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14. ABSTRACT We investigated how autophagy plays a role in accelerating or delaying recurrence of neu-overexpressing mouse mammary carcinoma (MMC) following adriamycin (ADR) treatment, and in affecting response to immunotherapy. We explored two strategies: 1) transient blockade of autophagy with chloroquine (CQ), which blocks fusion of autophagosomes and lysosomes during ADR treatment, and 2) permanent inhibition of autophagy by a stable knockdown of ATG5 (ATG5 _{KD}), which inhibits the formation of autophagosomes in MMC during and after ADR treatment. We found that while CQ prolonged tumor dormancy, but that stable knockdown of autophagy resulted in early escape from dormancy and recurrence. Interestingly, ATG5 _{KD} MMC contained an increased frequency of ADR-induced polyploid-like cells and rendered MMC resistant to immunotherapy. We also determined that expression of IFN-g Ra on tumor cells is not a major determinant of tumor progression and relapse. On the other hand, a transient blockade of autophagy did not affect the sensitivity of MMC to immunotherapy. Our observations suggest that while chemotherapy-induced autophagy may facilitate tumor relapse, cell-intrinsic autophagy delays tumor relapse, in part, by inhibiting the formation of polyploid-like tumor dormancy.					
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1. INTRODUCTION

The objective of the proposal is to understand the role of autophagy in chemotherapy induced tumor dormancy and recurrence.

2. KEYWORDS

tumor dormancy, tumor relapse, immunotherapy, immunoediting, autophagy

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

- 1) Understand the role of autophagy in chemotherapy-induced tumor dormancy (Aim 1)
- 2) Understand the role of tumor IFN-gamma Ra in determining tumor recurrence under immune pressure (Aim 2)

- **What was accomplished under these goals?**

ADR chemotherapy induces immunogenic apoptosis

It has been reported that calreticulin (CRT) is a marker of autophagy that contributes to activation of an immune response. We showed that ADR induced membrane translocation of calreticulin (CRT) on necrotic cells and late apoptotic cells (Figure 1). Unexpectedly, blockade of autophagy in the MMC cells by CQ did not affect ADR-induced CRT expression. We postulated that this may reflect the fact that the ADR-induced autophagy is not cytoprotective in this experimental model.

Adriamycin induces autophagy in MMC

In order to determine whether ADR induces autophagy and in turn establishes tumor dormancy, MMC cells were treated with ADR in the presence or absence of CQ, a pharmacological agent used to block the final stages of autophagy, specifically the fusion of autophagosomes with lysosomes that is necessary for digestion of the cargo in the autophagosomes (frequently termed “autophagic flux”). CQ blocked this autophagic flux as evidenced by the enhanced accumulation of acidic vesicles (red signals) (Figure 2A, ADR and

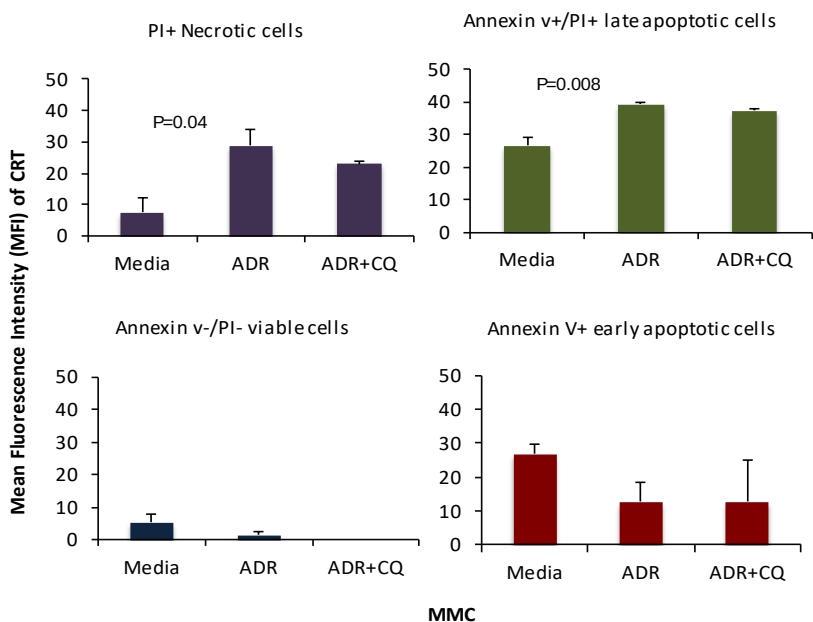


Fig. 1. ADR induces immunogenic apoptosis in MMC. MMC tumor cells were treated with a single dose of ADR alone (1 uM ADR for 2 hrs) (ADR) or in the presence of CQ (10 uM 3 hrs before ADR and 2hrs during ADR treatment) (ADR+CQ). Tumor cells were analyzed by Annexin v/PI staining prior to treatment (Media) and three days after the treatment (ADR and ADR+CQ). Experiments were performed in triplicates.

ADR+CQ). We further monitored degradation of the p62/SQSTM1 protein as a marker of autophagic flux, and LC3B expression as a marker of autophagosomes formation (since LC3 is a component of the autophagosomes). As shown in Figure 2B, ADR did not induce degradation of p62/SQSTM1 although it elevated LC3B, suggesting that ADR induces autophagy but fails to drive autophagy to completion and p62/SQSTM1 degradation.

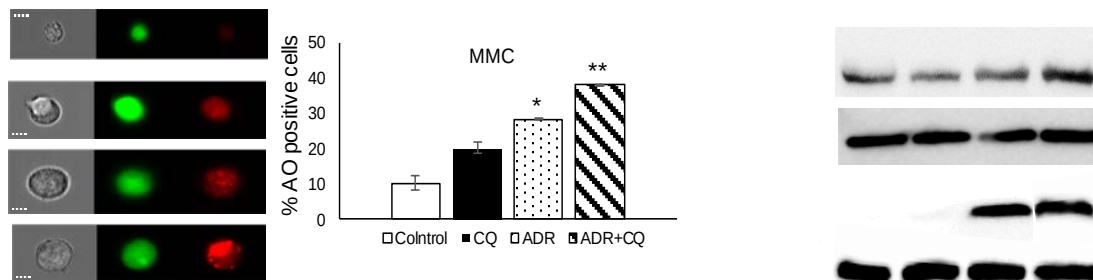


Figure 2. CQ blocks ADR-induced autophagy. MMC tumor cells received three daily doses of ADR alone (1 μ M ADR for 2 hrs) (ADR) or in the presence of CQ (10 μ M 3 hrs before ADR and 2hrs during ADR treatment) (ADR+CQ), washed after each daily treatment and analyzed by acridine orange (AO) one day after the last treatment. Untreated MMC (Medium) or MMC treated with CQ (CQ) served as controls. A) Acridine orange (AO) staining was analyzed for acidic vesicles (red) using image cytometry. Data represent triplicate experiments. B) Levels of p62/SQSTM1 and LC3B after treatment with ADR $\pm$ CQ indicative of autophagy induction in the absence of autophagic flux (B).

A transient blockade of autophagy by CQ during ADR treatment delays tumor relapse in vitro but not in vivo

Since CQ is being used to sensitize tumor cells susceptible to chemotherapy, we sought to determine whether blockade of autophagy by CQ during ADR treatment affects tumor dormancy and relapse. We showed that the presence of CQ during ADR treatment, in vitro, resulted in prolonging tumor dormancy such that, while ADR treated MMC resumed cell proliferation 6 weeks after the treatment, ADR+CQ treated MMC remained dormant (Figure 3A). In order to confirm tumor cell relapse after 6 weeks, flow cytometry analysis of ADR-treated MMC was performed, and indicated a shift of Ki67- non-proliferating cells to Ki67+ proliferating cells with a greater viability (Figure 3B). In fact, MMC cells remained apoptotic by producing floater dead cells following ADR treatment (Figure 4A), which compensated for cell proliferation and maintained tumor dormancy for 3 weeks after the completion of ADR treatment. Follow up studies on floater cells showed they were all apoptotic (Figure 4B). A transient blockade of autophagy by CQ did not affect susceptibility of tumor cells to ADR-induced apoptosis (Figure 5). On the other hand, a transient blockade of autophagy during ADR chemotherapy, in vivo, did not prolong tumor dormancy in FVBN202 mice (Figure 6).

A transient blockade of autophagy by CQ during ADR treatment does not change susceptibility of tumor cell to immunotherapy

In order to determine whether a transient blockade of autophagy during ADR treatment affects susceptibility of dormant MMC to immunotherapy, dormant MMC were cultured with either IFN- γ or MMC-reactive T cells three weeks after treatment with ADR or ADR+CQ. As shown in

Figure 7, untreated MMC or dormant MMC treated with ADR or ADR+CQ all remained susceptible to IFN- γ treatment or T cells.

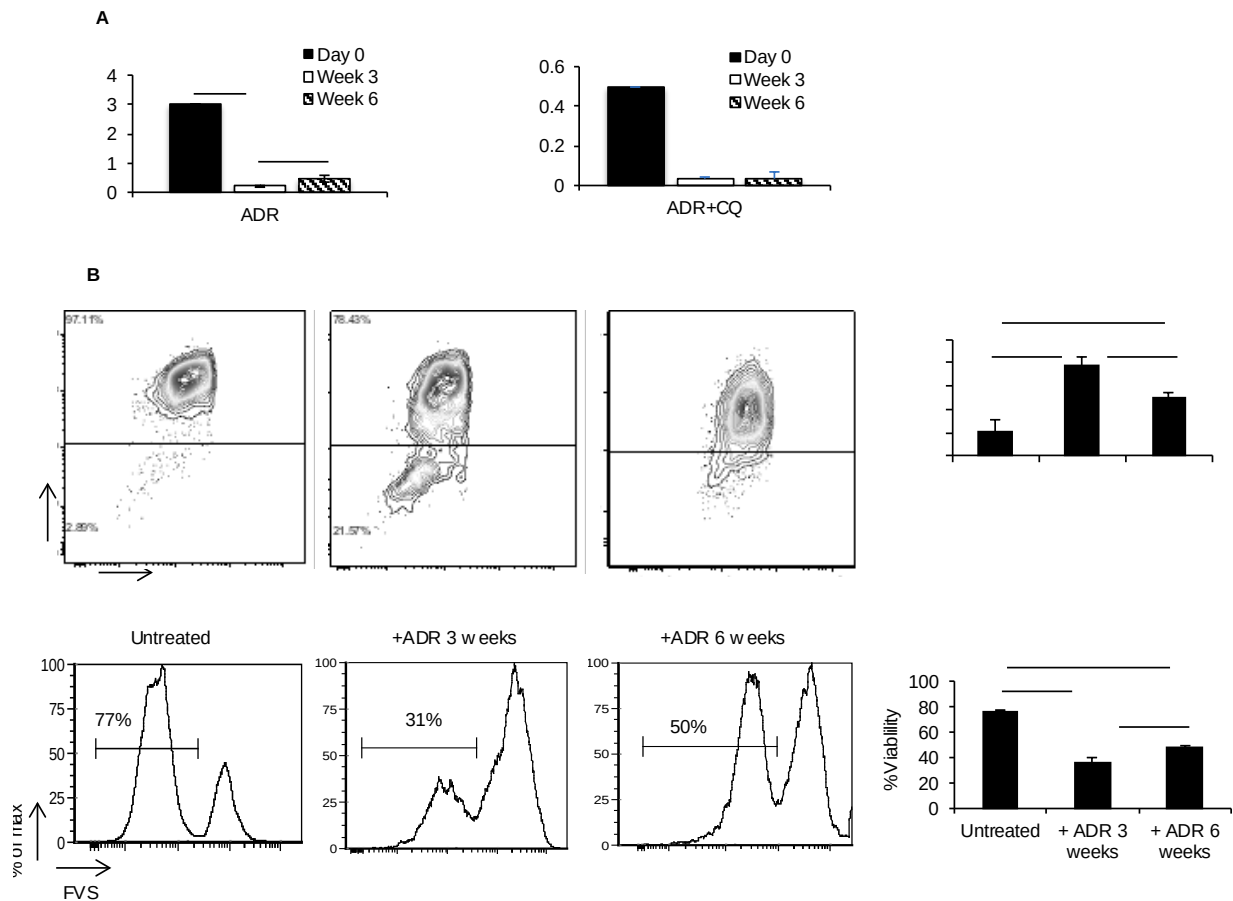
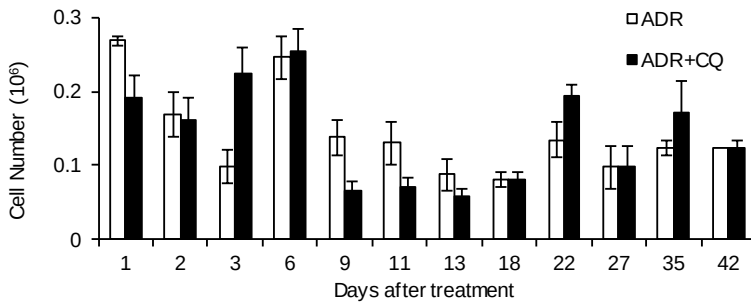


figure 3. ADR-induced dormant tumor cells remain dormant in the presence of CQ. MMC tumor cells were treated with doses of ADR (1 μ M for 2 hrs), with one group receiving CQ (10 μ M) 3 hrs prior to and during ADR treatment. Cells were treated for 3 weeks and 6 weeks, *in vitro*. A) Adherent viable cells were counted using trypan blue exclusion. Data represent 3 replicates $\pm$ SEM. B) At weeks 3 and 6 post-treatment, Ki-67 expression (upper panel) was quantified within the population of adherent tumor cells. Data represent 2-3 replicates $\pm$ SEM. Similar experiments have been carried out which have shown similar results.

A stable knockdown of autophagy reduces susceptibility of MMC to ADR treatment

CQ only transiently blocks fusion of autophagosomes and lysosomes during ADR treatment such that after removal of CQ, accumulated autophagosomes could eventually be fused with lysosomes to complete autophagy. In order to determine the role of autophagy in tumor dormancy or relapse, we used shRNA for a stable knockdown of ATG5 (ATG5^{KD}) which inhibits formation of autophagosomes in MMC. Scrambled shRNA was used as control (Figure 8A). The ATG5^{KD} MMC and scrambled control MMC were irradiated to confirm that ATG5^{KD} MMC cells were deficient in autophagy, using p62 and LC3B as read outs (Figure 8B). Tumor cells remained intact for the expression of neu antigen, as well as cell proliferation *in vitro* and *in vivo* following knockdown of autophagy (Figure 8C-E). Flow cytometry analysis determined a lower level of viability in MMC compared with ATG5^{KD} MMC following ADR treatment (Figure 9).

A



B

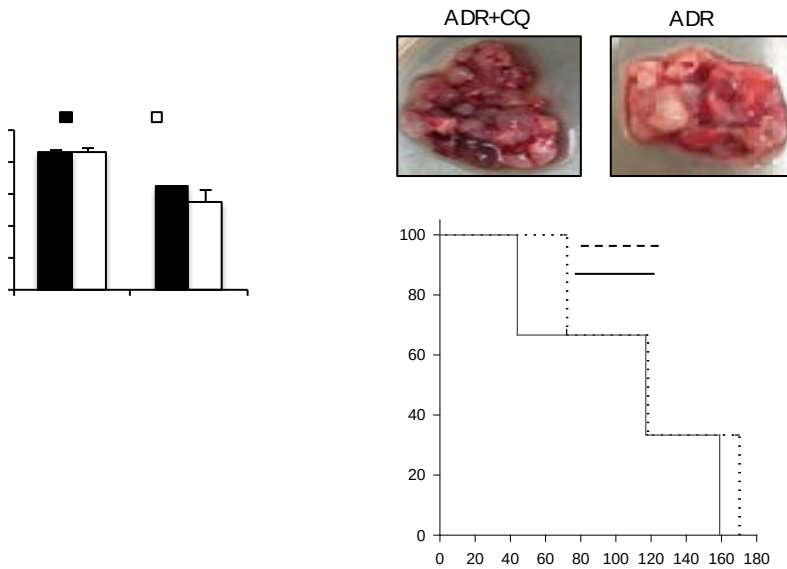
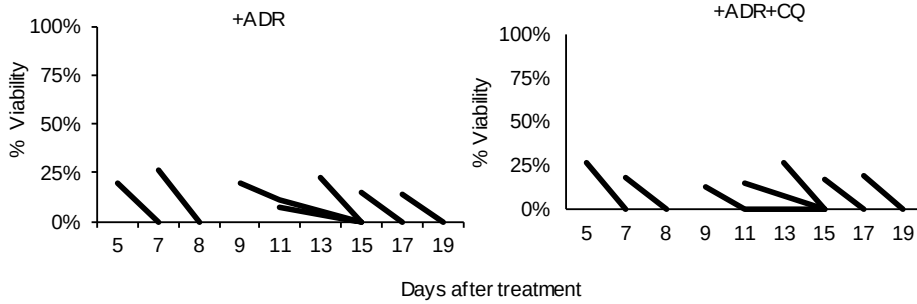


Figure 4. ADR-induced dormant tumor cells produce floater apoptotic cells, *in vitro*. MMC tumor cells (3×10^6 cells/flask) were treated with 3 daily doses of ADR (1 μ M for 2 hrs), with one group receiving CQ (10 μ M) 3 hrs prior to and during ADR treatment. Both groups remained untreated for 3 weeks and 6 weeks, *in vitro*. A) Floater cells were collected whenever culture medium was replaced and cell number and viability was assessed via trypan blue exclusion. Data represent 3 independent experiments and mean $\pm$ SEM. B) Floater cells were cultured separately for 2-3 days each time they were collected, and assessed for viability 2-4 days later by using trypan blue staining.

A stable knockdown of autophagy results in earlier tumor relapse associated with increased frequency of polyploid-like cells and resistance to immunotherapy

In order to determine whether a higher viability of ATG5^{KD} MMC following ADR treatment (Figure 9) facilitates an earlier tumor relapse compared with wild type MMC, follow up studies were performed for three weeks after ADR treatment. As shown in Figure 10A, ATG5^{KD} MMC survived better than autophagy-competent MMC following ADR treatment showing a significantly higher number of cells by 3 weeks after the treatment. Flow cytometry analysis of tumor cells showed greater levels of apoptosis in wild type MMC compared with ATG5^{KD} MMC (Figure 10B, $p < 0.001$). Interestingly, ATG5^{KD} MMC cells contained a higher number of polyploid-like cells following ADR treatment compared with autophagy-competent MMC (Figure 10B, $p < 0.03$).

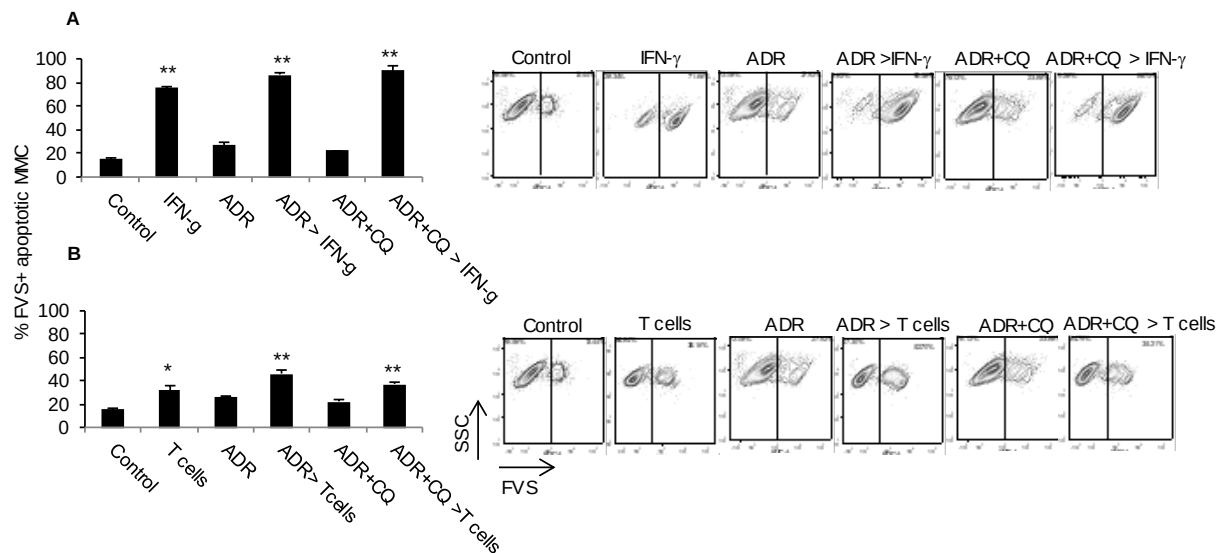


Figure 7. Dormant tumor cells established by ADR or ADR+CQ remain susceptible to immunotherapy. The in vitro tumor dormancy was established three weeks after three daily treatments of MMC with ADR or ADR+CQ. Untreated MMC cells were used as control. A) Apoptosis was determined by FVS viability staining in MMC (control), ADR-treated dormant MMC (ADR), ADR+CQ-treated dormant MMC (ADR+CQ), as well as control MMC cultured with three daily doses of IFN- γ and analyzed two days later (50 ng/ml) (IFN- γ), ADR-treated dormant MMC cultured with three daily doses of IFN- γ (50 ng/ml) and analyzed two days later (ADR > IFN- γ), or ADR+CQ-treated dormant MMC cultured with three daily doses of IFN- γ (50 ng/ml) and analyzed two days later (ADR+CQ > IFN- γ). B) Apoptosis was determined by FVS viability staining of MMC (control), MMC cultured with MMC-sensitized T cells for 48 hrs (T cells), ADR-treated dormant MMC (ADR), ADR-treated dormant MMC cultured with MMC-sensitized T cells for 48 hrs (ADR > T cells), ADR+CQ-treated dormant MMC (ADR+CQ), or ADR+CQ-treated dormant MMC cultured with MMC-sensitized T cells for 48 hrs (ADR+CQ > T cells). Splenic T cells were collected from MMC tumor-bearing FVBN202 mice.

In order to determine the in vivo relevance of our in vitro findings, FVBN202 mice were used. Tumor dormancy was first established by ADR treatment in vitro; FVBN202 mice ($n=7$ /group) were then challenged i.v. with one million viable dormant tumor cells. Animals were then sacrificed when they became moribund (lost 10% weight) as a result of massive lung metastasis. As can be seen in Figure 11A, animals that were challenged with ADR-treated ATG5^{KD} MMC developed lung metastasis significantly sooner than those that were challenged with ADR-treated MMC. Hematoxylin/eosin and immunohistochemistry analyses of tumor lesions determined a higher frequency of polyploid-like and Ki67+ tumor cells in animals that were challenged with ADR-treated ATG5^{KD} MMC (Figure 11B). Finally, ATG5^{KD} MMC were found to be resistant to T cell-induced apoptosis compared with autophagy-competent MMC (Figure 12).

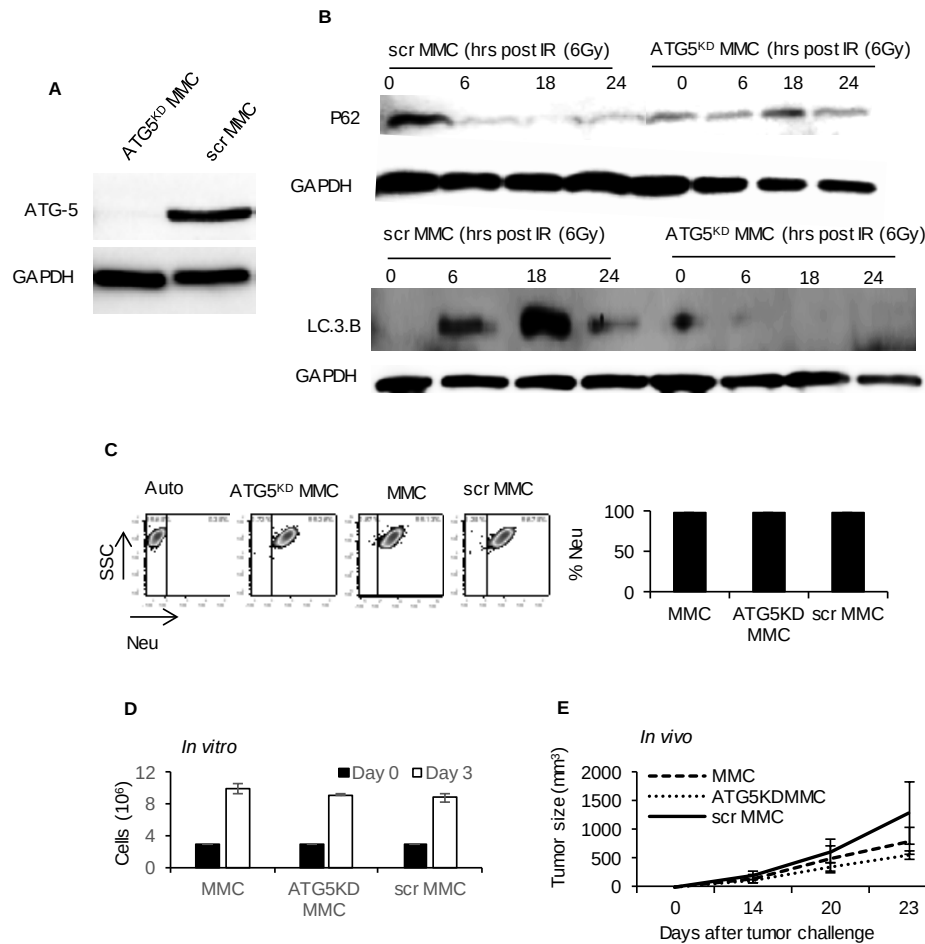


Figure 8. ATG5 knockdown tumor cells and wild type MMC show a similar pattern of growth. MMC cells were stably transfected with lentivirus expressing shRNA against ATG5 to establish autophagy-deficient cells (ATG5^{KD} MMC). Control MMC (MMC) were stably transfected with scrambled control vector as autophagy-competent cells (scr MMC). A) Cell lysates were collected and used for immunoblotting against ATG5. B) ATG5^{KD} MMC and scr MMC were treated with IR (6G) and cells lysates were collected at 6, 18, 24 hrs post treatment. Autophagy was determined by degradation of p62 and accumulation of LC3.B C) Expression of Neu protein was determined on autophagy-competent control MMC (MMC or scr MMC) and autophagy-deficient MMC (ATG5^{KD} MMC) using FACS analyses. D) Tumor cell proliferation was determined in a 3-day culture using trypan blue exclusion. E) FVB202 mice (n=3) were inoculated with autophagy-competent MMC (MMC or scr MMC) or autophagy-deficient MMC (ATG5^{KD} MMC) (3×10^6 cell/mouse, s.c. inoculation), and tumor growth was monitored by using a digital caliper. Data represents triplicate experiments.

Dormant MMC cells established by ADR or radiation therapy (RT) become resistant to higher doses of chemotherapy or RT, but remain sensitive to immunotherapy

In order to determine whether dormant MMC cells established by ADR treatment remain sensitive to tumor-reactive immune cells, dormancy was established by treating MMC with three daily doses of ADR (1 μ M/day for 2 hs); eight days after the final treatment, MMC cells received a high dose of ADR (1 μ M for 24 hs), or were cultured with tumor-reactive immune cells for 48 hs. ADR treatment induced apoptosis in MMC cells (Figure 13A-B, $p=0.01$). Tumor cells that survived apoptosis became chemo-refractory such that additional ADR treatment at a higher dose (1 μ M for 24 hs) did not induce cell death (Figure 13A-B, average 40% vs. 54%). However, they remained sensitive to tumor-reactive immune cells. In the presence of tumor-reactive immune cells, the frequency of viable ADR-treated dormant MMC dropped from 40% to 8% (Figure 13A-B, $p=0.003$). In fact, lymphocytes were more effective than a high dose of chemotherapy in inducing apoptosis in dormant MMC (Figure 13A-B, $p=0.02$). We also established dormant MMC by three daily doses of RT (2 Gy/day); again surviving dormant cells became refractory to RT. An additional RT at a higher dose (18 Gy) did not markedly decrease the frequency of viable tumor cells (Figure 13B-C, 53% vs. 52%). However, RT-refractory MMC cells remained sensitive to tumor-reactive lymphocytes as the viability dropped from 53% to 8% (Figure 13B-C, $p=0.002$). Recapitulating our results with chemotherapy-induced tumor cell dormancy, tumor-reactive immune cells were more effective than high dose RT at inducing apoptosis in dormant MMC (Figure 13B-C, $p=0.01$). In order to determine whether higher levels of apoptosis in

dormant tumor cells were due to their greater sensitivity to immune cells rather than a higher reactivity of the immune cells, IFN- γ ELISA was performed using re-programmed immune cells cultured with either MMC tumor cells or ADR-, RT-induced dormant MMC cells. As shown in Fig. 13D, tumor-reactive immune cells produced comparable levels of IFN- γ upon stimulation with MMC or dormant MMC (RT-MMC, ADR-MMC).

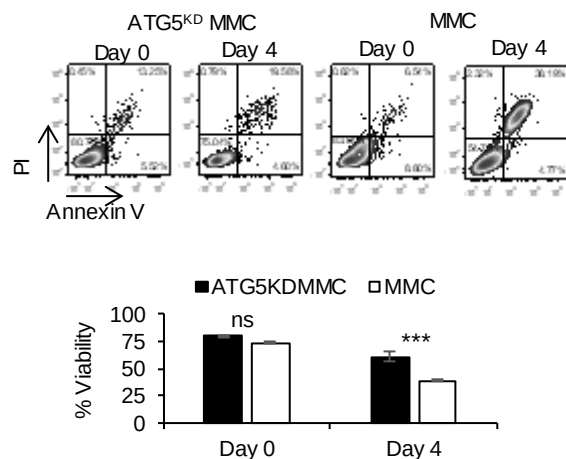


Figure 9. Autophagy knockdown tumor cells become less susceptible to ADR-induced apoptosis. Autophagy-deficient MMC (ATG5^{KD} MMC) or autophagy-competent MMC (MMC) were treated with a single dose of ADR alone (1 μ M ADR for 2 hrs). Tumor cells were analyzed by Annexin V/PI staining prior to treatment (Day 0) or three days after the treatment (Day 4). Experiments were performed in triplicates.

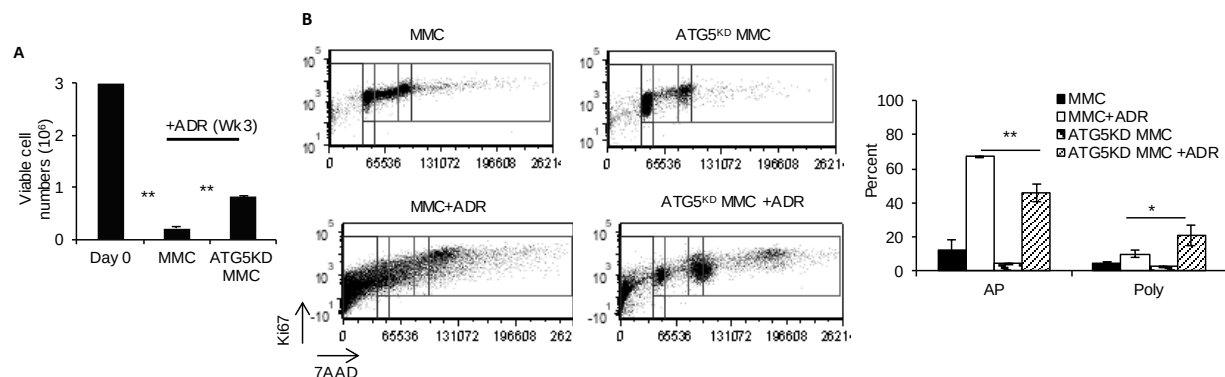


Figure 10. ADR-induced tumor dormancy in autophagy knockdown tumor cells with polyploid-like morphology compared with autophagy competent tumor cells, in vitro. MMC or ATG5^{KD} MMC tumor cells (3 million cells, Day 0) were treated with 3 daily doses of ADR (1 μ M for 2 hrs), and viable cells were counted at week 3 using trypan blue exclusion. Data represent triplicate experiments (A). Dot plots from each experimental group gated for cell cycle phase based upon DNA content (7-AAD) and Ki-67 expression. Events falling to the left of the G1/G0 gates are considered apoptotic cells (AP). Events falling to the far right of the G2/M gate are considered polyploid-like cells (Poly) (B). Three independent experiments have been performed and data represent 3 replicates $\pm$ SEM.

ADR induces two types of tumor dormancy: indolent and quiescent

In order to determine whether dormant MMC cells that were established by ADR were in the state of non-proliferative quiescent dormancy or were capable of sluggish proliferation (balanced proliferation and death), MMC cells were stained with a clinically relevant proliferation marker, Ki67. ADR treatment shifted Ki67 positive (Ki67+/high) highly proliferating MMC towards Ki67+/low indolent cells as shown by a significant drop in the Ki67's MFI (Figure 5, $p = 0.026$), as well as shifting towards Ki67 negative (Ki67-) quiescent cells (Fig. 14, 3% vs. 22%, $p = 0.01$). In order to determine whether indolent and quiescent types of tumor dormancy were present in vivo, FVBN202 mice were inoculated with MMC in the mammary gland (3×10^6 cells/mouse). Animals were either served as control (MMC) or received ADR chemotherapy when tumors became palpable (MMC+ADR). As shown in Figure 15, ADR chemotherapy inhibited tumor

growth and established local dormancy. Animals were then euthanized and their tumors subjected to H & E staining and IHC for Ki-67. Whereas all tumors in control group (MMC) were Ki-67+ (dark brown), tumors in the treatment group (MMC+ADR) were mostly Ki-67+/low indolent cells (light brown) and some Ki-67- quiescent cells (background blue color).

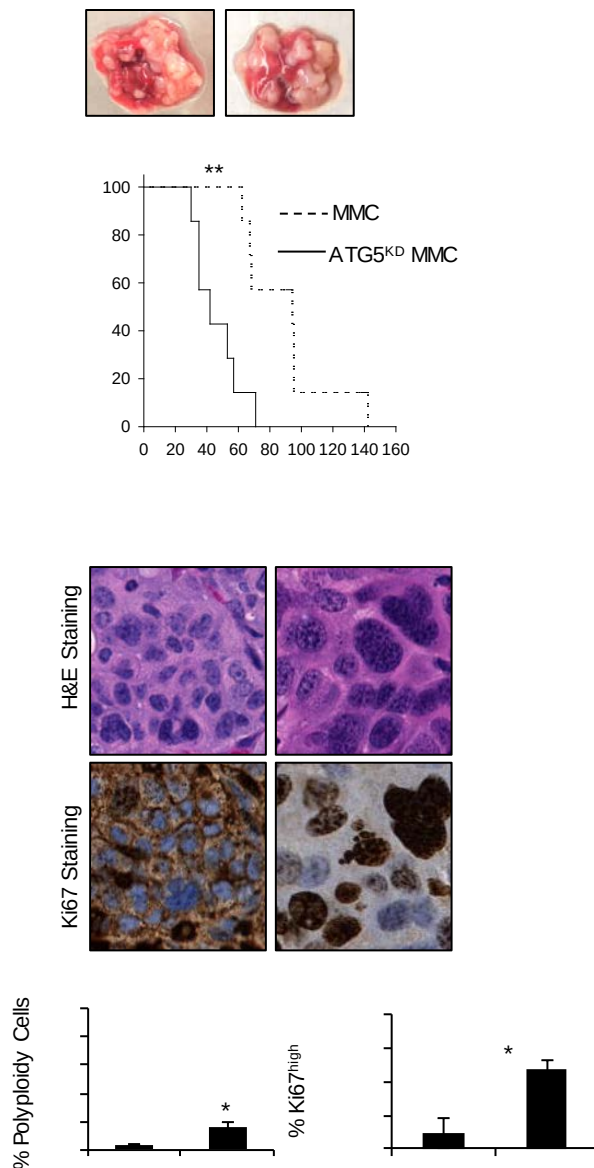


Figure 11. Earlier relapse of autophagy knockdown tumor cells with polyploid morphology compared with autophagy competent tumor cells, in vivo.

A) FVBN202 mice (n=7) were challenged i.v. with 10^6 cells ADR-treated dormant control MMC (MMC), or ADR-treated dormant ATG5^{KD} MMC (ATG5^{KD} MMC). Animals were euthanized as soon as they became moribund. Representative tumor relapse in the lung and survival curve are shown. B) Relapsed tumors were collected and immunohistochemistry slides were prepared by either staining samples with hematoxylin and eosin (H&E) or by Ki67 staining followed by subsequent digitization and analysis with NDP View software (Hamamatsu Photonics). At twenty-times magnification, three representative 0.02mm^2 areas were chosen from the H&E slides containing approximately 100 cells to measure nuclear envelope size. Cells containing a nuclear envelope equal to or greater than $16\mu\text{m}$ with visible multi-nuclei were considered polyploid-like or high grade cells. The corresponding cell was then analyzed on the Ki67 stained slide to determine Ki67 expression levels. Data was collected from three biological samples. Significance is based on a two-tailed t-test of $p<0.05$.

Indolent but not quiescent dormant tumor cells are prone to immunoediting and escape from immunotherapy

In order to determine sensitivity of dormant tumor cells to immunoediting and escape from immune response, expression of PD-L1, a suppressor of the immune response, was determined on dormant cells. Since IFN- γ was shown to be a major product of the immune response that induces tumor immunoediting, we wanted to determine if IFN- γ upregulates PD-L1 expression on Ki67+/low indolent dormant cells and/or Ki67- quiescent dormant tumor cells. First, ADR-induced tumor dormancy was established 3 weeks after the treatment cessation (Figure 16; + ADR). Dormant MMC were then treated with IFN- γ (+ ADR $\rightarrow$ IFN- γ) and analyzed for the expression of PD-L1 after 12 hrs. MMC cells (untreated) or MMC cells pulsed with IFN- γ (Untreated $\rightarrow$ IFN- γ) or ADR-treated dormant MMC (+ADR) served as controls. We detected the IFN- γ -induced upregulation of PDL-1 on Ki67+ untreated MMC and on Ki67+/low ADR-treated indolent dormant MMC (Figure 16B); however, IFN- γ did not upregulate PD-L1 on Ki67- quiescent tumor cells (Figure 16C).

