IC)-stimulation of leukocyte FcRγ is critical for establishing a pro-tumor microenvironment in premalignant tissue that directs not only recruitment of leukocytes from peripheral blood, but also leukocyte composition, phenotype and bioeffector functions once within neoplastic tissue. As such, proangiogenic and protumorigenic functions of mast cells and macrophages are differentially regulated by humoral immunity and functionally contribute to squamous carcinogenesis. These findings have broad clinical implications as they reveal critical signaling pathways regulated by humoral immunity and FcRγ to target therapeutically in patients suffering from chronic inflammatory diseases at risk for cancer, as well as individuals harboring premalignant lesions where chronic inflammation compromises tissue integrity and enhances risk of malignancy.

Summary: While many factors regulate leukocyte propensity to either promote or repress tumor development, in tissue- and/or oncogene-specific manners, polarized T_H2-type adaptive immune responses foster pro- as opposed to anti- tumor programming of myeloid cells that in turn directly regulate many of the "hallmarks" of solid tumor development[17]. Neutralizing these various "pro-tumor" regulatory pathways may provide relief for some aspects of late-stage cancer development as monotherapy, but more likely when combined with cytotoxic-, targeted- and/or immuno-therapy will provide a survival advantage by bolstering induction of anti-tumor bioactivities of tumor-associated leukocytes that extend efficacy of therapy.

References Cited:

1. de Visser, K.E., Eichten, A. & Coussens, L.M. Paradoxical roles of the immune system during cancer development. *Nature Reviews Cancer* **6**, 24-37 (2006).
2. Balkwill, F., Charles, K.A. & Mantovani, A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* **7**, 211-217 (2005).
3. Ruffell, B., Denardo, D.G., Affara, N.I. & Coussens, L.M. Lymphocytes in cancer development: Polarization towards pro-tumor immunity. *Cytokine Growth Factor Rev* (2009).
4. DeNardo, D.G. & Coussens, L.M. Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression. *Breast Cancer Res* **9**, 212 (2007).
5. Lin, E.Y., Nguyen, A.V., Russell, R.G. & Pollard, J.W. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* **193**, 727-740 (2001).
6. Lin, E.Y. & Pollard, J.W. Tumor-associated macrophages press the angiogenic switch in breast cancer. *Cancer Res* **67**, 5064-5066 (2007).
7. Gocheva, V.*, et al.* IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev* **24**, 241-255 (2010).
8. Wyckoff, J.B.*, et al.* Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res* **67**, 2649-2656 (2007).
9. Philippar, U.*, et al.* A Mena invasion isoform potentiates EGF-induced carcinoma cell invasion and metastasis. *Dev Cell* **15**, 813-828 (2008).

10. Guy, C.T., Cardiff, R.D. & Muller, W.J. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol Cell Biol* **12**, 954-961 (1992).
11. DeNardo, D.G.*, et al.* CD4+ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell* **16**, 91-102 (2009).
12. DiLillo, D.J., Matsushita, T. & Tedder, T.F. B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. *Ann N Y Acad Sci* **1183**, 38-57.
13. Mizoguchi, A. & Bhan, A.K. A case for regulatory B cells. *J Immunol* **176**, 705-710 (2006).
14. Coussens, L.M., Hanahan, D. & Arbeit, J.M. Genetic predisposition and parameters of malignant progression in K14- HPV16 transgenic mice. *Am J Path* **149**, 1899-1917 (1996).
15. de Visser, K.E., Korets, L.V. & Coussens, L.M. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell* **7**, 411-423 (2005).
16. Andreu, P.*, et al.* FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell* **17**, 121-134 (2010).
17. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).

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References Cited:

1. de Visser, K.E., A. Eichten, and L.M. Coussens, *Paradoxical roles of the immune system during cancer development.* Nature Reviews Cancer, 2006. **6**: p. 24-37.
2. Balkwill, F., K.A. Charles, and A. Mantovani, *Smoldering and polarized inflammation in the initiation and promotion of malignant disease.* Cancer Cell, 2005. **7**(3): p. 211-217.
3. Wu, J. and L.L. Lanier, *Natural killer cells and cancer.* Adv Cancer Res, 2003. **90**: p. 127-56.
4. Bui, J.D. and R.D. Schreiber, *Cancer immunosurveillance, immunoediting and inflammation: independent or interdependent processes?* Curr Opin Immunol, 2007. **19**(2): p. 203-8.
5. Dunn, G.P., L.J. Old, and R.D. Schreiber, *The immunobiology of cancer immunosurveillance and immunoediting.* Immunity, 2004. **21**(2): p. 137-148.
6. DeNardo, D.G. and L.M. Coussens, *Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression.* Breast Cancer Res, 2007. **9**(4): p. 212.
7. Coronella, J.A., et al., *Evidence for an antigen-driven humoral immune response in medullary ductal breast cancer.* Cancer Res, 2001. **61**(21): p. 7889-99.
8. Coronella-Wood, J.A. and E.M. Hersh, *Naturally occurring B-cell responses to breast cancer.* Cancer Immunol Immunother, 2003. **52**(12): p. 715-38.
9. Fernandez Madrid, F., *Autoantibodies in breast cancer sera: candidate biomarkers and reporters of tumorigenesis.* Cancer Lett, 2005. **230**(2): p. 187-98.
10. Kohrt, H.E., et al., *Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer.* PLoS Med, 2005. **2**(9): p. e284.
11. Bates, G.J., et al., *Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse.* J Clin Oncol, 2006. **24**(34): p. 5373-80.
12. Lin, E.Y., et al., *Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy.* J Exp Med, 2001. **193**(6): p. 727-740.
13. Lin, E.Y. and J.W. Pollard, *Tumor-associated macrophages press the angiogenic switch in breast cancer.* Cancer Res, 2007. **67**(11): p. 5064-6.
14. Gocheva, V., et al., *IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion.* Genes Dev, 2010. **24**(3): p. 241-55.
15. Wyckoff, J.B., et al., *Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors.* Cancer Res, 2007. **67**(6): p. 2649-56.
16. Philippar, U., et al., *A Mena invasion isoform potentiates EGF-induced carcinoma cell invasion and metastasis.* Dev Cell, 2008. **15**(6): p. 813-28.
17. Guy, C.T., R.D. Cardiff, and W.J. Muller, *Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease.* Mol Cell Biol, 1992. **12**(3): p. 954-61.
18. DeNardo, D.G., et al., *CD4+ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages.* Cancer Cell, 2009. **16**(2): p. 91-102.
19. DiLillo, D.J., T. Matsushita, and T.F. Tedder, *B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer.* Ann N Y Acad Sci. **1183**: p. 38-57.
20. Mizoguchi, A. and A.K. Bhan, *A case for regulatory B cells.* J Immunol, 2006. **176**(2): p. 705-10.