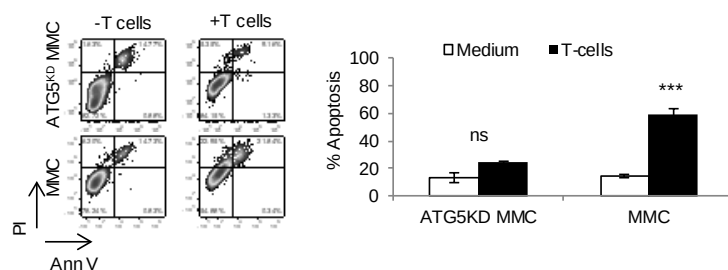


Figure 12. Autophagy knockdown tumor cells become resistant to T cell-induced apoptosis. Neu overexpressing autophagy-deficient MMC (ATG5^{KD} MMC) or autophagy-competent MMC (MMC) were co-cultured with MMC-sensitized T cells and then gated CD45-Neu⁺ tumor cells were analyzed by Annexin V/PI staining. Data represents triplicate experiments.

Dormant and relapsing MMC show unique inflammatory signature

We performed microarray analysis for each experimental group (n=3). Untreated MMC and MMC+CQ day 4 were included as controls. Fold change in gene expression for each group was determined by comparison with untreated MMC control. Unsupervised cluster analysis shows close clustering among the ADR+CQ groups at both the 3-week and 6-week time points, representing maintenance of dormancy. Conversely, the ADR groups at the 3-week and 6-week time points, representing dormancy and relapse, clustered apart from one another (Figure 17). We then sought to determine the gene profile unique to each experimental group. Venn diagram analysis showed 239 genes unique to dormancy when both dormant groups were compared (MMC+ADR week 3 vs MMC+ADR+CQ week 3) (Figure 18A upper panel). 682 genes unique to prolonged dormancy were found by comparing the fold changes of the relapsing group (MMC+ADR week 6) with the prolonged dormancy group (MMC+ADR+CQ week 6) (Figure 18A lower panel). The prolonged dormancy group (MMC+ADR+CQ week 6) and relapsing group (MMC+ADR week 6) shared 882 common probe sets. Each probe set group was then analyzed for disease function by Ingenuity Pathway Analysis (IPA). The 239 genes involved in dormancy showed a z-score increase in disease states related to acute inflammation, while the 682 genes unique to relapse showed predicted activation in disease states related to chronic inflammation (Figure 18B). The 882 genes shared by both week 6 groups, one relapsing and the other dormant, showed predicted activation of both chronic and acute disease states.

In order to pinpoint pathways and proteins involved in inflammation, all probe sets that showed a significant upregulation or downregulation for each experimental group were uploaded to IPA and comparison analysis on canonical pathways and upstream regulators was performed (Figure 18C). Most notably, RelA (NF- κ B p65) showed predicted activation (z-score=2.6) only in the MMC+ADR week 3 dormant group. NF- κ B (complex) showed predicted activation in all groups, however, the highest z-scores were in the MMC+ADR week 6 relapsing group and

MMC+ADR+CQ week 6 prolonged dormancy group (z-score=3.26 and 2.79). Type 1 interferons (alpha and beta) showed predicated activation in the MMC+ADR week 3 dormant group (z-score= 3.5 and 2.6) and the MMC+ADR+CQ week 3 dormant group (z-score=2.5 and 1.77). Interferon gamma (IFNG) also showed predicted activation in both dormancy groups (z-score=2.9 and 2.5). Interferon Signaling was shown to be highly activated (z-score=3.16) only in the MMC+ADR week 3 dormant group.

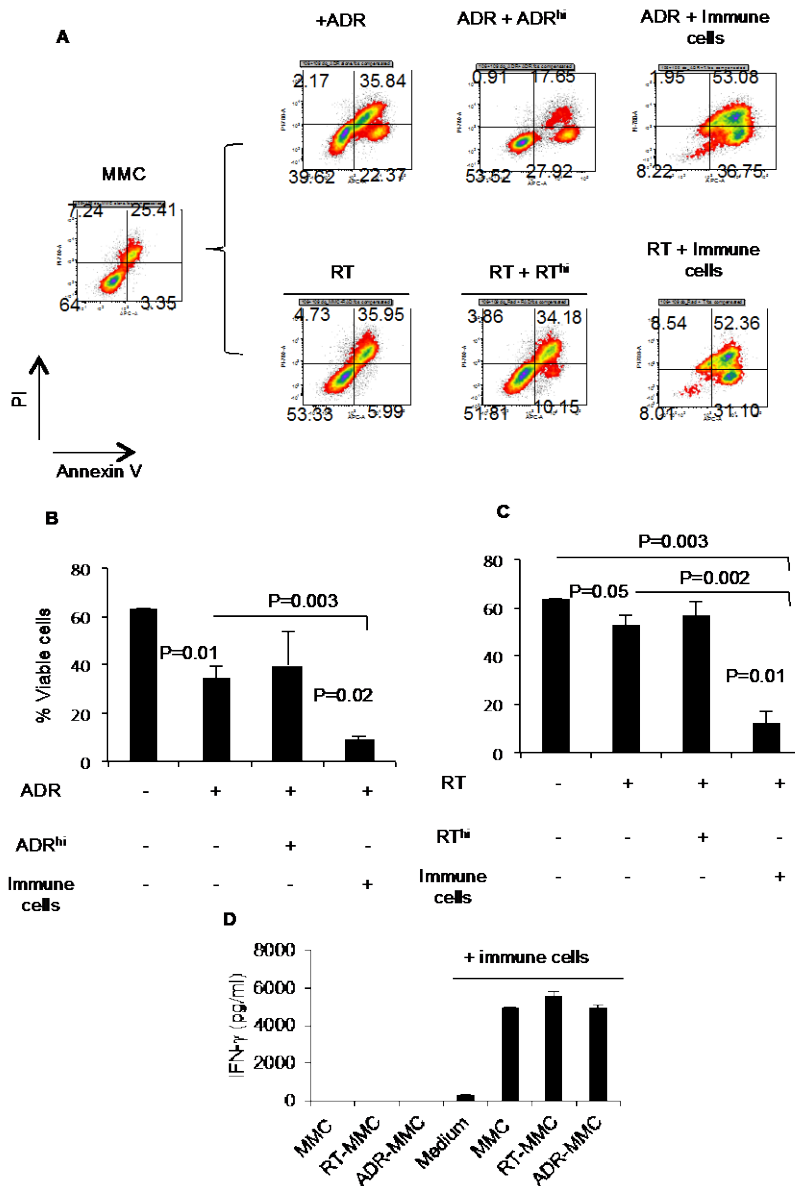


Figure 13. Immunotherapy displays cytotoxic function against treatment-refractory dormant tumor cells, *in vitro*.

A) MMC cells (n=3) treated with ADR (1 μ M, 2 hs) or 2 Gy RT (RT-treated MMC) for 3 consecutive days and remained in culture for 8 days total, in order to establish tumor cell dormancy *in vitro*. B) On day 8, these dormant tumor cells were treated with a high dose ADR (1 μ M, 24 hs) (ADR-treated MMC + ADR^{hi}) or reprogrammed immune cells (ADR-treated MMC + Immune cells; ADR-treated MMC + Immune cells). Two days later, cells were stained with Annexin V/PI and analyzed by flow cytometry. Data represent three biological repeats and mean $\pm$ SEM. C) On day 8, these dormant tumor cells were treated with 18 Gy RT (RT-treated MMC + RT^{hi}) or reprogrammed immune cells (RT-treated MMC + Immune cells). Two days later, cells were stained with Annexin V/PI and analyzed by flow cytometry. D) MMC tumor cells (MMC) or dormant MMC cells (RT-MMC, ADR-MMC) were cultured in the absence or presence of the reprogrammed immune cells in a 10:1 ratio for 24 hs. Control immune cells were cultured alone (Medium). IFN- γ release was detected in the supernatant using ELISA. Data represent two biological repeats and mean $\pm$ SEM.

In order to determine whether the predicted activation of RelA led to the secretion of inflammatory cytokines during dormancy (preceding relapse) a multiplex cytokine array was performed on the supernatant from the MMC+ADR week 3 group. Cytokines probes were chosen by analysis of robust multi-array average (RMA) expression data for inflammatory cytokines that showed unique upregulation during dormancy. Choice of cytokine was also limited based on market availability. The protein concentration corroborated mRNA expression

level from the microarray data for each cytokine, except RANTES, which shows a decrease in protein expression (Figure 19).

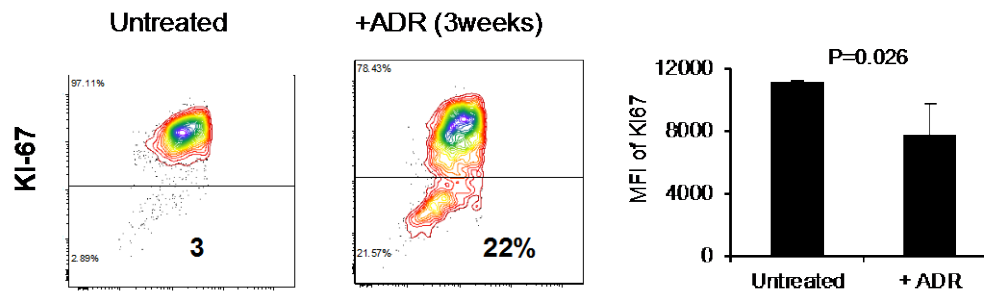


Figure 14. ADR induces indolent (Ki67+/low) and quiescent (Ki67-) types of tumor dormancy. MMC cells were treated with three daily doses of ADR (1 mM, 2hrs). Adherent MMC were analyzed for the expression of Ki67 before and 3 weeks after the treatment.

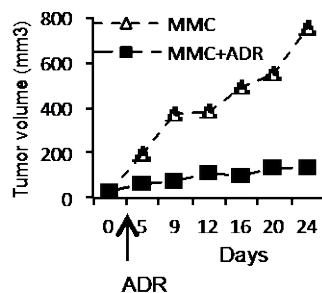
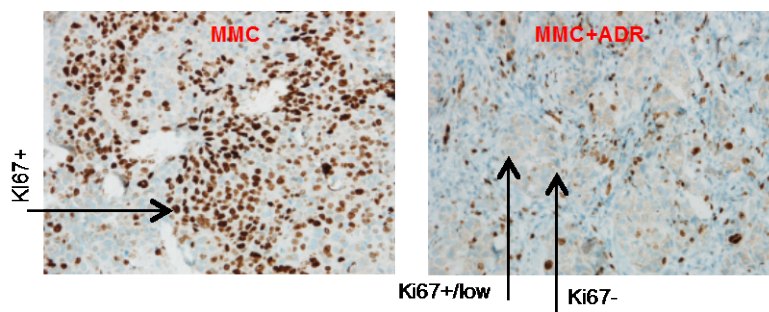


Figure 15. ADR treatment induces indolent and quiescent types of tumor dormancy, in vivo. FVBN202 mice were challenged with MMC (3x10⁶) in the mammary gland region; after tumors reached 30-50 mm³ animals were treated with ADR (MMC+ADR; 20 mg/kg; i.v.), or remained untreated (MMC). Tumor growth was monitored for 4 weeks, and tumors were removed and subjected to IHC analysis of Ki67.



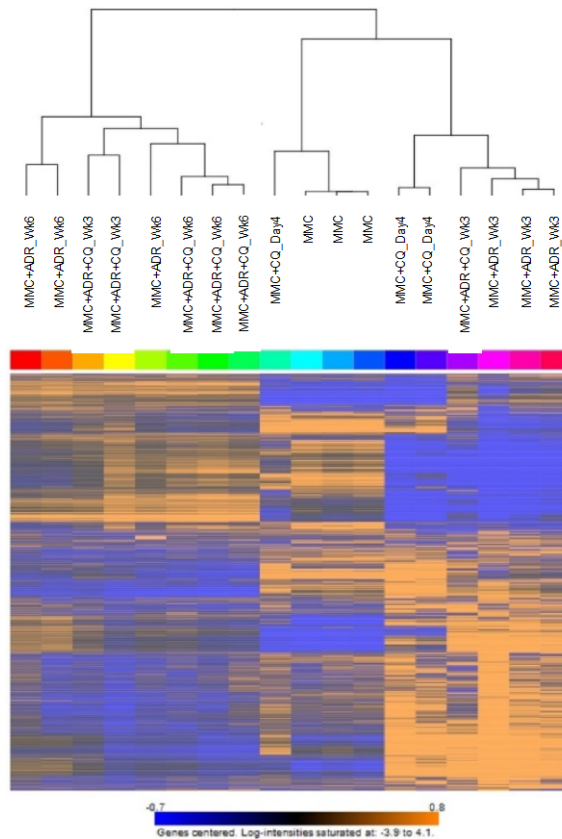
ADR-treated shNF- κ B p65 exhibit reduced growth and a reduced rate of relapse *in vitro* and *in vivo*.

Though CQ is most noted for its effects on blocking autophagy, unpublished data from our lab showed that autophagy protein 5 (ATG5) shRNA knockdown MMC resulted in a higher rate of relapse compared to control MMC. Therefore, transient blockade of autophagy alone during drug treatment could not be the cause for the delay in relapse of that group. In addition to blockade of autophagy, CQ has been shown to inhibit NF- κ B through blockade of I κ B degradation. Because of such findings, and IPA results suggesting unique NF- κ B p65 signaling pathways during dormancy, we created an shRNA knockdown of NF- κ B p65 in MMC in order to determine if prolonged dormancy in CQ-treated MMC was due to NF- κ B inhibition. MMC were transduced using lentiviral particles containing NF- κ B p65 shRNA (shNF- κ B p65) or SCR shRNA (SCR-

MMC) and remaining cells were subject to puromycin selection and western blot analysis (Figure 20A). Both groups showed similar rates of proliferation, *in vitro* (Figure 20B).

Fig. 17. Dormant MMC exhibit unique gene signature compared to relapsing MMC

Unsupervised cluster analysis shows a close clustering of experimental groups MMC +ADR+CQ at weeks 3 and 6 during the establishment and maintenance of tumor dormancy. However, tumor cells that enter the dormant state at week 3 and resumed a sluggish proliferation at week 6 (MMC +ADR week 3 and week 6) were clustered apart from each other. Control groups (MMC and MMC + C Q) were clustered together.



Growth rate and response to ADR treatment was determined *in vivo* by subcutaneously injecting FVBN202 mice with 3 million shNF- κ B p65 or SCR-MMC in the mammary fat pad (n=3). Tumor size initially showed no significant changes in growth between both groups (Figure 21A). However, when animals were treated intravenously with ADR (9mg/kg) every 3 days beginning on day 36, the shNF- κ B p65 tumors showed significantly (p=0.01) reduced growth compared to SCR-MMC tumors by day 54. Fractions of tumor cells were then cultured *in vitro* for 2 weeks upon resection from the animal in order to confirm stable shRNA knockdown of NF- κ B p65. Western blot analysis of each tumor shows maintained knock down of NF- κ B p65 to varying degrees (Figure 20B).

The shNF- κ B p65 MMC showed increased neu expression in response to ADR treatment *in vitro* and *in vivo*

We then sought to determine if tumor-intrinsic NF- κ B p65 signaling pathways had any effect on immunomodulation of ADR-treated MMC by analyzing neu, PD-L1, and MHCI expression. shNF- κ B p65 and SCR-MMC were treated with ADR as described above (n=3). On day 7 post treatment, cells were detached and analyzed for neu, PD-L1, and MHCI expression by flow cytometry. Mean florescence intensity (MFI) showed significant upregulation of neu expression in ADR-treated cells when compared to untreated control in both groups (p=0.0007 and p=0.01) (Figure 22A). However, shNF- κ B p65 displayed significantly higher upregulation when compared to SCR-MMC (p=0.006). Both shNF- κ B p65 and SCR-MMC showed no increase in

PD-L1 MFI after ADR treatment, and while MHC1 MFI did increase upon ADR treatment, there were no significant differences between shNF- κ B p65 and SCR-MMC. Due to significant changes in neu expression between ADR-treated shNF- κ B p65 and SCR-MMC, we chose to focus solely on neu expression for *in vivo* staining. Tumors resected from shNF- κ B p65 or SCR-MMC-inoculated mice treated with ADR (n=3) (described above), along with control mice (n=1), were stained for neu expression following the same protocol. Neu upregulation showed the same trend *in vivo*, with increased neu MFI in ADR-treated mice but a larger increase in the shNF- κ B p65 compared to SCR-MMC tumors (p<0.0001) (Figure 22B).

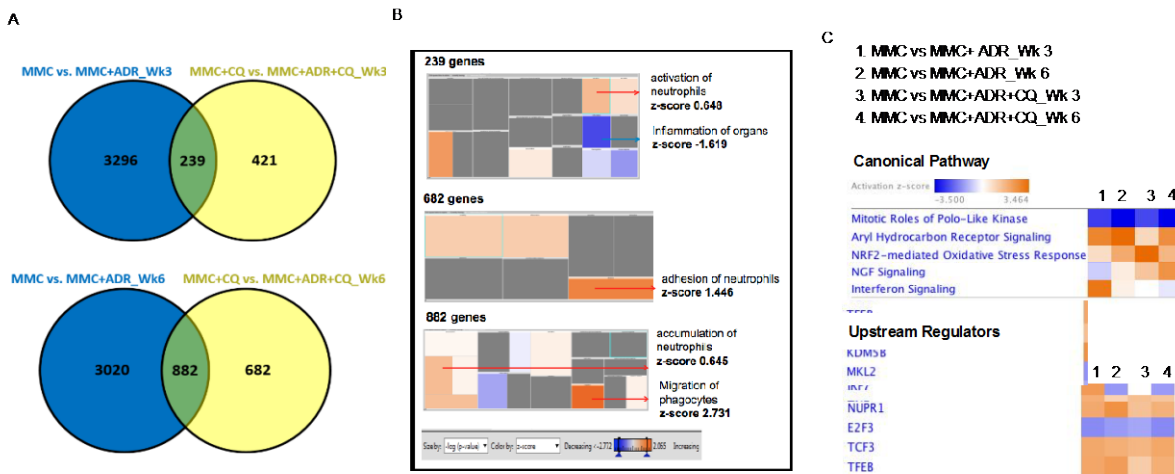


Figure 18. Inflammatory gene signature during dormancy and relapse. A) Venn Diagram analysis isolated 239 probe sets unique to dormancy when comparing both dormant groups (upper panel) and 682 unique probe sets unique to relapse when both week 6 groups were compared (lower panel). 882 probe sets were shared between the MMC+ADR week 6 relapsing group and the MMC+ADR+CQ week 6 prolonged dormancy group. Venn diagrams were generated from microarray fold-change expression data. Fold change was calculated by comparing each group to MMC control expression. B) Ingenuity Pathway Analysis (IPA) reveals genes among 239 shared probe sets involved in maintenance of dormancy shows predicted activation of disease states related to acute inflammation, 682 probe sets unique to relapse show predicated activation of disease states related to acute inflammation, and 882 shared probe sets involved in both maintenance and escape from dormancy show predicated activation of disease states related to acute and chronic inflammation. C) IPA comparison analysis shows predicted activation and inhibition of canonical pathways and upstream regulators based on z-score. RelA showed a significant (z-score=2.6) predicted activation in the dormant group (MMC+ADR week 3). All significant (p<0.0001) fold change expression data for each experimental group was included in comparison analysis.

The shNF- κ B p65 MMC show reduced tumor-infiltrate and a reduced immunostimulatory effects

We then sought to determine if tumor-intrinsic NF- κ B p65 signaling pathways had an effect on infiltration of CD45+ immune cells or the particular immune-cell type. shNF- κ B p65 or SCR-MMC tumors resected from ADR-treated mice (n=3) were stained with CD45 and compared with that of non-treated mice (n=1) (Figure 23A). Additional staining for CD11b, GR1, CD3, CD4, CD8, B220, CD49b was performed only on non-treated mice from each group. Log₂ frequency ratios showed the fold change increase or decrease in percentages of each cell type and was calculated by comparing the log₂ ratio of shNF- κ B p65 sample percentages to those of SCR-MM. While total CD45+ infiltrate showed no significant differences between the ADR-treated groups, the ADR-treated wild type tumors (Scr-MMC) showed greater CD45+ infiltrates than their untreated control cells, when compared with the shNF- κ B p65 MMC tumor cells. The Scr-MMC tumor site contained an increased CD11b+GR1+ MDSCs compared to the SCR-MMC tumor. In addition, the shNF- κ B p65 MMC tumors showed a decreased CD8+ T cell infiltrates.

In order to investigate the role of NF- κ B p65 in the anti-tumor immune response, splenocytes were collected from ADR-treated mice which had been inoculated with either shNF- κ B p65 or SCR-MMC tumors (n=3). Reprogramming of tumor-sensitized immune cells was done *ex vivo*,

as previously described by our group. Splenocytes from shNF- κ B p65 tumors showed significant ($p=0.05$) reduction in expansion, based on final cell count, compared with those from SCR-MMC tumor mice (Figure 23B). MMC remained sensitive to tumor-reactive lymphocytes taken from SCR-MMC tumor mice, with 65% apoptosis of target MMC. However, tumor-sensitized lymphocytes isolated from shNF- κ B p65 tumor mice showed reduced cytotoxic function against MMC, inducing 49% apoptosis ($p=0.05$).

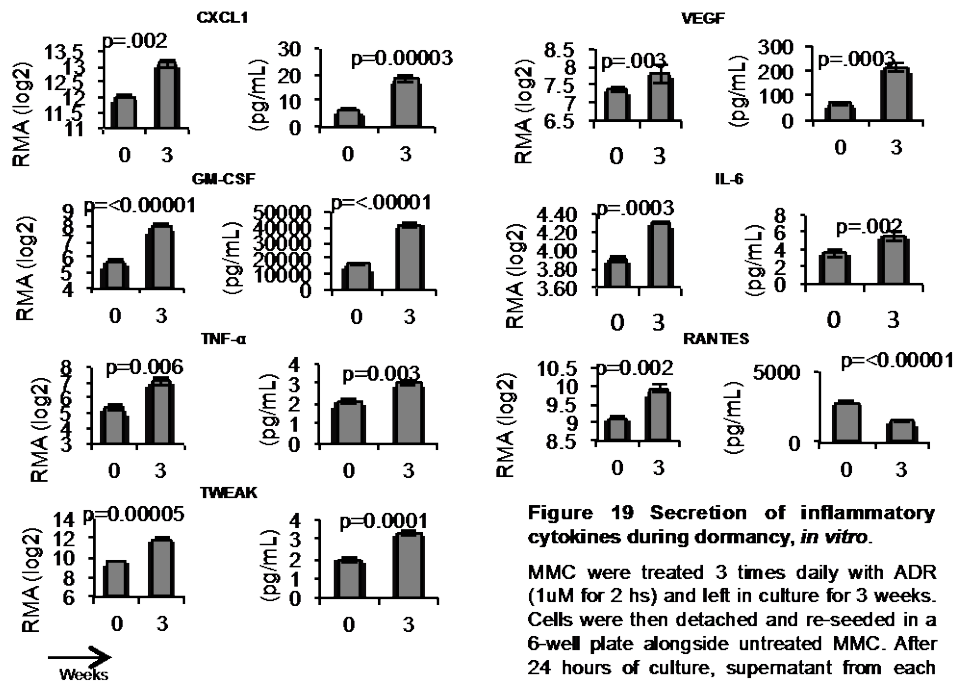


Figure 19 Secretion of inflammatory cytokines during dormancy, *in vitro*.

MMC were treated 3 times daily with ADR (1 μ M for 2 hs) and left in culture for 3 weeks. Cells were then detached and re-seeded in a 6-well plate alongside untreated MMC. After 24 hours of culture, supernatant from each well was collected. RMA (log) value from microarray data and concentration (pg/mL) of each cytokine are compared side-by-side. Targets were chosen based on microarray data and commercial availability.

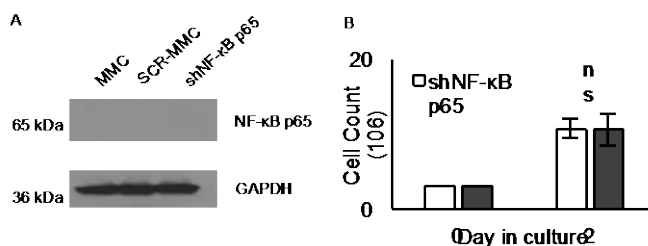


Figure 20. NF- κ B p65 knockdown in MMC reduces the rate of relapse, *in vitro*. A) Western Blot analysis shows knock down of NF- κ B p65 shRNA-transduced MMC, after puromycin selection, alongside control MMC and SCR-MMC. GAPDH was used as the housekeeping control. B) shNF- κ B p65 and SCR-MMC were seeded at 3×10^6 cells/flask and left in culture for 48 hours. Cells were detached and counted via trypan blue exclusion. Data represent 3 independent experiments and mean $\pm$ SEM.

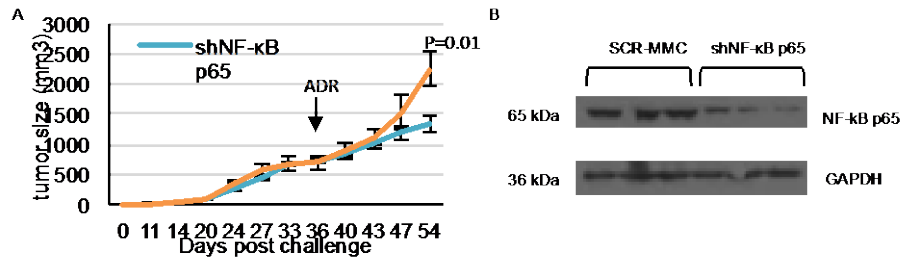


Figure 21. The shNF-κB p65 tumors show reduced growth in response to ADR treatment, in vivo . A) 3×10^6 shNF-κB p65 or SCR-MMC were injected subcutaneously into the mammary fat pad of FVB/N202 naïve mice (n=3). Mice were treated every 3 days with 9mg/kg of ADR beginning 36 days post challenge, when tumors has reached 800mm³, and euthanized on day 54 when tumors had reached greater than 2000mm³. Tumors were measured twice weekly by digital caliper. B) Remaining tumor samples were cultured for two weeks and western blot analysis was performed separately for each animal.

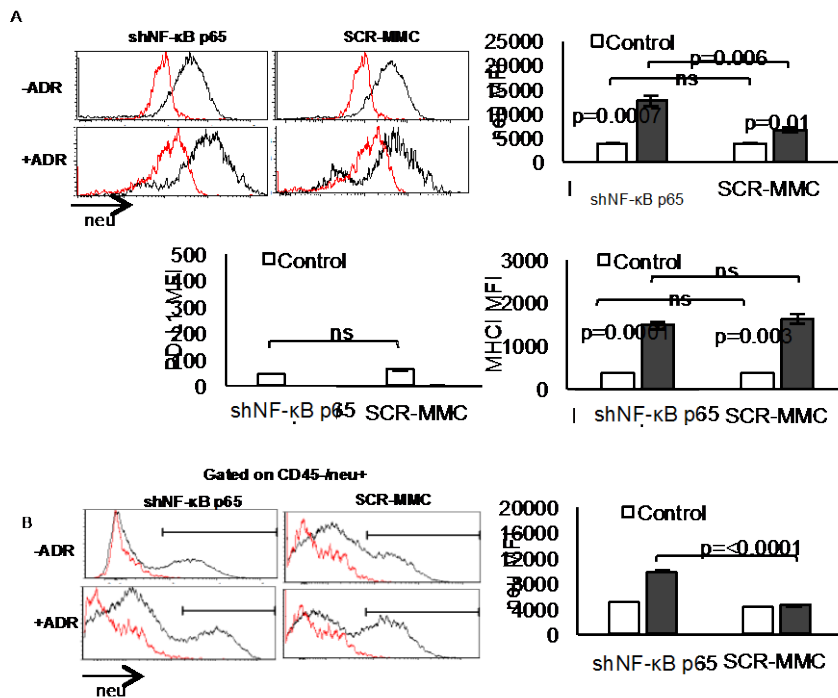


Figure 22. The shNF-κB p65 MMC show higher upregulation of neu in response to ADR treatment. A) shNF-κB p65 and SCR-MMC were treated 3 times daily with ADR (1uM for 2 hrs) and left in culture for 1 week. Neu, PD-L1, and MHC1 expression was determined by MFI B) 3×10^6 shNF-κB p65 or SCR-MMC were injected subcutaneously into the mammary fat pad of FVB/N202 naïve mice. Mice were treated every 3 days with 9mg/kg ADR 36 days post challenge, when tumors has reached 800mm³, and euthanized on day 54 when tumors had reached greater than 2000mm³. Control mice were injected with 5×10^6 of each and euthanized when tumors had reached >800mm³. Tumors were resected from mice and measured for neu expression by flow cytometry. MFI was determined by gating on CD45-neu+ cells only.

Level of the expression of IFN-γ Ra on tumor cells do not protect them from tumor progression

Since IFN-g is involved in tumor immunoediting which in turn could facilitate tumor progression and relapse, we sought to determine whether lack or overexpression of IFN-g Ra on tumor cells may render them susceptible to endogenous immune response and inhibit tumor relapse. We established MMC tumor cells overexpressing IFN- γ R α^{high} by stable transfection of pcDNA3 vector (Invitrogen) containing mouse IFN- γ R α ORF construct. For this, cDNA of IFN- γ R α was amplified using mouse mammary tumor cDNA library as a template in PCR reaction with proofreading polymerase (Accuzyme, BioLine). PCR conditions were as follows: 94°C 3 min, 94°C 30 s, 48°C 30 s, 68°C 2 min (10 cycles); 94°C 30 s, 60°C 30 s, 68°C 2 min (20 cycles) followed by 10 min extension at 68°C. Primers used in this reaction: sense: 5'-TTTATGGTACCATGGGCCCCGAGGCGGCA-3' and antisense: 5'-TTAGATATCTTAGGACAGCTCCTGGGCCTC-3' containing restriction sites for KpnI and EcoRV, respectively (underlined). Amplified cDNA fragment and pcDNA3+ vector (Invitrogen) were then digested with KpnI and EcoRV endonucleases (NEB BioLabs) for preparing compatible ends for ligation reaction. After ligation, constructs with insert were isolated and sequenced to confirm intact expression of the gene. Transfection of MMC cells with construct was done using Lipofectamine 2000 Reagent (Invitrogen) according to manufacture protocol. Tumor cells were maintained in RPMI 1640 supplemented with 10% FBS. We used G418 antibiotic at a 200 $\mu\text{g/ml}$ (Gibco) for the selection. We also established MMC cell line expressing dominant negative IFN-g Ra (dnIFN-g Ra) as a model to inhibit IFN-g signaling in tumor cells. The dnIFN- γ R α and IFN- γ R α vectors were gifts from Dr. William Lee of the U Penn. Challenge of FVBN202 mice with IFN- γ R α^{high} MMC or dnIFN-g Ra MMC inhibited tumor growth compared with those of wild type MMC, but did not fully protect animals from tumor progression.

- **What opportunities for training and professional development has the project provided?**
 - Four PhD students, one Masters student, have been trained.
 - Five MD and one MD/PhD students have been trained.
 - Two medical oncology fellows have been trained.
 - Two visiting scientists from China have done fellowship training.
 - Five undergraduate students have been trained.
- **How were the results disseminated to communities of interest?**
 - 1) Concepts that are proposed in this project were used to formulate two undergraduate lectures on tumor dormancy and autophagy. As guest speakers, the initiating PI and collaborating PI each presented 1/5 hours lecture to over 200 undergraduate students in BIOL450 (Biology of Cancer). Among 15 guest lecturers, the initiating PI and collaborating PI were rated by the students as best basic science lecturers.
 - 2) Concepts that are proposed in this project were used to formulate two graduate level lectures- advanced immunology and molecular biology of cancer- related to cancer dormancy.
 - 3) As an invited speaker, Dr. Manjili gave a talk on “*Immunotherapy for cancer dormancy*”. The 2016 Controlling Cancer Summit, London, UK, May 17-19, 2016.

- 4) As an invited speaker, Dr. Manjili gave a lecture on tumor dormancy in the University of Connecticut, Title: *"The inherent premise of immunotherapy for cancer dormancy"*, Hartford, CT, May 5, 2016.
- 5) As an invited speaker, Dr. Manjili gave a lecture on tumor dormancy in the University of South Carolina, Title: *"Current status and future prospects of immunotherapy: Targeting cancer dormancy"*, Columbus, SC, September 4, 2015.
- 6) As an invited speaker, Dr. Manjili gave a lecture on tumor dormancy in the Cancer Cell Signaling group meeting, Massey Cancer Center, Title: *Immune-mediated tumor dormancy and inflammation*, September 2015
- 7) Concepts that are proposed in this project were used to formulate two graduate level lectures-advanced immunology and molecular biology of cancer- related to cancer dormancy.
- 8) As an invited speaker, Dr. Manjili gave a lecture on tumor dormancy in Mayo Clinic, Rochester MN. Title: *Immunotherapy is the only option for the treatment of cancer dormancy*". July 2017
- 9) As an invited speaker, Dr. Manjili gave a lecture on tumor dormancy in The VCU Institute of Molecular Medicine (VIMM) and Human & Molecular Genetics (HMG) seminar, VCU School of Medicine, Richmond VA. Title: *Paradigm shifts in cancer immunotherapy: targeting tumor dormancy*. May 2017
- 10) Two posters were presented at IMMUNOLOGY 2017TM AAI Annual Meeting, Washington D.C., May 12-16, 2017.
- 11) Concepts that are proposed in this project were used to formulate two graduate level lectures-advanced immunology and molecular biology of cancer- related to cancer dormancy.
- 12) As an invited speaker and keynote speaker, Dr. Manjili gave lectures on tumor dormancy at 14th International Congress of Immunology and Allergy (ICIA) in April 2018 Keynote speech: *"Theoretical framework for the efficacy of cancer immunotherapy"* (April 26, 2018); Plenary speech: *"The promise and challenges of cancer immunotherapy: The adaptation model of immunity"* (April 28, 2018)
- 13) One poster was presented at IMMUNOLOGY 2018TM AAI Annual Meeting, Austin, TX, May 4-8, 2018 [received 2018 AAI Laboratory Travel Grant Award]
- 14) One oral presentation was accepted at IMMUNOLOGY 2019TM AAI Annual Meeting, San Diego, CA, May 9-13, 2019 [received 2019 AAI Laboratory Travel Grant Award for oral and poster presentation]

- **What do you plan to do during the next reporting period to accomplish the goals?**

Nothing to Report

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

- Dr. Manjili provided expert commentary in a Twitter Chat hosted by the National Cancer Institute in April 2015 following the national airing of the PBS documentary cancer: the Emperor of All Maladies. The subject was "immunotherapy of cancer".