21. Coussens, L.M., D. Hanahan, and J.M. Arbeit, *Genetic predisposition and parameters of malignant progression in K14- HPV16 transgenic mice.* Am J Path, 1996. **149**(6): p. 1899-1917.
22. de Visser, K.E., L.V. Korets, and L.M. Coussens, *De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent.* Cancer Cell, 2005. **7**(5): p. 411-423.
23. Andreu, P., et al., *FcRgamma activation regulates inflammation-associated squamous carcinogenesis.* Cancer Cell, 2010. **17**: p. 121-134.
24. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.

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LISA M. COUSSENS, PH.D.

Cold Spring Harbor Laboratory, CSH NY USA

Inflammation and Cancer:

Polarized Immune Responses Regulate Cancer Development

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The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have recently appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion during skin carcinogenesis is B cell, Ig and FcγR–dependent, during mammary carcinogenesis by contrast, T_H2-polarized $CD4^+$ T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of M2-polarized macrophages and dendritic cells, that together regulate metastasis of malignant mammary epithelial cells to lung. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and thoughts on how these properties can be harnessed for effective anticancer therapeutics.

Funding from the NIH/NCI and Department of Defense Era of Hope Scholar Award and Program in Mesothelioma

Oral Presentation at the 2010 Annual Meeting of the American Association for Cancer Research (AACR), Washington DC, USA.

Targeting Macrophages as a Novel Therapeutic Approach for Malignant Pleural Mesothelioma

Nikita Kolhatkar[1,3], Magnus Johansson[1], Dario Barbone[3], Adam Yagui-Beltrán[2], Raphael Bueno[4], David J. Sugarbaker[4], David M Jablons[2,5], V. Courtney Broaddus[3], Lisa M Coussens[1,5]

Departments of [1]Pathology, [2]Surgery, [3]Lung Biology Center, and [5]Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco. [4]Division of Thoracic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts.

Mesothelioma is a life-threatening tumor, induced by inhalation of asbestos fibers, which is largely resistant to most chemotherapeutic approaches. One feasible approach could be to harness the power of the immune system to increase the chemosensitivity of mesotheliomas. Using a combination of immunohistochemistry and flow cytometry to analyze the leukocyte compositions of human mesotheliomas, we have found that 1) epithelioid and mixed mesothelioma tumor subtypes have a higher degree of immune cell Infiltration, when compared to sarcomatous tumors, and 2) mesothelioma tumors have large infiltrations of macrophages (31 ± 4.6% of the inflammatory cell population [$CD45^+$]). Indeed, the percentage of macrophages in mesotheliomas exceeded that found in other thoracic malignancies thus far evaluated (NSCLC cancer, 9%; esophageal, 4%). In view of recent data indicating that macrophages can be targeted therapeutically to minimize some aspects of cancer development, we investigated whether macrophages could be targeted to enhance chemosensitivity of human mesotheliomas. To address this question, we adapted a 3-dimensional spheroid growth model, enabling heterotypic culture of mesotheliomal cells with macrophages. We found that mesothelioma chemoresistance can be lowered by co-incubation with macrophages. However, the magnitude of the response was dictated by specific macrophage phenotype. Macrophage phenotype and bioactivity is modulated by Th1 versus Th2 cytokine exposure that in turn regulate either an M1 (IFN-γ & LPS) or M2 (IL-4) phenotype. M1-polarized macrophages increased the response of malignant mesothelioma spheroids to pro-apoptotic chemotherapeutic agents, such as TRAIL and gemcitabine. Furthermore, our preliminary data indicate that primary human tumor-associated macrophages, isolated from untreated malignant mesotheliomas, have similar pro-apoptotic effects when polarized with M1 cytokines, suggesting that cytokine re-polarization of macrophages in mesothelioma tumors to an M1 phenotype could augment therapeutic efficacy.

This work was supported by grants from the NCI and a DoD Mesothelioma Program grant PR080717 to Broaddus and Coussens

Abstract for Invited Presentation: International Mesothelioma Interest Group International Meeting

August 31- Sept. 2, 2010
Kyoto, JAPAN

Targeting macrophages as a novel therapeutic approach for malignant pleural mesothelioma

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Mesothelioma is a life-threatening tumor, induced by inhalation of asbestos fibers, which is largely resistant to most chemotherapeutic approaches. One feasible approach could be to harness the power of the immune system to increase the chemosensitivity of mesotheliomas. Using a combination of immunohistochemistry and flow cytometry to analyze the leukocyte compositions of human mesotheliomas, we have found that 1) epithelioid and mixed mesothelioma tumor subtypes have a higher degree of immune cell Infiltration, when compared to sarcomatous tumors, and 2) mesothelioma tumors have large infiltrations of macrophages (31 +/- 4.6% of the inflammatory cell population (CD45+)). Indeed, the percentage of macrophages in mesothelioma exceeded that found in other thoracic malignancies thus far evaluated (NSCLC cancer, 9%; esophageal, 4%). In view of recent data indicating that macrophages can be targeted therapeutically to minimize some aspects of cancer development, we investigated whether macrophages could be targeted to enhance chemosensitivity of human mesotheliomas. To address this question, we adapted a 3-dimensional spheroid growth model, enabling heterotypic culture of mesotheliomal cells with macrophages. We found that mesothelioma chemoresistance can be lowered by co-incubation with macrophages. However, the magnitude of the response was dictated by macrophage phenotype. Macrophage phenotype and bioactivity is modulated by Th1 versus Th2 cytokine exposure that in turn regulate either an M1 (IFN-gamma & LPS) or M2 (IL-4) phenotype. M1-polarized macrophages increased the response of malignant mesothelioma spheroids to pro-apoptotic agents, such as TRAIL plus anisomycin. Furthermore, our preliminary data indicate that primary human tumor-associated macrophages, isolated from malignant mesotheliomas, have similar pro-apoptotic effects when polarized with M1 cytokines, suggesting that cytokine re-polarization of macrophages in mesothelioma tumors to an M1 phenotype could augment therapeutic efficacy.

This work was supported by a DoD Mesothelioma Program grant PR080717 (to Broaddus and Coussens).

Abstract for: MRS-AACR Joint Conference on Metastasis and the Tumor Microenvironment

September 7-15, 2010, Philadelphia PA

Inflammation and Cancer: Reprogramming the immune microenvironment as an anti-cancer therapeutic strategy

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The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have recently appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion during skin carcinogenesis is B cell, Ig and FcγR–dependent, during mammary carcinogenesis by contrast, T_H2-polarized $CD4^+$ T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of M2-polarized macrophages and dendritic cells, that together regulate metastasis of malignant mammary epithelial cells to lung. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and thoughts on how these properties can be harnessed for effective anticancer therapeutics.

LMC acknowledges generous support from the NIH and NCI, and Department of Defense Era of Hope Scholar Award (W81XWH-06-1-0416) and Investigator-Initiated Research Award in Mesothelioma (PR080717).

Abstract for: NCI, **IMMUNITY, INFLAMMATION, AND CANCER CONFERENCE**
September 23-24, 2010, Bethesda MD USA

Inflammation and Cancer: Reprogramming the immune microenvironment as an anti-cancer therapeutic strategy

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LMC acknowledges generous support from the NIH and NCI, and Department of Defense Era of Hope Scholar Award (W81XWH-06-1-0416) and Investigator-Initiated Research Award in Mesothelioma (PR080717).

Abstract for: Nature CNIO Cancer Symposium "Frontiers in Tumour Progression"
24th – 27th October 2010
Palacete de los Duques de Pastrana, Madrid, Spain

Inflammation and Cancer: Reprogramming the immune microenvironment as an anti-cancer therapeutic strategy

Lisa M. Coussens. Department of Pathology, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, 513 Parnassus Ave., HSW-450C San Francisco, CA 94143-0502 USA; Phone: 001-415-502-6378; e-mail: Lisa.Coussens@ucsf.edu

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Targeting Macrophages in a Preclinical Model of Mesothelioma

Collin M. Blakely[1], Nikita Kolhatkar[2,4], Magnus Johansson[2], Dario Barbone[4], Adam Yagui-Beltrán[3], Raphael Bueno[5], David J. Sugarbaker[5], Thierry M. Jahan[1,6], David M. Jablons[3,6], V. Courtney Broaddus[4], and Lisa M. Coussens[2,6]

Departments of [1]Medicine, Division of Hematology/Oncology, [2]Pathology, [3]Surgery, [4]Lung Biology Center, and [6]Helen Diller Family Comprehensive Cancer Center, University of California San Francisco. [5]Division of Thoracic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts.

Background: Malignant mesothelioma is a debilitating, incurable cancer that exhibits a high degree of resistance to standard chemotherapy. Novel therapeutic approaches to treat this disease are desperately needed. We have found that mesothelioma tumors resected from patients exhibit a high rate of infiltration by macrophages. Increasing evidence suggests that tumor-associated macrophages (TAMs) secrete proangiogenic, prosurvival, and proinvasive factors that act to promote tumor formation and progression. Novel approaches designed to target TAMs have been developed. These include depletion of macrophages with the bisphosphonate clodronate or targeting the Colony Stimulating Factor-1 Receptor (CSF-1R), which is important for monocyte/macrophage-lineage recruitment to tumors.
Objective/Hypothesis: We hypothesize that mesothelioma-associated macrophages promote resistance to the cytotoxic effects of chemotherapy and that inhibition of macrophage infiltration of tumors, or their protumor bioactivity, will improve the efficacy of chemotherapy.
Specific Aims: (1) Determine the functional significance of macrophage depletion on the effects of chemotherapy in an orthotopic mouse model of mesothelioma. (2) Determine the functional significance of CSF-1R inhibition on the effects of chemotherapy in an orthotopic mouse model of mesothelioma. (3) Determine the functional significance of CSF-1R inhibition on the effects of chemotherapy in a de novo mouse model of mesothelioma.
Study Design: To assess the effects of macrophage depletion on mesothelioma responsiveness to chemotherapy we will conduct three independent preclinical trials. In the first experiment we will assess the effects of depleting macrophages with clodronate on mesothelioma responsiveness to chemotherapy. Mice will be injected intraperitoneally with a syngeneic mesothelioma cell line to establish tumors. Tumor-bearing mice will be randomized into 1 of 4 treatment groups: 1) clodronate liposomes alone; 2) control liposomes alone; 3) clodronate liposomes + carboplatin/pemetrexed chemotherapy; 4) control liposomes + carboplatin/pemetrexed. In the second experiment we will assess the effects of CSF-1R inhibition on mesothelioma responsiveness to chemotherapy by using the novel, orally bioavailable, small molecule CSF-1R kinase inhibitor, PLX3397 (Plexxikon Inc.). Mice with orthotopic mesothelioma tumors will be randomized into 1 of 4 treatment groups: 1) PLX3397 chow alone; 2) control chow alone; 3) PLX3397 chow + carboplatin/pemetrexed; 4) control chow + carboplatin/pemetrexed. Finally, to assess the effects of CSF-1R inhibition in a de novo model of mesothelioma, we will treat $NF2^{+/KO}$ mice with asbestos to induce mesothelioma tumor formation. Mice with mesothelioma will be randomized into 1 of 4 treatment groups to receive chemotherapy with or without PLX3397 as above. In each experiment, mice will be followed for tumor progression by optical imaging and/or ultrasound as well as for overall survival. Tumors will be analyzed histopathologically, and by flow cytometry (FACS) analysis for changes in vasculature, proliferation, apoptosis, and immune cell infiltration. Statistical analysis will be performed to identify significant changes in any of these parameters between treatment groups.
Cancer Relevance: This study will provide a thorough preclinical assessment of the potential benefits of targeting macrophages in mesothelioma. We predict that these results will be readily translated into novel therapies for mesothelioma patients. Many other cancers, including lung and breast cancer, are also infiltrated with inflammatory cells. As such, the potential benefit of studying this new method of targeting cancer is enormous.

This work was supported by an NIH T32 training grant to Dr. Blakely, and by a DoD Mesothelioma Program grant (PR080717) to Drs. Broaddus and Coussens.

Abstract for poster presentation: Annual meeting of the Radiological Society of North America (RSNA), Nov. 28- Dec. 3, 2010, Chicago, IL.

MR Imaging of Tumor Associated Macrophages with Ferumoxytol

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Purpose: The presence of tumor-associated macrophages (TAM) in breast cancer correlates strongly with poor outcome. The purpose of this study was to develop a clinically applicable, non-invasive diagnostic assay for selective targeting and visualization of TAMs in breast cancer, based on magnetic resonance (MR) imaging and the clinically applicable iron oxide nanoparticle compound ferumoxytol.

Methods: F4/80-negative cancer cells and F4/80-positive TAM were incubated with ferumoxytol and were compared regarding MR signal changes and iron uptake. Mice with MMTV PyMT breast cancers underwent nanoparticle-enhanced MR up to 1 hour (h) and at 24 h post injection (p.i.). The tumor enhancement on MR images was correlated with the presence of TAMs on histopathology.

Results: In vitro studies revealed that the clinically applicable iron oxide nanoparticle compound ferumoxytol is preferentially phagocytosed by TAMs, but not by tumor cells. *In vivo*, all tumors demonstrated an initial contrast agent perfusion on immediate postcontrast T2-weighted MR images with gradual transendothelial leakage into the tumor interstitium. At 24 h p.i., all tumors demonstrated a persistent signal decline on MR scans. Detection of iron using DAB-enhanced Prussian Blue staining, and immunodetection of $CD68^+$ TAMs in tumor tissue sections localized iron oxide nanoparticles to $CD68^+$ TAMs, indicating that the MR signal effects on delayed MR images were largely due to TAM-mediated uptake of contrast agent.

Conclusion: Ferumoxytol nanoparticles can selectively target and visualize of TAMs in breast cancer on MR images.

Clinical Implication: These data indicate that tumor-enhancement with clinically applicable iron oxide nanoparticles may serve as a new biomarker for long-term prognosis, related treatment decisions and the development of new immune-targeted therapies.