- Dr. Manjili provided expert commentary on "Accelerating Progress against Cancer" in a Twitter Chat hosted by ABC News (April 19, 2016)

- Our paper from this project was the most read article over the last six months of 2016 in Journal of Leukocyte Biology. First author of the manuscript, Kyle Payne, was invited to speak at the 2017 Annual Meeting of the Society for Leukocyte Biology (SLB) to be held in Toronto, October 5-7, 2017.
- Received AAI Laboratory Travel Award to present an abstract in the AAI Annual Meeting in 2018

- **What was the impact on other disciplines?**

The results had an impact on environmental science by linking how chemicals can cause tumor dormancy or escape from dormancy and result in recurrence. As a moderator, Dr. Manjili disseminated the results of tumor dormancy during the roundtable group that assessed data needed to better inform the low-dose mixture theory, Low-dose mixtures and cancer highlighted at NIEHS symposium, Durham, North Carolina, August 2015. (<http://www.niehs.nih.gov/news/newsletter/2015/9/spotlight-mixtures/index.htm>)

- **What was the impact on technology transfer?**

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS

- **Changes in approach and reasons for change**

Nothing to report

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report

- **Changes that had a significant impact on expenditures**

Nothing to report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report

- **Significant changes in use or care of human subjects**

Nothing to report

- **Significant changes in use or care of vertebrate animals**

Nothing to report

- **Significant changes in use of biohazards and/or select agents**

Nothing to report

6. PRODUCTS

- **Publications, conference papers, and presentations**

- **Journal publications**

1. Payne KK, Aqbi HF, Butler SE, Graham L, Keim RC, Wan W, Idowu MO, Bear HD, Wang XY, **Manjili MH**. Gr1^{-/low}CD11b^{-/low}MHCII⁺ myeloid cells boost T cell anti-tumor efficacy. *J Leukoc Biol* 104:1215-1228, 2018 PMID: 29985529
2. Aqbi HF, Tyutyunyk-Massey L, Keim RC, Butler SE, Thekkudan T, Joshi S, Smith TM, Bandyopadhyay D, Idowu MO, Bear HD, Payne KK, Gewirtz DA, **Manjili MH**. Autophagy-deficient breast cancer shows early tumor recurrence and escape from dormancy. *Oncotarget* 9(31): 22128-22137, 2018 PMID: 29774126
3. Aqbi HF, Wallace M, Sappal S, Payne KK, **Manjili MH**. IFN- γ orchestrates tumor elimination, tumor dormancy, tumor escape and progression. *J Leukoc Biol* 2018 Feb 22. doi: 10.1002/JLB.5MIR0917-351R. [Epub ahead of print] PMID: 29469956
4. **Manjili MH**. A theoretical basis for the efficacy of cancer immunotherapy and immunogenic tumor dormancy: The adaptation model of immunity. *Adv Cancer Res* 137:17-36, 2018 PMID: 29405975
5. Shah SA, Zarei M, Manjili SH, Guruli G, Wang XY, **Manjili MH**. Immunotherapy of cancer: targeting cancer during active disease or during dormancy? *Immunotherapy* 9 (11): 943-949, 2017 PMID: 29338608
6. Benson Z, Manjili SH, Habibi M, Guruli G, Toor AA, Payne KK, **Manjili MH**. Conditioning neoadjuvant therapies for improved immunotherapy of cancer. *Biochem Pharmacol* 145:12-17, 2017 PMID: 28803721
7. **Manjili MH**. Tumor dormancy and relapse: from a natural by-product of evolution to a disease state. *Cancer Res* 77 (10) 2564-2569, 2017 PMID: 28507050
8. **Manjili MH** and Payne KK. Immune regulatory function of Tregs. *Immunol Invest*. 45(8):708-711, 2016. PMID: 27775448
9. **Manjili MH** and Butler SE. Role of Tregs in cancer dormancy or recurrence. *Immunol Invest* 45(8):759-766, 2016
10. Payne KK, Keim RC, Graham L, Idowu MO, Wan W, Wang XY, Toor AA, Bear HD, **Manjili MH**. Tumor-reactive immune cells protect against metastatic tumor and induce immunoediting of indolent but not quiescent tumor cells. *J Leukoc Biol* 100(3):625-35, 2016 PMID:26928306
11. **Manjili MH**, Payne KK. Prospects in cancer immunotherapy: treating advanced stage disease or preventing tumor recurrence? *Disc Med* 19: 427-431, 2015 PMID: 26175400

12. Goodson III WH, Lowe L, Carpenter DO, Gilbertson M, Ali AM, López de Ceráin Salsamendi A, Lasfar A, Carnero A, Azqueta A, Amedei A, Charles AK, Collins AR, Ward A, Salzberg AC, Colacci A, Olsen AK, Berg A, Barclay BJ, Zhou BP, Blanco-Aparicio C, Baglione C, Dong C, Mondello C, Hsu CW, Naus CC, Yedjou C, Curran CS, Laird DW, Koch DC, Carlin DJ, Felsher DW, Roy D, Brown D, Ratovitski E, Ryan E, Corsini E, Rojas E, Moon EY, Laconi E, Marongiu F, Al-Mulla F, Chiaradonna F, Darroudi F, Martin FL, Van Schooten FJ, Goldberg GS, Wagemaker G, Nangami G, Rice G, Calaf GM, Williams G, Wolf GT, Koppen G, Brunborg G, Lysterly HK, Krishnan H, Ab Hamid H, Yasaei H, Sone H, Kondoh H, Salem HK, Hsu HY, Park HH, Kotubash I, Miousse IR, Scovassi I, Klaunig JE, Vondráček J, Raju J, Roman J, Wise Sr. JP, Whitfield JR, Woodrick J, Christopher J, Ochieng J, Martinez-Leal JF, Weisz J, Kravchenko J, Sun J, Prudhomme KR, Narayanan KB, Cohen-Solal KA, Moorwood K, Gonzalez L, Soucek L, Jian L, D'Abronzio LS, Lin LT, Li L, Gulliver L, McCawley LJ, Knudsen LE, Memeo L, Vermeulen L, Leyns L, Zhang L, Valverde M, Khatami M, Romano MF, Chapellier M, Williams MA, **Manjili MH**, Leonart M, Xia M, Gonzalez MJ, Karamouzis MV, Kirsch-Volders M, Vaccari M, Kuemmerle NB, Singh N, Cruickshanks N, Kleinstreuer N, van Larebeke N, Ahmed N, Ogunkua O, Krishnakumar PK, Vadgama P, Marignani PA, Ghosh PM, Ostrosky-Wegman P, Thompson P, Dent P, Heneberg P, Darbre P, Leung PS, Nangia-Makker P, Cheng Q, Robey RB, Al-Temaimi R, Roy R, Andrade-Vieira R, Sinha RK, Mehta R, Vento R, Di Fiore R, Ponce-Cusi R, Dornetshuber R, Nahta R, Castellino RC, Palorini R, Hamid RA, Langie SAS, Eltom S, Brooks SA, Ryeom S, Wise SS, Bay SN, Harris S, Papagerakis S, Romano S, Pavanello S, Eriksson S, Forte S, Casey SC, Luanpitpong S, Lee TJ, Otsuki T, Chen T, Massfelder T, Sanderson T, Guarnieri T, Hultman T, Dormoy V, Odero-Marah V, Sabbisetti V, Maguer-Satta V, Rathmell WK, Engström W, Decker WK, Bisson WH, Rojanasakul Y, Luqmani Y, Chen Z, Hu Z. Assessing the Carcinogenic Potential of Low Dose Exposures to Chemical Mixtures in the Environment: The Challenge Ahead. *Carcinogenesis* 36 Suppl 1:S254-96, 2015 PMID:26106142

- **Books or other non-periodical, one-time publications**

Nothing to Report

- **Other publications, conference papers, and presentations.**

- **Presentations (invited speaker):**

- 1) Manjili MH. *Immunotherapy of cancer dormancy*. Seminar presentation at the Molecular Biology & Genetics (MBG) Seminar Series, VCU School of Medicine, Richmond VA. (December 2014).
- 2) Manjili MH. Immunotherapy of breast cancer dormancy, 2014 Annual Meeting for Oncology Branch of Beijing Medical Association & 2nd Annual TARGET' China Cancer Congress 2014, Beijing, China November 1-3, 2014.
- 3) Manjili MH. "*Immunotherapy for cancer dormancy*". The 2016 Controlling Cancer Summit, London, UK, May 17-19, 2016.
- 4) Manjili MH. "*The inherent premise of immunotherapy for cancer dormancy*" Hartford, CT, May 5, 2016.
- 5) Manjili MH. "*Current status and future prospects of immunotherapy: Targeting cancer dormancy*", The University of South Carolina, Columbus, SC, September 4, 2015.
- 6) Manjili MH. *Immune-mediated tumor dormancy and inflammation*, Cancer Cell Signaling group meeting, Massey Cancer Center, September 2015
- 7) Invited speaker, Mayo Clinic, Rochester MN. Title: *Immunotherapy is the only option for the treatment of cancer dormancy*", July 2016

- 8) Invited speaker, The VCU Institute of Molecular Medicine (VIMM) and Human & Molecular Genetics (HMG) seminar, VCU School of Medicine, Richmond VA. Title: *Paradigm shifts in cancer immunotherapy: targeting tumor dormancy*, May 2017
 - 9) Invited speaker, 14th International Congress of Immunology and Allergy (ICIA), Tehran, Iran, April 26-28, 2018. Keynote speech: “*Theoretical framework for the efficacy of cancer immunotherapy*” (April 26, 2018), Plenary speech: “*The promise and challenges of cancer immunotherapy: The adaptation model of immunity*” (April 28, 2018)
- Aqbi HF, Coleman C, Idowu M, **Manjili MH**. Low-dose neoadjuvant chemotherapy dominates Ki67⁺ quiescent tumor dormancy for an effective immunotherapy of breast cancer, IMMUNOLOGY 2019TM
 - AAI Annual Meeting, San Diego, CA, May 9-13, 2019 [received 2019 AAI Laboratory Travel Grant Award for oral and poster presentation]
 - Aqbi HF, Smith TM, Idowu MO, Butler SB, Payne KK, **Manjili MH**. Autophagy-deficient breast cancer shows early escape from dormancy and recurrence following chemotherapy, IMMUNOLOGY 2018TM AAI Annual Meeting, Austin, TX, May 4-8, 2018 [received 2018 AAI Laboratory Travel Grant Award]
 - Aqbi HF, Smith TJ, McKiver B, Joshi S, Keim R, Idowu MO, Guo C, Wang XY, Payne KK, **Manjili MH**. Autophagy and chemotherapy-induced tumor dormancy. Cancer Immunology & Immunotherapy: from conception to delivery. NIH, Washington D.C., October 12-13, 2017.
 - Aqbi HF, Butler SE, Keim R, Idowu MO, **Manjili MH**. Chemotherapy-induced tumor dormancy and relapse. IMMUNOLOGY 2017TM AAI Annual Meeting, Washington D.C., May 12-16, 2017.
 - Smith TM, Butler SE, Wang XY, **Manjili MH**. Low-dose chemotherapy induces immunogenic tumor dormancy in mouse model of mammary carcinoma cells. IMMUNOLOGY 2017TM AAI Annual Meeting, Washington D.C., May 12-16, 2017. [received 2017 AAI Trainee Poster Award]
 - Payne KK, Graham L, Bear HD, **Manjili MH**. Adoptive cellular therapy containing T cells and CD25⁺ NKT cells modulates myeloid cells and stimulates endogenous anti-tumor immune function. IMMUNOLOGY 2015TM AAI Annual Meeting, New Orleans, LA, May 8-12, 2015. [selected for oral presentation and received AAI Laboratory Travel Grant Award and AAI Trainee Abstract Award]
 - Payne KK, Graham L, Bear HD, **Manjili MH**. Adoptive immunotherapy containing T cells and CD25⁺ NKT cells modulates myeloid cells to stimulate endogenous anti-tumor immune response. 11th Annual VCU Women’s Health Research Day, Richmond, VA, April 2, 2015.
 - **Website(s) or other Internet site(s)**
 - VCU Massey Cancer Center Research Report, November 2018. Research Highlights: Manjili discovers new form of immune cells with implications for treating cancer.
 - VCU Institute of Molecular Medicine (VIMM) News and Views, Issue No. 13, January 2018. Tumor dormancy: a natural byproduct of evolutionary survival mechanism.
 - Research Report, VCU Massey Cancer Center (March 2017): Manjili’s study most read article over the last six months in JLB: <http://myemail.constantcontact.com/Massey-Research-Report-for-March-2017.html?soid=1101948063140&aid=H9ev46PEC3Y>

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 - o <http://www.journalofclinicalpathways.com/article/immunotherapy-plus-chemotherapy-kills-more-cancer-cells-chemotherapy-alone>

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Rebecca Keim
Project Role:	Research fellow
Researcher Identifier:	
Nearest person month worked:	6
Contribution to Project:	Ms. Keim has performed in vitro studies of chemotherapy-induced tumor dormancy as well as generating anti-CD4 Ab

	for in vivo T cell depletion studies in FVB mice.
Funding Support:	DoD

Name:	Kyle Payne
Project Role:	Graduate Student
Researcher Identifier:	
Nearest person month worked:	15
Contribution to Project:	Mr. Payne has performed in vivo studies of chemotherapy-induced tumor dormancy, and in vitro studies of tumor dormancy.
Funding Support:	AAI Fellow ship award and DoD

Name:	Supriya Joshi
Project Role:	Graduate Student
Researcher Identifier:	
Nearest person month worked:	9
Contribution to Project:	Ms. Joshi has performed in vitro studies of chemotherapy-induced tumor dormancy.
Funding Support:	First year of graduate students are supported by the school of Medicine. She will be supported by this grant this year.

Name:	Savannah Butler
Project Role:	Lab specialist/graduate student
Researcher Identifier:	
Nearest person month worked:	18
Contribution to Project:	Ms. Butler has performed in vitro studies of chemotherapy-induced tumor dormancy
Funding Support:	DoD, MCC Pilot Project Award

Name:	Hussein Aqbi
Project Role:	Graduate Student
Researcher Identifier:	
Nearest person month worked:	36
Contribution to Project:	Mr. Aqbi has performed in vitro and in vivo studies associated with chemotherapy- and RT-induced tumor dormancy, and immune response studies.
Funding Support:	DoD, PhD scholarship

Name:	Yibin Xie
Project Role:	Visiting Fellow
Researcher Identifier:	
Nearest person month worked:	6
Contribution to Project:	Dr. Xie has performed in vivo studies of breast cancer dormancy.
Funding Support:	DoD, fellowship award

Name:	Timothy Smith, PhD
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Project Role:	Graduate student
Researcher Identifier:	
Nearest person month worked:	9
Contribution to Project:	Mr. Smith has performed in vitro studies of chemotherapyinduced tumor dormancy.
Funding Support:	DoD, PhD scholarship

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report

- **What other organizations were involved as partners?**

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS**

The partnering PI, Dr. David Gewirtz, is submitting an independent progress report

- **QUAD CHARTS**

N/A

9. APPENDICES

Documents attached.

ARTICLE

Gr1^{-/low}CD11b^{-/low}MHCII⁺ myeloid cells boost T cell anti-tumor efficacy

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Abstract

Conventional APCs that express MHC class II (MHCII) and co-stimulatory molecules include dendritic cells (DCs) and macrophages. Beyond these conventional APCs, immune stimulatory cells have been more recently shown to extend to a class of atypical APCs, composed of mast cells, basophils, and eosinophils. Here, we describe a unique type of APC, Gr1^{-/low}CD11b^{-/low} cells with a granularity and size characteristic of myeloid cells and with the ability to present Ag for crosspresentation. These cells constitutively express MHCII and the costimulatory molecules, CD80, CD86, and CD40. They do not express pan markers of myeloid DCs (CD11c), plasmacytoid DCs (Ly6C), or macrophages (F4/80), and their frequency is inversely correlated with myeloid-derived suppressor cells (MDSCs) in tumor-bearing mice. Among splenocytes, they are more abundant than DCs and macrophages, and they exhibit antitumor immune stimulatory function at a steady state without further activation, ex vivo. They are also found within the tumor bed where they retain their immune stimulatory function. Our findings suggest the use of these novel APCs in additional preclinical studies to further investigate their utility in APC-based cancer immunotherapies.

KEYWORDS

adoptive immunotherapy, Ag presenting cells, breast cancer, cancer vaccine, myeloid-derived suppressor cells

1 | INTRODUCTION

Dendritic cells (DCs) play a central role in inducing immune responses against infectious diseases and cancer. However, their efficacy as a cell-based vaccine is limited despite continued optimization of various vaccination parameters. This is in part due to the host-derived

immune suppressive cells such as myeloid-derived suppressor cells (MDSCs). The accumulation of MDSCs hinders protective immune responses to cancer and infectious diseases such as tuberculosis,^{1,2} AIDS,³⁻⁵ hepatitis C,^{6,7} hepatitis B,^{8,9} pneumonia,^{10,11} and *Staphylococcus aureus* infection.¹² Importantly, an elevation of MDSCs is associated with a reduced efficacy of vaccines.^{13,14} In addition, the generation of monocyte-derived DCs or bone marrow-derived DCs requires extensive ex vivo culturing, conceivably hampering

Abbreviations: AIT, adoptive immunotherapy; DC, Dendritic cell; ILC, innate lymphoid cell; MDSC, myeloid-derived suppressor cell

the immunogenicity of the vaccine. Recent studies, therefore, have focused on vaccines that make use of primary DCs.¹⁵ For instance, Sipuleucel-T is the only FDA-approved therapeutic vaccine for metastatic prostate cancer.¹⁶ The vaccine uses readily isolated circulating DCs cultured with prostate tumor Ag and GM-CSF. However, circulating DCs are very rare and tumor-induced immune suppressive cells, such as MDSCs, limit their efficacy in inducing a sustained antitumor immune response. Therefore, there is an urgent need to identify a new class of APC that are highly efficient in orchestrating profound antitumor immunity to facilitate the development of a new class of cell-based cancer vaccines.

In recent years, there has been a rapid increase in our understanding of the biology of cells with APC characteristics, namely the ability to activate T cells. For instance, mouse neutrophils can induce Th1 and Th17 responses^{17,18} and tumor-associated neutrophils have been demonstrated to stimulate T cell responses in early-stage human lung cancer.¹⁹ A recent review discusses a number of atypical APCs including mast cells, basophils, eosinophils, and innate lymphoid cells (ILC).^{20,21} However, these APCs are rare in the circulation and their maintenance of effective antitumor immune responses is likely to be inhibited due to high frequencies of MDSCs in locations of T cell priming. Very recently, it was reported that activated NKT cells decrease the frequency and immunosuppressive activity of MDSCs in tumor-bearing mice.²² In an animal model, activated NKT cells converted MDSCs into immunogenic APCs.²³ Using peripheral blood mononuclear cells (PBMC) of patients with early stage breast cancer, we also demonstrated that conversion of MDSCs to CD33⁺CD11b^{-/low}HLA-DR⁺ APCs, *in vitro*, was associated with an increased frequency of CD25⁺ NKT cells in reprogrammed immune cells.²⁴

In an effort to understand this MDSC-APC axis during the application of adoptive immunotherapy (AIT) to treat breast cancer, we identified a class of Gr1^{-/low}CD11b^{-/low} MHCII⁺ APCs. These cells retain their immune stimulatory function during tumor progression and are inversely correlated to the frequency of splenic and tumor-infiltrating MDSCs. Importantly, we identified the presence of these cells in nonpathological conditions, whereupon we confirmed their ability to cross-present Ag to stimulate T cells. Therefore, these APCs offer a potentially novel APC-based vaccine for cancer therapy.

2 | MATERIALS AND METHODS

2.1 | Mouse model

FVBN202 transgenic female mice (The Jackson Laboratory; Bar Harbor, ME) were used between 8 and 12 weeks of age throughout these experiments. These mice overexpress a nonmutated, nonactivated rat neu transgene under the regulation of the mouse mammary tumor virus promoter.²⁵ These mice develop premalignant mammary hyperplasia similar to ductal carcinoma *in situ* prior to the development of spontaneous carcinoma.²⁶ Premalignant events in FVBN202 mice include the accumulation of endogenous MDSCs.²⁶ These studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

2.2 | Tumor cell lines

The neu overexpressing mouse mammary carcinoma (MMC) cell line was established from a spontaneous mammary tumor harvested from FVBN202 mice. Tumor cells were maintained in RPMI 1640 supplemented with 10% FBS.

2.3 | Ex vivo reprogramming and expansion of splenocytes

Reprogramming of tumor-sensitized immune cells was performed as previously described by our group.⁵ Briefly, FVBN202 transgenic mice were inoculated in the mammary fat pad with 3×10^6 MMC cells. Tumor growth was monitored by digital caliper, and tumor volumes were calculated by volume ($v = (L [\text{length}] \times W [\text{width}]^2)/2$). As previously described,¹¹ splenocytes were harvested 21–25 days after tumor challenge, when the tumor had reached $\geq 1000 \text{ mm}^3$. Splenocytes were then cultured in complete medium (RPMI 1640 supplemented with 10 % FBS, L-glutamine (2 mM), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ Streptomycin) and were stimulated with Bryostatin 1 (2 nM; Sigma, Saint Louis, MO), Ionomycin (1 μM ; Calbiochem, San Diego, CA), and 80 U/ml/ 10^6 cells of IL-2 (Peprotech) for 16–18 h.^{24,27} Lymphocytes were then washed thrice and cultured at 10^6 cells/ml in complete medium with IL-7 and IL-15 (20 ng/ml of each cytokine, Peprotech, Rocky Hill, NJ). After 24 h, 20 U/ml of IL-2 was added to the complete medium. The following day, the cells were washed and cultured at 10^6 cells/ml in complete medium with 40 U/ml of IL-2. After 48 h, cells were washed and cultured at 10^6 cells/ml in complete medium with 40 U/ml of IL-2. After 24 h, lymphocytes were again washed and cultured at 10^6 cells/ml in complete medium with 40 U/ml of IL-2. Lymphocytes were harvested 24 h later on the sixth day and were then either used in AIT or analyzed *ex vivo*. Reprogramming of splenocytes consistently yielded 5-fold expansion with greater than 40% memory T cells and 35% CD25⁺ NKT cells.²⁷

2.4 | Adoptive cellular therapy

Twenty-four hours prior to AIT, FVBN202 mice were injected *i.p.* with CYP (100 mg/kg) to induce lymphopenia. Approximately 18 h later FVBN202 mice were challenged *i.v.* with MMC cells (1×10^5). Mice then received adoptive transfer of reprogrammed splenocytes *i.v.* at a dose of $70 \times 10^6/\text{mouse}$ later the same day (AIT), or remained untreated (Control). The study end-point and euthanasia occurred when the animals were considered moribund upon losing 10–20% of their initial body weight due to disease progression.

2.5 | Characterization of splenocytes and tumor-infiltrating leukocytes

Spleens and metastases of tumor-bearing FVBN202 mice were harvested when the animals became moribund, and were then homogenized into a single cell suspension as described previously²⁸ and below; single cell suspensions were then characterized using flow cytometry. Reagents used for flow cytometry: anti-CD16/32 Ab (93); FITC-CD11b (M1/70); PE-GR-1 (RB6-8C5); PE-CD11c (N418); PE-F4/80 (BM8);

PE-CD25 (3C7); Allophycocyanin-CD49b (DX5); Allophycocyanin-Annexin V; Alexa Fluor 647-I-Aq (KH116); Alexa Fluor 700 Ly-6G (1A8); PercP/CY5.5-CD86 (GL-1); PercP/CY5.5-Rat IgG2a, k Isotype Control (RTK2758); PE-Dazzle-CD80 (16-10A1); PE-Dazzle-Armenian Hamster IgG Isotype Control (HTK888); PE/CY7-CD40 (3/23); PE/CY7-Rat IgG2a, k Isotype Control (RTK2758); Brilliant Violet 510 Ly-6C (HK1.4); Brilliant Violet 605-CD45 (30-F11); BV421-CD20 (SA275A11); BV711-Ly6C (HK1.4); BV510-CD11b (M1/70); and BV785-CD86 (GL-1), all of which were purchased from Biolegend (San Diego, CA). BD Horizon V450-Annexin V and BUV395-CD3 (SK7) were purchased from BD Biosciences (Franklin Lakes, NJ). Propidium Iodide (PI) was purchased from Sigma. (All reagents were used at the manufacturer's recommended concentration. Cellular staining was performed as previously described by our group.²⁴ Multicolor data acquisition was performed using a LSRFortessa X-20 (BD Biosciences) and a ImageStreamX Mark II Imaging Flow Cytometer (Millipore Sigma, Billerica, MA). Data was analyzed using FCS Express v4.07 and v5.0 (De Novo Software; Glendale, CA).

2.6 | Sorting of myeloid cells by FACS

Splenocytes were stained for surface expression of CD11b and Gr1 as described above. Isolated cells were gated on the myeloid cell population based on their inherent light scattering properties²⁹ thereby excluding cells of lymphoid origin. Gr1^{−/low}CD11b^{−/low} myeloid cells from the Control and AIT groups were then sorted into independent populations using a FACS Aria (BD Biosciences) as previously described.³⁰ Purity of sorted cells was consistently greater than 90%.

2.7 | IFN- γ ELISA

Splenocytes from the Control and AIT groups were independently cultured in serum-free RPMI 1640 in order to enrich for nonadherent cells.³¹ After 2 h, nonadherent lymphocytes were cultured in complete medium with irradiated MMC cells (140 Gy) at a 10:1 ratio, and with or without sorted Gr1^{−/low}CD11b^{−/low} myeloid cells at a 2:1 ratio, for 20 h. Also, sorted Gr1^{−/low}CD11b^{−/low} cells or bone marrow-derived DCs were pulsed with recombinant rat Neu extracellular domain (50 μ g/ml) in the presence of GM-CSF (20 ng/ml) for 24 h, washed of free protein, and co-cultured with tumor-sensitized, reprogrammed T cells (1:3) for 20 h. Irradiated MMC (140 Gy) were used as positive target for tumor-sensitized reprogrammed T cells (1:10 ratio). Supernatants were then collected and stored at -80°C until assayed. IFN- γ was detected in the supernatant using a Mouse IFN- γ ELISA kit (BD Biosciences), according to the manufacturer's protocol.

2.8 | In vitro Ag uptake

Splenocytes (10^6 cells/ml) of naïve FVB/N202 mice were pulsed with 50 μ g/ml Alexa Fluor 488 (AF488)-conjugated ovalbumin (ThermoFisher Scientific) in RPMI1640 supplemented with 10% FBS for 5 or 16 h. Cells were then washed and stained for FVS, CD11c, CD11b, Gr1. Gated FVS⁺ viable cells were subgated for CD11c⁺ DCs

or Gr1^{−/low}CD11b^{−/low} myeloid cells, and analyzed for Alexa Fluor 488 as a reporter of OVA internalization.

2.9 | Cytotoxicity assay

Antitumor efficacy of T cells was determined in a cytotoxicity assay, in vitro, using flow cytometry as previously described by our group³² with minor modifications. The ex vivo expanded tumor reactive T cells were cultured in complete medium with MMC cells (10:1 E:T ratio) in the presence or absence of sorted Gr1^{−/low}CD11b^{−/low} cells at a 5:1 ratio (five T cells vs. one APCs), for 48 h. Cells were collected and stained with Annexin V, PI, anti-CD45 and anti-Neu Abs immediately prior to flow cytometry acquisition.

2.10 | Isolation of tumor-infiltrating leukocytes from lung metastases

Lungs were harvested from the Control and AIT groups after animals became moribund. Metastatic lesions were individually excised from the residual lung tissue, and were minced and digested in Trypsin-EDTA (0.25%; Life Technologies) overnight at 4°C . The following day, the suspension was incubated at 37°C for 30 min, followed by gentle tissue homogenization to create a cellular suspension. The cell suspension was then washed twice with RPMI supplemented with 10% FBS. Residual red blood cells were then lysed using ACK lysing buffer, followed by an additional wash with RPMI 10% FBS. 10^6 cells of the suspension were then stained for surface molecules as described above. All analysis was performed by gating on viable leukocytes (CD45⁺ Annexin V[−]).

2.11 | Statistical analysis

Outcomes are summarized by basic descriptive statistics such as mean and SEM; differences between groups are illustrated using graphical data presented as mean $\pm$ SEM. Statistical comparisons between groups were made using one-tailed and two-tailed Student's *t*-test per the specific hypothesis. Time to death in the in vivo survival studies was calculated from baseline to the date of death. Mice were euthanized when they had a weight loss of $\geq 10\%$. Kaplan-Meier curves and log-rank tests are used to illustrate time to death and to test the difference between each group. A *P*-value ≤ 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Gr1^{−/low}CD11b^{−/low} cells demonstrate characteristics of professional APCs

Antitumor immune responses are often corrupted in tumor bearing hosts due to pathological emergency myelopoiesis, which leads to the accumulation of MDSCs in secondary lymphoid organs and tumor beds.^{33,34} However, it has been reported that lymphoid effectors, namely NKT cells, functionally alter MDSC function by promoting an immunostimulatory, rather than suppressive, phenotype in the context of antitumor immunity.^{24,27} Therefore, we sought to gain an

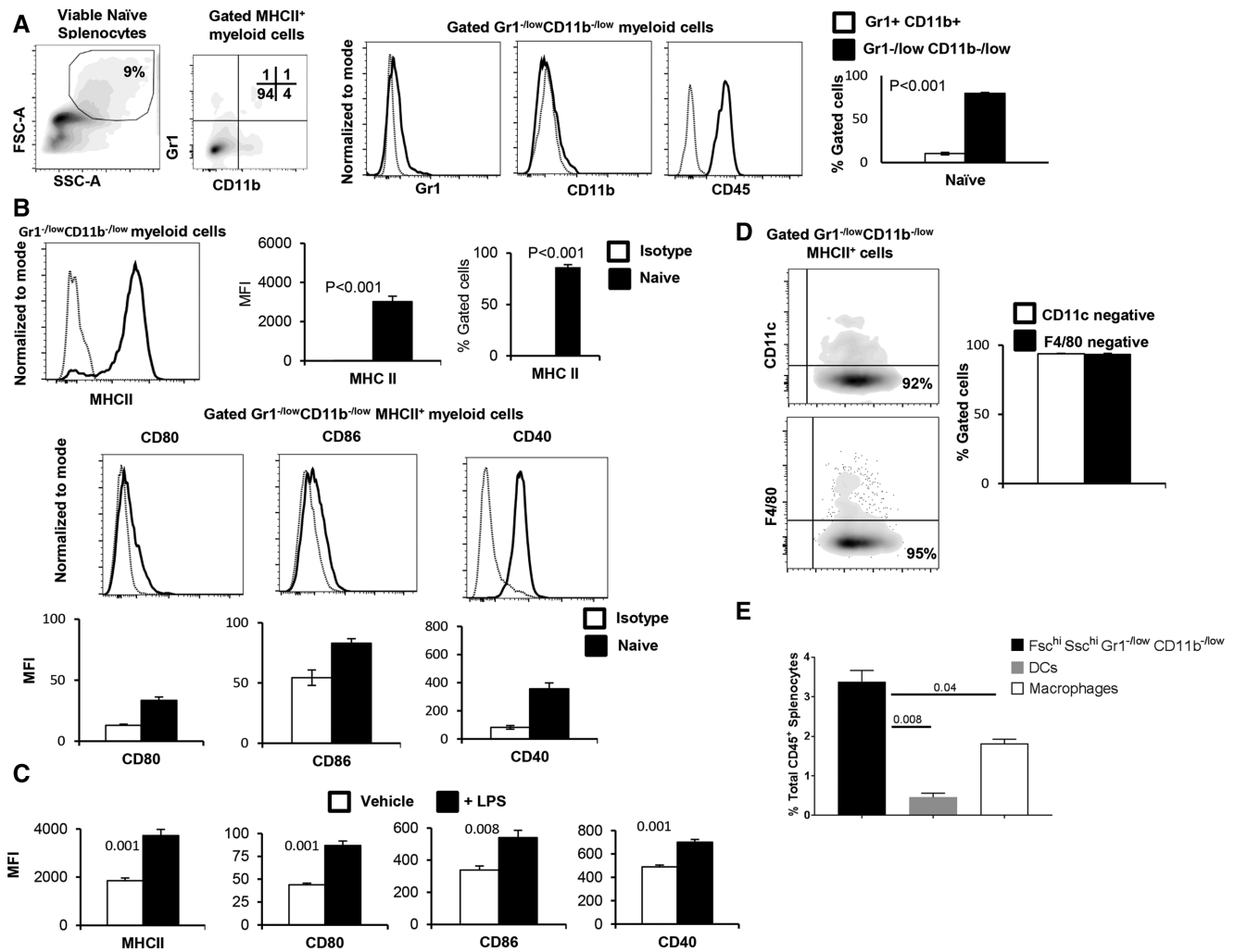


FIGURE 1 Splenic Gr1^{-low}CD11b^{-low} cells show characteristics of APCs. (A) Splenocytes of naïve FVB/N202 mice ($n = 3$) were gated within the myeloid cell region based on forward-scatter and side-scatter, and were analyzed for the expression of Gr1 and CD11b. The proportion of the splenic Gr1^{-low}CD11b^{-low} myeloid cells and Gr1⁺CD11b⁺ myeloid cells was determined. (B) Gated Gr1^{-low}CD11b^{-low} cells were analyzed for the expression of MHC class II (MHCII). Gated Gr1^{-low}CD11b^{-low}MHCII⁺ cells were also analyzed for the expression of the co-stimulatory molecules, CD80, CD86, and CD40. Mean fluorescence intensity (MFI) of the co-stimulatory molecules showed a significant shift compared with isotype control. (C) Sorted Gr1^{-low}CD11b^{-low}MHCII⁺ cells were cultured in the absence (-LPS) or presence of LPS (+LPS, 1 μ g/ml) for 24 h. Gated Gr1^{-low}CD11b^{-low}MHCII⁺ cells were analyzed for the expression of MHCII as well as CD80, CD86, or CD40. MFI was calculated after the subtraction of isotype control. (D) Gated Gr1^{-low}CD11b^{-low}MHCII⁺ cells were analyzed for the expression of CD11c or F4/80. (E) Percent total frequency of MHCII⁺ Gr1^{-low}CD11b^{-low}, DCs and macrophages in the spleen. Data represent mean $\pm$ SEM. Data are representative of at least 3 independent experiments

understanding of the biology of myeloid cells under nonpathological conditions in order to appreciate their functional plasticity. First, we observed that the splenic Fsc^{hi} Ssc^{hi} myeloid cell compartment of naïve mice was dominated by a population of Gr1^{-low}CD11b^{-low} cells (Fig. 1A, right panel; $P = 0.00002$), which were of hematopoietic origin. Furthermore, these Gr1^{-low}CD11b^{-low} cells demonstrated expression of MHC class II (MHCII; $P = 0.0002$) and the co-stimulatory molecules, CD80 ($P = 0.001$), CD86 ($P = 0.009$), and CD40 ($P = 0.0003$), as shown in Fig. 1B. LPS stimulation induced the maturation of Gr1^{-low}CD11b^{-low} cells (Fig. 1C) by up-regulating the expression of MHCII (MFI: 1851 vs. 3732, $P = 0.001$), CD80 (MFI: 44 vs. 87, $P = 0.001$), CD86 (MFI: 338 vs. 541, $P = 0.008$) and CD40 (MFI: 488 vs. 800, $P = 0.001$). Despite displaying such classical characteristics of APCs, Gr1^{-low}CD11b^{-low} cells did not express pan mark-

ers of DCs, CD11c, or macrophages, F4/80 (Fig. 1D). Importantly, however, these Fsc^{hi} Ssc^{hi} Gr1^{-low}CD11b^{-low} myeloid cells possess a similar size and granularity, and express similar levels of MHCII as well as costimulatory molecules to total splenic macrophages and dendritic cells (Supplementary Fig. 1). The total frequency of Fsc^{hi} Ssc^{hi} Gr1^{-low}CD11b^{-low} APCs was significantly higher than all DCs and macrophages in the spleen (Fig. 1E, $P = 0.008$ and $P = 0.04$, respectively). Additionally, morphological studies of these cells using Diff-Quick staining demonstrated the presence of both monoblast-like (large cells), and lymphocyte-like (small cells) within the Fsc^{hi} Ssc^{hi} Gr1^{-low}CD11b^{-low} gate (Supplementary Fig. 2).

Given that Diff-Quick staining revealed the presence of lymphocyte-like cells among sorted Gr1^{-low}CD11b^{-low} cells from naïve mice, we sought to further determine the phenotype

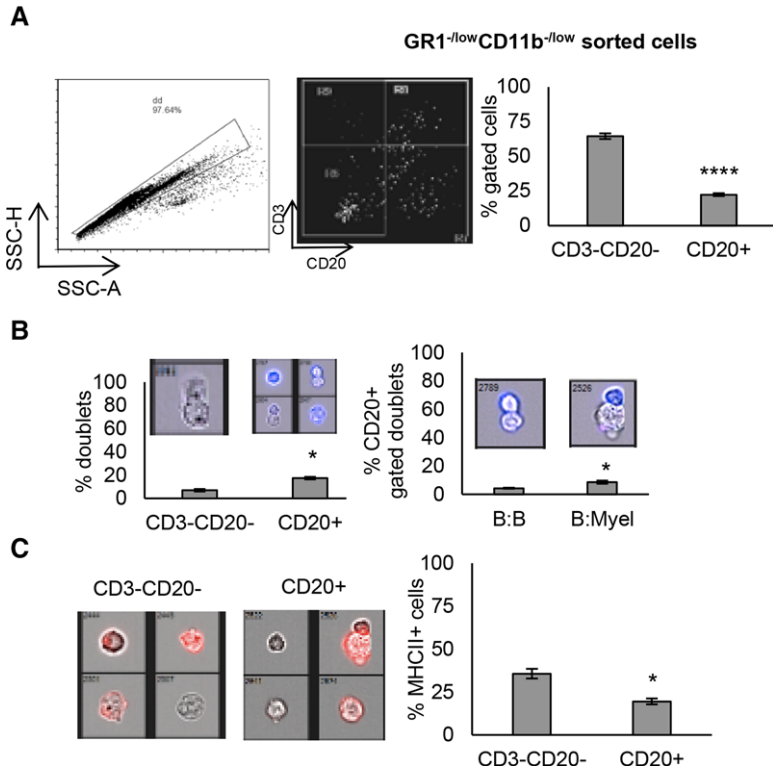


FIGURE 2 $GR1^{-/low}CD11b^{-/low}$ cells contain myeloid cells and B cells. $GR1^{-/low}CD11b^{-/low}$ cells within the myeloid region of the scatter plot were sorted and analyzed via Image Stream. (A) After excluding doublets, cells were analyzed for CD3 and CD20 expression to determine if T and B cells were still falling within the myeloid gate. (B) Hundred images/events from the $CD3^{-}CD20^{-}$ and $CD20^{+}$ populations were analyzed for doublets by inspecting each image manually. Also, doublets within $CD20^{+}$ cells were analyzed based on morphology showing B cell:B cells (B:B) or B cells:Myeloid cells (B:Myel) interactions. (C) MHCII (red) expression on $CD3^{-}CD20^{-}$ and $CD20^{+}$ populations. Data represent mean $\pm$ SEM of triplicate experiments

and frequency of these cells within the sorted population. We found that a majority of gated $Gr1^{-/low}CD11b^{-/low}$ cells lacked expression of lineage markers for T or B cells ($CD3^{-}CD20^{-}$), although 22% of cells included $CD20^{+}$ B cells (Fig. 2A). We then hypothesized that the presence of residual B-cells in the $Fsc^{hi} Scc^{hi}$ myeloid region was due to cell-to-cell interactions between B cells and myeloid cells. To investigate this, ImageStreamX analysis was performed. The total events were analyzed for percentage of events that had two cells contained in one event by observing each event manually. The number of doublets containing the $CD20^{+}$ population was significantly higher in comparison to the $CD3^{-}CD20^{-}$ doublets (Fig. 2B, left and middle panels, 7% vs. 17.5%). Among $CD20^{+}$ B cells in this population, the majority of cell-to-cell contacts were shown to be B cell:myeloid cell interactions (B:Myel), rather than B cell:B cell (B:B) interactions (Fig. 2B, right panel, 9% vs. 4%). We then determined the source of MHCII expression among these interacting cells. As can be seen in Fig. 2C, myeloid cells ($CD20^{-}CD3^{-}$) had significantly higher percent of MHCII expression compared to $CD20^{+}$ cells. Taken together, our data suggest the presence of a unique lineage of myeloid-derived APC, which demonstrates characteristics of classical APC:B cell interactions in naïve mice.^{35,36}

3.2 | $Gr1^{-/low}CD11b^{-/low}$ MHCII⁺ cells are heterogeneous populations that are both lineage committed and noncommitted

To further unravel the biology of $Gr1^{-/low}CD11b^{-/low}$ MHCII⁺ cells, we found that approximately 50% of these cells expressed Ly6G, indicative of a commitment to the granulocyte lineage, while the remainder of this population was negative for both Ly6G and Ly6C (Fig. 3A). Accordingly, the $Ly6G^{+}Ly6C^{-}$ subset displayed a more

mature phenotype than the $Ly6G^{-}Ly6C^{-}$ subset, expressing significantly higher levels of MHCII ($P = 0.001$), CD80 ($P = 0.03$), CD86 ($P = 0.0006$), and CD40 ($P = 0.025$; Fig. 2B). As the $Ly6G^{-}Ly6C^{-}$ subset did not demonstrate a specific myeloid-cell lineage commitment by any parameter that we tested, we hypothesized that this population would respond more robustly to activating stimuli due to a presumed lack of maturity. Indeed, as shown in Fig. 3C, the $Ly6G^{-}Ly6C^{-}$ subset showed a stronger response to LPS stimulation when compared to vehicle treatment than the $Ly6G^{+}Ly6C^{-}$ subset. This suggests that under non-pathological conditions there exists a population of both lineage committed and noncommitted splenic $Gr1^{-/low}CD11b^{-/low}$ MHCII⁺ cells, which possess the potential to perform professional Ag-presenting cellular functions.

3.3 | Adoptive immunotherapy modulates $Gr1^{-/low}CD11b^{-/low}$ APCs

It has been reported that activated NKT cells can convert MDSCs into immune-stimulatory APCs.^{22,23} We have reported that reprogrammed lymphocytes containing $CD25^{+}$ NKT cells can induce maturation of human $CD33^{+}CD11b^{+}HLA-DR^{-}$ MDSCs into stimulatory $CD33^{+}CD11b^{-/low}HLA-DR^{+}$ APCs, *in vitro*.^{24,27} Given the inverse correlation between $Gr1^{+}CD11b^{+}$ cells and $Gr1^{-/low}CD11b^{-/low}$ APCs in naïve mice (Fig. 1A), we sought to determine the impact of tumor burden as well as AIT, containing conventional tumor-specific T cells and $CD25^{+}$ NKT cells, on the modulation of $Gr1^{-/low}CD11b^{-/low}$ APCs, *in vivo*. FVB/N202 mice were challenged i.v. with Neu-overexpressing MMC tumor cells, and then either remained untreated (control) or were subjected to an adoptive transfer of tumor-sensitized reprogrammed T cells and NKT cells.³² Animals were sacrificed upon disease progression culminating in metastases in the lung. As shown

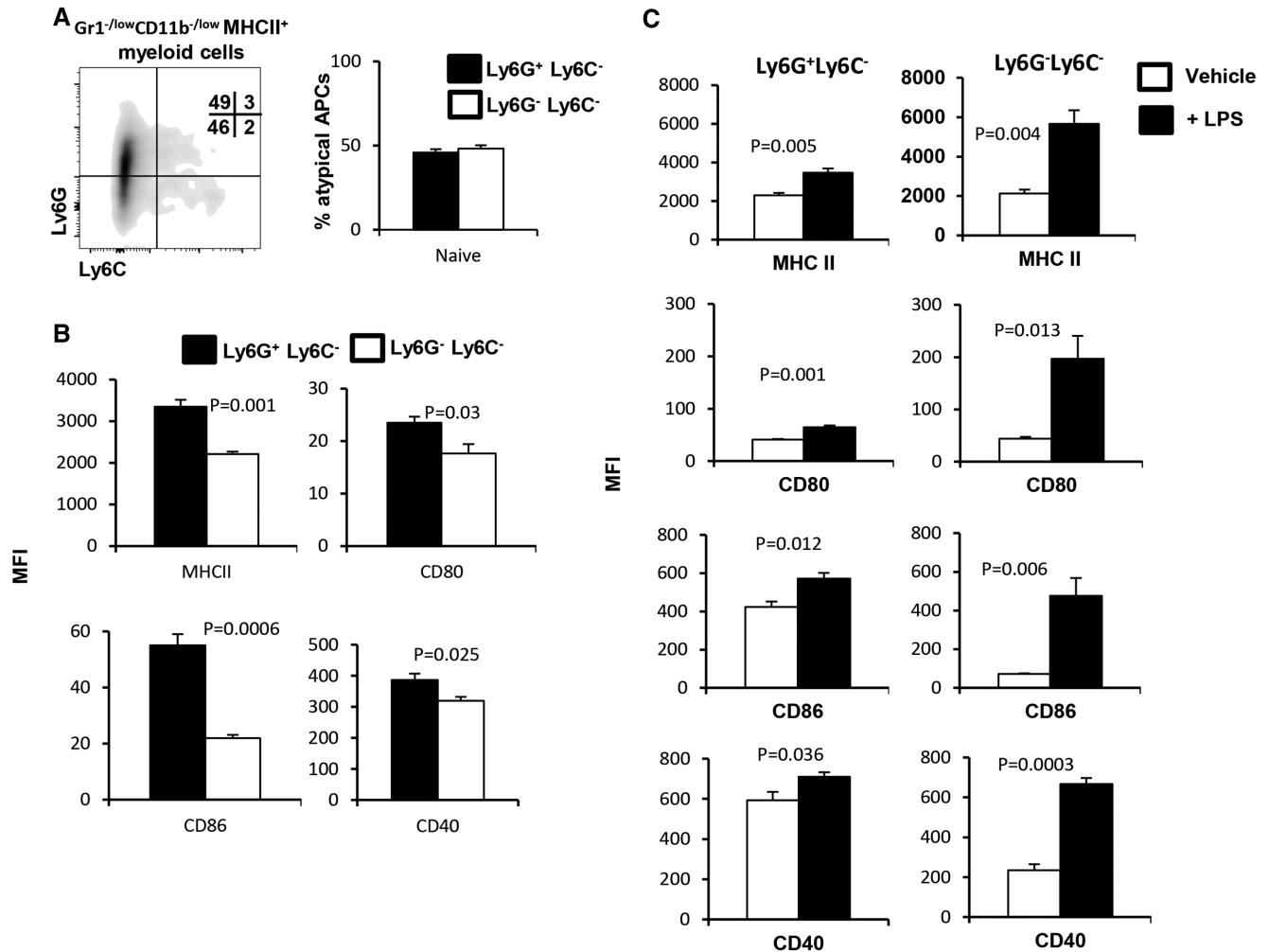


FIGURE 3 $Gr1^{-/low}CD11b^{-/low}MHCII^{+}$ myeloid cells contain $Ly6G^{+}Ly6C^{-}$ and $Ly6G^{-}Ly6C^{-}$ subsets. (A) Splenocytes of naïve FVB/N202 mice ($n = 3$) were gated within the myeloid cell region and expression of Ly6G and Ly6C was determined on gated $Gr1^{-/low}CD11b^{-/low}MHCII^{+}$ cells. (B) Expression of MHCII and co-stimulatory molecules was determined on gated $MHCII^{+}Ly6G^{+}Ly6C^{-}$ and $MHCII^{+}Ly6G^{-}Ly6C^{-}$ cells. (C) Expression of MHCII and co-stimulatory molecules was determined on gated $Ly6G^{+}Ly6C^{-}$ or $Ly6G^{-}Ly6C^{-}$ subsets after 24 h stimulation in the absence (-LPS) or presence of LPS (+LPS, 1 μ g/ml). MFI were calculated after subtraction of isotype control. Data represent mean $\pm$ SEM. Data are representative of at least 3 independent experiments

in Fig. 4A, AIT significantly prolonged animal survival ($P = 0.015$). Such antitumor protection was associated with modulation of the myeloid cell compartment, resulting in a significantly increased frequency of $Gr1^{-/low}CD11b^{-/low}$ APCs (Fig. 4B, 56% vs. 38%); the frequency of these cells dominated $Gr1^{+}CD11b^{+}$ MDSCs in the AIT group compared to the control group (Fig. 4B, 56% vs. 33%), even at equally advanced stages of tumor progression. Unlike naïve mice and AIT recipients, the myeloid cellular compartment of the untreated control group mainly consisted of MDSCs (Fig. 4B, $P = 0.03$). The emergence of $Gr1^{-/low}CD11b^{-/low}$ APCs in the animals treated with AIT was associated with a significantly increased frequency of splenic $CD25^{+}$ NKT cells compared with the control group (Supplementary Fig. 3, $P = 0.037$). Further analyses showed similar levels of MHCII expression (MFI and % gated) in both groups, though those treated with AIT had a significantly higher frequency of $Gr1^{-/low}CD11b^{-/low}MHCII^{+}$ APCs among all splenocytes (Fig. 4C, $P = 0.001$). AIT also resulted in the up-regulation of CD86 (Fig. 4D, MFI: 32 vs. 66, $P = 0.01$)

and down-regulation of CD40 (Fig. 4D, 616 vs. 278, $P = 0.001$) on $Gr1^{-/low}CD11b^{-/low}$ cells. In fact, AIT restored the frequency of $Gr1^{-/low}CD11b^{-/low}MHCII^{+}$ APCs and the expression of CD40 to the levels similar to those in naïve mice, though CD86 expression was uniquely up-regulated following AIT (Supplementary Fig. 4A). AIT also resulted in a significantly increased frequency of splenic $CD11c^{+}$ DCs and $F4/80^{+}$ macrophages (Supplementary Fig. 4B, $P = 0.001$ and $P = 0.018$, respectively). In order to determine whether $Gr1^{-/low}CD11b^{-/low}$ APCs of the control and AIT groups had the capacity to respond to inflammatory stimuli and undergo maturation, LPS stimulation was performed in vitro. While LPS stimulation resulted in similar trends for both groups, as shown in Fig. 4E, tumor burden with or without AIT resulted in a unique pattern of maturation; we observed that $Gr1^{-/low}CD11b^{-/low}$ cells of the AIT group increased the expression of CD86 (MFI: 360 vs. 667, $P = 0.022$) and CD40 (MFI: 662 vs. 902, $P = 0.023$) whereas those of the control group increased the expression of MHCII (MFI: 2200 vs. 5647,

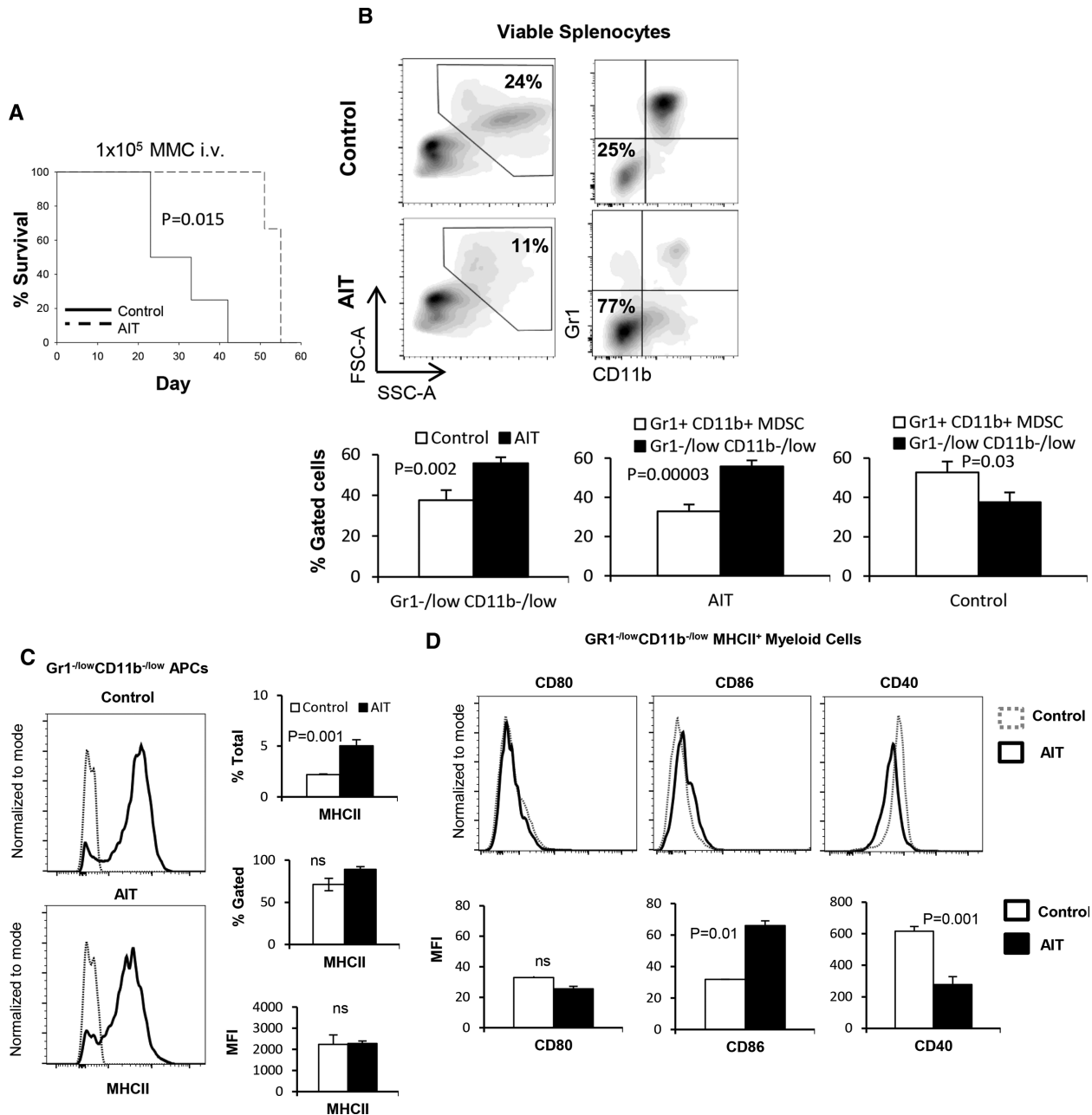


FIGURE 4 $Gr1^{-/low}CD11b^{-/low}$ myeloid cells are modulated during tumor challenge or AIT. (A) Kaplan-Meier analysis of survival in FVB/N202 mice that were injected with 10^5 MMC cells i.v.; animals were sacrificed when they became moribund due to lung metastases. (B) Splenocytes of the control and AIT groups were analyzed by flow cytometry after staining with fluorescently labeled anti-Gr1 and anti-CD11b Abs. Data show the frequency of the splenic $Gr1^{-/low}CD11b^{-/low}$ cells and MDSCs in the control and AIT groups. (C) Frequency and expression levels of MHCII were determined on gated $Gr1^{-/low}CD11b^{-/low}MHCII^{+}$ cells of the AIT and control groups. (D) Gated $Gr1^{-/low}CD11b^{-/low}$ cells were analyzed for the expression of co-stimulatory molecules in the spleens of the AIT and control groups. (E) $Gr1^{-/low}CD11b^{-/low}$ cells were sorted from the spleens of the AIT and control groups and cultured for 24 h in the presence or absence of LPS (+LPS and -LPS). Gated cells were then analyzed for the expression of MHCII and co-stimulatory molecules. Data represent mean $\pm$ SEM of triplicate experiments

$P = 0.02$), CD80 (MFI: 53 vs. 107, $P = 0.053$), and CD86 (MFI: 282 vs. 525, $P = 0.042$).

As the $Ly6G^{+}Ly6C^{-}$ subset had a higher expression of co-stimulatory molecules than the $Ly6G^{-}Ly6C^{-}$ subset in naïve mice (Fig. 3), we sought to determine whether this trend was also present during tumor burden or following AIT. Subset analysis of $Gr1^{-/low}CD11b^{-/low}$ APCs showed the emergence of a $Ly6G^{+}Ly6C^{-}$ cell population in tumor-bearing mice that received AIT when com-

pared with the control group (Fig. 5A, 35% vs. 7%). Unlike untreated tumor-bearing mice, animals receiving AIT showed a similar trend with naïve mice in regards to the frequency of $Ly6G^{+}Ly6C^{-}$ myeloid cells (Supplementary Fig. 5). Whereas both subsets showed comparable levels of the expression of MHCII, CD80, and CD40 in the control and AIT groups, the $Ly6G^{+}Ly6C^{-}$ subset exhibited a significantly higher level of CD86 expression (Fig. 5B, Control, MFI: 17 vs. 27; AIT, MFI: 16 vs. 41). As expected, the mature $Ly6G^{+}Ly6C^{-}$ subset

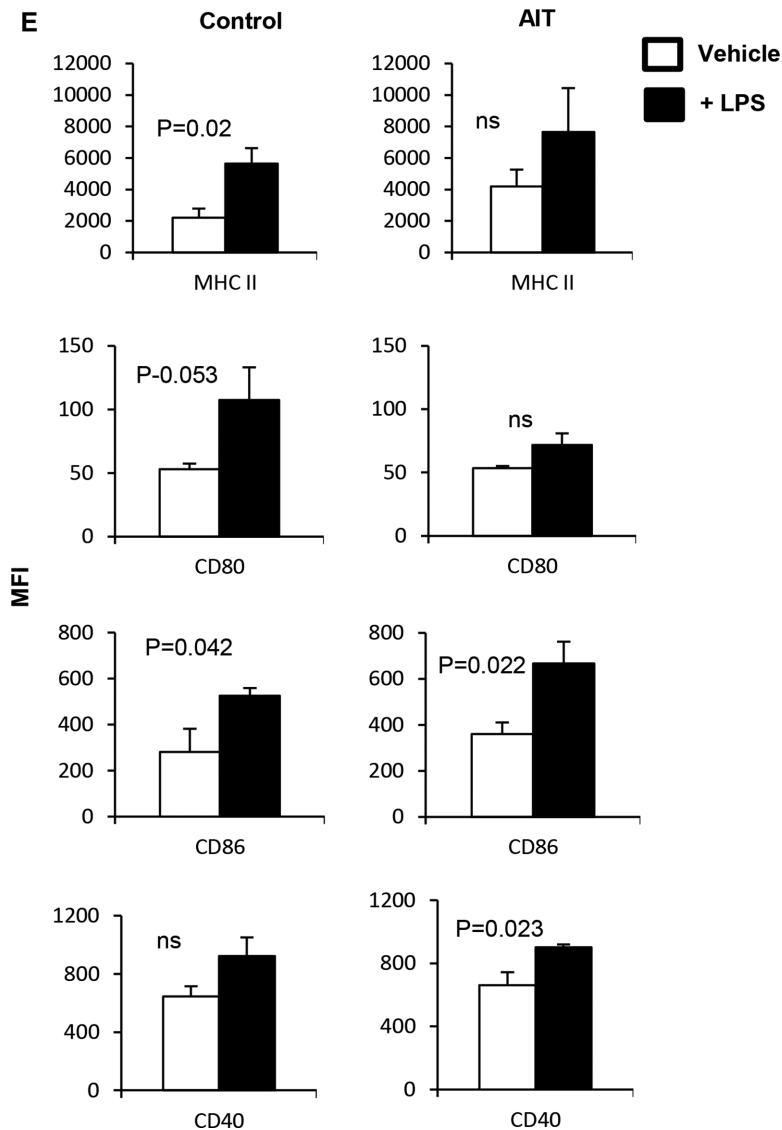


FIGURE 4 Continued

did not result in an increase in the expression of CD86 following LPS stimulation. However the $\text{Ly6G}^-\text{Ly6C}^-$ subset in the control group and in the AIT group did experience increases in the expression of CD80 (Fig. 4C, $P = 0.042$ and $P = 0.058$) and CD86 (Fig. 4C, $P = 0.004$ and $P = 0.058$). The $\text{Ly6G}^-\text{Ly6C}^-$ subset within control mice also increased the expression of MHCII ($P = 0.021$) and CD40 ($P = 0.05$) following LPS stimulation. Therefore, these data suggest that AIT rescues the myeloid compartment of tumor-bearing animals by promoting the maturation of myeloid cells to the frequency and functional potential observed in naïve mice.

3.4 | $\text{Gr1}^{-/\text{low}}\text{CD11b}^{-/\text{low}}\text{Ly6G}^+\text{Ly6C}^-$ APCs are present within the tumor bed

To determine whether $\text{Gr1}^{-/\text{low}}\text{CD11b}^{-/\text{low}}$ APCs are present in the tumor bed, tumor lesions of both the AIT and control groups were analyzed when animals were euthanized due to tumor progression with similar tumor burden. As in the spleen, we again found that $\text{Gr1}^{-/\text{low}}\text{CD11b}^{-/\text{low}}$ cells dominated the tumor-infiltrating $\text{Fsc}^{\text{hi}}\text{Ssc}^{\text{hi}}$ myeloid cell compartment within the AIT group, where they demon-

strated a greater than 3-fold increase in frequency over $\text{Gr1}^+\text{CD11b}^+$ MDSCs (Supplementary Fig. 6A, 14% vs. 46%, $p = 0.016$). Such differences were, again, not observed in the control group. These $\text{Gr1}^{-/\text{low}}\text{CD11b}^{-/\text{low}}$ APCs had similar pattern of maturation between the AIT and control groups to that of the spleen, as shown by comparable levels of the expression of MHCII, CD80, CD86, and CD40 (Supplementary Fig. 6B). Within the tumor bed, the $\text{Ly6G}^+\text{Ly6C}^-$ subset was clearly dominant within the AIT group (Supplemental Fig. 6C, 63% vs. 16%; $P = 0.014$). Whereas both subsets showed comparable levels of costimulatory molecule expression, the $\text{Ly6G}^+\text{Ly6C}^-$ subset demonstrated more robust MHCII expression at the tumor site of the AIT group (Supplementary Fig. 6D, MFI: 11984 vs. 4739, $P = 0.026$).

3.5 | $\text{Gr1}^{-/\text{low}}\text{CD11b}^{-/\text{low}}$ APCs exhibit immune stimulatory function

In order to determine if $\text{Gr1}^{-/\text{low}}\text{CD11b}^{-/\text{low}}$ APCs possess immune stimulatory function during tumor burden and/or following AIT, splenic lymphocytes from the AIT and control group were independently cultured with MMC tumor cells in the presence or absence of

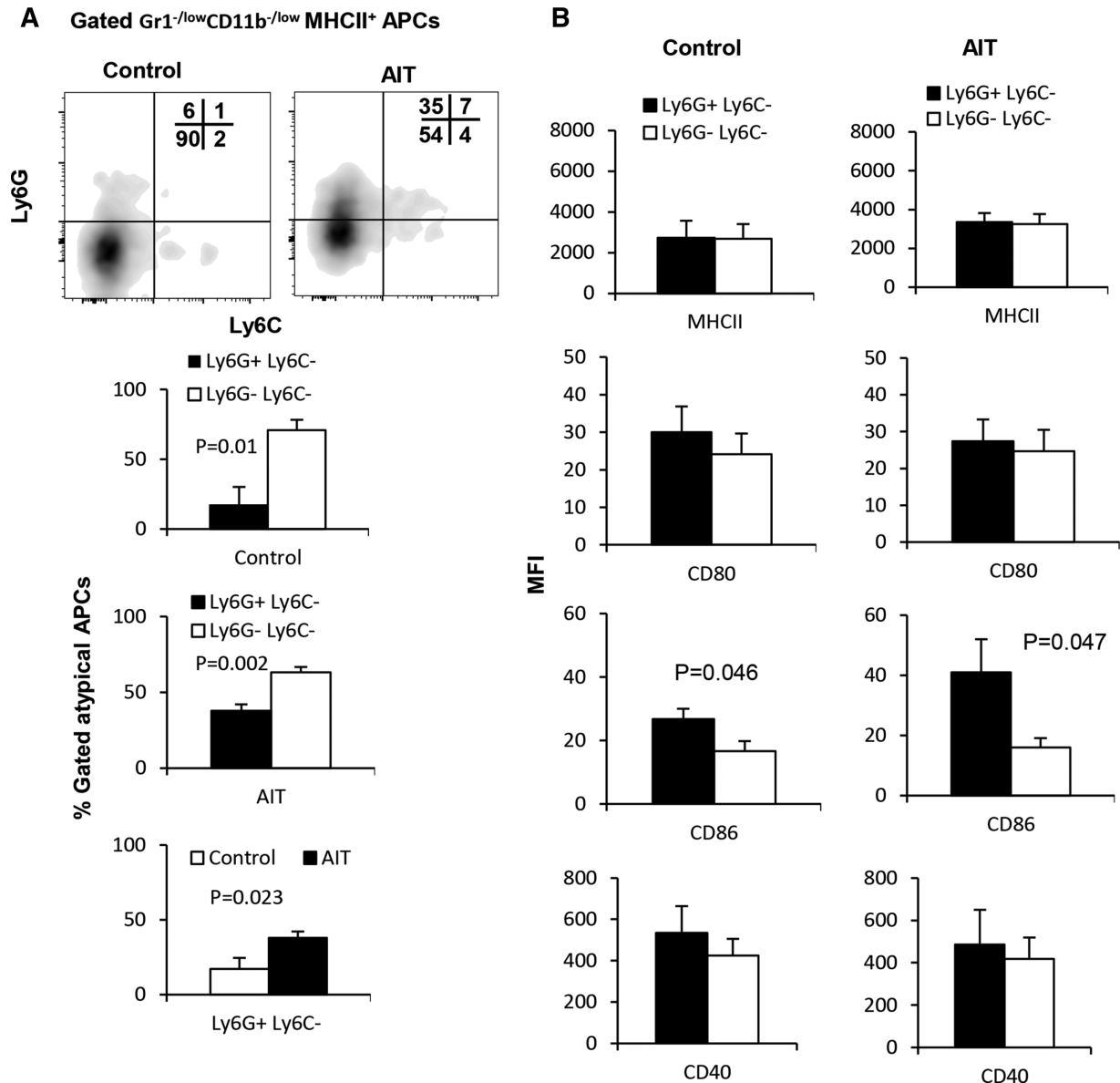


FIGURE 5 Tumor burden or AIT modulates Gr1^{low}CD11b^{low} myeloid cells. (A) Splenocytes of FVBN202 mice bearing metastatic tumor in the lung without treatment (Control) or after AIT (AIT) were subjected to analysis by flow cytometry. (A) Comparative analysis of Ly6G⁺Ly6C⁻ and Ly6G⁻Ly6C⁻ subsets among gated APCs of control and AIT groups. (B) Expression of MHCII and co-stimulatory molecules on Ly6G⁺Ly6C⁻ and Ly6G⁻Ly6C⁻ subsets in gated APCs of control and AIT groups. Gated cells were then analyzed for the expression of MHCII and co-stimulatory molecules. (C) Gr1^{low}CD11b^{low} APCs were sorted from the spleens of the AIT and control groups, and cultured for 24 h in the presence or absence of LPS (+LPS and -LPS). Data represent mean $\pm$ SEM of triplicate experiments

sorted autologous Gr1^{low}CD11b^{low} cells. As shown in Fig. 6A, lymphocytes derived from the AIT group released IFN- γ in the presence of Neu⁺ MMC cells ($p = 0.0001$). Importantly, the IFN- γ producing immune response to MMC was significantly boosted by autologous Gr1^{low}CD11b^{low} APCs (Fig. 6A, $p = 0.015$). On the other hand, lymphocytes derived from the control group did not demonstrate significant IFN- γ release in the presence of MMC; the addition of autologous Gr1^{low}CD11b^{low} APCs did not enhance this response (Fig. 6B). In order to determine if Gr1^{low}CD11b^{low} myeloid cells from the control group retained their immune stimulatory function, they were co-cultured with tumor-reactive T cells from the AIT group in the presence or absence of MMC. We hypothesized that T cell

specific killing of MMC cells from the AIT group could facilitate cross presentation of tumor Ags by Gr1^{low}CD11b^{low} APCs, resulting in the enhancement of the immune response. As shown in Fig. 6C, the presence of Gr1^{low}CD11b^{low} APCs boosted tumor-reactive IFN- γ production by splenic T cells derived from the AIT group ($P = 0.0002$). This was associated with the induction of apoptosis in MMC by reprogrammed T cells that were used for AIT compared with those of the control group (Fig. 6D, $P = 0.0004$). To assess the possibility of Gr1^{low}CD11b^{low} myeloid cells to potentially uptake and cross-present Ag to T cells, we first pulsed these cells with ovalbumin conjugated to a fluorophore. As shown in Fig. 6E, Gr1^{low}CD11b^{low} myeloid cells demonstrated the ability to uptake this protein, with

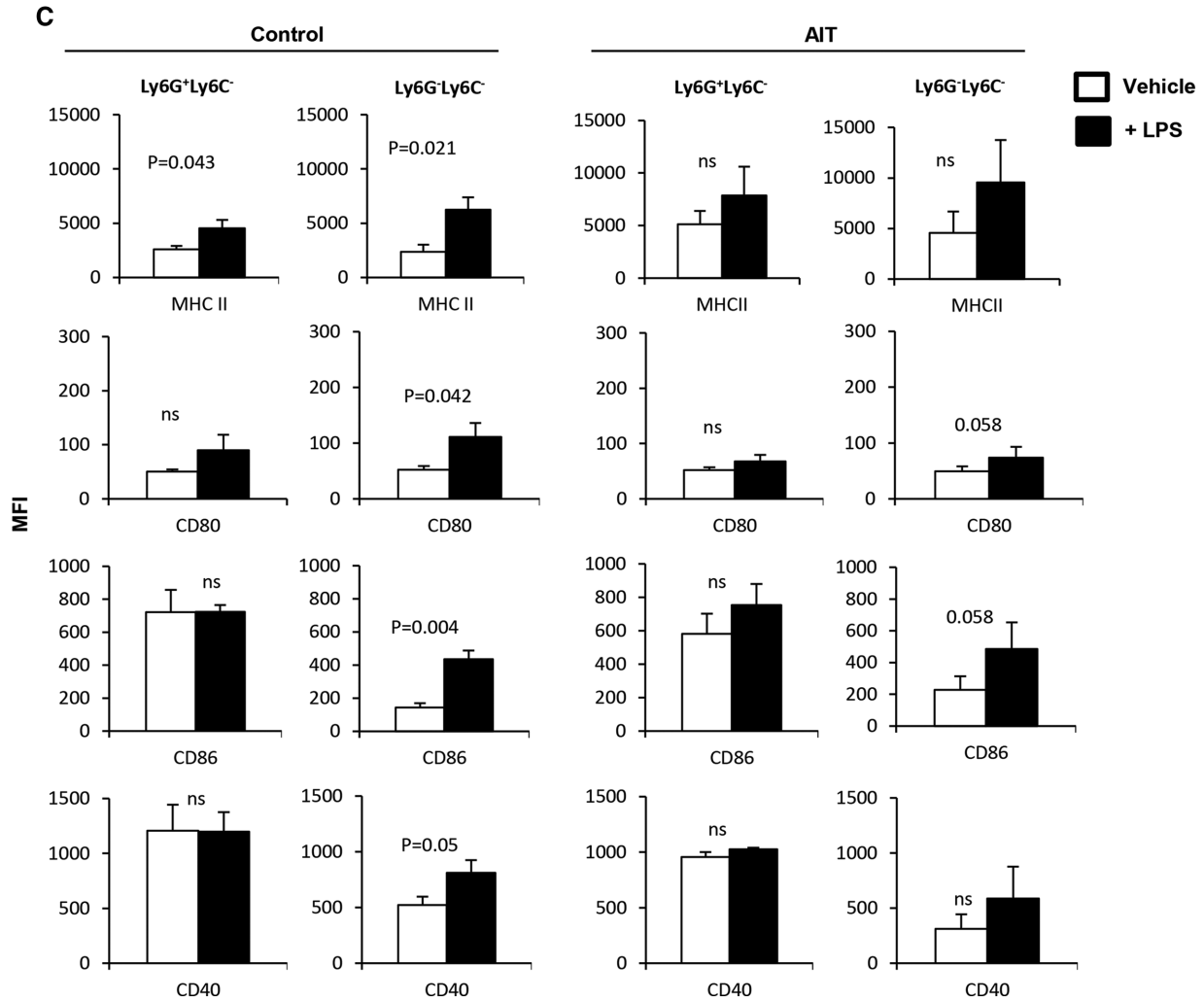


FIGURE 5 Continued

increased fluorescence intensity over time. Although it appears these cells have a reduced efficiency to uptake this Ag compared to DCs, these data suggest that Gr1^{-/low}CD11b^{-/low} myeloid cells may potentially function to cross-present processed Ag to T cells.

Thus, to specifically determine if Gr1^{-/low}CD11b^{-/low} myeloid cells could cross-present Ag to provoke a T cell response, we sorted splenic Gr1^{-/low}CD11b^{-/low} myeloid cells and pulsed them with recombinant Neu ECD protein, followed by a culturing period with tumor-sensitized T cells. In fact, as can be seen in Fig. 6F, Gr1^{-/low}CD11b^{-/low} myeloid cells were able to induce IFN- γ production from tumor-sensitized T cells only after they were pulsed with Neu ECD, suggesting these cells possess Ag-processing and presentation functionality. Bone marrow-derived DCs were used as a positive control for Ag cross presentation; irradiated MMC cells were used as a specificity control for assessing Neu-reactive T cell function. We then utilized a direct cytotoxicity assay to demonstrate that sorted Gr1^{-/low}CD11b^{-/low} myeloid cells from tumor-bearing mice boosted tumor-reactive T cell-mediated killing of MMC target cells, ex vivo (Fig. 7A, $P = 0.001$). These data suggest that although tumor burden drives the expansion of MDSCs and suppresses the expansion of mature Ly6G⁺ Ly6C⁻ APCs, these Gr1^{-/low}CD11b^{-/low} cells retain their immune stimulatory function,

but may not become fully functional in the presence of a weak antitumor immune response.