The authors acknowledge support from the Department of Defense (W81XWH-06-1-0416, PR080717) to LMC and grants from NIH/NCI R01CA140943 to LMC and HED

Abstract for Invited Talk
Cancer, Immunity, & Microenvironment Program
March 1, 2011
UCSF

A Role for Macrophages in a Recalcitrant Tumor, Mesothelioma

V. Courtney Broaddus, MD
Invited Speaker
March 1, 2011

Mesothelioma is highly resistant to treatment, a problem thought to be due to the resistance to apoptosis. In our collaboration with Dr. Lisa Coussens, we are now investigating the underpinning of this resistance and have focused on the role of the tumor-associated macrophages. We have now shown that mesothelioma contains a predominance of macrophages, more evident in mesothelioma than in other thoracic malignancies such as lung cancer or esophageal cancer. Perhaps the significance of macropahges may stem from their initial involvement in the response to asbestos, the only known etiology of mesothelioma. In hybrid spheroids that we are constructing to study the interaction of mesothelioma cells and macrophages, we have learned that macrophages can be reprogram ed by cytokines to a pro-apoptotic phenotype (M1) which enhances the chemosensitivity of mesothelioma cells to therapy. Most recently, we have shown that this repolarization can be accomplished in tumor fragment spheroids, leading to clear increases in the apoptotic response of the mesothelioma cells ex vivo to standard chemotherapeutic agents (cisplatin or carboplatin plus pemetrexed).

This work was supported by the Simmons Mesothelioma Foundation and a DoD Mesothelioma Program grant PR080717.

Abstract for: American Association of Cancer Research (AACR)
April 2-6, 2011 Orlando, FL

Macrophage-depletion as a Novel Therapeutic Approach for Malignant Pleural Mesothelioma

Collin M. Blakely[1], Nikita Kolhatkar[2,4], Magnus Johansson[2], Dario Barbone[4], Adam Yagui-Beltrán[3], Raphael Bueno[5], David J. Sugarbaker[5], Thierry M. Jahan[1,6], David M. Jablons[3,6], V. Courtney Broaddus[4], and Lisa M. Coussens[2,6]

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Malignant pleural mesothelioma (MPM) is a debilitating incurable cancer that exhibits a high degree of resistance to standard chemotherapy; thus, we sought to identify novel therapeutic targets for treatment. In the vast majority of cases, MPM is associated with exposure to asbestos fibers, which result in a chronic pro-inflammatory state. As such, we hypothesized that MPMs were infiltrated by leukocytes possessing tumor-potentiating activities. To address this, we evaluated MPMs, resected from 26 patients, by flow cytometry and found that tumors were highly infiltrated with macrophages (31 ± 4.6% of total $CD45^+$), a percentage that is significantly higher that that observed in other thoracic malignancies (NSCLC cancer, 9%; esophageal, 4%). Since recent experimental data has revealed that tumor-associated macrophages (TAMs) secrete proangiogenic, prosurvival, and proinvasive factors that foster tumor progression, we evaluated macrophage-depletion in MPM as a novel therapeutic strategy. Using a syngeneic murine transplantation model and liposomal-encapsulated clodronate that efficiently deplete phagocytic macrophages, as monotherapy, and in combination with chemotherapy, we revealed a significant decrease in tumor growth and a decrease in tumor-burden in tumor-bearing mice depleted of macrophages. However, when macrophage-depletion was combined with the cytotoxic agents gemcitabine and TRAIL, an even greater reduction in tumor burden was observed as compared to mice treated with either agent alone. These studies indicate that: 1) mesothelioma-associated macrophages provide a protumor function, and 2) depletion of mesothelioma-associated macrophages may improve efficacy of cytotoxic chemotherapy. Ongoing studies to reveal the effect of macrophage-depletion plus chemotherapy in limiting MPM development in $NF2^{ko/+}$ mice exposed to asbestos are underway.

This work was supported by an NIH T32 training grant to Dr. Blakely, and by a DoD Mesothelioma Program grant (PR080717) to Drs. Broaddus and Coussens.

Abstract for Invited Talk: UCSF Radiation Therapy Grand Rounds
April 15, 2011 San Francisco, CA

Malignant Mesothelioma: What is the role of radiotherapy?

V. Courtney Broaddus, MD
Invited Speaker
April 15, 2011

In this Grand Rounds, I will cover the background of the etiology of mesothelioma, updates in diagnosis and staging, a current assessment of treatment options including radiation therapy, and insights from our laboratory about novel approaches to treatment of mesothelioma. Mesothelioma is induced by asbestos probably as it lodges in the parietal pleural lymphatics, most likely explaining why mesothelioma originates in the parietal pleura. As it develops over decades, it becomes apparent in imaging (plain films, CT scans, PET scans and MRI) at a point when it is incurable by surgical resection. Diagnosis is still difficult although using panels of immunohistochemical markers has made a positive identification of mesothelioma more accurate. Staging criteria are not universally accepted and different systems are used; a new system is under evaluation by the IASLC together with iMig. Therapy used today is generally a multimodality therapy, including surgery, chemotherapy and radiation. Radiation therapy has a long history but not a clear evidence of effectiveness. In our 3D models, radiation of spheroids has encountered resistance due to acquired multicellular resistance of cells in 3D. Our effort to undermine this resistance, by blocking the Bcl-2 anti-apoptotic repertoire or by blocking or repolarizing the macrophages within the tumors, may improve the response of mesothelioma to therapy, including radiation therapy.

This work was supported by the Simmons Mesothelioma Foundation and a DoD Mesothelioma Program grant PR080717.

Abstract for Oral Presentation: 14th World Conference on Lung Cancer (WCLC), International Association for the Study of Lung Cancer
July 3- 7, 2011 Amsterdam, The Netherlands

Tumor-associated Macrophages can be Polarized to Reduce the Chemoresistance of Malignant Mesothelioma

V. Courtney Broaddus[3,4], Sailaja Battula[3], Dario Barbone[3], Collin Blakely[1], Raphael Bueno[5], Nikita Kolhatkar[1,3], David J. Sugarbaker[5], David M Jablons[2,4], Lisa M Coussens[1,4]

Departments of [1]Pathology, [2]Surgery, [3]Lung Biology Center, and [4]Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco. [5]Division of Thoracic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts.

Background: Mesothelioma is highly resistant to chemotherapy. One feasible approach could be to harness the immune system to undermine the chemoresistance of mesothelioma. We studied the chemoresistance of mesothelioma cells in a spheroid model wherein mesothelioma cells acquire multicellular apoptotic resistance that resembles that of the solid tumor.

Methods: Immunohistochemistry and flow cytometry were used to analyze the leukocyte compositions of human mesotheliomas. Macrophages, either derived from human mesothelioma, THP-1-derived or matured from peripheral blood monocytes, were polarized to a Th1 or Th2 phenotype by exposure for 48 h to cytokines (IFN-gamma & LPS or IL-4 respectively). Polarized macrophages were then grown with mesothelioma cells either together in heterotypic 3-dimensional spheroids or in a Transwell system in which macrophages were grown separately from mesothelioma spheroids by a 3 micron filter. After 24 h, mesothelioma spheroids with and without macrophages were treated with TRAIL plus gemcitabine or carboplatin plus pemetrexed for 48 h and studied for apoptosis by analysis of Hoescht-stained nuclear morphology.