4 | DISCUSSION

Here, we describe a new class of APC, Gr1^{-/low}CD11b^{-/low} cells that do not express pan markers of myeloid DCs (CD11c), plasmacytoid DCs (Ly6C) or macrophage (F4/80). Characterization of these cells demonstrated their expression of MHCII and the costimulatory molecules CD80, CD86, and CD40 at the steady state. Further characterization of this population revealed that while the majority of Gr1^{-/low}CD11b^{-/low} myeloid cells do not express T or B cell lineage markers, we found that B cells interact with this myeloid APC population, a phenomenon that has classically been described to occur between DCs and B cells.^{35,36} This interaction may contribute to the immune stimulatory function of these atypical APCs, as such an interaction has been reported to boost immune stimulatory function of conventional DCs.³⁷ The frequency of Fc γ ch⁺ Ssc⁺ Gr1^{-/low}CD11b^{-/low} APCs was significantly greater than that of DCs in the spleen. Importantly, Gr1^{-/low}CD11b^{-/low} APCs were also present at the tumor site

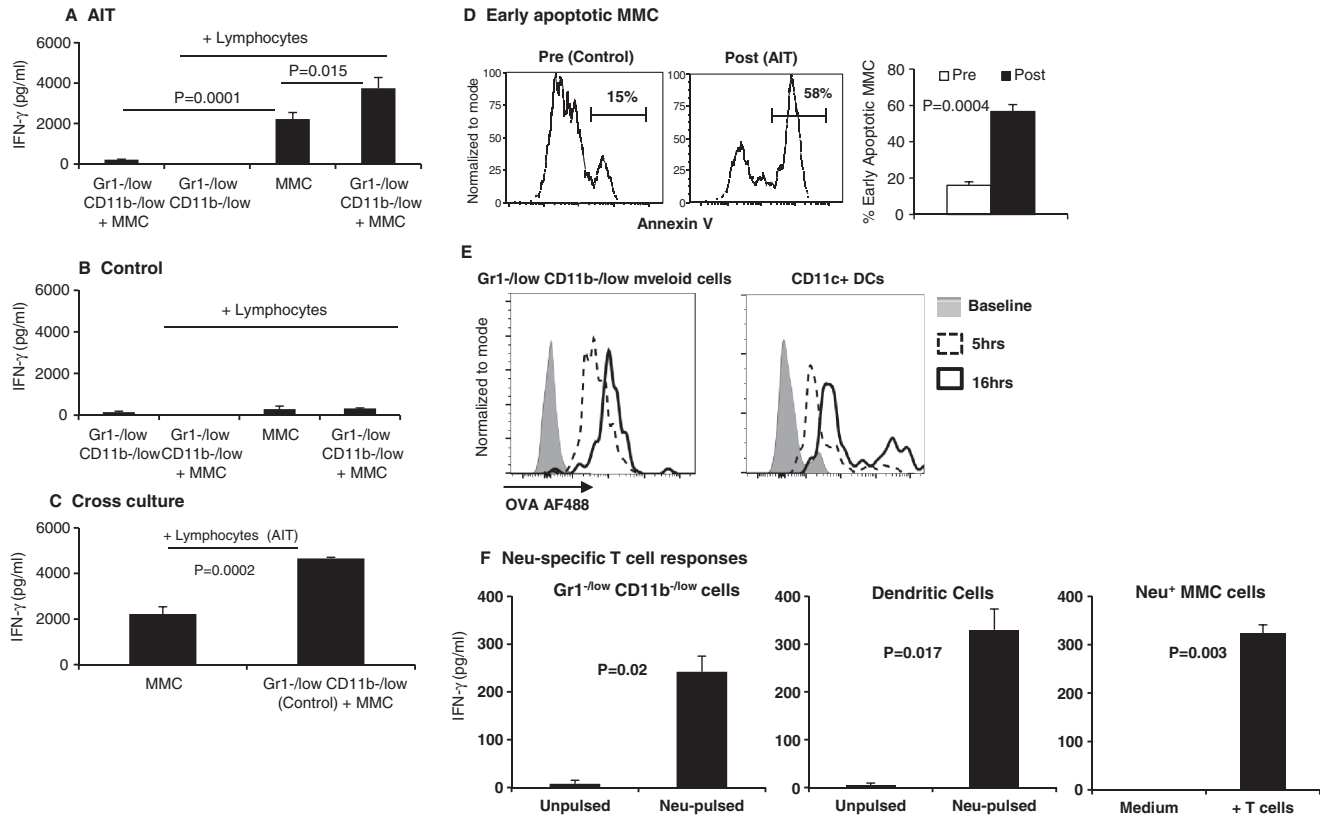


FIGURE 6 Gr1^{-low}CD11b^{-low} myeloid cells retain their immune stimulatory function during tumor burden and display characteristics of Ag-presentation. FACS sorted splenic Gr1^{-low}CD11b^{-low} cells from (A) AIT recipients or (B) Control mice were co-cultured without or with MMC (5:1) and without or with endogenous splenic lymphocytes (1:2) for 20 h; supernatant IFN- γ concentration was determined by ELISA. (C) Lymphocytes of the AIT group were cultured with MMC in the presence or absence of sorted Gr1^{-low}CD11b^{-low} cells of the control group. Data represent mean $\pm$ SEM after subtracting background signal from control conditions. (D) Quantification of Annexin V⁺ early apoptotic MMC cells after culture with freshly isolated lymphocytes of tumor-bearing control mice prior to the ex vivo re-programming (Pre) or with re-programmed lymphocytes used for AIT (Post). Data represent quadruplicate experiments. (E) Splenocytes (10⁶ cells/ml) of naïve FVB/N202 mice were pulsed with 50 μ g/ml Alexa Fluor 488 (AF488)-conjugated ovalbumin in RPMI1640 supplemented with 10% FBS for 5 or 16 h. Unpulsed cells were used as control (Baseline). Gated FVS⁻ viable cells were subgated for CD11c⁺ DCs or Gr1^{-low}CD11b^{-low} cells, and analyzed for intensity of Alexa Fluor 488 as a marker of ovalbumin internalization. (F) Sorted Gr1^{-low}CD11b^{-low} splenic cells or bone marrow-derived CD11c⁺ DCs were pulsed with Neu ECD and cultured with tumor-sensitized T cells. Irradiated MMC target cells were used as a positive control

of animals bearing lung metastases at a frequency that was inversely proportional to that of MDSCs. Interestingly AIT drove the accumulation of Gr1^{-low}CD11b^{-low} APCs while concomitantly reducing the frequency of Gr1⁺CD11b⁺ MDSCs both in the spleen and within the tumor bed; this was associated with an improved survival of tumor-bearing animals.

Gr1^{-low}CD11b^{-low} APCs were abundant in the steady state in naïve mice in vivo and had antitumor immune stimulatory function without any need for further ex vivo activation, although stimulation by LPS suggested they maintain the potential for further activation. These data suggest that Gr1^{-low}CD11b^{-low} APCs may be optimal performers in terms of Ag uptake as well as Ag presentation. In fact, Gr1^{-low}CD11b^{-low} myeloid cells were capable of Ag uptake and cross-presentation with similar efficiency to CD11c⁺ DCs. This paradoxical property of Gr1^{-low}CD11b^{-low} myeloid cells was associated with the presence of two subsets; a Ly6G⁺Ly6C⁻ subset and a Ly6G⁻Ly6C⁻ subset. While the Ly6G⁺Ly6C⁻ subset showed higher basal maturity, the emergence of which was associated with prolonged

survival of tumor-bearing mice, the Ly6G⁻Ly6C⁻ subset showed less maturity and higher responsiveness to LPS stimulation.

Tumor burden altered the frequency of Gr1^{-low}CD11b^{-low} myeloid cells but did not impair their immune stimulatory function; these cells, when derived from either the control group or the AIT group, were able to boost tumor-reactive T cell responses. Interestingly, AIT during tumor burden resulted in the modulation of the myeloid cell compartment, revealing an inverse relationship between Gr1^{-low}CD11b^{-low} myeloid cells and MDSCs. Such modulation of Gr1^{-low}CD11b^{-low} cells by AIT was associated with a significantly higher frequency of the Ly6G⁺Ly6C⁻ subset and splenic CD25⁺ NKT cells, which increased survival of animals. These observations are supported by previous work from our group and others.^{23,24,27,38} It has previously been shown by our group that MDSCs can be rendered immune stimulatory in the presence of CD25⁺ NKT cells. The removal of NKT cells from tumor-reactive lymphocytes resulted in the inability of AIT to modulate MDSCs to become immune stimulatory, and failed to protect animals from tumor challenge.²⁷ Similar observations were

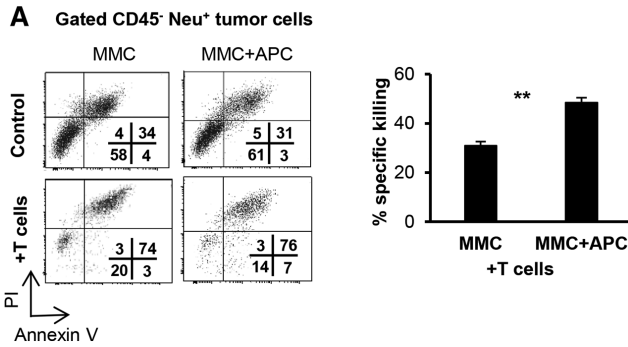


FIGURE 7 Gr1^{-low}CD11b^{-low} APCs boost antitumor function of T cells. (A) Tumor-reactive T cells derived and expanded from FVBN202 mice were co-cultured with MMC (10:1 ratio) in the presence or absence of sorted Gr1^{-low}CD11b^{-low} cells (5:1 ratio). Tumor cell cytotoxicity was determined on gated CD45-Neu⁺ tumor cells using control tumor cells alone, or in the presence of sorted Gr1^{-low}CD11b^{-low} APCs, T cells, or sorted Gr1^{-low}CD11b^{-low} APCs and T cells. Percent increased apoptosis of tumor cells by T cells in the absence (MMC) or presence of Gr1^{-low}CD11b^{-low} APCs (MMC+APC) was calculated by normalizing to the respective control. Data represent mean $\pm$ SEM of triplicate experiments

made using PBMCs of patients with breast carcinoma showing that HER-2/Neu-specific T cell responses were sustained in the presence of MDSCs; these sustained T cell responses were associated with the loss of CD11b and the up-regulation of HLA-DR on MDSCs, as well as the presence of CD25⁺ NKT cells.²⁴ Therefore, our current results suggest that a sufficient frequency of activated NKT cells in secondary lymphoid organs as well as the tumor microenvironment may modulate the myeloid cell compartment in tumor bearing mice to reduce the suppressive capacity of MDSCs, while also driving the emergence of Ly6G⁺Ly6C⁻Gr1^{-low}CD11b^{-low} immune stimulatory APCs.

The immune stimulatory function of Gr1^{-low}CD11b^{-low} APCs was also associated with the induction of specific tumor cell killing by Ag-sensitized T cells. In fact, our data suggest that Gr1^{-low}CD11b^{-low} myeloid cells function as APCs to process and cross-present tumor Ags to tumor-reactive T cells, resulting in the promotion of antitumor immune responses. This was further confirmed by showing a higher antitumor function of T cells in the presence of Gr1^{-low}CD11b^{-low} myeloid cells, as well as the ability of these cells to uptake Ag, and to cross-present to tumor-reactive T cells. These properties of Gr1^{-low}CD11b^{-low} APCs make them a potential candidate for a cell-based immunotherapy of cancer without having limitations of DC-based vaccines. Such impaired DC function is attributed to MDSCs both in vivo³⁹ and in vitro.⁴⁰ Similar MDSC-mediated suppressive function of macrophages has been reported in cancer patients.⁴¹ Furthermore, DC-intrinsic immune suppressive activity has been reported in cancer patients as well as in animal models of transplanted and spontaneous carcinoma.^{42–46}

In summary, we have identified Gr1^{-low}CD11b^{-low} myeloid cells that possess characteristics of APCs that are unique in the following ways: (i) they are more abundant than DCs, (ii) they are heterogeneous making them highly effective in both Ag uptake and Ag presentation simultaneously, (iii) they retain their immune stim-

ulatory function during tumor burden, and are inversely correlated with MDSCs, and (iv) their frequency is increased in the presence of CD25⁺ NKT cells. Moreover, human CD33⁺CD11b^{-low}HLA-DR⁺ myeloid cells appear to have similar immune stimulatory function as murine Gr1^{-low}CD11b^{-low} APCs.²⁴

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DISCLOSURES

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Department of Defense. The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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Autophagy-deficient breast cancer shows early tumor recurrence and escape from dormancy

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ABSTRACT

Breast cancer patients who initially respond to cancer therapies often succumb to distant recurrence of the disease. It is not clear why people with the same type of breast cancer respond to treatments differently; some escape from dormancy and relapse earlier than others. In addition, some tumor clones respond to immunotherapy while others do not. We investigated how autophagy plays a role in accelerating or delaying recurrence of neu-overexpressing mouse mammary carcinoma (MMC) following adriamycin (ADR) treatment, and in affecting response to immunotherapy. We explored two strategies: 1) transient blockade of autophagy with chloroquine (CQ), which blocks fusion of autophagosomes and lysosomes during ADR treatment, and 2) permanent inhibition of autophagy by a stable knockdown of ATG5 (ATG5^{KD}), which inhibits the formation of autophagosomes in MMC during and after ADR treatment. We found that while CQ prolonged tumor dormancy, but that stable knockdown of autophagy resulted in early escape from dormancy and recurrence. Interestingly, ATG5^{KD} MMC contained an increased frequency of ADR-induced polyploid-like cells and rendered MMC resistant to immunotherapy. On the other hand, a transient blockade of autophagy did not affect the sensitivity of MMC to immunotherapy. Our observations suggest that while chemotherapy-induced autophagy may facilitate tumor relapse, cell-intrinsic autophagy delays tumor relapse, in part, by inhibiting the formation of polyploid-like tumor dormancy.

INTRODUCTION

Autophagy plays a paradoxical role in the promotion and inhibition of cancer. On the one hand,

autophagy has a cancer-promoting role by protecting tumor cells from chemotherapy or providing a source of energy for tumor cells to survive under hypoxic and acidic conditions despite the lack of mature vessels [1].

On the other hand, inhibition of autophagy by disruption of *Beclin 1* or deletion of *ATG5* increases the frequency of spontaneous malignancies [2] or liver tumor [3], respectively. Recently, four different mechanisms have been proposed to describe paradoxical functions of autophagy in cancer, which include cytotoxic, cytostatic, cytoprotective and non-protective autophagy [4]. There are also three major types of autophagy which include micro-autophagy involving the direct engulfment of cytosolic material by lysosomes through invagination, chaperone-mediated autophagy involving HSP70 and the lysosomal membrane associated protein 2 A (LAMP2A), and macro-autophagy which is a highly conserved pathway involving the formation of autophagosomes, which fuse with lysosomes. To this end, *ATG5* is involved in the elongation of autophagosomes to engulf toxic material for degradation. A stable knockdown of *ATG5* results in the inhibition of the formation of autophagosomes and progression of macro-autophagy [5]. Chloroquine (CQ), on the other hand, does not have any effects on autophagosomes but it blocks the fusion of autophagosomes and lysosomes, thereby preventing the completion of macro-autophagy. In order to investigate the role of macro-autophagy in tumor dormancy and relapse, we performed a transient inhibition of macro-autophagy by means of CQ during chemotherapy, which mainly inhibits chemotherapy-induced autophagy while cell-intrinsic autophagy will be restored after the completion of chemotherapy. We also performed a permanent inhibition of cell-intrinsic macro-autophagy by a stable knockdown of *ATG5* in tumor cells. We demonstrated that cell-intrinsic, but not chemotherapy-induced, autophagy can inhibit tumor relapse.

RESULTS

Adriamycin induces autophagy in MMC

In order to determine whether ADR induces autophagy and in turn establishes tumor dormancy, MMC cells were treated with ADR in the presence or absence of CQ, a pharmacological agent used to block the final stages of autophagy, specifically the fusion of autophagosomes with lysosomes that is necessary for digestion of the cargo in the autophagosomes (frequently termed “autophagic flux”). CQ blocked this autophagic flux as evidenced by the enhanced accumulation of acidic vesicles (red signals) (Figure 1A, ADR and ADR+CQ). We further monitored degradation of the p62/SQSTM1 protein as a marker of autophagic flux, and LC3B expression as a marker of autophagosomes formation (since LC3 is a component of the autophagosomes). As shown in Figure 1B, ADR did not induce degradation of p62/SQSTM1 although it elevated LC3B, suggesting that ADR induces autophagy but fails to drive autophagy to completion and p62/SQSTM1 degradation.

A transient blockade of autophagy by CQ during ADR treatment delays tumor relapse *in vitro* but not *in vivo*

Since CQ is being used to sensitize tumor cells susceptible to chemotherapy [6], we sought to determine whether blockade of autophagy by CQ during ADR treatment affects tumor dormancy and relapse. We showed that the presence of CQ during ADR treatment, *in vitro*, resulted in prolonging tumor dormancy such that, while ADR treated MMC resumed cell proliferation 6 weeks after the treatment, ADR+CQ treated MMC remained dormant (Figure 2A). In order to confirm tumor cell relapse after 6 weeks, flow cytometry analysis of ADR-treated MMC was performed, and indicated a shift of Ki67- non-proliferating cells to Ki67+ proliferating cells with a greater viability (Figure 2B). In fact, MMC cells remained apoptotic by producing floater dead cells following ADR treatment (Supplementary Figure 1A) which compensated for cell proliferation and maintained tumor dormancy for 3 weeks after the completion of ADR treatment. Follow up studies on floater cells showed they were all apoptotic (Supplementary Figure 1B). A transient blockade of autophagy by CQ did not affect susceptibility of tumor cells to ADR-induced apoptosis (Supplementary Figure 2). On the other hand, a transient blockade of autophagy during ADR chemotherapy, *in vivo*, did not prolong tumor dormancy in FVBN202 mice (Supplementary Figure 3).

A transient blockade of autophagy by CQ during ADR treatment does not change susceptibility of tumor cell to immunotherapy

In order to determine whether a transient blockade of autophagy during ADR treatment affects susceptibility of dormant MMC to immunotherapy, dormant MMC were cultured with either IFN- γ or MMC-reactive T cells three weeks after treatment with ADR or ADR+CQ. As shown in Figure 3, untreated MMC or dormant MMC treated with ADR or ADR+CQ all remained susceptible to IFN- γ treatment or T cells.

A stable knockdown of autophagy reduces susceptibility of MMC to ADR treatment

CQ only transiently blocks fusion of autophagosomes and lysosomes during ADR treatment such that after removal of CQ, accumulated autophagosomes could eventually be fused with lysosomes to complete autophagy. In order to determine the role of autophagy in tumor dormancy or relapse, we used shRNA for a stable knockdown of *ATG5* (*ATG5^{KD}*) which inhibits formation of autophagosomes in MMC. Scrambled shRNA was used as control (Supplementary Figure 4A). The *ATG5^{KD}* MMC and scrambled control MMC were irradiated to confirm that *ATG5^{KD}* MMC cells were deficient in autophagy, using p62

and LC3B as read outs (Supplementary Figure 4B). Tumor cells remained intact for the expression of neu antigen, as well as cell proliferation *in vitro* and *in vivo* following knockdown of autophagy (Supplementary Figure 4C–4E). Flow cytometry analysis determined a lower level of viability in MMC compared with ATG5^{KD} MMC following ADR treatment (Figure 4).

A stable knockdown of autophagy results in earlier tumor relapse associated with increased frequency of polyploid-like cells and resistance to immunotherapy

In order to determine whether a higher viability of ATG5^{KD} MMC following ADR treatment (Figure 4) facilitates an earlier tumor relapse compared with wild type MMC, follow up studies were performed for three weeks after ADR treatment. As shown in Figure 5A, ATG5^{KD} MMC survived better than autophagy-competent MMC following ADR treatment showing a significantly higher number of cells by 3 weeks after the treatment. Flow cytometry analysis of tumor cells showed greater levels of apoptosis in wild type MMC compared with ATG5^{KD} MMC (Figure 5B, $p < 0.001$). Interestingly, ATG5^{KD} MMC cells contained a higher number of polyploid-like cells following ADR treatment compared with autophagy-competent MMC (Figure 5B, $p < 0.03$).

In order to determine the *in vivo* relevance of our *in vitro* findings, FVB/N202 mice were used. Tumor dormancy was first established by ADR treatment *in vitro*; FVB/N202 mice ($n = 7$ /group) were then challenged i.v. with one million viable dormant tumor cells. Animals were then sacrificed when they became moribund (lost 10% weight) as a result of massive lung metastasis. As can be seen in Figure 6A, animals that were challenged with ADR-treated ATG5^{KD} MMC developed lung metastasis significantly sooner than those that were

challenged with ADR-treated MMC. Hematoxylin/eosin and immunohistochemistry analyses of tumor lesions determined a higher frequency of polyploid-like and Ki67+ tumor cells in animals that were challenged with ADR-treated ATG5^{KD} MMC (Figure 6B). Finally, ATG5^{KD} MMC were found to be resistant to T cell-induced apoptosis compared with autophagy-competent MMC (Figure 7).

DISCUSSION

Cell-intrinsic autophagy is an ongoing process, which regulates cellular metabolism and homeostasis. Autophagy is also induced by insults such as chemotherapy. Here, we studied a paradoxical role of autophagy in tumor promotion and tumor inhibition by a transient inhibition of autophagy only during chemotherapy or a stable knockdown of autophagy in MMC tumor cells. While the former transiently blocked autophagy and cell-intrinsic autophagy was restored after the completion of chemotherapy, the latter permanently blocked chemotherapy-induced autophagy and cell-intrinsic autophagy. We demonstrated that inhibition of chemotherapy-induced autophagy by CQ did not increase susceptibility of tumor cells to chemotherapy-induced apoptosis. Nevertheless, chemotherapy-induced autophagy appeared to accelerate tumor relapse such that use of CQ during chemotherapy delayed tumor relapse *in vitro*. Our observation is consistent with other reports showing that increased autophagy in residual breast cancer after neoadjuvant chemotherapy was correlated with increased risk of tumor relapse [7]. A transient blockade of autophagy during chemotherapy of tumor-bearing animals did not affect tumor relapse, perhaps, because tumor inhibitory effects of *in vivo* chemotherapy was not as effective as *in vitro* drug treatment. Also, chemotherapy-induced autophagy did not affect the sensitivity of tumor cells to apoptosis induced by IFN- γ or tumor-reactive T cells.

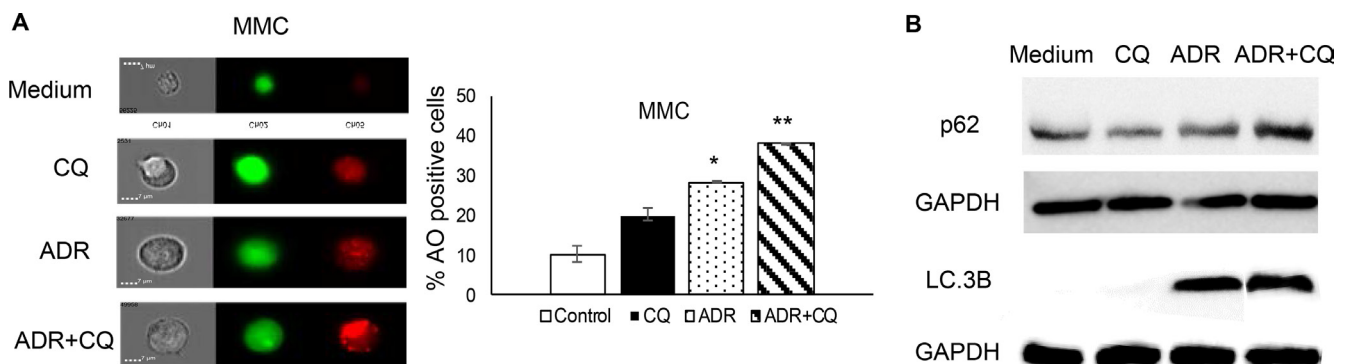


Figure 1: CQ blocks ADR-induced autophagy. MMC tumor cells received three daily doses of ADR alone (1 μ M ADR for 2 hrs) (ADR) or in the presence of CQ (10 μ M 3 hrs before ADR and 2 hrs during ADR treatment) (ADR+CQ), washed after each daily treatment and analyzed by acridine orange (AO) one day after the last treatment. Untreated MMC (Medium) or MMC treated with CQ (CQ) served as controls. (A) Acridine orange (AO) staining was analyzed for acidic vesicles (red) using image cytometry. Data represent triplicate experiments. (B) Levels of p62/SQSTM1 and LC3B after treatment with ADR $\pm$ CQ indicative of autophagy induction in the absence of autophagic flux (B).

We also demonstrated that, unlike chemotherapy-induced autophagy, cell-intrinsic autophagy accelerated tumor relapse. A stable knockdown of cell-intrinsic autophagy by ATG5 shRNA resulted in a reduced sensitivity of tumor cells to chemotherapy- or T cell-induced apoptosis, and accelerated tumor relapse *in vivo*. These effects coincided with an increased frequency of multinuclear polyploid-like dormant cells. These observations suggest that chemotherapy-induced autophagy could have tumor-promoting effects and facilitate tumor relapse, whereas cell-intrinsic autophagy could synergize with cancer therapeutics and delay tumor relapse. In fact, cell-intrinsic autophagy would seem to inhibit the formation of multinuclear cells following chemotherapy, and to prevent chemotherapy-induced genetic instability associated with resistance to cancer therapeutics. Similar observations have been made in other breast tumor models by showing that CQ but not knockdown of Beclin 1 or ATG12 sensitized the tumor to chemotherapy [8]. Therefore, anti-tumor effects of

autophagy inhibitors such as CQ is likely to be because of the inhibition of chemotherapy-induced autophagy while anti-tumor effects of autophagy inducers such as rapamycin may result from enhanced cell-intrinsic autophagy [9, 10]. It has been reported cancer stem cells play a role in tumor dormancy [11] and drug resistance [12], and that immunotherapeutic targeting of breast cancer stem cells inhibits growth of mammary carcinoma [13]. However, we did not detect the enrichment of CD44⁺CD24⁻ cancer stem cells following ADR-induced tumor dormancy (data not shown).

Anticancer drugs and ionizing radiation tend to induce autophagy in tumor cells [14]. Treatment-induced autophagy could lead to apoptosis [15] and tumor cell dormancy [16]. We have already reported that dormant tumor cells established by ADR treatment or radiation therapy, *in vitro*, developed resistance to these treatments but remained susceptible to immunotherapy [17]. Therefore, evaluation of apoptosis or tumor growth inhibition as a single factor without evaluating

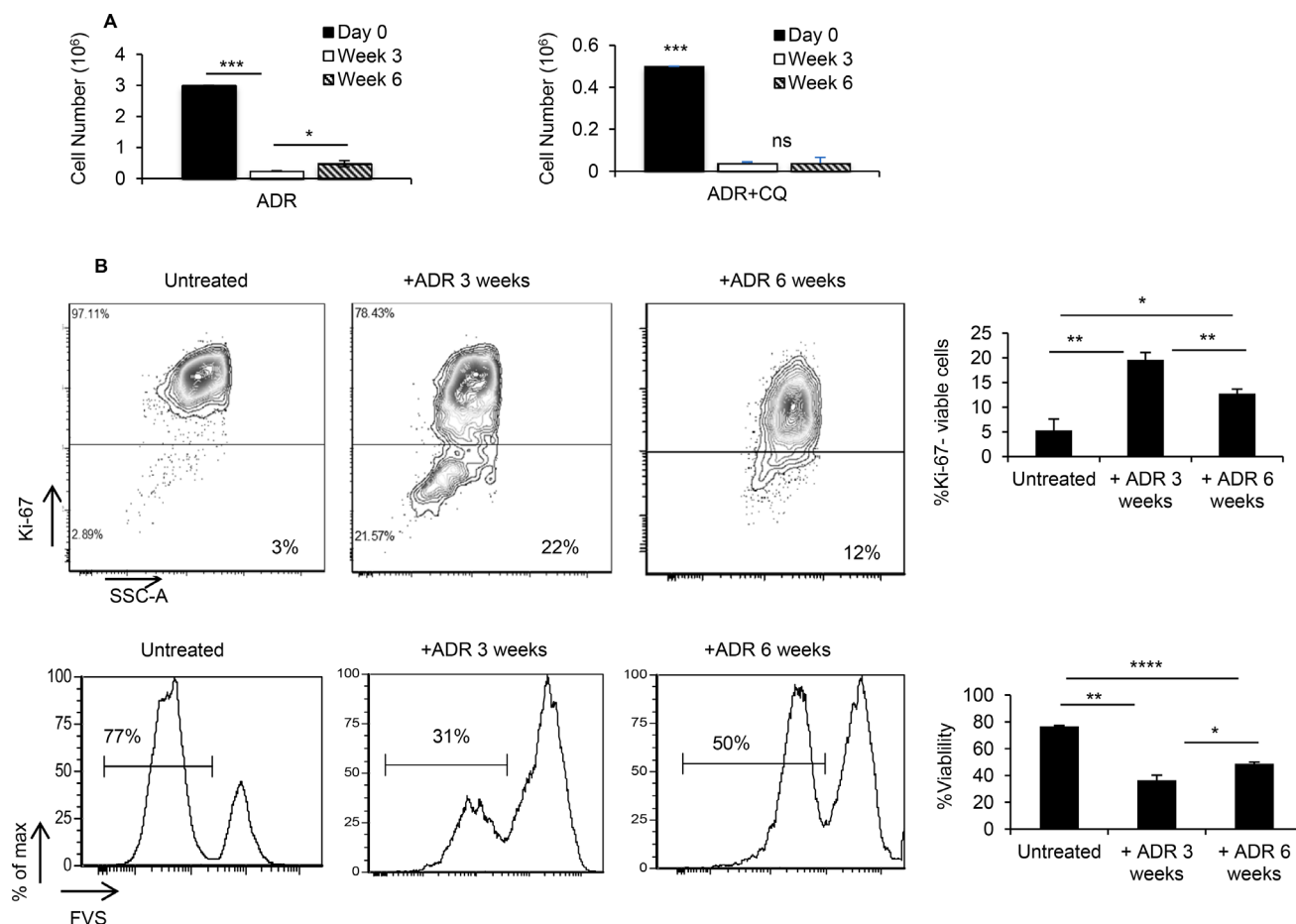


Figure 2: ADR-induced dormant tumor cells remain dormant in the presence of CQ. MMC tumor cells were treated with 3 daily doses of ADR (1 μ M for 2 hrs), with one group receiving CQ (10 μ M) 3 hrs prior to and during ADR treatment. Both groups remained untreated for 3 weeks and 6 weeks, *in vitro*. (A) Adherent viable cells were counted using trypan blue exclusion at various time points. Data represent 3 replicates $\pm$ SEM. (B) At weeks 3 and 6 post-treatment, Ki-67 expression (upper panel) and viability (lower panel) were quantified within the population of adherent tumor cells. Data represent 2–3 replicates $\pm$ SEM. Four independent experiments have been carried out which have shown similar results.

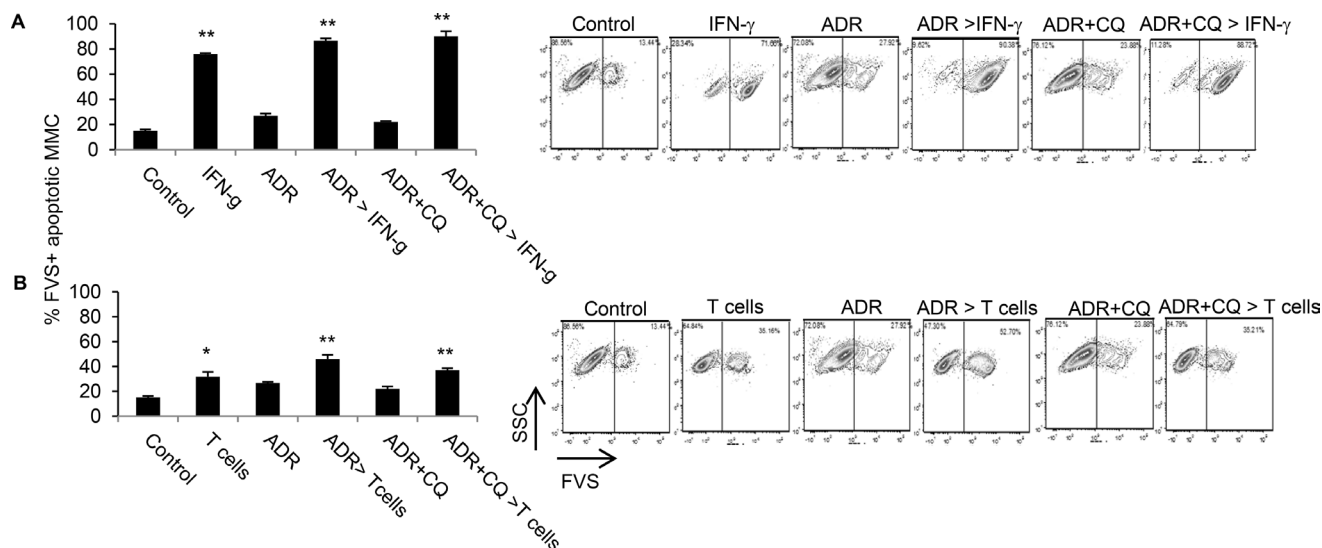


Figure 3: Dormant tumor cells established by ADR or ADR+CQ remain susceptible to immunotherapy. The *in vitro* tumor dormancy was established three weeks after three daily treatments of MMC with ADR or ADR+CQ. Untreated MMC cells were used as control. **(A)** Apoptosis was determined by FVS viability staining in MMC (control), ADR-treated dormant MMC (ADR), ADR+CQ-treated dormant MMC (ADR+CQ), as well as control MMC cultured with three daily doses of IFN-g and analyzed two days later (50 ng/ml) (IFN-g), ADR-treated dormant MMC cultured with three daily doses of IFN-g (50 ng/ml) and analyzed two days later (ADR > IFN-g), or ADR+CQ-treated dormant MMC cultured with three daily doses of IFN-g (50 ng/ml) and analyzed two days later (ADR+CQ > IFN-g). **(B)** Apoptosis was determined by FVS viability staining of MMC (control), MMC cultured with MMC-sensitized T cells for 48 hrs (T cells), ADR-treated dormant MMC (ADR), ADR-treated dormant MMC cultured with MMC-sensitized T cells for 48 hrs (ADR > T cells), ADR+CQ-treated dormant MMC (ADR+CQ), or ADR+CQ-treated dormant MMC cultured with MMC-sensitized T cells for 48 hrs (ADR+CQ > T cells). Splenic T cells were collected from MMC tumor-bearing FVB/N202 mice.

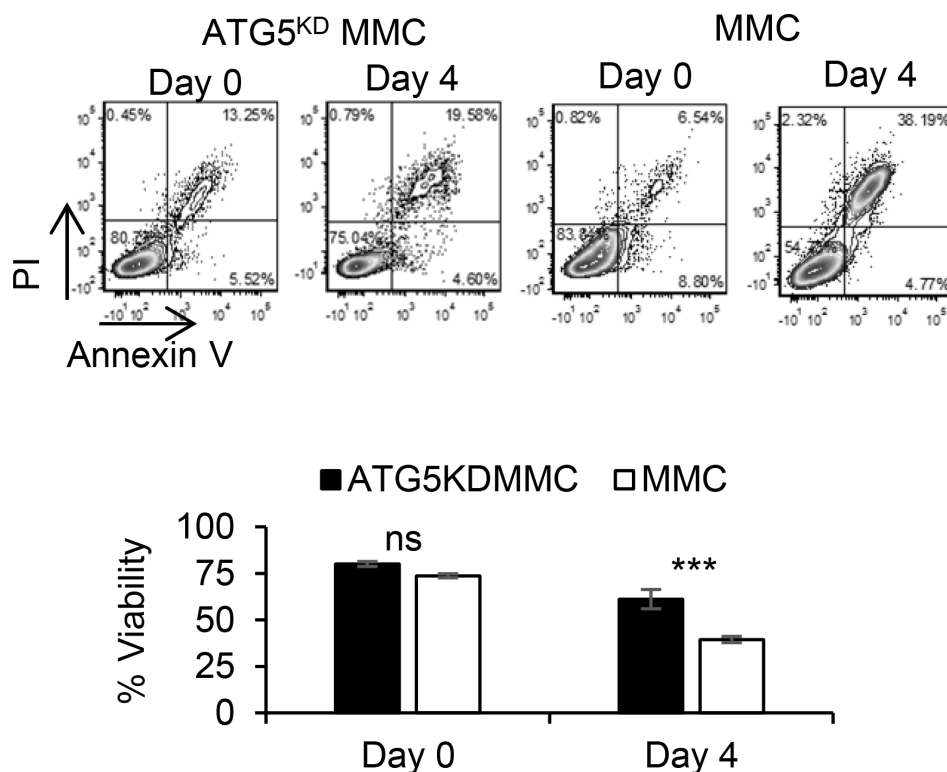


Figure 4: Autophagy knockdown tumor cells become less susceptible to ADR-induced apoptosis. Autophagy-deficient MMC (ATG5^{KD} MMC) or autophagy-competent MMC (MMC) were treated with a single dose of ADR alone (1 μ M ADR for 2 hrs). Tumor cells were analyzed by Annexin V/PI staining prior to treatment (Day 0) or three days after the treatment (Day 4). Experiments were performed in triplicates.

tumor dormancy and relapse may not be sufficient for understanding anti-cancer efficacy of autophagy inhibitors such as CQ. Inhibition of autophagy by CQ during chemotherapy diminishes the expression of DNA repair proteins, resulting in tumor growth inhibition in carboplatin-resistant BRCA1 wild-type TNBC orthotopic xenografts [18]. In triple negative breast cancer, CQ sensitizes tumor cells to paclitaxel chemotherapy [19]. In several tumor models, CQ synergistically augmented sunitinib cytotoxicity on tumor cells [6]. However, the role of CQ in inhibiting tumor recurrence has yet to be determined.

Cells that are deficient in autophagy show increased levels of reactive oxygen species which result in the accumulation of DNA damage, increased double-strand breaks and polyploid nuclei [20, 21]. To this end, cell-intrinsic autophagy protects the cell from genomic instability induced by the accumulation of toxins within the cell [22]. It has been reported that Beclin1 knockout mice fail to maintain genomic integrity by increasing DNA double stranded breaks and gene amplifications [20]. A higher expression of Beclin 1 in healthy breast tissue than in breast cancer suggests a deficiency in cell-intrinsic autophagy in tumors [23], which could contribute to genomic instability during tumorigenesis. In breast cancer patients who received adjuvant chemotherapy, presence of tumor cell intrinsic autophagy contributed to reduced risk of tumor relapse [24]. Expression of ATG5 in the tumor specimens is also associated with relapse-free survival in breast cancer patients [25]. In glioma, reduced tumor cell progression and relapse by knockdown of CDGSH iron sulfur domain 2 (CISD2) was associated with the activation of Beclin 1-mediated autophagy [26].

Our observations suggest that any deficiency in tumor cell-intrinsic autophagy could result in a reduced sensitivity of breast cancer to chemotherapy or immunotherapy. Therefore, IHC analysis of tumor biopsies

before and after neoadjuvant or adjuvant chemotherapy could determine cell-intrinsic and chemotherapy-induced autophagy, respectively, and in turn might predict the risk of distant recurrence of the diseases accordingly. In future studies, other murine and human breast tumor cell lines as well as other types of carcinoma cells should be used in order to determine whether our findings offer a general mechanism of autophagy-associated tumor dormancy and relapse, or it might be a cancer specific phenomenon.

MATERIALS AND METHODS

Tumor cell line

The neu overexpressing mouse mammary carcinoma (MMC) cell line was established from spontaneous mammary tumors harvested from FVBN202 mice [27]. Tumor cells were maintained in RPMI 1640 supplemented with 10% FBS.

Genetic silencing of ATG5 in MMC

Mission shRNA bacterial stocks for ATG5 and scrambled Control were purchased from Sigma Aldrich. Lentiviruses were produced in HEK 293TN cells co-transfected using Endo F ectinTM Lenti Transfection Reagent (GeneCopoeia, 1001-01) with a packaging mixture of psPAX2 and pMD2.G constructs (Addgene). Media containing the viruses was used to infect MMC cells; puromycin (1 μ g/ml) was used as a selection marker to enrich for infected cells.