Results: We found that all subtypes of mesothelioma harbor a large macrophage population (31±4.6% of the inflammatory cell population [$CD45^+$]). Indeed, the percentage of macrophages in mesotheliomas exceeded that found in other thoracic malignancies thus far evaluated (NSCLC cancer, 9%; esophageal, 4%). We found that Th1-polarized macrophages increased the response of mesothelioma spheroids to TRAIL plus gemcitabine by 155% and to carboplatin plus pemetrexed by 47.6% respectively, compared to Th2 polarization. The pro-apoptotic effect was similar whether macrophages were grown together with or separately from the mesothelioma cells, suggesting the mediation of macrophage-derived cytokines. The pro-apoptotic effect of Th1 polarization depended on the presence of macrophages: mesothelioma spheroids without macrophages exposed to Th1 cytokines showed no increase in apoptosis.

Conclusion: Polarization of macrophages within mesothelioma tumors to an Th1 phenotype could enhance the efficacy of standard and novel therapies.

This work was supported by grants from the NCI and a DoD Mesothelioma Program grant PR080717 to Broaddus and Coussens

Accepted for the AACR International Conference:
April 2-6, 2011
Orlando, FL

Targeting Macrophages in a Preclinical Model of Malignant Mesothelioma

Collin M. Blakely[1], Nikita Kolhatkar[2,4], Magnus Johansson[2], Dario Barbone[4], Adam Yagui-Beltrán[3], Raphael Bueno[5], David J. Sugarbaker[5], Thierry M. Jahan[1,6], Brian L. West[7], David M. Jablons[3,6], V. Courtney Broaddus[4], and Lisa M. Coussens[2,6]

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Malignant pleural mesothelioma (MPM) is a debilitating, incurable cancer that exhibits a high degree of resistance to standard chemo-therapy; thus, we sought to identify novel therapeutic targets for treatment. In the vast majority of cases, MPM is associated with prior exposure to asbestos fibers, resulting in a chronic pro-inflammatory state in pleura. As such, we hypothesized that MPMs were infiltrated by leukocytes possessing tumor-potentiating activities. To address this, we evaluated MPMs, resected from 24 patients, by flow cytometry and found that tumors were highly infiltrated by macrophages (31 ± 4.6% of total CD45+), a percentage significantly higher than that observed in other thoracic malignancies (NSCLC cancer, 9%; esophageal, 4%). Since recent experimental data has revealed that tumor-associated macrophages (TAMs) secrete proangiogenic, prosurvival, and pro-invasive factors that foster tumor progression, we evaluated macrophage depletion in MPM as a novel therapeutic strategy. Using a syngeneic murine transplantation model, and liposomal-encapsulated clodronate that efficiently depletes phagocytic macrophages. These studies revealed a significant decrease in tumor growth and a decrease in tumor-burden in mice depleted of macrophages. A similar effect was observed when MPM-bearing mice were treated with an anti-CD11b neutralizing mAB in combination with cytotoxic chemotherapy. Finally, we revealed that an orally bioavailable tyrosine kinase inhibitor of CSF-1R (PLX3397) that decreases macrophage infiltration, also reduces MPM as mono-therapy. These studies indicate that: 1)macrophages potentiate mesothelioma development, and 2) depletion of mesothelioma-associated macrophages improve efficacy of cytotoxic chemotherapy and may provide a survival advantage.

This work was supported by an NIH T32 training grant to Dr. Blakely, and by a DoD Mesothelioma Program grant (PR080717) to Drs. Broaddus and Coussens.

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University of California San Francisco
San Francisco, CA
November 14, 2011

Th1-Polarized Macrophages Enhance the Apoptotic Response To Chemotherapy In Mesothelioma

Sailaja Battula[1], Dario Barbone[1], David J. Sugarbaker[5], Raphael Bueno[5], David M Jablons[3, 4], Lisa M. Coussens[2,4], V. Courtney Broaddus[1,4]

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The tumor microenvironment (TME) may contribute to tumor chemoresistance. Manipulating the TME may be one of the strategies to target mesothelioma, a highly chemoresistant tumor. We found that macrophages constitute a high percentage of inflammatory cells in mesothelioma. Our aim is to understand whether macrophages influence the tumor cells and whether alteration of macrophage phenotype could enhance the chemoresponsiveness of the tumor. Two 3D models of mesothelioma were used: 1) the multicellular spheroid, in which mesothelioma cells are grown alone or co-cultured with macrophages derived from peripheral blood monocytes and 2) the tumor fragment spheroid (TFS) where the small fragments of fresh tumor from mesothelioma patients are grown in culture. The macrophages were polarized to Th0 (no cytokines), Th1 (by exposure to LPS & IFNγ) or Th2 (by exposure to IL-4 &IL-13) phenotype and subjected to various chemotherapeutic agents. Polarization of the macrophages to their respective phenotype was confirmed by qPCR showing an increase in M1 phenotype markers (TNF, IL-12A, IFNγ) and M2 phenotype markers (CD206, IL10) in presence of their respective cytokines. We found that, compared to Th0- or Th2-polarized macrophages, Th1-polarized macrophages increased the apoptotic response of multicellular spheroids to carboplatin and pemetrexed by 81%. The presence of macrophages is essential to elicit the pro-apoptotic effect because the cytokines have no effect on the chemoresponsiveness of the mesothelioma cells alone. Moreover, Th1-polarized macrophages enhanced chemoresponsiveness even when separated from the target mesothelioma cells by a filter, suggesting that the effect is mediated by soluble factors. Furthermore, in TFS from patients, Th1-polarization increased mesothelioma cell apoptosis alone and after chemotherapy. Therefore, our results demonstrate that manipulating the TME may be an effective therapeutic approach to mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and the T32 Training Grant to Battula.