Antibodies

All antibodies were purchase from Biolegend (San Diego, CA, USA) unless otherwise stated. Antibodies were used as instructed by the supplier. Antibodies

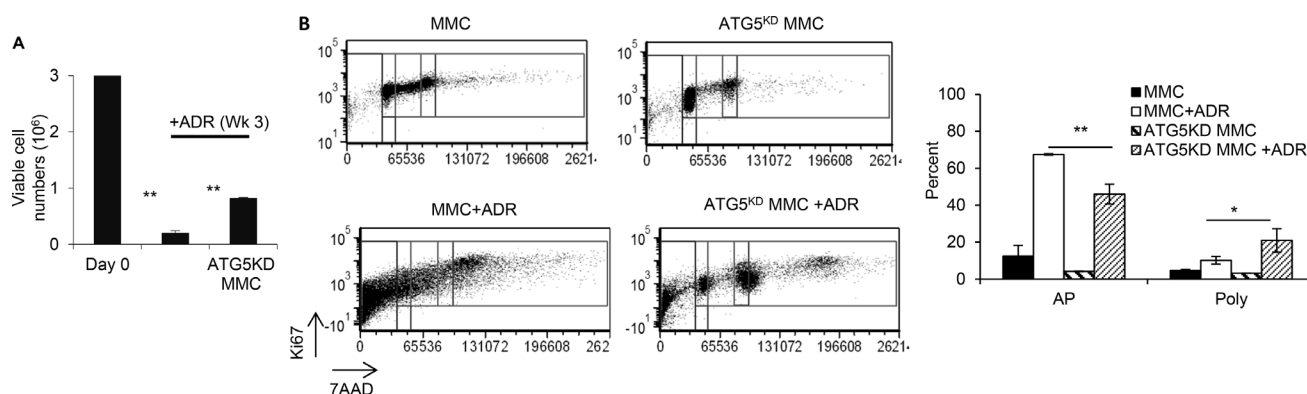


Figure 5: ADR-induced tumor dormancy in autophagy knockdown tumor cells with polyploid-like morphology compared with autophagy competent tumor cells, *in vitro*. MMC or ATG5^{KD} MMC tumor cells (3 million cells, Day 0) were treated with 3 daily doses of ADR (1 μ M for 2 hrs), and viable cells were counted at week 3 using trypan blue exclusion. Data represent triplicate experiments (A). Dot plots from each experimental group gated for cell cycle phase based upon DNA content (7-AAD) and Ki-67 expression. Events falling to the left of the G1/G0 gates are considered apoptotic cells (AP). Events falling to the far right of the G2/M gate are considered polyploid-like cells (Poly) (B). Three independent experiments have been performed and data represent 3 replicates $\pm$ SEM.

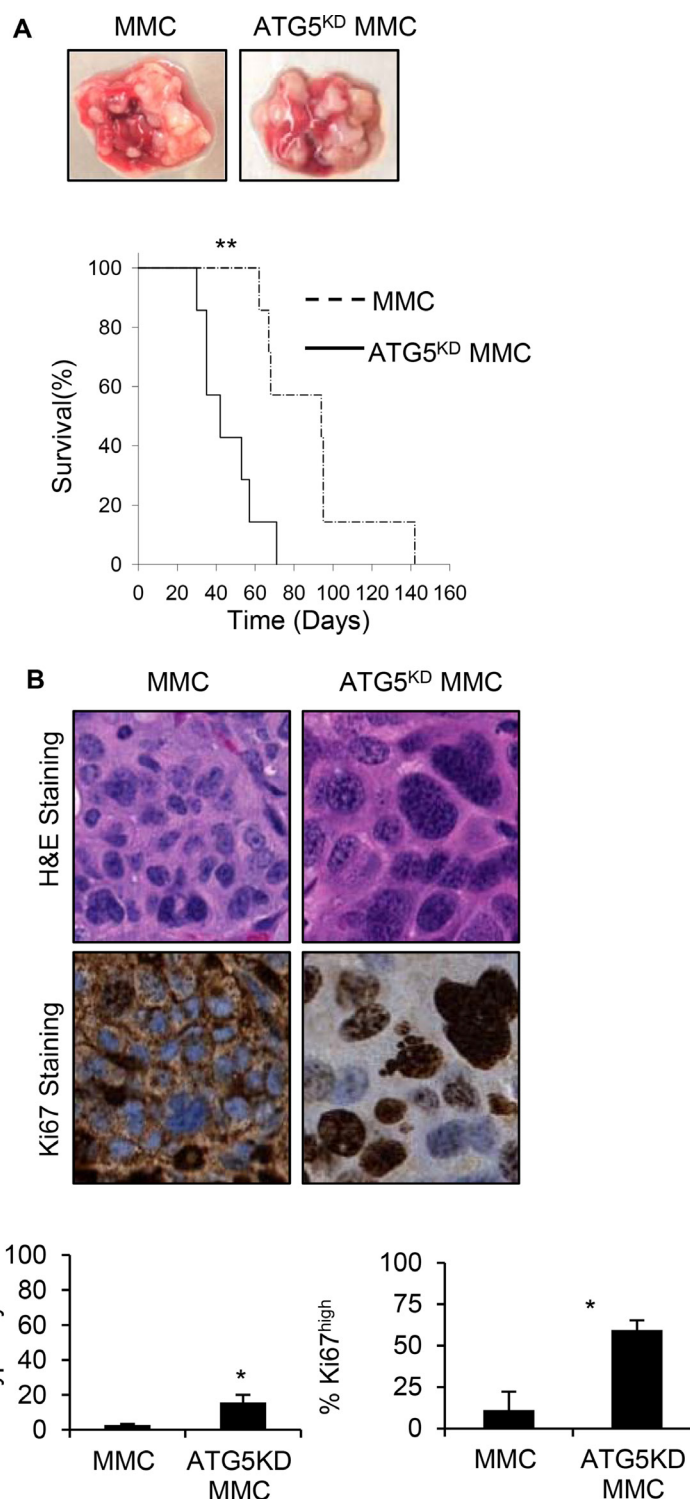


Figure 6: Earlier relapse of autophagy knockdown tumor cells with polyploid morphology compared with autophagy competent tumor cells, *in vivo*. (A) FVBN202 mice ($n = 7$) were challenged i.v. with 10^6 cells ADR-treated dormant control MMC (MMC), or ADR-treated dormant ATG5^{KD} MMC (ATG5^{KD} MMC). Animals were euthanized as soon as they became moribund. Representative tumor relapse in the lung and survival curve are shown. (B) Relapsed tumors were collected and immunohistochemistry slides were prepared by either staining samples with hematoxylin and eosin (H&E) or by Ki67 staining followed by subsequent digitization and analysis with NDP View software (Hamamatsu Photonics). At twenty-times magnification, three representative 0.02 mm² areas were chosen from the H&E slides containing approximately 100 cells to measure nuclear envelope size. Cells containing a nuclear envelope equal to or greater than 16 μ m with visible multi-nuclei were considered polyploid-like or high grade cells. The corresponding cell was then analyzed on the Ki67 stained slide to determine Ki67 expression levels. Data was collected from three biological samples. Significance is based on a two-tailed t -test of $p < 0.05$.

include: anti-CD16/32 (clone 93), APC-anti-mouse IgG (Poly4053), PE-Ki67 (16A8), Alexa flour 488-Ki67 (11F6), Brilliant Violet 605-CD45 (30-F11), FITC-Annexin V, APC-Annexin V, 7-AAD viability staining solution and Propidium Iodide solution (PI), mouse anti-rat neu (anti-c-Erb2/c-Neu; 7.16.4, Calbiochem, Billerica, MA, USA), FITC-FVS (BD Biosciences). All reagents were used at the manufacturer's recommended concentration.

Mice

FVBN202 transgenic female mice (The Jackson Laboratory; Bar Harbor, ME, USA) were used. These mice overexpress non-mutated, non-activated rat neu transgene under the regulation of the mouse mammary tumor virus promoter [28]. These mice develop premalignant mammary hyperplasia similar to ductal carcinoma *in situ* prior to the development of spontaneous carcinoma [29]. These studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Experimental tumor dormancy

In vitro tumor dormancy was established by the treatment of MMC or ATG5^{KD} MMC tumor cells with 3 daily doses of ADR (Sigma-Aldrich, 1uM for 2 hrs). During ADR treatment, MMC tumor cells were cultured without or with CQ (Sigma-Aldrich, 10 uM, 3 hrs prior to and during ADR treatment). By 2 weeks after the treatment, all groups did not show any increases in the number of adherent cells, which is the characteristic of tumor dormancy. For *in vivo* induction of tumor dormancy, FVBN202 mice were challenged with ADR-treated dormant MMC or ATG5^{KD} MMC (i.v. injection of 1 million viable cells), or untreated MMC followed by 3

weekly treatments of ADR (i.v., 9 mg/kg) or with 3 weekly treatment of ADR + 60 mg/kg CQ (i.p.).

Cytotoxicity assay

Freshly isolated tumor-primed splenic T cells or *ex vivo* expanded splenic T cells were cultured with MMC at a 10:1 E:T ratio in 3 ml complete medium (RPMI-1640 supplemented with 100 U/ml of penicillin, 100 µg/ml streptomycin, 10% FBS, 10 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol) with 20U/ml of IL-2 (Peprotech) in 6 well culture dishes. After 48 hs cells were harvested and stained for neu (anti-c-Erb2/c-Neu, Calbiochem), Annexin V and PI according to the manufacturer's protocol (BD Pharmingen). Flow cytometry was used to analyze the viability of neu positive cells [17, 30].

IFN-γ ELISA. Reprogrammed immune cells were cultured in complete medium with irradiated (140 Gy) tumor cells, ADR-treated dormant MMC or ADR+CQ-treated dormant MMC at a 10:1 ratio for 20 hrs. Supernatants were then collected and stored at -80°C until assayed. IFN-γ was detected using a Mouse IFN-γ ELISA kit (BD Biosciences), according to the manufacturer's protocol [30].

Statistical analysis

Data are summarized as means and standard errors of the mean (SEM) with differences between groups being illustrated with graphical data presented as mean ± SEM. Statistical comparisons were made using a one-tailed or two-tailed Student *t* test and a *p*-value < 0.05 was considered significant (*: < 0.05, **: < 0.005, ***: < 0.0005, ****: < 0.00005).

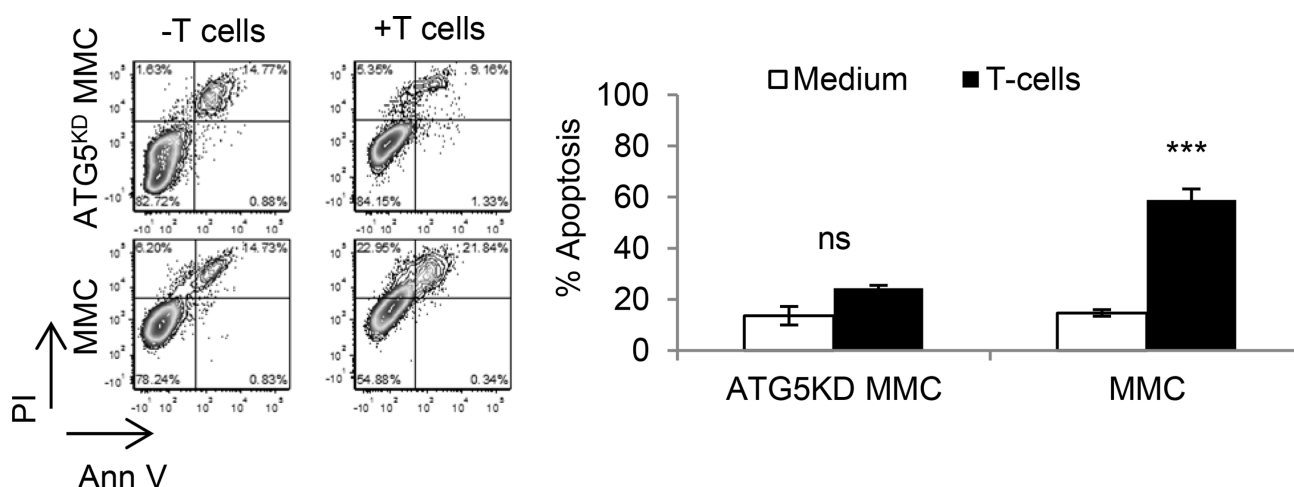


Figure 7: Autophagy knockdown tumor cells become resistant to T cell-induced apoptosis. Neu overexpressing autophagy-deficient MMC (ATG5^{KD} MMC) or autophagy-competent MMC (MMC) were co-cultured with MMC-sensitized T cells and then gated CD45-Neu⁺ tumor cells were analyzed by Annexin V/PI staining. Data represents triplicate experiments.

Abbreviations

ADR, Adriamycin, ATG5, Autophagy-related gene 5, BRCA1, Breast cancer gene 1, CQ, chloroquine, HSP70, Heat shock protein 70, IHC, Immunohistochemistry LAMP2A, Lysosomal membrane associated protein 2 A, MMC, neu-overexpressing mouse mammary carcinoma, TNBC, Triple negative breast cancer.

Author contributions

M.H.M., D.A.G., K.K.P. contributed to the study's conception, design, experimental and analytical performance, and writing of the manuscript. H.F.A., L.T-M., T.T., R.C.K., S.J., S.E.B., T.M.S. contributed to the study's experimental and analytical performance and writing of the manuscript. D.B. contributed to statistical analysis and writing of the manuscript. H.D.B. and M.O.I. contributed to analytical performance and writing of the manuscript.

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CONFLICTS OF INTEREST

Authors have no potential conflicts of interest to disclose.

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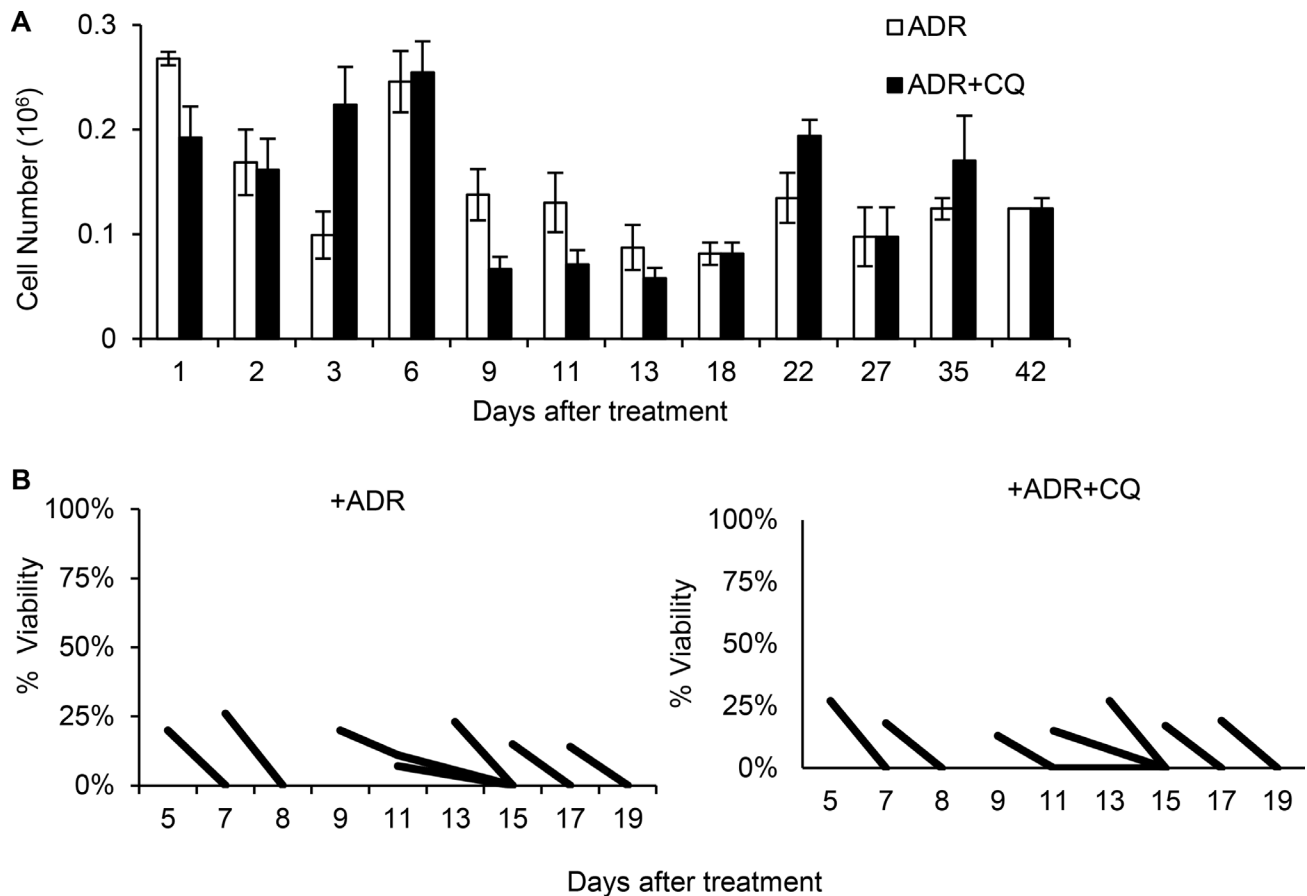
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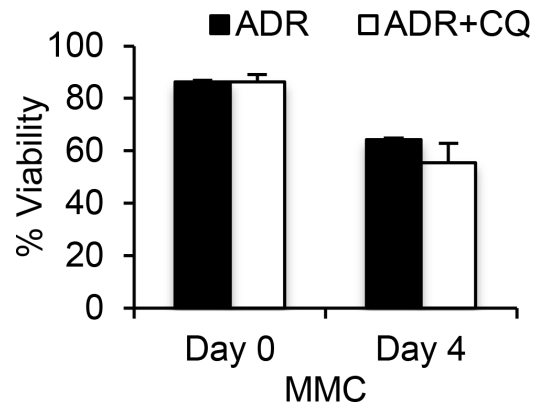
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Autophagy-deficient breast cancer shows early tumor recurrence and escape from dormancy

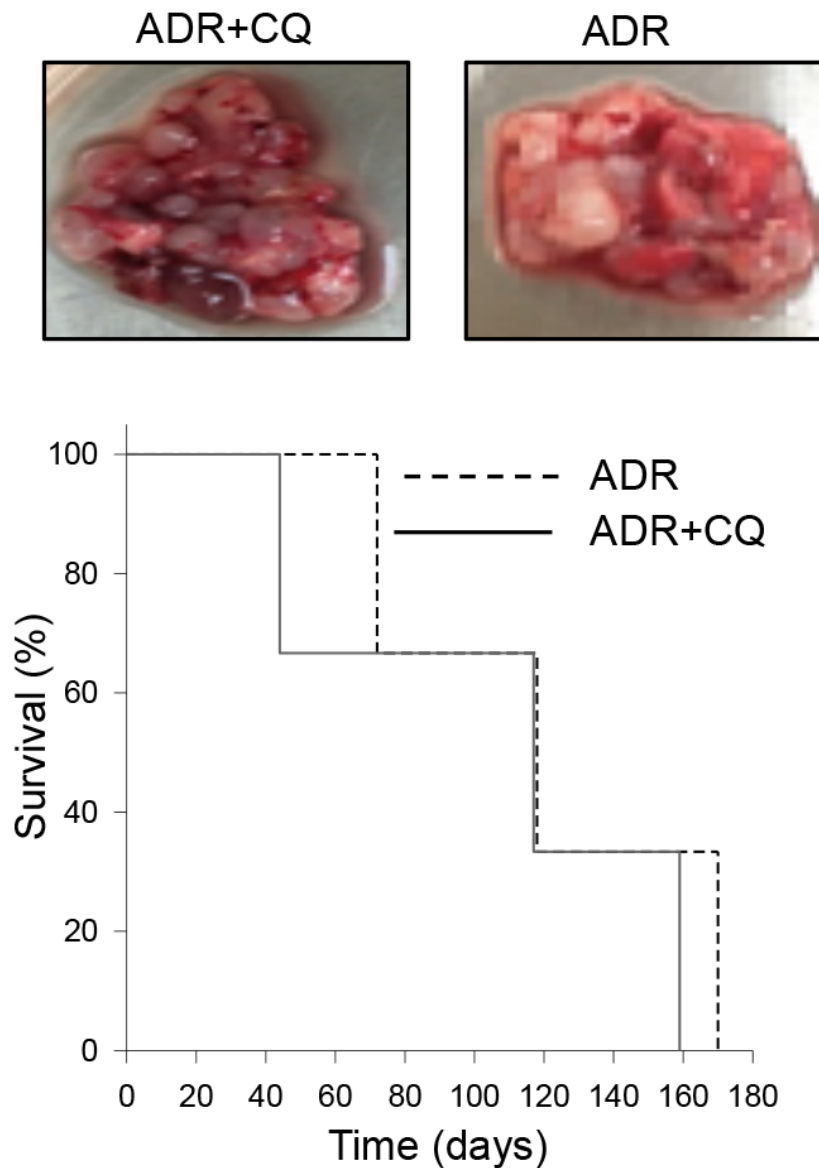
SUPPLEMENTARY MATERIALS



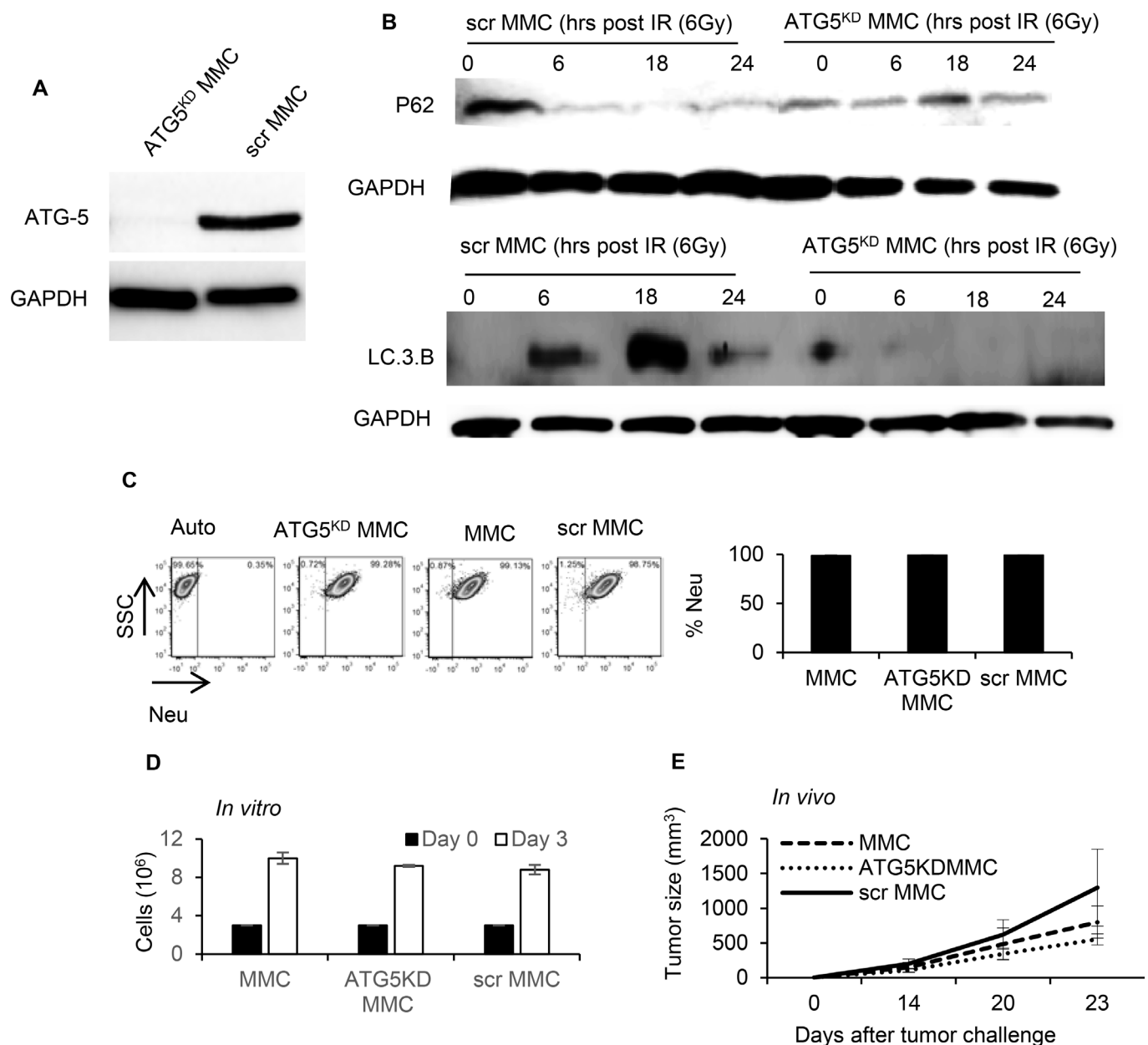
Supplementary Figure 1: ADR-induced dormant tumor cells produce floater apoptotic cells, *in vitro*. MMC tumor cells (3×10^6 cells/flask) were treated with 3 daily doses of ADR (1 μ M for 2 hrs), with one group receiving CQ (10 μ M) 3 hrs prior to and during ADR treatment. Both groups remained untreated for 3 weeks and 6 weeks, *in vitro*. (A) Floater cells were collected whenever culture medium was replaced and cell number and viability was assessed via trypan blue exclusion. Data represent 3 independent experiments and mean $\pm$ SEM. (B) Floater cells were cultured separately for 2–3 days each time they were collected, and assessed for viability 2–4 days later by using trypan blue staining.



Supplementary Figure 2: A transient blockade of autophagy by CQ did not change the susceptibility of MMC to ADR treatment. MMC tumor cells were treated with ADR alone (1 uM ADR for 2 hrs) (ADR) or in the presence of CQ (10 uM 3 hrs before ADR and 2 hrs during ADR treatment) (ADR+CQ). Tumor cells were analyzed by Annexin v/PI staining prior to treatment (Day 0) or three days after the treatment (Day 4). Experiments were performed in triplicates.



Supplementary Figure 3: A transient blockade of autophagy by CQ during ADR treatment fails to maintain tumor dormancy, *in vivo*. FVBN202 mice ($n = 3/\text{group}$) were challenged with MMC (i.v. injection of 1 million viable cells), and three days after tumor challenge animals were split into two groups: one group received 3 weekly treatments of ADR (i.v., 9 mg/kg), and another group received 3 weekly treatment of ADR + 60 mg/kg CQ (i.p.). Animals were sacrificed when they became moribund. Figure shows Kaplan-Meier survival curve and tumors in the lung.



Supplementary Figure 4: ATG5 knockdown tumor cells and wild type MMC show a similar pattern of growth. MMC cells were stably transfected with lentivirus expressing shRNA against ATG5 to establish autophagy-deficient cells (ATG5^{KD} MMC). Control MMC (MMC) were stably transfected with scrambled control vector as autophagy-competent cells (scr MMC). (A) Cell lysates were collected and used for immunoblotting against ATG5. (B) ATG5^{KD} MMC and scr MMC were treated with IR (6G) and cells lysates were collected at 6, 18, 24 hrs post treatment. Autophagy was determined by degradation of p62 and accumulation of LC3.B (C) Expression of Neu protein was determined on autophagy-competent control MMC (MMC or scr MMC) and autophagy-deficient MMC (ATG5^{KD} MMC) using FACS analyses. (D) Tumor cell proliferation was determined in a 3-day culture using trypan blue exclusion. (E) FVB/N202 mice ($n = 3$) were inoculated with autophagy-competent MMC (MMC or scr MMC) or autophagy-deficient MMC (ATG5^{KD} MMC) (3×10^6 cell/mouse, s.c. inoculation), and tumor growth was monitored by using a digital caliper. Data represents triplicate experiments.

REVIEW

IFN- γ orchestrates tumor elimination, tumor dormancy, tumor escape, and progression

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Abstract

Tumor immunoediting consisting of three phases of elimination, equilibrium or dormancy, and escape has been supported by preclinical and clinical data. A comprehensive understanding of the molecular mechanisms by which antitumor immune responses regulate these three phases are important for developing highly tailored immunotherapeutics that can control cancer. To this end, IFN- γ produced by Th1 cells, cytotoxic T cells, NK cells, and NKT cells is a pleiotropic cytokine that is involved in all three phases of tumor immunoediting, as well as during inflammation-mediated tumorigenesis processes. This essay presents a review of literature and suggests that overcoming tumor escape is feasible by driving tumor cells into a state of quiescent but not indolent dormancy in order for IFN- γ -producing tumor-specific T cells to prevent tumor relapse.

KEYWORDS

IFN- γ , immunotherapy, tumor dormancy, tumor immunoediting

1 | INTRODUCTION

Tumors display high levels of heterogeneity because of genetic instability, a characteristic of malignancy.¹ This results in a multitude of responses of tumor to the host immune responses or immunotherapeutics such that some tumor clones undergo apoptosis while other clones lay dormant and may later escape from the immune response and lead to distant metastasis. Antitumor immune responses utilize four major pathways to fight the tumor. Firstly, activated lymphocytes produce perforin to poke a hole in the extracellular membrane of target tumor cells as well as granzyme B to enter tumor cells and cleave caspases for the induction of apoptosis.² Secondly, they also express Fas-L to engage with Fas receptor on tumor cells and induce apoptosis.² Thirdly, they produce TNF-related apoptosis-inducing ligand (TRAIL) to engage with TRAIL receptors on tumor cells and in turn induce tumor cell apoptosis.³ Finally, activated lymphocytes produce IFN- γ , which is a pleiotropic cytokine with a wide range of activities; IFN- γ simultaneously induces apoptosis, tumor dormancy, and immunoediting in tumor cells that could lead to tumor relapse and progression.^{4–8} Paradoxically, chronic exposure of cells to IFN- γ facilitates the devel-

opment of hepatocellular carcinoma (HCC),⁹ colorectal carcinoma,¹⁰ and papilloma.¹¹ Therefore, understanding the distinct mechanisms by which IFN- γ affects the tumor could lead to the development of highly tailored immunotherapeutics that could control the tumor without inducing tumor escape and relapse. IFN- γ is primarily produced by T cells, NK cells, and NKT cells. The receptor for IFN- γ is composed of two subunits, which include IFN- γ receptor alpha (IFN- γ R α) and IFN- γ receptor beta (IFN- γ R β). Binding of IFN- γ to its cell surface receptor IFN- γ R α induces dimerization of IFN- γ R α , thereby forming a site for the assembly with IFN- γ R β . Upon heterodimerization of IFN- γ R α /IFN- γ R β , their intracellular janus family kinases, JAK1 and JAK2, respectively, dimerize and become phosphorylated. This phosphorylation creates binding sites for the signal transducer and activator of transcription (STAT) proteins, primarily STAT1.¹² Phosphorylated STAT1 homodimers are then translocated into the nucleus to bind the IFN regulatory factor-1 (IRF-1) gene gamma-activated sequence sites on the promoters of downstream target genes.¹³ This, in turn, activates diverse pathways in different tumor clones.

2 | IFN- γ INDUCES APOPTOSIS IN TUMOR CELLS

IFN- γ exerts its tumor killing function directly by the induction of apoptosis or by facilitating nonapoptotic cell death, as well as indirectly

Abbreviations: BCL, B-cell lymphoma; DR5, death receptor 5; HCC, hepatocellular carcinoma; IFN- γ R α , IFN- γ receptor alpha; IFN- γ R β , IFN- γ receptor beta; ROS, reactive oxygen species; RNI, reactive nitrogen intermediates; STAT, signal transducer and activator of transcription; TRAIL, TNF-related apoptosis-inducing ligand; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis

by rendering tumor cells susceptible to apoptosis-inducing function of the immune response or chemotherapies. For instance, IFN- γ induces IRF1, a tumor suppressor gene, which in turn reduces B-cell lymphoma 2 (BCL2) and increases Bak. These events facilitate the release of cytochrome c from mitochondria and activation of caspases, resulting in apoptosis.¹⁴ Reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) at low concentrations are associated with cell proliferation. However, tumor cells that produce high amounts of RNI and ROS in response to IFN- γ tend to undergo apoptosis.¹⁵ IFN- γ can also induce nonapoptotic cell death through the induction of autophagy in human HCC.¹⁶ IFN- γ -induced activation of STAT1 enhances the expression of the death receptor FAS and its ligand FAS-L in hepatoma and colon adenocarcinoma cells,¹⁷ and of TRAIL and its receptor death receptor 5 (DR5) in human tumor cell lines.^{18–20} Accordingly, activated STAT1 sensitizes tumor cells to FAS or TRAIL-mediated apoptosis. Also, activation of STAT1 by IFN- γ inhibits the expression of the p53 inhibitor murine double minute 2, thereby enhancing p53-induced apoptosis by doxorubicin and cisplatin.²¹

3 | IFN- γ ARRESTS CANCER GROWTH BY DRIVING TUMOR CELLS INTO A STATE OF DORMANCY

Although the IFN- γ /STAT1 pathway induces tumor cell apoptosis, activation of STAT1 can also result in the inhibition of tumor cell growth and establishment of dormancy. In melanoma, activation of the IFN- γ /STAT1 pathway results in the down-regulation of cyclin E and cyclin A with consequent tumor cell dormancy.²² Activated STAT1 can also interact with cyclins D1, D2, D3, and CDK4 and results in cell cycle arrest in fibrosarcoma cells.²³ Tumor inhibitory function of IFN- γ -induced STAT1 activation is also mediated by the up-regulation of the miRNA-29 family and a consequent down-regulation of CDK6 in melanoma cells.²⁴ IFN- γ -mediated tumor dormancy can also be induced independent from STAT1 signaling. Tumor clones that highly express indolamine 2,3-dioxygenase 1 and kynurenine-aryl hydrocarbon receptor respond to IFN- γ by upregulating the cell cycle inhibitor p27, consequently preventing STAT1 signaling and inducing tumor dormancy.²⁵ In fact, p21 and p27 facilitate hypophosphorylation of the tumor suppressor Rb, thereby suppressing the activity of E2F transcription factor and inhibiting the activation of genes involved in cell proliferation. In a T-antigenTag-induced multistage carcinogenesis in pancreatic islets, IFN- γ -producing CD4⁺ T cells inhibit tumor cell proliferation and establish tumor dormancy without destroying malignant cells.²⁶ It was also reported that CD8⁺ T cells maintain murine BCL1 in the state of dormancy by producing IFN- γ .⁶ Radiation-induced tumor dormancy is also mediated by the production of IFN- γ in BALB/c neu transgenic mice such that neutralization of IFN- γ reversed radiation-induced tumor dormancy and resulted in tumor relapse.²⁷ It has been demonstrated that levels of the expression of IFN- γ R α on mammary tumor cells determine whether IFN- γ eliminates the tumor or establishes tumor dormancy. While low expression of IFN- γ R α in tumor cells results in tumor dormancy, high levels of IFN- γ R α expression result in tumor elimination in the presence of IFN- γ -producing neu-specific

CD8⁺ T cell responses in FVB mice.⁷ Given that STAT1 activation by IFN- γ results in the up-regulation of MHC class I molecules, which present antigens to T cells,²⁸ dormant tumor cells could become more susceptible to the immune surveillance.

4 | IFN- γ EDITS TUMOR CELLS AND FACILITATES TUMOR ESCAPE AND RELAPSE

In addition to apoptosis-inducing and tumor inhibitory functions, IFN- γ can also induce aberrant DNA methylation^{29,30} or genetic alteration in tumor cells,⁴ resulting in tumor progression and relapse. IFN- γ -induced tumor immunoediting is mediated through several mechanisms, which include the induction of tumor antigen loss,^{30–34} up-regulation of PD-L1 in tumor cells,³⁵ recruitment of myeloid-derived suppressor cells, and tumor-associated macrophages to the tumor site.^{36,37} IFN- γ -induced HER2/neu loss has been reported in FVBN202 transgenic mouse model of breast cancer,³⁰ and in patients with HER2/neu positive ductal carcinoma in situ or breast cancer.^{32–34} Activation of STAT1 by IFN- γ results in the induction of the immune checkpoint protein PD-L1 in tumor cells.³⁸ In addition, chronic IFN- γ signaling in tumor cells increases resistance to immune checkpoint blockade through STAT1-related epigenetic and transcriptomic alterations, rendering melanoma resistant to radiation therapy and immune checkpoint inhibitors.³⁹ It was suggested that the genomic instability induced by IFN- γ during tumor progression is due to adaptation of the tumor to an immunologically hostile microenvironment.⁴ This phenomenon has been predicted by the adaptation model of immunity.^{40,41} Recent studies suggested that the state of tumor dormancy could determine whether IFN- γ may keep dormant cells in check or may edit dormant tumor cells and result in tumor relapse. Specifically, Ki67^{low} indolent tumor cells are susceptible to immunoediting and escaping from immunotherapy, whereas Ki67[–] quiescent dormant cells fail to undergo immunoediting and thus remain dormant by IFN- γ -producing T cells.⁸ Quiescent dormancy is due to lack of tumor cell proliferation and tumor cell arrest in G₀ phase, whereas indolent dormancy is due to a balance between tumor cell apoptosis and proliferation. As genetic and epigenetic changes take place during cell division, indolent cells remain susceptible to immunoediting and escape from immunotherapy. We have reported that IFN- γ induces the expression of PD-L1 on Ki67^{low} indolent, but not on Ki67[–] quiescent dormant cells.⁸ The detection of circulating tumor cells in breast cancer survivors even after 22 years of mastectomy without clinical evidence of disease⁴² suggests the existence and maintenance of tumor dormancy in cancer survivors.