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San Francisco, CA

Colony-Stimulating Factor-1 Receptor Blockade by the Tyrosine Kinase Inhibitor PLX3397 Reprograms Malignant Mesothelioma Tumor Microenvironments and Decreases Tumor Growth

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Malignant mesothelioma (MM) is a debilitating, frequently incurable cancer that exhibits a high degree of resistance to standard cytotoxic chemotherapy (CTX). Novel therapeutic approaches to treat this disease are desperately needed. In the vast majority of cases, MM is associated with prior exposure to asbestos fibers, resulting in a chronic pro-inflammatory state in pleura. As such, we hypothesized that MMs are infiltrated by leukocytes possessing tumor-potentiating activities. To address this, we evaluated MMs resected from patients (n=16) and compared the complexity of immune cells infiltrating MM to those found in normal pleura (n=4). Using polychromatic fluorescent-activated cell sorting (FACS) on freshly-resected whole tissues, we found a significant increased presence of $CD11b^+CD14^+HLA\text{-}DR^+$ monocytes/macrophages in MM (37.3 ± 4.4% of total $CD45^+$ cells) as compared to normal pleural tissue (13.8 ± 6.5% of total $CD45^+$ cells), and an even more significant increase in MM resected from patients treated with CTX (48.8 ± 5.5% of $CD45^+$ cells)**.** To determine if increased presence of $CD11b^+CD14^+HLA\text{-}DR^+$ cells was associated with varied expression of cytokine signaling genes, we examined mRNA expression of tissues/tumors. We found increased expression of *Colony Stimulating Factor 1 (CSF1)*, and *Colony-Stimulating Factor-1 Receptor* (*CSF-1R*) mRNA, a critical cytokine-signaling axis regulating monocyte/macrophage differentiation and recruitment into tumors, in MM tumors compared to normal pleura, and even higher levels in tumors resected from patients treated with CTX. Since recent experimental data has revealed that tumor-associated macrophages (TAMs) secrete proangiogenic, prosurvival, and pro-invasive factors that foster tumor progression, we evaluated macrophage depletion in MM as a novel therapeutic strategy. We conducted studies evaluating PLX3397 (Plexxikon Inc., Berkeley, CA), a novel, orally bioavailable, small molecule tyrosine kinase inhibitor of CSF-1R. Using a syngeneic orthotopic murine model of MM, we found that treatment of mice with PLX3397 alters the tumor immune microenvironment by decreasing TAM infiltration and increasing the proportion of $CD8^+$ cytotoxic T lymphocytes within tumors. This reprogramming of the tumor immune microenvironment was associated with alterations in the tumor microvasculature as evidenced by a decrease in $CD31^+$ structures, as well as a decrease in *VEGFA* mRNA expression. Ultimately, these changes resulted in an increase in tumor cell apoptosis and a decrease in tumor burden. These studies indicate that: 1) macrophages potentiate mesothelioma development, and 2) depletion of mesothelioma-associated macrophages may improve the efficacy of cytotoxic chemotherapy and provide a survival advantage.

This work was supported by an NIH T32 training grant to Dr. Blakely, and by a DoD Mesothelioma Program grant (PR080717) to Drs. Broaddus and Coussens.

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SAN ANTONIO BREAST CANCER CONFERENCE, DECEMBER 2011

MODULATING IMMUNE RESPONSE TO IMPROVE THERAPY FOR BREAST CANCER

LISA M. COUSSENS, PH.D.

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BWhile BC has not historically been linked to underlying inflammation or infection, it exhibits tumor-associated inflammation marked by infiltration of innate and adaptive immune cells into developing tumors. In BC, macrophages are one of the most abundant innate immune cells present. BC-associated macrophages are regulated in part by colony stimulating factor 1 (CSF1), a key cytokine involved in monocyte/macrophage maturation, recruitment and activation, and its cognate receptor CSF1R. Macrophage presence in BC correlates with increased CSF1, increased vascular density, and worse clinical outcome. We reported that $CD4^+$ T cells promote invasion and metastasis of mammary adenocarcinomas by directly regulating macrophage phenotype that in turn fosters invasive tumor growth, presence of circulating tumor cells and pulmonary metastasis. This preclinical data implied that women with BC heavily infiltrated by macrophages would have a worse clinical outcome as compared to tumors not heavily infiltrated with macrophages. We evaluated survival outcomes in 698 women with invasive BC treated with surgery alone and found that recurrence-free survival could be stratified based upon macrophage and T cell infiltration. Thus, we investigated CSF1 and CSF1R antagonists, in combination with standard-of-care chemotherapy (CTX) in mouse models of mammary carcinogenesis. We found that when macrophage infiltration in mammary adenocarcinomas was blocked, paclitaxel (PTX) chemosensitivity was increased, accompanied by development of productive anti-tumor immune responses and $CD8^+$ cytotoxic T cell (CTL) infiltration. The combined effects of these changes were reduced primary tumor growth, 85% reduction in metastases and increased survival. In collaboration with clinical colleagues, we are currently evaluating the clinical benefit of macrophage modulation in preclinical models of BC to facilitate biomarker identification, and inform clinical trials of CTX in combination with macrophage-antagonists. Based on our preliminary data, we hypothesize that components of macrophage responses in BC can be identified to serve as biomarkers for risk stratification. And, that these components can be effectively targeted for therapeutic intervention, resulting in reduced late-stage BC development and metastasis when combined with CTX.

LMC acknowledges generous support from the NIH/NCI (R01CA130980, R01CA13256, R01CA140943, R01CA15531), the Department of Defense (W81XWH-09-1-0342, W81XWH-10-BCRP-EOHS-EXP) and the Susan G Komen Foundation (KG111084)

Australian Society for Immunology, December 2011

MODULATING IMMUNE RESPONSE TO IMPROVE CANCER THERAPY

LISA M. COUSSENS

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The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion during skin carcinogenesis are B cell, Ig and FcγR–dependent, during mammary carcinogenesis by contrast, T_H2-$CD4^+$ T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of macrophages and dendritic cells, that together regulate metastasis of malignant mammary epithelial cells to lung. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and new studies evaluating how neutralizing selective aspects of pro-tumor immunity can be exploited to enhance therapeutic responses to cytotoxic therapy and in general bolster tumor-suppressive anti-tumor immune responses.

LMC acknowledges generous support from the NIH/NCI (R01CA130980, R01CA13256, R01CA140943, R01CA15531), the Department of Defense (W81XWH-09-1-0342, W81XWH-10-BCRP-EOHS-EXP) and the Susan G Komen Foundation (KG111084)

Abstract for
American Association of Cancer Research
March 31 – April 4, 2012 Chicago, IL

Manipulation of Macrophage Phenotype Enhances the Apoptotic Response To Chemotherapy In Mesothelioma

V. Courtney Broaddus[1,5], Sailaja Battula[1], Dario Barbone[1], Collin Blakely[4], David J. Sugarbaker[6], Raphael Bueno[6], Lisa M. Coussens[2,5,7].

[1]San Francisco General Hospital, and Departments of [2]Pathology, [3]Surgery and [4]Medicine, [5]Helen Diller Family Comprehensive Cancer Center, Univ. California SF, CA. [6]Division of Thoracic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, [7]Department of Cell & Developmental Biology, Knight Cancer Institute, Oregon Health 7 Sciences University, Portland OR.