5 | CHRONIC EXPOSURE TO IFN- γ FACILITATES TUMORIGENESIS

Although IFN- γ is known for its antitumor function during antitumor immune responses, chronic exposure of normal cells to IFN- γ can also facilitate malignant transformation. In fact, IFN- γ appears to be

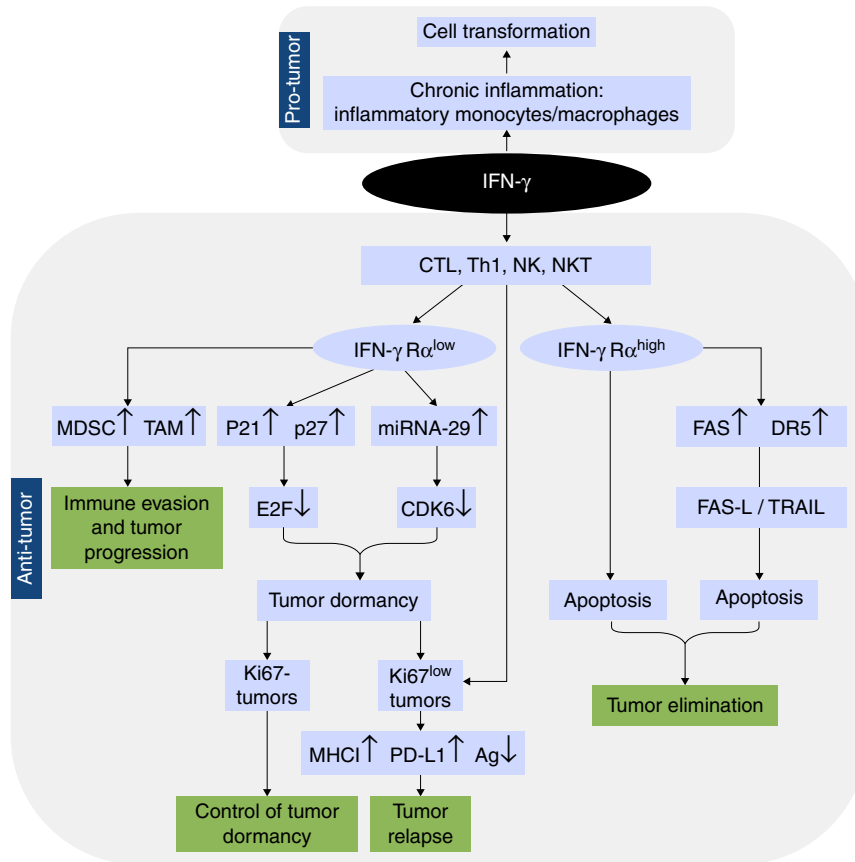


FIGURE 1 Multifaceted role of IFN- γ in cancer. Pro-tumor function of IFN- γ is mediated by chronic inflammation involving inflammatory monocytes and macrophages. Antitumor function of IFN- γ is mediated by cells of the adaptive immune system (CTL and Th1), NK cells, and NKT cells. The outcome of antitumor immune responses is determined by the status of the expression of IFN- γ R α on target cells such that high levels of IFN- γ R α render the tumor susceptible to apoptosis, while low levels of IFN- γ R α could result in tumor immunoediting and relapse or maintenance of immunogenic tumor dormancy depending on the type of tumor dormancy being Ki67[−] quiescent or Ki67^{low} indolent, respectively

protumorigenic early during cell transformation, whereas it manifests antitumor function against established tumors. For instance, IFN- γ has been reported to be involved in the initiation stage, but not in the promotion stage, of diethylnitrosamine-induced HCC due to its inflammatory function.⁹ Suppressors of cytokine signaling-1 (SOCS1) deficient mice are not able to inhibit IFN- γ inflammatory signaling. These mice develop spontaneous colorectal carcinoma because of the IFN- γ -induced hyperactivation of STAT1, which results in the induction of carcinogenesis-related enzymes, cyclooxygenase-2, and inducible nitric oxide synthase.¹⁰ In the 12-O-tetradecanoylphorbol-13-acetate-induced papilloma model, IFN- γ is involved in the development of papilloma by enhancing a Th17-associated inflammatory response.¹¹ IFN- γ -producing macrophages were detected in 70% of human melanomas.⁴³ To this end, UV-induced cutaneous malignant melanoma can be abolished by systemic blockade of IFN- γ .⁴³ Nonalcoholic fatty liver disease (NAFLD) is also associated with the dominance of M1 macrophages that produce inflammatory cytokines, including IFN- γ .^{44,45} In fact, IFN- γ -induced protein 10 is elevated in patients with progressive NAFLD.⁴⁶ Dietary saturated fatty acids are major contributors to NAFLD through the activation of NF- κ B, which is a key transcription factor for M1 macrophage activation.^{44,47} This, in turn, leads to inflammation-induced liver damage in nonalcoholic

steatohepatitis (NASH) disease⁴⁵ and consequent progression to HCC.^{48,49} Even in the absence of NF- κ B signaling, IFN- γ -producing NKT cells actively participate in the pathogenesis of NASH disease.⁵⁰ Also, a higher frequency of IFN- γ -producing Th1 cells is evident as NAFLD progresses to NASH disease.⁵¹

6 | CONCLUDING REMARKS

IFN- γ is a pleiotropic cytokine that could manifest opposing effects on host cells ranging from cell transformation in the context of chronic inflammation, monocytes/macrophages, to antitumor effects, cytotoxic T cells (CTL), Th1, NK, NKT cells, during the immune response (Fig. 1). The antitumor function of IFN- γ also varies depending on heterogeneity of the tumor cells and tumor microenvironment. IFN- γ can induce tumor cell apoptosis, directly or indirectly by upregulating the expression of FAS and DR5 on tumor cells. This cytokine can also induce cell cycle arrest and establish tumor cell dormancy. A dual function of IFN- γ appears to be due to low expression of IFN- γ R α in tumor cells. Depending on the type of tumor dormancy, IFN- γ -producing T cells can maintain tumor dormancy or result in tumor escape and relapse. In fact, IFN- γ could induce tumor immunoediting

in indolent dormant cells (Ki67^{low}), whereas it maintains quiescent dormant cells (Ki67⁻) in the state of dormancy without clinical evidence of disease. To this end, CD8⁺ T cells, Th1 cells, NK cells, NKT cells could be involved in the process of tumor immunoediting. Therefore, we suggest that establishment of quiescent tumor dormancy in residual disease by novel therapeutics may render dormant cells highly responsive to immunotherapy without risk of recurrence.

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DISCLOSURES

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Department of Defense. The authors declare no conflicts of interest.

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A Theoretical Basis for the Efficacy of Cancer Immunotherapy and Immunogenic Tumor Dormancy: The Adaptation Model of Immunity

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Abstract

In the past decades, a variety of strategies have been explored to cure cancer by means of immunotherapy, which is less toxic compared with chemotherapy or radiation therapy, and could establish memory for long-lasting protection against tumor recurrence. These endeavors have been successful in offering therapeutic antibodies, vaccines, or cellular immunotherapies, which resulted in prolonging survival of some cancer patients; however, complete cures have not been consistently achieved. The conception, design,

and implementation of these promising immunotherapeutic strategies have been influenced by two schools of thought in immunology, which include the “self–nonself” (SNS) model and the “danger” model. Further progress in cancer immunotherapy to achieve consistent cancer cures requires an evolution in our understanding of how the immune system works. The purpose of this review is to revisit premises and limitations of the SNS and danger models based on the outcomes of cancer immunotherapies by suggesting that both models are two sides of the same coin describing how the immune response is induced against cancer. However, neither explains how the immune response succeeds or fails in eliminating the tumor. To this end, the adaptation model has been proposed to explain efficacy of the immune response for achieving cancer cure.



1. INTRODUCTION

The “self–nonself” (SNS) model (Janeway, 1992) and the “danger” model (Matzinger, 2002) of immunity appear to be on opposite sides of thought in describing how the immune system functions. However, growing evidence suggests that both concepts are complementary when it comes to describing how an immune response is induced against cancer rather than how it succeeds or fails to eliminate cancer. For an antitumor immune response, T cells must receive two signals. Signal I is provided by the presentation of tumor antigens to T cells in the context of major histocompatibility complex/T cell receptor (MHC/TcR) interaction, and signal II is provided by T helper cells (Bretscher & Cohn, 1970) or costimulatory molecules such as B7.1/B7.2–CD28 (Janeway, 1992). Although the original SNS model (Bretscher & Cohn, 1970) does not have an explanation for signal II, an evolved version of the SNS model suggests that signal II is also induced by foreign proteins recognized by pathogen-associated molecular patterns (PAMPs) on the immune cells (Janeway, 1992). However, PAMPs such as toll-like receptors (TLR) also recognize self-proteins or endogenous ligands (Yu, Wang, & Chen, 2010). In some classifications, cytokine signaling during T cell activation or differentiation is considered as signal III; however, the proposed classification is that signals I and II are involved in T cell activation and differentiation. Therefore, both costimulatory molecules and cytokine signaling are considered as signal II. The SNS model solely emphasizes foreignness and focuses on the affinity of T cell receptor for the antigen. This model proposes that foreign antigens usually have a stronger affinity for T cell activation because self-antigen-educated T cells develop tolerance in the thymus. The danger model emphasizes on danger signals in response to any damage being harmful to the host and which induces signal II. The

Table 1 Three Signals During Antitumor Immune Responses

Models	Signals	Molecules	Function	Outcomes
SNS	Signal I	MHC-TcR	Antigen recognition	T cell activation and differentiation
Danger	Signal II	B7.1/B7.2-CD28	T cell activation	
		Cytokines	T cell differentiation	
Adaptation	Signal III	AR-AL	T cell function	Success or failure of the immune response

danger signals include damage-associated molecular pattern; PAMP could also be considered as danger signal because of being expressed on pathogens that are harmful to humans. Without signal II, signal I induces tolerance toward antigens. In fact, the danger model is the evolution of the SNS model by theorizing the entity of signal II in the induction of the immune response regardless of the self or nonself entity of signal I, the antigen. The evolutionary relationship between the SNS model and the danger model is similar to that of tumor immunosurveillance and tumor immunoediting theories (Dunn, Bruce, Ikeda, Old, & Schreiber, 2002). Vaccines have been designed based on the inspiration from the SNS model by including highly immunogenic antigens as signal I, and from the danger model by including adjuvants, regardless of the self or nonself entity of adjuvants, to induce signal II. To understand how an antitumor immune response succeeds or fails in eliminating the tumor, a signal III has to be involved. Signal III is a communication signaling that determines whether tumor cells die, proliferate, or become dormant following vaccination or immunotherapy (Table 1). The adaptation model proposes that this communication signaling has to be orchestrated through adaptation receptors (ARs) and adaptation ligands (ALs) that are distinct from costimulation (Manjili, 2014).



2. OUTCOME OF CANCER IMMUNOTHERAPIES INSPIRED BY THE SNS AND DANGER MODELS

2.1 Targeting Tumor-Associated Antigens or Tumor-Specific Antigens?

The SNS model suggests that the sequence or nature of tumor antigens determines the strength of an antitumor immune response. Whereas

tumor-associated antigens (TAAs) are thought to be weakly immunogenic, tumor-specific antigens (TSAs) are considered to be highly immunogenic. This assumption is based on the SNS model without empirical evidence demonstrating that immunotherapeutic targeting of TSAs or foreign-like antigens is more effective than that of targeting TAAs or self-antigens. Although targeting mutant neoantigens is a viable immunotherapeutic strategy supported by the SNS model, it is not more effective than targeting TAAs. To target TAAs or TSAs in a vaccine formulation, the danger model provides a conceptual framework emphasizing the use of an adjuvant in order to induce signal II (Gallucci, Lolkema, & Matzinger, 1999). The danger model suggests that the use of an effective adjuvant and continuous vaccination is important for antitumor efficacy of a vaccine (Gallucci et al., 1999; Matzinger, 2002). Immunotherapeutics that target TAAs have been approved by the FDA based on prolonging survival of patients with carcinomas when used in a therapeutic setting. For instance, prostatic acid phosphatase is a TAA being used in sipuleucel-T (Provenge) vaccine against asymptomatic or minimally symptomatic metastatic hormone refractory prostate cancer, and extended survival of patients by a median of 4.1 months (Kantoff et al., 2010). HER2/neu is another TAA being used as a target for antibody therapy of metastatic breast cancer. Addition of anti-HER2/neu antibody therapy to chemotherapy prolonged a median survival of 5.1 months (Slamon et al., 2001). Two FDA-approved HPV and EBV vaccines containing TSAs—nonself viral antigens—have been tested in prophylactic settings for the prevention of cervical cancer and liver cancer, respectively. Importantly, the efficacy of these vaccines has more to do with their use in prophylactic settings, rather than the nature of the antigen being foreign entity or an adjuvant being a strong inducer of danger signals.

2.2 Allogeneic Cancer Vaccines

To enhance immunogenicity of cancer vaccines, an allogeneic system has been designed and tested in a randomized phase III clinical trial using Canvaxin (Kelland, 2006). The vaccine consists of allogeneic, living whole melanoma cells, as a source of foreign antigens, and BCG as adjuvant. According to the SNS model, the inclusion of foreign antigens (Bretscher & Cohn, 1970) and a foreign adjuvant (Janeway, 1992) was expected to induce robust antitumor immune responses. However, the trial was discontinued prematurely because survival benefit was unlikely to be achieved (Kelland, 2006). Another allogeneic vaccine called GVAX (Cell Genesys, Inc.) consisting of allogeneic pancreatic cancer cell lines

transfected with a human *GM-CSF* gene as adjuvant. GVAX was tested in combination with CTLA4 blockade in patients with previously treated advanced pancreatic ductal adenocarcinoma and resulted in prolonging a median overall survival of only 5.7 vs 3.6 months for CTLA4 schedule alone (Le et al., 2013). However, no complete cures were achieved. It has been suggested that the inclusion of foreign helper epitopes should be sufficient to induce an effective antitumor CD8+ T cell response (Anderson, 2014) without overloading the immune system with foreign antigens. Despite an improved efficacy, this strategy did not provide a complete protection against the tumor in animal models (Snook, Magee, Schulz, & Waldman, 2014; Steinaa, Rasmussen, Rygaard, Mouritsen, & Gautam, 2007).

2.3 Allogeneic Stem Cell Transplantation

Allogeneic stem cell transplantation (SCT) is a promising immunotherapeutic approach for the treatment of patients with hematological malignancies. This strategy is based on the SNS model, proposing that donor T cells will recognize recipient tumor cells as nonself entities and attack them. The treatment has to be performed in the setting of donor recipient being matched in major histocompatibility antigens, HLA-A, -B, -C, DR, and ideally DQ. However, mismatch in minor histocompatibility antigens could induce an alloreactive immune response, which is often associated with graft vs host disease (GVHD). Allogeneic SCT is usually given along with irradiation or chemotherapy to the recipient, which could potentially function as adjuvant depending on the immunogenic nature of some chemotherapies or radiation therapies at certain doses. The danger model proposes that signal II is readily induced in organs such as the skin and the gut because these organs are exposed to the external world, commensals and pathogens, which cause damage and induce danger (Matzinger, 2012); this could act as adjuvant or danger for allogeneic SCT and result in GVHD in these organs. However, these alarming conditions also exist in recipients of autologous SCT without causing severe GVHD. What has been less appreciated is the role of conditioning regimens in disrupting homeostatic cellular adaptation that contributes to the development of tissue-specific GVHD (Manjili & Toor, 2014). Treatment for GVHD is also inspired by the SNS model, assuming that alloreactive T cells are responsible for GVHD; therefore, immunosuppressive drugs are given as GVHD prophylaxis or as therapeutic regimens, rendering patients susceptible to infections and increasing the risk of tumor relapse. The SNS model has not been able to offer an effective therapeutic strategy for GVHD without compromising

the patient immune response. The danger model suggests that the high frequency of GVHD in the gut, the skin, and the liver is because these organs are most in contact with commensals and pathogens producing danger signals. That is why allogeneic SCT fails to induce severe GVHD in germ-free animals (Matzinger, 2012). However, similar danger signals are present in the gut, the skin, and the liver following autologous SCT without causing a severe GVHD.

2.4 Neoantigen Cancer Vaccines and Engineered TcR

The next-generation cancer vaccines that have been conceived based on the SNS and danger models contain mutant neoantigens and adjuvant. The idea is based on the understanding that cancer cells usually undergo somatic mutations resulting in the expression of mutant antigens that can be considered as nonself, because they are not expressed during central tolerance. Mutant tumor antigens have been detected in cancer patients (Assadipour et al., 2017; Verdegaal et al., 2016), though they do not induce tumor rejection. Vaccination with defined neoantigens in combination with poly I:C adjuvant has shown some efficacy in mice when combined with immune-checkpoint inhibitors (Gubin et al., 2014). Thus far, no human data are available to confirm antitumor efficacy of neoantigen vaccines. Another immunotherapeutic strategy inspired by the SNS model is enhancing affinity of T cells for target antigens by means of engineered TcR. This strategy can be combined with targeting neoantigens. A combination of two strategies by targeting KRAS-mutant neoantigens and using T cells engineered to express TcR specific for the appropriate KRAS mutations was elegantly tested in mice (Wang et al., 2016). Adoptive transfer of the KRAS-mutant-specific transduced T cells significantly reduced pancreatic tumor growth in nonobese diabetic scid gamma mice, but the treatment did not eliminate the tumors (Wang et al., 2016). Such outcomes have been attributed to the neoantigen immunoediting by T cells, and it was suggested that induction of broad neoantigen-specific T cell responses should be used to avoid tumor resistance (Verdegaal et al., 2016).



3. BEYOND THE SNS AND DANGER MODELS: TUMOR ESCAPE AND IMMUNE EVASION

Immunotherapeutic strategies that have been inspired by the SNS and danger models have shown limited efficacy against cancer. Such outcomes have been attributed to tumor escape and immune evasion, which cannot be

directly explained by either the SNS or danger models. In fact, these models can explain the induction of the immune response rather than predicting its outcome. To overcome a single tumor antigen loss, multiple tumor antigens have been used and epigenetic modulators have been tested to induce the expression of a panel of cancer testis antigens (CTAs) so as to overcome a single antigen loss during immunotherapy. A randomized phase II clinical trial of multiepitope vaccine in patients with stage IV melanoma increased median overall survival by a few months (Slingluff et al., 2013). A combination of decitabine to induce CTAs and a vaccine targeting NY-ESO1 in ovarian cancer resulted in a partial response (Odunsi et al., 2014). In patients with stage IV melanoma, a combination vaccine comprised of six HLA-DR-restricted peptides increased median overall survival of 4.1 years compared with control arm (Hu, Kim, Blackwell, & Slingluff, 2015). Immune evasion mechanisms have also been targeted by various strategies. For instance, tumor-induced immunosuppressive cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) have been targeted in combination with immunotherapy, yet cancer cure has not been achieved. In patients with head and neck squamous cell carcinoma, tadalafil treatment significantly reduced both MDSCs and Tregs and increased tumor-specific immune responses, but no objective response was reported (Weed et al., 2015). In the 4T1 murine mammary tumor model, decitabine combined with adoptive immunotherapy (AIT) resulted in tumor inhibition and an increased rate of cure (Terracina et al., 2016), though its therapeutic efficacy against locally advanced tumor or established tumor metastasis has not been shown. In an animal model of HER2/neu-positive mammary carcinoma, depletion of MDSCs and induction of the expression of a panel of CTAs by decitabine, combined with AIT, resulted in prolonging survival of animals carrying metastatic breast cancer in the lung, although animals eventually succumbed to the tumor (Payne et al., 2016). In addition, targeting immune-checkpoint pathways of immune evasion by using anti-CTLA4 or anti-PD-1/PD-L1 antibody resulted in prolonging survival of cancer patients (Achkar & Tarhini, 2017), but again, a consistent and complete remission has yet to be achieved. Therefore, tackling several tumor escape pathways during immunotherapeutic regimens that were inspired by the SNS model or the danger model could improve the clinical outcome for cancer patients but could not consistently achieve a cancer cure. A continuous immunization, as suggested by Matzinger, may maintain anti-tumor immune responses, but it could not offer a cure for cancer because of tumor escape mechanisms.



4. DISCOVERY AND MODULATION OF TUMOR ADAPTATION RECEPTORS

The adaptation model of immunity was recently proposed to explain efficacy of the immune response during cancer, infectious diseases, allergy, and autoimmune diseases (Manjili, 2014). The model proposes a different theoretical perspective in tumor immunology and immunotherapy by suggesting that dysregulation of target tissues for the expression of ARs and ALs renders them susceptible or resistant to ongoing immune responses.

4.1 Central Tolerance and the Adaptation Model

Positive selection results in the maturation of CD4+ CD8+ T cells into a single-positive CD4+ or CD8+ T cells via MHC class II or MHC class I restriction, respectively. During positive selection, MHC/self-peptide complex (signal I) selects and supports survival of T cells that are self-reactive. However, the affinity of these T cells for self-antigens is low due to the nature of cortical thymic epithelial cells (cTECs) expressing wobbly or private peptides that bind MHC molecules weakly. The cTECs express $\beta 5t$ -containing thymoproteasomes, which inefficiently cleave substrates adjacent to hydrophobic amino acids of self-peptides, and as a result create wobbly binding of $\beta 5t$ -derived peptides with a faster TcR off-rate (Murata et al., 2007; Ziegler, Muller, Bockmann, & Uchanska-Ziegler, 2009). On the other hand, medullary TEC or DCs express $\beta 5i$ -containing immunoproteasomes, which are efficient in cleaving substrates adjacent to hydrophobic amino acids and create high-affinity MHC/self-peptides for all positively selected T cells. Therefore, similar peptides can have different affinities during positive and negative selections. Medullary DCs also express costimulatory molecules such as CD40, B7-1, and B7-2 (signal II) (Klein, Hinterberger, Wirnsberger, & Kyewski, 2009). Around two-thirds of medullary DCs are CD11c^{high} DCs, which contain CD8 α + thymic resident DCs, which are efficient in antigen cross-presentation, and CD8 α - migratory DCs (Li, Park, Foss, & Goldschneider, 2009). Medullary DCs express a wide array of tissue-specific antigens regulated by the autoimmune regulator (*AIRE*) gene as well as *AIRE*-independent mechanisms (Derbinski, Schulte, Kyewski, & Klein, 2001; Takaba et al., 2015). Negative selection is a mystery that has not been fully understood by the SNS or the danger model. A classical explanation is that T cells die because of the high affinity for antigens, while those with a low-affinity survive. This explanation raises some questions: (i) theoretically, all positively selected T cells recognizing

$\beta 5t$ -derived peptides should have a higher affinity for the $\beta 5i$ -derived peptides in the medulla, so why do some T cells die and some survive during negative selection? (ii) Why do high-affinity T cells die upon activation in the thymus, but they survive in the periphery? T cells that were matured from double-positive into single-positive cells in the cortex should function like alloreactive T cells after activation upon recognizing high-affinity antigens. T cell activation also takes place in the medulla in the absence of any danger signals; (iii) why do surviving T cells not get activated upon receiving signal I and signal II in the medulla, but they do get activated in the periphery? The $\beta 5i$ -containing immunoproteasomes in the medulla increase the affinity of self-peptides for surviving T cells, while they also receive signal II, yet they do not get activated. The adaptation model (Manjili, 2014) proposes that negatively selected T cells in the medulla express ARs and thus survive upon antigen recognition, whereas defective T cells that lack ARs will be eliminated upon antigen recognition; if these T cells escape from negative selection, they would die in the periphery upon activation. Therefore, the purpose of negative selection is to eliminate faulty T cells and select functional T cells that are able to survive upon activation. Autoreactive T cells could not be the otherwise deleted T cells because thymic emigration decreases in AIRE^{-/-} mice (Jin et al., 2017), suggesting that autoreactivity is not because of the escape of otherwise deleted T cells and their addition to the pool of surviving T cells. On the other hand, autoreactive T cells are perhaps those that do not die during negative selection in spite of recognizing MHC/self-antigens. In the periphery, upon engagement of ALs on DCs with ARs on T cells during activation, ARs transduce survival signals in T cells by inducing the expression of antiapoptotic proteins, such as cFLIP and Bcl-xL (Paulsen & Janssen, 2011). Lack of expression of ALs by APCs could also result in activation-induced cell death (AICD) in T cells. For instance, hepatic DCs induce apoptosis in T cells during activation, whereas splenic DCs support survival of activated T cells (Bertolino, Trescol-Biemont, & Rabourdin-Combe, 1998).

4.2 ARs and ALs: (i) The Endothelin Axis

Cancer patients often harbor preexisting antitumor immune responses that fail to protect the patients from cancer (Lu et al., 2012). Also, immunotherapy as a single agent often fails to eliminate the tumor. Similar observations were made in different diseases. For instance, healthy individuals and patients with multiple sclerosis (MS) harbor T cells that recognize myelin basic proteins (MBPs), but a pathogenic manifestation of the immune

response is evident only in MS patient (Martin, Whitaker, Rhame, Goodin, & McFarland, 1994). Similarly, preexisting anti-DNA autoantibodies were detected in healthy individuals and patients with lupus erythematosus with a pathogenic manifestation only in the latter (Martin et al., 1994). Th1 and Th17 inflammatory cells in the gut can protect the host from *Helicobacter pylori* infection without any toxicity to the tissue (Ding et al., 2013), but they become destructive during Crohn's disease. These paradoxical observations suggest that the immune response alone is not the primary factor in the pathogenesis of autoimmune diseases or inefficacy in cancer patients; rather, alterations in the expression of AR on the target cells could render them susceptible or resistant to the immune response. In fact, an altered gut microbiome profile is associated with Crohn's disease such that nutritional therapy can modulate pediatric Crohn's disease (de la Cruz-Merino et al., 2011), again suggesting that gut microbiome is an important factor in regulating the expression of ARs in the tissue. Tumor cells that arise from normal cells, perhaps, retain their ARs to survive immune surveillance. One candidate for the AR/AL is the endothelin axis, which includes the endothelin (ET) containing ET-1, ET-2, and ET-3 isoforms as ALs, and the ET receptor A (ET_A) as an AR. Activation of the ET_A AR by the ET-1 AL can lead to the induction of survival pathways, whereas activation of the ET_B, which antagonizes the ET_A, results in apoptosis (Nelson, Udan, Guruli, & Pflug, 2005). ETs are expressed by a variety of cell types including endothelial cells, macrophages, astrocytes, and neurons (Simonson, 1993). The ET_A receptor has a greater affinity for ET-1, and the ET_B receptor binds to all three ET isoforms equally (Arai, Hori, Aramori, Ohkubo, & Nakanishi, 1990). ET-1 is upregulated by astrocytes in a number of brain pathologies, including MS (D'haeseleer et al., 2013) and Alzheimer's disease (Palmer, Barker, Kehoe, & Love, 2012), as well as in rheumatoid arthritis (Haq, El-Ramahi, Al-Dalaan, & Al-Sedairy, 1999) and cancer (Wulfing et al., 2004). ET_B is upregulated in active MS lesions (Yuen et al., 2013), and ET-1 acts almost exclusively through ET_B, and not ET_A, on astrocytes to inhibit remyelination (Hammond et al., 2015). Therefore, it is reasonable to predict that alterations in the balance between the ET_A AR and its antagonist receptor, the ET_B, render the nervous system susceptible to anti-MBP immune responses. In humans, ET_A acts as an AR by inducing the expression of antiapoptotic genes in prostate cancer (Nelson et al., 2005). Its ligand, ET-1, acts as an AL and is produced by the prostate epithelia (Nelson et al., 2005). The ET-1/ET_A pathway is involved in the inhibition of apoptosis in melanocytes during UV irradiation (Swope & Abdel-Malek, 2016). In fact, a higher

responsiveness of melanoma patients to immunotherapy compared with patients with prostate cancer or ovarian cancer could be because the ET_A AR is upregulated in prostate and ovarian cancers but not in melanoma (Nelson, Bagnato, Battistini, & Nisen, 2003). The ET-1 AL is produced by the prostate epithelia (Nelson et al., 2005); in prostate cancer, not only a key component of ET-1 clearance, the ET_B receptor, is diminished (Nelson et al., 1996), but also the ET_A AR is upregulated (Nelson et al., 2003). These could make tumor-infiltrating T cells ineffective in patients with prostate cancer. Human DCs also produce ET-1 upon activation (Spirig et al., 2009), which in turn support survival of T cells during activation as well as tumor cells that express ET_A. ET-1 is also involved in the survival of activated T cells during autoimmune systemic sclerosis (Elisa et al., 2015). In rats, the ET-1/ET_A pathway is critical for thymocyte proliferation (Malendowicz, Brelinska, De Caro, Trejer, & Nussdorfer, 1998).

4.3 ARs and ALs: (ii) The PD-L1/PD-1 Checkpoint Pathway

The programmed cell death-1 (PD-1) receptor is expressed on activated T cells. Its ligands, PD-L1 and PD-L2, are commonly expressed on dendritic cells or macrophages. PD-L1 is a bidirectional membrane protein acting as a ligand to induce anergy in PD-1-positive T cells and acting as an AR to induce antiapoptotic genes in PD-L1-positive target cells (Azuma et al., 2008). Constitutive expression of PD-L1 in the immune-privileged sites such as cornea and retina protects them from GVHD following corneal allograft, despite infiltration of CD4+ T cells; however, blockade of PD-L1 accelerates allograft rejection (Hori et al., 2006). In a murine model, PD-L1 deficiency in pancreatic beta-cells triggers their destruction by CD8+ T cells (Rajasalu et al., 2010). An altered expression of PD-L1 correlates with not only autoimmune diseases but also cancer progression. For instance, PD-L1 loss was reported in children with systemic lupus erythematosus, and expression of PD-L1 is restored only during disease remission (Mozaffarian, Wiedeman, & Stevens, 2008). The expression of PD-L1 on activated T cells supports their survival such that PD-L1-deficient T cells express lower Bcl-xL, which is an antiapoptosis gene, than wild-type cells and are more sensitive to apoptosis in vivo (Pulko et al., 2011). Tumor cells exploit this pathway by the expression of PD-L1 in order to survive immune surveillance. Antitumor T cells can upregulate PD-L1 on tumor cells through the production of IFN- γ . For instance, upregulation of PD-L1 is only detected in tumor cells that are adjacent to IFN- γ -producing TILs in melanoma patients (Taube et al., 2012). Of note, tumor cells also

utilize IFN- γ -independent pathways for the expression of PD-L1 which involve *PTEN* (Parsa et al., 2007) or EGFR (Akabay et al., 2013). In phase I clinical trial, anti-PD-1 therapy showed cumulative response rates of 18%, 28%, and 27% among patients with non-small-cell lung cancer, melanoma, and renal cell carcinoma, respectively (Topalian et al., 2012). More recently, an objective response rate of 30%–40% in melanoma patients has been reported (Robert et al., 2015; Topalian et al., 2014). A high variety of response rates among different types of cancers to PD-1 immune-checkpoint inhibition therapy suggest the involvement of additional ARs that support tumor cell survival when the PD-L1 pathway is blocked. According to the adaptation model of immunity, anti-tumor efficacy of anti-PD-1/PD-L1 immunotherapy as a single agent is mainly due to the blockade of antiapoptotic gene expression downstream of PD-L1 on tumor cells. Therefore, the model predicts a higher efficacy of anti-PD-L1 therapy than anti-PD-1 therapy. In fact, some types of anti-PD-L1 antibodies can inhibit the interaction of not only PD-L1 and PD-1 but also PD-L1 and CD80 (Keir, Butte, Freeman, & Sharpe, 2008). On the other hand, blockade of PD-1 can rescue effector T cells from suppression, but the engagement of PD-L1 on tumor cells with CD80 on APCs can still induce survival signaling in tumor cells, facilitating resistance of tumor cells to antitumor effector T cells (Fig. 1). However, studies performed

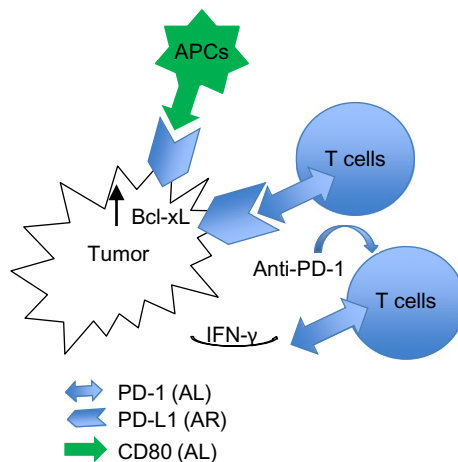
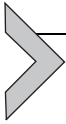


Fig. 1 PD-L1 acts as an AR on tumor cells. Anti-PD-1 could block PD-1/PD-L1 interaction and result in rescuing T cells from suppression. However, the engagement of CD80 on APCs with PD-L1 on tumor cells can upregulate the antiapoptotic gene Bcl-xL in tumor cells and support their survival in the presence of IFN- γ -producing antitumor T cells.

in the context of SNS model pay more attention to rescuing T cells from the suppression rather than blocking survival signaling in tumor cells following anti-PD-1/PD-L1 immunotherapy.



5. IMMUNOGENIC DORMANCY OF OCCULT TUMOR CELLS THROUGH ADAPTATION

An effective antitumor immune response, which is capable of inducing tumor regression, cannot guarantee elimination of tumor dormancy. In fact, immune responses induce the expression of an AR, PD-L1, on tumor cells through secretion of IFN- γ (Payne et al., 2016). IFN- γ is a dual-edged cytokine capable of inducing apoptosis and also facilitating tumor dormancy (Liu et al., 2017). Immunogenic tumor dormancy has been documented during unintentional transplantation of cancer into immunocompromised recipients from organ donors who were in clinical remission (Kauffman, McBride, & Delmonico, 2000) or with no clinical history of cancer (Myron Kauffman et al., 2002). Immunogenic tumor dormancy is defined by the expression of mutant antigens, increased MHC-I, cell membrane translocation of calreticulin, release of ATP, release of nonhistone chromatin-binding protein high-mobility group box 1, and secretion of immunostimulatory cytokines such as type I interferons (Michaud et al., 2011, 2014; Sistigu et al., 2014). A mechanism of immunogenic tumor dormancy was demonstrated in an animal model of methylcholanthrene-induced sarcoma (Koebel et al., 2007). Immunogenic dormancy is also evident in *Mycobacterium tuberculosis* infection keeping the infectious agent in dormant or latent state, thus protecting the host from active disease. Long latency before the appearance of AIDS is also evident in the presence of the immune response (Goonetilleke et al., 2009). HIV-infected CD4+ T cells express PD-L1 (Trabattoni et al., 2003), which could be kept dormant by HIV-specific PD-1^{low} CD8+ T cells during the latency period. Whereas PD-1^{high} effector T cells can be suppressed through PD-L1 engagement allowing tumor growth, the PD-1^{low} effector T cells could remain active and push PD-L1-positive tumor cells into the state of immunogenic dormancy by producing IFN- γ ; dormant tumor cells will remain in check by the immune response until they escape from dormancy. Thus far, two types of tumor dormancy have been reported, which include Ki67⁻ quiescent dormancy and Ki67^{low} indolent dormancy (Payne et al., 2016). Similar to actively proliferating tumor cells, the indolent, but not quiescent, dormant cells can evolve through immunoediting and escape from the

immune response. Recently, an elegant study by Dr. Restifo's group demonstrated that tumor necrosis releases an intracellular ion, potassium, into the extracellular fluid at the tumor site and results in the suppression of effector T cells. They showed that ionic reprogramming of tumor-specific T cells can improve their effector functions and prolong survival of melanoma-bearing mice (Eil et al., 2016). In clinical settings, targeting neoantigens by immunotherapy resulted in the stabilization of metastatic cholangiocarcinoma for 13 months, and then, disease progression was observed in the lungs (Tran et al., 2014). In a separate study, adoptive T cell therapy using a polyclonal CD8+ TIL recognizing mutant KRAS G12D in a patient with metastatic colorectal cancer resulted in the regression of lung metastatic lesions. However, one lesion escaped through loss of heterozygosity of the copy of chromosome 6 that encoded HLA-C*08:02 (Tran et al., 2015; Tran et al., 2016). Complete regression of neuro-expressing mammary carcinoma and subsequent relapse of antigen-negative tumor variant have been reported in a semiallogeneic model in which T cells and tumor cells were matched in major but not minor histocompatibility antigens (Kmieciak, Knutson, Dumur, & Manjili, 2007; Santisteban et al., 2009). Effectiveness of immunotherapy in some cancer patients but not others perhaps results from differences in the expression of ARs and/or ALs regulated by different oncogenes or epigenetic alterations. The adaptation model can also explain sterile chronic inflammation where the immune response to self-antigens is induced in the presence of signals I and II, but rather than destroying target organs, it initially inhibits cell growth because of the presence of ARs on target tissues, and eventually facilitates escape of natural malignant cells from dormancy (Manjili, 2017). Advances in our understanding of the AR/AL pathways are expected to lead to a breakthrough in immunotherapeutic treatment of cancer.

In summary, the adaptation model of immunity proposes that the status of ARs/ALs on tumor cells and T cells, respectively, determines the outcome of antitumor immune responses. There are four scenarios predicted by the adaptation model of immunity (Table 2). Tumor cells expressing ARs (ARs+) will receive survival signals from T cells by engaging with ALs on T cells (ALs+) and as a result become dormant as long as antitumor effector T cells are present. Other tumor-infiltrating cells such as myeloid cells could also express PD-1. Also, tumor cells expressing ALs will induce survival signals in effector T cells that express ARs (Scenario 1). Alterations in the expression of ARs/ALs on tumor cells could change the outcome, leading to the elimination of tumor cells that lack ARs (ARs-) by effector T cells (Scenarios 2). Tumor cells that do not express ALs fail to induce

Table 2 Outcomes of Antitumor Immune Responses

Scenarios	Tumor	Effector T Cells	Outcomes
1	AR +	AL +	Tumor dormancy
	AL +	AR +	T cell survival
2	AR–	AL +/–	Tumor elimination
	AL +	AR +	T cell survival
3	AR–	AL +/–	Tumor elimination
	AL–	AR +/–	T cells undergo AICD
4	AR +	AL +	Tumor escape and relapse
	AL–	AR–/+	T cells undergo AICD

survival signals in antitumor T cells, and these T cells will undergo AICD if they do not receive survival signals from stromal cells (Scenario 3). Finally, dormant tumor cells could escape from the immune response by down-regulating the expression of ALs on antitumor T cells (ALs–) and relapse (Scenario 4). Advances in our understanding and identification of ARs and ALs could lead to targeted therapies for epigenetic silencing of ARs on tumor cells, thereby rendering them vulnerable to immunotherapy.