Macrophages within the tumor microenvironment (TME) may contribute to tumor chemoresistance. We have found that macrophages constitute a significant percentage of the infiltrating leukocytes in mesothelioma, a highly chemoresistant tumor. We asked whether reprogramming of macrophage phenotype from a Th2 (pro-tumor) to a Th1 (anti-tumor) phenotype would alter the mesothelioma chemoresponsiveness. To address this, we used 3D organotypic models of mesothelioma growth: 1) multicellular spheroids, with mesothelioma cells grown alone or co-cultured with macrophages derived from peripheral blood monocytes and, 2) primary tumor fragment spheroid (TFS) derived from small fragments of freshly isolated human mesothelioma. Macrophages were incubated with Th1 (LPS & IFNγ) or Th2 (IL-4 &IL-13) cytokines prior to co-culture with mesothelioma spheroids, followed by exposure to standard-of-care chemotherapy, cisplatin plus pemetrexed. Gene expression was evaluated in macrophages to affirm Th1-type (TNF, IL-12, IFNα) versus Th2-type (CD206, IL10) programming. In both spheroid approaches, Th1-type macrophage programming significantly increased the apoptotic response of mesothelioma cells to chemotherapy. Moreover, when CSF1R signaling in macrophages was inhibited by incubation with a small molecular weight kinase antagonist, GW2850, chemoresponsiveness was significantly increased as evidenced by increased presence of apoptotic tumor cells in spheroids. Enhanced chemoresponsiveness of mesothelioma tumor cells was dependent on presence of macrophages because incubation of tumor cells with Th1-type cytokines or GW2850 were without effect. We conclude that manipulating the TME may be a promising therapeutic approach in mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and a T32 Training Grant to Battula and Blakely.

Invited Presentation
2nd International Symposium on Lung-sparing Therapies for Malignant pleural Mesothelioma
May 12, 2012
Santa Monica, CA

MANIPULATING THE CORE APOPTOTIC MACHINERY IN MESOTHELIOMA

V. Courtney Broaddus

Thank you for this opportunity to present our research and to describe how it may improve the therapeutic options in mesothelioma. Along with members of my laboratory, Drs. Dario Barbone and Sailaja Battula, and our major collaborators, Dr. Lisa Coussens, we have made promising strides in understanding the core apoptotic resistance in mesothelioma and how the resident macrophages can be manipulated to tilt the balance from chemoresistance to chemosensitivity.

For many years now, our major effort has been in uncovering the apoptotic resistance of malignant pleural mesothelioma, a particularly recalcitrant tumor. Our studies in 3D mesothelioma spheroids have indicated roles for certain pathways and proteins that would not have been recognized by studies in 2D monolayers. Some of these findings are the role played by mTOR and by the Bcl-2 family of pro- and anti-apoptotic proteins. Our current studies using macrophages in co-culture with mesothelioma cells have illustrated the potential role of macrophages in enhancing apoptosis. In fact, these studies have helped advance clinical trials planned to use inhibitors to a key macrophage receptor, the CSF1R, in patients with mesothelioma.

In this talk, I will present findings in the 3D spheroids and in the hybrid spheroids co-cultured with mesothelioma cells plus macrophages. This will then lead to parallel studies in the mouse mesothelioma model and the use of this anti-CSF1R therapeutic agent to enhance chemosensitivity. We hope that these pre-clinical studies will speed clinical trials in which this non-toxic approach can be tested in patients with mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and a T32 Training Grant to Battula and Blakely.

Cancer Immunotherapy Consortium's 2012 Colloquium, April 2012

Inflammation and Cancer: Reprogramming the immune microenvironment as an anti-cancer therapeutic strategy

Lisa M. Coussens. Department of Cell & Developmental Biology, Knight Cancer Institute, Oregon Health & Sciences University, 3181 SW Sam Jackson Park Road, Portland OR 97239-3098. Email: coussenl@ohsu.edu

The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion during skin carcinogenesis are B cell, Ig and FcγR–dependent, during mammary carcinogenesis by contrast, T_H2-$CD4^+$ T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of macrophages and dendritic cells, that together regulate metastasis of malignant mammary epithelial cells to lung, as well as responses to cytotoxic therapies. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and new studies evaluating how attenuating protumor properties of myeloid cells can be exploited to enhance therapeutic responses to cytotoxic therapy.

LMC acknowledges generous support from the NIH / NCI, and Department of Defense Era of Hope Scholar Award Expansion Award, Susan G. Komen Foundation for a Komen Promise award, and an Investigator-Initiated Research Award in Mesothelioma from the DoD.

International Symposium of the Collaborative Research Center 832, Bad Neuenahr Ahrweiller, Germany, May 2012

Inflammation and Cancer: Reprogramming the immune microenvironment as an anti-cancer therapeutic strategy

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The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion during skin carcinogenesis are B cell, Ig and FcγR–dependent, during mammary carcinogenesis by contrast, T_H2-$CD4^+$ T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of macrophages and dendritic cells, that together regulate metastasis of malignant mammary epithelial cells to lung, as well as responses to cytotoxic therapies. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and new studies evaluating how attenuating protumor properties of myeloid cells can be exploited to enhance therapeutic responses to cytotoxic therapy.

LMC acknowledges generous support from the NIH / NCI, and Department of Defense Era of Hope Scholar Award Expansion Award, Susan G. Komen Foundation for a Komen Promise award, and an Investigator-Initiated Research Award in Mesothelioma from the DoD.

Accepted for an Oral Presentation
American Thoracic Society
San Francisco, CA
May 23, 2012

Abstract 33655

Th1-Polarized Macrophages Enhance The Apoptotic Response To Chemotherapy In Mesothelioma

S. Battula[1], D. Barbone[1], C. Blakely[1], R. Bueno[2], D. Sugarbaker[2], D. Jablons[3], L.M. Coussens[1], V.C. Broaddus[1]
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Type:	**Scientific Abstract**
Category:	**09.25 - Pleural Diseases/Mesothelioma (CP)**
Status:	**ACCEPTED**
Session Code:	**D14**
Session Title:	**ADVANCES IN PLEURAL INFECTION AND MALIGNANCY**
Session Day/Date:	**Wednesday, May 23, 2012**
Session Starting/Ending Times:	**8:15am - 10:45am**
Session Type:	**Mini Symposium - Oral Presentation**

RATIONALE The tumor microenvironment (TME) may contribute to tumor chemoresistance. As a result, manipulating the TME may be a means of targeting mesothelioma, a highly chemoresistant tumor. We found that macrophages constitute a high percentage of inflammatory cells in mesothelioma. Our aim is to understand whether macrophages influence the tumor cells and whether alteration of the macrophage phenotype could enhance the chemoresponsiveness of the tumor.

METHODS Two 3D models of mesothelioma were used: 1) the multicellular spheroid, in which mesothelioma cells are grown alone or co-cultured with macrophages derived from peripheral blood monocytes and 2) the tumor fragment spheroid (TFS) in which the small fragments of fresh tumor from mesothelioma patients are grown ex vivo in culture. The macrophages were polarized to Th0 (no cytokines), Th1 (by exposure to LPS & IFNγ) or Th2 (by exposure to IL-4

& IL-13) phenotypes and subjected to various chemotherapeutic agents.

RESULTS Polarization of the macrophages to their respective phenotype was confirmed by qPCR showing an increase in Th1 phenotype markers (TNF, IL-12A, IFNγ) and Th2 phenotype markers (CD206, IL10) in presence of their respective cytokines. We found that, compared to Th0- or Th2-polarized macrophages, Th1-polarized macrophages increased the apoptotic response of multicellular spheroids to carboplatin and pemetrexed by 80%. The presence of macrophages was essential for the pro-apoptotic effect because the cytokines had no effect on the chemoresponsiveness of the mesothelioma cells alone. Th1-polarized macrophages enhanced chemoresponsiveness even when separated from the target mesothelioma cells by a filter, suggesting that the effect was mediated by soluble factors. Most interestingly, in TFS grown from patient tumors, Th1-polarization significantly increased mesothelioma cell apoptosis to chemotherapy, suggesting that macrophages in the actual tumors can be manipulated to enhance tumor chemoresponsiveness.

CONCLUSION Therefore, our results demonstrate that manipulating the tumor microenvironment by altering the macrophage to a Th1 phenotype may be an effective therapeutic approach to mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and a T32 Training Grant to Battula and Blakely.

Invited Presentation
Medical Grand Rounds, Massachusetts General Hospital, June 14, 2012
Boston, MA

Asbestos and its toxic relationship with the pleura: update and future concerns.

V. Courtney Broaddus, MD

In this presentation, I will describe current understanding of the interaction of asbestos fibers with the parietal pleura ultimately leading to pleural disease, especially pleural mesothelioma. Initially, asbestos fibers are inhaled into the airways, reaching the airspaces due to their unique shape. There they attract macrophages which enhance an innate inflammatory response to the fibers. However, many fibers translocate to the pleura by moving directly across the visceral pleura, due to their migration toward the negative pressure of the pleural space, and the 'milking' motion of the lung in ventilation. In animal studies, asbestos fibers can be shown to reach the pleural space within hours to days. There, the fibers likely move to the parietal pleura along with the absorption of pleural liquid. In early studies, I have shown that the pleural liquid is absorbed by bulk flow, into lymphatic stomata of the parietal pleura. Later studies have shown that carbon also collects at these locations and, in a study by Boutin using VATS biopsies, asbestos fibers were also found to accumulate at these discrete locations. There, the inflammatory stimulus of the asbestos fibers continues over decades. The fibers cannot negotiate the lymphatic openings and remain stuck in the parietal pleura, macrophages are recruited but cannot digest or remove the fibers, and thus inflammation is unchecked. Presumably, the inflammation along with the oxygen radicals and chromosomal damage induced by the asbestos fibers leads to malignant change. Such insights have led to current efforts to inhibit macrophage function and clinical trials that are being developed to block the macrophage receptor, CSF1R. Early studies using co-culture of macrophages with mesothelioma calls and animal studies with asbestos-induced and orthotopic mesothelioma have shown promising results suggesting that targeting the macrophage and its inflammatory responses may be an effective strategy for fighting mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and a T32 Training Grant to Battula and Blakely.

Invited Presentation
Harvard Lung Conference, Harvard Combined Program, June 14, 2012
Boston, MA

Spheroids and 3D Insights into Apoptotic Resistance: Mesothelioma and Lung Cancer

V. Courtney Broaddus, MD

In this presentation, I will describe our laboratory's evolution into the study of tumor biology using 3D spheroids. Our major effort has been in uncovering the apoptotic resistance of malignant pleural mesothelioma, a particularly recalcitrant tumor, but we have also found application of our findings and approaches to lung cancer. Our studies in 3D mesothelioma spheroids have indicated roles for certain pathways and proteins that would not have been recognized by studies in 2D monolayers. Some of these findings are the role played by mTOR and by the Bcl-2 family of pro- and anti-apoptotic proteins. Our current studies using macrophages in co-culture with mesothelioma cells have illustrated the potential role of macrophages in enhancing apoptosis. In fact, these studies have helped advance clinical trials planned to use inhibitors to a key macrophage receptor, the CSF1R, in patients with mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and a T32 Training Grant to Battula and Blakely.

Accepted for Oral Presentation: International Mesothelioma Interest Group
Sept. 10-14, 2012
Boston, MA

Macrophages can be Manipulated to Enhance the Apoptotic Response To Chemotherapy In Mesothelioma

Sailaja Battula[1], Collin Blakely[4,] Dario Barbone[1], David J. Sugarbaker[6], Raphael Bueno[6], Lisa M. Coussens[2,5,7,] V. Courtney Broaddus[1,5].

[1]San Francisco General Hospital, and Departments of [2]Pathology, [3]Surgery and [4]Medicine, [5]Helen Diller Family Comprehensive Cancer Center, Univ. California SF, CA. [6]Division of Thoracic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, [7]Department of Cell & Developmental Biology, Knight Cancer Institute, Oregon Health 7 Sciences University, Portland OR.

Background: Macrophages within the tumor microenvironment (TME) may contribute to tumor chemoresistance. We have found that macrophages constitute a significant percentage of the infiltrating leukocytes in mesothelioma, a highly chemoresistant tumor. We asked whether reprogramming of macrophage phenotype from a Th2 (pro-tumor) to a Th1 (anti-tumor) phenotype or blockade of macrophages by inhibition of a key macrophage receptor, CSF1R, would alter the chemoresponsiveness of mesothelioma.

Methods: To address this, we used 3D organotypic spheroid models of mesothelioma growth: 1) multicellular spheroids, with mesothelioma cells grown alone or co-cultured with macrophages derived from peripheral blood monocytes and, 2) primary tumor fragment spheroid (TFS) derived from small fragments of resected viable human mesothelioma. Macrophages were incubated with Th1 (LPS & IFNγ) or Th2 (IL-4 &IL-13) cytokines prior to co-culture with mesothelioma spheroids, followed by exposure to standard-of-care chemotherapy, carboplatin plus pemetrexed. Gene expression was evaluated in macrophages to confirm Th1-type (TNF, IL-12, IFNα) versus Th2-type (CD206, IL10) programming. Mice with syngeneic orthotopic mesothelioma were treated with carboplatin plus pemetrexed with and without GW2850, a small molecule inhibitor of CSF1R.

Results: In both spheroid models, Th1-type macrophage programming significantly increased the apoptotic response of mesothelioma cells to chemotherapy. Moreover, when CSF1R signaling in macrophages was inhibited by incubation with GW2850, chemoresponsiveness was significantly increased as evidenced by increased presence of apoptotic tumor cells in spheroids. Enhanced chemoresponsiveness of mesothelioma tumor cells was dependent on presence of macrophages because incubation of tumor cells with Th1-type cytokines or GW2850 was without effect. We extended these findings using a syngeneic orthotopic murine model of malignant mesothelioma. Treatment of tumor-bearing mice with GW2580 in combination with carboplatin plus pemetrexed chemotherapy resulted in a significant decrease in tumor burden and an increase in tumor cell apoptosis compared to treatment of mice with chemotherapy or GW2580 alone.

Conclusion: We conclude that manipulating the macrophage within the TME may be a promising therapeutic approach in mesothelioma.

This work was supported by a DOD Mesothelioma Program grant PR80717 to Broaddus and Coussens and a T32 Training Grant to Battula and Blakely.