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DISCLOSURES

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Immunotherapy of cancer: targeting cancer during active disease or during dormancy?

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Immunotherapeutic targeting of advanced stage cancers has prolonged the survival of cancer patients, yet its curative efficacy is limited due to tumor immunoediting and escape. On the other hand, human vaccines have been able to eradicate smallpox and control several other infectious diseases. The success has resulted from the administration of vaccines in prophylactic settings, or during latency periods in order to protect an individual during future exposure to the disease rather than curing an established disease. Therefore, administration of immunotherapy at the right time is the key to success. However, instead of focusing on the prevention of cancer, current cancer immunotherapies are often being used in a therapeutic setting with the goal of eliminating tumor cells. The present review of evidence related to cancer immunotherapeutics suggests that immunotherapeutic targeting of tumor dormancy could be more promising than targeting of advanced stage disease to achieve a cure for cancer.

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Current strategies for improving the efficacy of cancer immunotherapy rely on: strengthening antitumor immune responses by modulating tumor cells to become highly immunogenic and/or reprogramming of T cells to increase their affinity and avidity for tumor antigens as well as their sustainability in the host in order to improve humoral and cell-mediated immune responses, overcoming immune suppressive pathways by targeting Tregs and myeloid-derived suppressor cells (MDSCs), and overcoming immune tolerance by the blockade of the immune checkpoint pathways. These strategies, alone or in combination, have shown promising results against established cancers in some, but not all, patients. Very recently, attempts were made to identify and target mutated neoantigens in order to develop personalized immunotherapy, and thus, make it effective for all cancer patients. Here, we provide a review of literature highlighting the challenges that these strategies are facing. This review demonstrates that immunotherapeutic strategies that improve efficacy of tumor-reactive T cells, modulate the tumor-immune cells crosstalk or target some tumor escape mechanisms can at best prolong survival of cancer patients and cannot guarantee cancer cure. Based on recent observations that quiescent dormant tumor cells are not able to undergo immunoediting [1], we suggest that the immunotherapeutic targeting of tumor dormancy with the goal of maintaining tumor dormancy and preventing cancer recurrence, would be an effective strategy in containment or cure of cancer.

Immunotherapeutic targeting of advanced cancer prolongs patient survival but comes short from achieving cancer cure

Studies which demonstrated that the cellular arm of the immune system might be responsible for tissue rejection [2] led investigators to postulate the use of immune cells for the treatment of tumors. The first clinical study in humans demonstrating immune responses generated by tumor infiltrating lymphocytes (TIL) against autologous

tumors was published in 1987 [3]. TILs have been detected in the stroma of various cancers, and have been harnessed for adoptive cellular therapy (ACT). Conditioning the host environment by a nonmyeloablative (NMA) lymphodepleting regimen (cyclophosphamide and fludarabine) prior to ACT showed increased tumor responses [4]. In order to ascertain the degree of the effect of lymphodepletion, a pivotal follow-up study by Goff *et al.* randomized 51 patients to receive an NMA lymphodepleting regimen (cyclophosphamide and fludarabine) and 50 patients to receive an NMA regimen along with 1200 cGy of total body irradiation (TBI) prior to receiving autologous TIL. The results revealed that even though the objective response (OR) rate was higher in the NMA + 1200 cGy arm (62%) compared with the NMA arm alone (45%), both regimens had almost identical complete response (CR) rates of 24% [5]. In a prior study by the same group, the degree of lymphodepletion (chemotherapy alone) was noted to show increasing CRs of 12, 20 and 40%, respectively [5]. All these patients were previously heavily treated with other regimens for advanced melanoma (high-dose IL-2, anti-CTLA-4, anti-PD-1, a combination of anti-CTLA-4, anti-PD-1, IFN- α , dacarbazine, temizolamide, small molecule inhibitors and biochemotherapy). None of the prior treatment strategies were reflective of any correlation to observed tumor responses in either arm on subgroup analysis [5]. The duration of ongoing CR was 53.4 months as of the date of publication and one patient with CR recurred at 19 months. Even though these studies showed successful ACT and improvement in degree of tumor response with increasing lymphodepletion, this was not sustained in the partial responders and did not reach statistical significance. In a Phase II clinical trial, Chandran *et al.* [6] evaluated the effect of autologous CD8⁺ T cells clones against MART-1 or gp100 in patients with refractory metastatic melanoma. Fifteen patients treated with these highly avid clones resulted in immune-mediated targeting of skin melanocytes in 11 patients (73%) with minor transient tumor response by Response Evaluation Criteria In Solid Tumors criteria [7] but no OR in spite of successful clonal repopulation and engraftment in the host [6]. Multiple studies in both murine and human models have shown that younger the T cells are the higher the likelihood of antitumor efficacy is [8–10]. In a pilot study, 33 patients were treated with lymphodepleting chemotherapy alone followed by CD8⁺ enriched young TIL and 23 patients received lymphodepleting chemotherapy and 6Gy TBI followed by CD8⁺ enriched young TIL (longer telomeres, higher expression of CD27/28). Nineteen of the 33 patients (58%) showed OR by Response Evaluation Criteria In Solid Tumors criteria, including three CR (9%) and 16 partial responders (48%). In the arm receiving additional TBI, 11 out of 23 patients showed an OR (48%) including two patients with CR (9%), with CR similar to previous cohort receiving lymphodepleting chemotherapy alone. It was noticed that in comparison to prior standard TIL therapies, this study cohort that received younger TIL following transfer showed higher level of absolute lymphocyte count on reconstitution suggesting as increased capacity for *in-vivo* expansion for younger TIL compared with selected TIL previously described [11,12]. Analysis among subsets of memory T cells in different studies has indicated that central memory T (T_{CM}) cells are more efficient in antitumor activity in comparison to effector memory T (T_{EM}) cells [13–15]. Among CD8⁺ memory T cells, T memory stem cells (T_{SCM}) have been identified with even superior antitumor properties compared with other subsets of memory T cells [16].

Modulating the crosstalk between T cells & tumor cells improves the efficacy of cancer immunotherapy but could also induce tumor immunoediting & escape

According to the self-nonsel theory of immunity, tumors are often incapable of inducing an effective antitumor immune response because of the expression of self-antigens. Therefore, enhancing immunogenicity of tumor cells and increasing the affinity of T cells for the antigen are expected to modulate the crosstalk between tumor cells and T cells, thereby improving the efficacy of cancer immunotherapy. To test this hypothesis, Yu and colleagues used double-transgenic mice engineered to express both human T-cell receptor chains against gp100 antigenic peptides in T cells and human MHC-I domains in somatic cells. They demonstrated that a mutant gp100 peptide serving as a foreign-like antigen, induced a stronger immune response leading to tumor inhibition compared with a native peptide. However, a complete regression of the tumor was not achieved [17]. In clinical settings, targeting mutant peptides or neopeptides by means of adoptive T-cell therapy resulted in the stabilization of metastatic cholangiocarcinoma for 13 months, and then, disease progression was observed in the lungs [18]. In a separate study, adoptive T-cell therapy using a polyclonal CD8⁺ TIL recognizing mutant KRAS G12D in a patient with metastatic colorectal cancer resulted in the objective regression of all seven lung metastatic lesions. However, one lesion escaped through loss of heterozygosity of the copy of chromosome 6 that encoded HLA-C*08:02 [19,20]. We also observed complete regression of neu overexpressing mammary carcinoma in wild-type FVB mice in a T-cell-dependent manner recognizing the rat neu protein as a foreign protein. However, a fraction of animals experienced tumor recurrence due to neu antigen loss [21,22]. Similar observations were made in a preclinical

model of breast cancer, and in patients with multiple myeloma when tumor cells were epigenetically modulated by the administration of hypomethylating drugs in order to express cancer testis antigens (CTA) [1,23]. ACT by means of genetically engineered T cell receptor recognizing a cancer testes antigen NY-ESO in patients with either melanoma or synovial cell sarcoma, showed an OR of nine out of 17 patients (52%). Five patients with metastatic melanoma showed OR including two CR (on going at 22, 20 months as of the date of publication), and four out of six patients (66%) with synovial sarcoma showed OR though partial with one lasting 18 months [24]. In the FVBN202 transgenic mouse model of breast carcinoma, adoptive T-cell therapy combined with decitabine prolonged survival of animals bearing lung metastasis, but animals eventually succumbed to metastatic tumors due to tumor immunoediting characterized by the downregulation and loss of tumor antigens as well as upregulation of PD-L1 [1]. In patients with multiple myeloma, use of azacytidine resulted in the expression of CTA in tumor cells and the induction of CTA-reactive immune responses, leading to tumor regression following autologous stem cell transplantation [23]. However, some patients experienced tumor relapse associated with loss of CTA in their tumor cells (Payne *et al.*, Unpublished Data). To this end, modulation of the antigenic profile of tumors improved the efficacy of immunotherapy but was not able to overcome tumor immunoediting and escape from immunotherapy. Similar results were obtained using engineered T cells. Chimeric antigen receptor (CAR) T-cell therapy targeting CD19 resulted in complete remissions in some patients with relapsed/refractory acute lymphocytic leukemia (ALL) [25,26]. This therapy also induced CD19 loss, which is a limiting factor for its therapeutic efficacy. In two patients with refractory CD19⁺ ALL, CAR T-cell therapy led to a complete remission, which was sustained in one patient during a follow-up period of 9 months, and led to relapse of CD19 negative ALL after 1 month [27]. To overcome tumor escape, T cells were collected from patients whose tumors lost CD19, and modified to target CD22. Again, tumor relapse was evident as a result of CD22 downregulation or total loss [28]. It appears that IFN- γ produced by T cells is responsible for inducing tumor immunoediting [29,30]. Such tumor immunoediting has not been observed in adults with chronic lymphocytic leukemia [31]. This could be due to the state of dormancy in residual tumor cells since CAR therapy was used after the establishment of stable disease by using bendamustine with rituximab chemotherapies in adults with chronic lymphocytic leukemia. The study did not examine whether stable disease was in the state of cellular dormancy. We have recently reported that quiescent, but not indolent, dormant tumor cells are resistant to immunoediting [1].

Targeting tumor escape mechanisms: MDSCs, Tregs and immune checkpoints

Active solid tumors often induce and recruit MDSCs and/or Tregs, thereby inhibiting the efficacy of antitumor immune responses. A meta-analysis of eight studies that included 442 patients with solid tumors showed that MDSCs were associated with poor overall survival [32]. In patients with advanced non-small-cell lung cancer (NSCLC), multivariate analysis revealed an independent association of MDSCs with decreased progression free-survival and overall survival [33]. A meta-analysis of 18 published studies that included 8562 patients with breast cancer showed an association between Tregs infiltration and poorer prognosis [34]. Similar results were reported from patients with prostate cancer [35]. Analysis of the peripheral blood of 41 patients with prostate cancer and 36 healthy controls showed an increased frequency of MDSCs and Tregs in patients with prostate cancer associated with poor prognosis [35]. In addition, FOXP3 immunohistochemistry analysis of tissue microarray from 2002 prostate cancer patients revealed a higher number of intratumoral FOXP3⁺ Tregs associated with a more advanced tumor stage [36]. Although, control of MDSCs and Tregs restored antitumor immune responses, it did not produce a curative outcome in cancer patients. In order to target MDSCs and Tregs as well as to increase the efficacy of adoptively transferred TIL, conditioning regimens were used prior to ACT. Murine models and follow-up human studies demonstrated that use of lymphodepletion prior to cell transfer increased the effectiveness of ACT significantly [11]. Lymphodepleting regimens could increase the persistence of transferred T cells [4], deplete endogenous lymphocytes and myeloid cells containing Tregs [37], increase levels of homeostatic cytokines (IL-7 and IL15) as well as remove their sink as seen in both murine and human studies [38]; and finally, they enhance the efficacy of ACT by activating antigen presenting cells via stimulation of toll-like receptors resulting from translocation of commensal microflora across mucosal barriers [39]. Addition of the immune checkpoint inhibitors, however, produced OR in some patients. Use of the phosphodiesterase-5 inhibitor tadalafil has also been associated with depletion in MDSCs [40]. In patients with head and neck squamous cell carcinoma, tadalafil treatment significantly reduced both MDSCs and Tregs, and increased tumor-specific immune responses, though no OR was reported [41]. Therapeutic targeting of immune checkpoints pathways has found to be effective in producing objective clinical responses. The use of neoadjuvant anti-CTLA4, ipilimumab, in patients with regionally advanced melanoma resulted in elevated T-cell responses

against NY-ESO-1, MART-1 and gp100 antigens associated with decreased tumor infiltrating Tregs and MDSCs, and improved progression-free survival for 1 year [42]. Anti-PD1 and anti-PD-L1 immunotherapies have been highly effective for patients with NSCLC, bladder cancer, head and neck cancer and Merkel cell carcinoma. These immune checkpoint inhibitors are the only US FDA approved drugs for bladder cancer in the past 20 years [43].

Immunotherapeutic targeting of tumor dormancy

Four decades ago, Gray & Watkins published a comprehensive review article related to cancer immunotherapy in which they attributed spontaneous regression of neuroblastoma, hypernephroma, choriocarcinoma and melanoma as well as the existence of tumor dormancy to the host-immune system [44]. The notion that tumor dormancy is controlled by the immune system was further supported in six cases of NSCLC exhibiting strong delayed hypersensitivity reactions to the soluble tumor antigens following immunotherapy. These patients ended up with tumor recurrence after an immunosuppressive event or drug treatment [45]. It was also reported that immunization by means of irradiated tumor cells can establish and maintain tumor dormancy in a murine model of B-cell leukemia/lymphoma [46]. Antibody response [47,48] and IFN- γ producing CD8⁺ T cells [49] were found to be responsible for maintaining the murine B-cell lymphoma in a dormant state. In breast cancer patients, presence of tumor dormancy in the bone marrow was associated with an increase in CD8⁺ T memory cells that were reactive against HLA-A2/HER-2/neu(p369–377) tumor antigen [50]. Two FDA-approved monoclonal antibodies, trastuzumab and pertuzumab, targeting HER2/neu can also prolong tumor dormancy as evidenced by delaying tumor recurrence and increasing progression free survival and overall survival of patients with invasive breast cancer [51]. Similar observations were made in patients with prostate cancer. Approximately, 70% of patients with prostate cancer have disseminated dormant cells in the bone marrow [52]. Recently, TGF- β was reported to be involved in maintaining prostate cancer dormancy in the bone marrow [53]. It remains to be determined whether TGF- β producing Tregs may contribute to prostate cancer dormancy.

Recent reviews of literature on tumor dormancy and immune response suggest tumor dormancy as the best target for immunotherapeutic prevention of tumor recurrence and advanced disease prophylaxis [54–56]. This is because dormant tumor cells that have been established by chemotherapy or radiation therapy remain susceptible to immunotherapy [1]. A prospective, randomized, multicenter Phase II clinical trial evaluated the efficacy of GP2+GM-CSF vaccine in HLA-A2+, HER2⁺, node-positive and high-risk node-negative breast cancer patients. The vaccine was administered when patients were found to be disease-free, though might have harbored dormant tumor cells, in other words micrometastatic disease. This vaccination during tumor dormancy resulted in 5-year disease-free survival in 100% of HER2⁺ patients compared with 89% disease-free in control patients [57]. However, the caveat is that dormant tumor cells could undergo immunoediting and eventually escape and relapse. In particular, indolent dormant cells are susceptible to immunoediting and escape. High grade tumor clones that are susceptible to chemotherapy or radiation therapy could become dormant but low grade tumor clones that do not respond well to these treatments could establish micrometastatic minimal residual disease. While dormant cells contain Ki67^{low} indolent and Ki67[–] quiescent tumor cells [1], minimal residual disease is composed of indolent tumor cells, and more susceptible to immunoediting compared with dormant cells. In general, proliferating tumor cells either in the form of active disease or in the form of minimal residual disease or indolent dormancy are prone to immunoediting depending on the selective therapeutic pressure. Cancer therapeutics that could induce G0 cell cycle arrest could establish a quiescent type of tumor dormancy that is incapable of change and escape from therapy. It was reported that IFN- γ producing CD8⁺ T cells were responsible for establishing and maintaining tumor dormancy, as well as inducing tumor immunoediting and subsequent tumor recurrence [1,21,29]. A very recent report identified IFN- γ as a key cytokine responsible for tumor immunoediting [30]. To this end, we reported that Ki67[–] quiescent, but not Ki67^{low} indolent, dormant cells were resistant to immunoediting [1]. Therefore, the challenge in immunotherapeutic targeting of tumor dormancy is to dominate a quiescent type of tumor dormancy by means of conditioning regimens prior to immunotherapy in order to overcome tumor immunoediting and escape from immunotherapy. Alternatively, combination of targeted therapies with immunotherapy could inhibit certain immunoediting pathways in indolent dormant tumor cells. For instance, MYC inhibitors could prevent the expression of PD-L1 and CD47, because these immunoediting pathways are regulated by MYC [58]. Also, immunotherapeutic targeting of escape mechanisms such as PD-L1 or CTLA-4 expression could be overcome by immune checkpoint inhibitors [59]. The challenge is that tumor cells utilize numerous escape mechanisms; thus, some tumor clones could still escape from targeted therapies or immune checkpoint inhibitors.

Future perspective

Recently, there have been significant advances in the field of cancer immunotherapy. However, these advances have been limited to increasing patients' survival for a limited period of time when immunotherapeutics are administered in a therapeutic setting against advanced stage disease. For instance, T-cell-based therapies could produce CRs, yet they could not overcome tumor escape and recurrence in some patients. Similar observations were made in other immunotherapeutic approaches when targeting advanced stage diseases. For instance, Sipuleucel-T (Provenge) has extended survival of patients with metastatic prostate cancer by median 4.1 months [60]. The significance of immune checkpoint inhibitors is an increased survival tail in some patients with certain types of cancer, which has not been achieved by standard-of-care chemotherapies. Cumulative response rates for the anti-PD-1 antibody among patients with NSCLC, melanoma and renal cell cancer were 18, 28 and 27%, respectively. Responses were durable such that 20 of 31 responses lasted 1 year or more in patients with 1 year or more of follow-up [61]. To increase the size of survival tails, other checkpoint pathways should be identified and targeted; yet, immune checkpoint inhibitors cannot work for certain types of cancer that are weakly immunogenic to induce antitumor immune responses. To this end, immunogenic chemotherapies or radiation therapies should be considered to render all types of cancer responsive to immune checkpoint inhibitors. Alternatively, administration of immunotherapy including immune checkpoint inhibitors during tumor dormancy as a relapse prophylaxis regimen could be more effective, as prophylactic vaccines have been successful against many infectious diseases, as well as against HPV-associated cervical cancer [62]. In addition, application of stem cell transplantation and donor-derived lymphocyte infusion is successful only against minimal residual disease rather than against active and advanced stage disease. Therefore, it is reasonable to expect that the administration of immunotherapy during minimal residual disease or tumor dormancy could deliver a curative outcome.

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Commentary

Conditioning neoadjuvant therapies for improved immunotherapy of cancer



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ABSTRACT

Recent advances in the treatment of melanoma and non-small cell lung cancer (NSCLC) by combining conventional therapies with anti-PD1/PD-L1 immunotherapies, have renewed interests in immunotherapy of cancer. The emerging concept of conventional cancer therapies combined with immunotherapy differs from the classical concept in that it is not simply taking advantage of their additive anti-tumor effects, but it is to use certain therapeutic regimens to condition the tumor microenvironment for optimal response to immunotherapy. To this end, low dose immunogenic chemotherapies, epigenetic modulators and inhibitors of cell cycle progression are potential candidates for rendering tumors highly responsive to immunotherapy. Next generation immunotherapeutics are therefore predicted to be highly effective against cancer, when they are used following appropriate immune modulatory compounds or targeted delivery of tumor cell cycle inhibitors using nanotechnology.

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1. Introduction

Combinatorial cancer therapies such as chemo-immunotherapy, radio-immunotherapy, or targeted therapies combined with immunotherapy have been rationally designed to impinge on different pathways of tumor growth in order to achieve additive or synergistic anti-tumor effects. For instance, patients with HER2/neu overexpressing breast cancer receive chemotherapy and anti-HER2/neu antibody therapy using Trastuzumab and Pertuzumab. Chemotherapeutics such as doxorubicin increase free radicals that cause DNA damage, as well as intercalate into DNA and disrupt the DNA repairing function of topoisomerase-II [1]. Trastuzumab induces antibody dependent cellular cytotoxicity (ADCC), increases endocytotic destruction of the receptor, and inhibits shedding of the extracellular domain of HER2/neu [2] while Pertuzumab inhibits homo- and hetero-dimerization of HER2/neu, thereby blocking signalling pathways of tumor cell proliferation [3]. The caveat for such traditional chemo-immunotherapies is that standard dose chemotherapies are highly toxic to the host immune system and thus less effective for being simultaneously combined with immunotherapy (Table 1). Recent advances in our understanding of the mechanisms of action of low dose versus high dose chemotherapies are changing the concept of and approaches to chemo-immunotherapeutic design. Many studies demonstrated that certain chemotherapeutics at low doses induce immunogenic tumor cell death (ICD) and confer immune stimulatory effects. Therefore, the rationale for low dose chemotherapies is to condition tumor cells to become highly responsive to immunotherapies. A similar concept applies to the combined use of other cancer therapies, particularly those that induce cell cycle arrest, as conditioning regimens for an effective immunotherapy of cancer. The new chemo-immunotherapeutic approaches are predicted to make immunotherapies highly effective against cancer (Table 1).

2. Low dose metronomic (LDM) chemotherapy for an effective immunotherapy of cancer

Standard chemotherapy dosing regimens have traditionally used the maximum tolerated dose (MTD) of a drug administered with acceptable side effects as determined through clinical trials. In addition to targeting the malignant cells, the nonspecific cytotoxic drugs damage healthy cells with a high proliferation rate such as gastrointestinal mucosal and immune cells. Consequently, an extended time period is required between treatments in order to allow for tissue recovery. LDM chemotherapy is an alternative dosing regimen that is characterized by administering a cytotoxic drug at a low dose scheduled at a regular interval in order to minimize the drug-free time periods. Metronomic dosing schedules aim to achieve adequate disease control with less toxicity than MTD chemotherapy. The rationale for LDM is to not only inhibit tumor growth but also induce ICD and anti-tumor immune responses [4–7] to make patients highly responsive to immunotherapies. A LDM chemotherapy can control tumor progression in patients with early stage as well as those with advanced-stage cancers [8].

2.1. Non-immunogenic mechanisms of LDM chemotherapy

Proliferating malignant cells' oxygen requirements are met by forming inappropriate vascularization to the tumor. Tumor hypoxia results in the production and release of angiogenic cytokines, which leads to resistance to both antiangiogenic and chemotherapeutic regimens [9,10]. One of the earliest studies using low dose chemotherapy at regular intervals referred to the dosing regimen as antiangiogenic scheduling [11]. The study found that low dose cyclophosphamide given at regular schedule was able to kill cells that were resistant to a standard dose chemotherapy. The results have been reproducible [12,13], though the efficacy of low dose chemotherapy as a first line treatment for untreated cancers is yet to be determined. The tumor regression was attributed to sustained endothelial cell apoptosis that occurred due to the higher frequency dosing, which did not occur during the drug-free periods used in MTD chemotherapy. In fact, circulating endothelial cells are released from the bone marrow as an adaptive response to marrow suppression induced by MTD chemotherapy, allowing for damaged tumor cells to regenerate. In this aspect LDM chemotherapy has a unique mechanism in suppressing vasculogenesis by suppressing the source of vascular growth factors [14]. Promotion and maintenance of angiogenesis involves a balance of proangiogenic and antiangiogenic molecules acting within the tumor microenvironment. One of the earliest growth factors released from the tumor site in response to hypoxia is the transcriptional regulator, HIF-1 α . Doxorubicin at a LDM regimen has been reported to block this transcription factor, the inhibition of which has been shown to overcome resistance to antiangiogenic therapies and promote tumor regression [15,16]. LDM chemotherapy has been shown to decrease expression of proangiogenic molecules VEGF and VEGF receptor 2 [17] and increase the expression of the antiangiogenic thrombospondin 1 [18]. Taken together these data indicate that LDM chemotherapies suppress the tumor microenvironment's response to hypoxia by suppressing angiogenesis.

2.2. Immunogenic mechanisms of LDM chemotherapy

Certain chemotherapies at the MTD have been associated with immune stimulation through the induction of ICD. The term ICD was first introduced over a decade ago by Dr. Kroemer's group to indicate a functionally peculiar type of cell death induced by certain chemotherapeutics that can elicit an immune response against damage associated molecular patterns (DAMPs) in the absence of any adjuvant [19]. Inducers of ICD include doxorubicin, cyclophosphamide, epirubicin, idarubicin, mitoxantrone, bleomycin, bortezomib, 5-fluorouracil, paclitaxel and oxaliplatin [20,21]. On the other hand, some other chemotherapeutics such as cisplatin fail to induce ICD [22]. Animals challenged with doxorubicin-sensitized tumor cells were able to mount anti-tumor immune responses that protected them from re-challenge with tumor cells of the same type [19]. Recent studies demonstrated that the lack of ICD is correlated with poor prognosis for breast cancer patients

Table 1
Current concepts on combinatorial cancer immunotherapies.

Concept	Objective	Approach	Weakness	Strength
Traditional	To impinge on different pathways of tumor growth in order to achieve additive or synergistic anti-tumor effects	Adjuvant therapies at maximum tolerated doses	Toxicity Immune suppression	Tackle multiple drug resistant mechanisms
New	To condition the tumor microenvironment and make tumor cells highly responsive to immunotherapy	Low dose neoadjuvant conventional therapies and standard dose adjuvant immunotherapy	Tumor immunoediting and escape	Immune stimulatory Safe

[23], and ongoing clinical studies have identified some standard-of-care chemotherapeutics that induce ICD [20,24].

Molecular components of DAMPs that induce ICD following chemotherapy have been identified as: i) cell surface expression of endoplasmic reticulum (ER) chaperones such as calreticulin (CRT), ii) release of ATP, iii) release of non-histone chromatin-binding protein high mobility group box 1 (HMGB1), and iv) secretion of immunostimulatory cytokines such as type I interferons [25–27]. ICD is induced even prior to cell death such as during autophagy or senescence [26]. Such chemotherapy-induced ICD recruited dendritic cells (DCs) to the tumor site and activated them to take up dead-cell associated antigens. The activated DCs undergo maturation and present tumor antigens to T cells, resulting in the induction of tumor-specific immune responses [26]. CRT, HMGB1, and ATP interact with CD91, TLR-4, and purinergic P2RX7 receptors on DCs, respectively. These interactions, in turn, result in antigen uptake, antigen presentation and production of IL-1 β by DCs [22,28,29]. Any defects in the DAMP-sensing machinery, such as type I interferon receptor alpha and beta, CD91, TLR4 or P2RX7 could alter the immune response to chemotherapy-induced ICD. Other chaperones such as HSP70 and HSP90, as well as uric acid are also considered as markers of ICD that interact with CD91 or TLRs [30]. Unfortunately, standard chemotherapies are also known to compromise immune surveillance by killing proliferating effector T cells, and contribute to treatment resistance [31]. On the other hand, LDM chemotherapies can induce ICD and also confer additional immune stimulatory effects without the significant killing of anti-tumor T cells. This immune stimulatory function of LDM chemotherapies is important because tumor cells are able to unleash an immunosuppressive network of cells composed of M2-polarized macrophages, regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs) [32], which leads to tumor cell evasion by dampening anti-tumor immune responses. Breast cancer patients who were treated with low dose cyclophosphamide showed decreased Tregs and increased effector T cells as well as NK-cell-dependent anti-tumor immunity [4,5]. Cyclophosphamide also enhances Th-17 and Th1 immune responses, and expands NK-cell and DCs in multiple mouse tumor models [33,34]. Other immune stimulatory chemotherapies that deplete circulating or tumor-infiltrating Tregs and/or circulating MDSCs include 5-fluorouracil [35,36], gemcitabine [37,38], oxaliplatin [39], paclitaxel [40], and docetaxel [41], and decitabine [42]. In addition, oxaliplatin promotes anti-tumor function of macrophages and neutrophils [43]; paclitaxel induces maturation of DCs [44] and tumor infiltration of NK cells in breast cancer patients [45]. In fact, the anti-tumor efficacy of doxorubicin has been suggested to depend on the host immune system [46] such that depletion of T cells compromises anti-tumor efficacy of doxorubicin [47]. LDM chemotherapy also has been shown to be a suitable preparative regimen for vaccination approach in order to boost anti-tumor immune responses against dormant cells [7]. Similarly, whereas fractionated radiation therapy (RT) is immunogenic and generates abscopal responses in mice, single high-dose RT fails to do so [48]. This failure is because of the upregulation of three prime repair exonuclease 1 (TREX1) which in turn inhibits type I interferon secretion, an ICD signal, by irradiated tumor cells [49].

3. Epigenetic targeting of tumor cells for immune modulation against cancer

Spontaneous cancers arise in immunocompetent individuals with active immunoeediting mechanisms that make tumor cells weakly immunogenic [50]. Therefore, improving the immunogenicity of cancer is essential to improving cancer immunotherapy. Epigenetic modulators such as azacytidine (Aza) and decitabine

(Dec) function as cytosine analogs, which lead to their incorporation into newly synthesized DNA strands during S phase of the cell cycle; these agents have been shown to enhance immunogenicity of tumor cells by inducing the expression of a panel of highly immunogenic cancer testis antigens (CTAs), and result in improved immunotherapy of cancer [51,52]. Both Aza and Dec also induce the expression of tumor suppressor gene p53 [53] and the death receptor Fas [54] on tumor cells. These functions are attributed to the capacity of these agents to mechanistically operate as potent DNA methyltransferase (DNMT) inhibitors through the formation of a covalent complex with a cysteine residue at the active site of DNMT1. This results in CpG island demethylation during cellular proliferation, which, in turn, results in hypomethylation within the promoter of tumor suppressor genes as well as highly immunogenic CTAs [55,56], leading to their enhanced transcription. Ultimately, the use of such epigenetic modulating agents renders tumor cells susceptible to CTA-reactive immune responses while potentially reducing the proliferative capacity of tumor cells by restoring p53 expression. In fact, aberrant CTA expression has been shown to elicit CTA-specific cytotoxic T cell responses in melanoma; treatment of CTA-expressing metastatic melanoma with autologous CTA-specific T cells has elicited long-term complete remission [57,58]. Dec in particular is an attractive therapeutic because it requires activation by deoxycytidine kinase (DCK), an enzyme preferentially expressed in tumor cells and myeloid cells. Therefore, it is expected to specifically kill tumor cells and MDSCs while leaving T and B cells unharmed. In addition, DCK has been found to be overexpressed in poor outcome breast cancer [59], suggesting that epigenetic therapy to induce CTA expression may prove to be an efficacious approach in breast cancer patients with poor prognosis. A low dose regimen of Dec was shown to render mouse mammary carcinoma highly susceptible to immunotherapy [52]. In colorectal cancer, Aza at a low dose increased type I interferon production within the tumor through the re-activation of endogenous retroviruses, and as a result enhanced anti-tumor immune responses [60]. In patients with solid tumors, low dose Dec increased TcR diversity, which is important for T cells to respond to antigenic diversity of tumor cells [61]. A low dose regimen of Dec was also reported to induce the expression of CD80 co-stimulatory molecule on tumor cells associated with enhanced anti-tumor immune responses [62]. Aza therapy in patients with relapsed or refractory Hodgkin lymphoma resulted in a complete response to immune checkpoint inhibitors [63]. We have also reported that the use of Aza combined with the immune modulatory lenalidomide induced the expression of CTAs within tumor cells, and generated CTA-specific immune responses in patients with multiple myeloma [64]. Similar results were observed in a mouse model of experimental metastatic breast cancer [65]. Therefore, Dec is an attractive candidate as a neoadjuvant immune modulator when combined with immunotherapy.

4. Control of cell cycle progression prior to immunotherapy

The dysregulation of the cell cycle is a classic hallmark of cancer growth and metastasis. Cyclin-dependent kinases (CDKs) are a family of multifunctional enzymes that can modify various protein substrates involved in cell cycle progression. All eukaryotic cells have multiple cyclins, which act during a specific stage of the cell cycle. Common cyclins include G0/G1-phase cyclins, G1/S-phase cyclins, S-phase cyclins, and M-phase cyclins. CDK4 and CDK6 are important for progression during the G₁ cell cycle phase [66], CDK2 is important for transition from G₁ to S-phase [67], and CDK1 is important during G₂ and M progression [68]. The inhibition of tumor cell cycle progression through CDK inhibitors has emerged as an attractive option for targeted cancer therapy. Three

specific CDK4/6 inhibitors palbociclib, abemaciclib and ribociclib have been successfully tested in patients with hormone receptor-positive HER2-negative breast cancer [69]. Palbociclib inhibits cell growth and DNA replication in a number of retinoblastoma (Rb) proficient human cancer cells, including breast cancer because over 70% of breast cancers are Rb proficient [70]. Palbociclib is a well-tolerated cancer therapeutic [71] that induces G0/G1 arrest in HER2/neu– and HER2/neu+ breast tumor cells [69,72] as well as in neu positive murine mammary tumor cells [73]. Palbociclib can be administered at a concentration of 150 mg/kg through oral gavage and given daily for 3–4 weeks either alone or after the completion of chemotherapy in order to further reduce tumor cell burden. Palbociclib and chemotherapies may not be used simultaneously, because G0/G1 arrest by Palbociclib could render tumor cells resistant to cytotoxic function of chemotherapy [74]. Palbociclib does not induce apoptosis in bone marrow hematopoietic cells, and its function as a cell cycle arresting agent is reversible upon its removal [75]. Unlike pan-CDK inhibitors, palbociclib is not toxic to T cells [76]; thus, it can be used with immunotherapeutics. Roscovitine is a selective CDK5 inhibitor that is able to induce the apoptosis of drug-resistant breast cancer cells [77]. A sequential use of chemotherapy and roscovitine can induce G2/M arrest and apoptosis in highly invasive triple negative breast cancers [78]. Roscovitine is not toxic for tumor-reactive T cells [79] and could sensitize breast cancer cells to immunotherapy by TRAIL-induced apoptosis [80]. Very recently, it was reported that IFN- γ -induced immunoediting via PDL-1 expression is a CDK5-dependent event; thus, roscovitine can suppress IFN- γ -induced expression of PD-L1 [81].

The rationale for use of CDK inhibitor is to push tumor cells towards dormancy so that the immune system can control tumor growth by inducing tumor cell death. It has been reported that dormant tumor cells, while become refractory to chemotherapy, remain susceptible to immunotherapy [65]. Therefore, the next generation immunotherapeutics are expected to be highly effective against cancer, when combined with immune modulatory compounds.

5. Targeted delivery of tumor cell inhibitors: nanotechnology

Although LDM chemotherapies were found to be immunogenic, they could still affect the normal cells because of their administration over an extended period of time. Therefore, tumor immune modulatory chemotherapies that induce ICD and increase the expression of MHC or other immune modulatory receptors such as Fas or CD80 on tumor cells would be more effective through targeted delivery than systemic administration of the drug. To this end, targeted delivery of cell cycle inhibitors prior to immunotherapy would be desirable. Folate (vitamin B9) receptor (FR) is an attractive target because of high level of expression on tumors of epithelial origin compared to normal tissue, including ovarian, breast, brain, lung and colorectal cancers [82]. Folate-conjugated nanoparticles that carry desirable drugs are internalized upon binding to FR and the acidic microenvironment inside tumor cells dissociates FR from the drug-carrying nanoparticles resulting in drug-induced apoptosis or inhibition of tumor cell proliferation. Cytotoxic drugs have been successfully delivered to tumor cells via targeting the FR and using nanoparticles as a potent drug carrier. In a breast tumor model, paclitaxel-loaded folate modified lipid-shell and polymer-core nanoparticles (FLPNPs) showed similar antitumor efficacy but lower toxicity compared to paclitaxel. The paclitaxel-loaded FLPNPs confer a higher tumor inhibitory effect than the nontargeted paclitaxel-loaded LPNPs [83]. Cases of successful oral delivery of Dec to abdominal tumors by means of nanostructured lipid carrier have been reported [84]. It was

demonstrated that nanoconjugated delivery of Dec to human glioblastoma cells could overcome chemo-resistance by rendering tumor cells susceptible to alkylating chemotherapy [85]. In humans, carbon nanoparticles have been successfully used to protect parathyroid glands in patients with thyroid cancer. Such a nanotechnology approach reduced incidence of hypoparathyroidism and hypocalcemia after surgical removal of thyroid tumor [86]. Safety and efficacy of SGT-53 liposomal nanoparticle delivery of p53 gene into refractory solid tumors in combination with chemotherapy have also been established [87]. Very recently, therapeutic nanoparticles plus trastuzumab with and without cyclophosphamide were successfully tested through enhanced permeability and retention in patients with HER2 positive metastatic breast cancer [88]. These trials suggest the feasibility of tumor-targeted nanoparticle drug delivery using cell cycle inhibitors as a conditioning regimen for immunotherapy of cancer. However, the feasibility and efficacy of such targeted delivery of tumor immune modulators in combination with immunotherapy remain to be investigated.

Conflict of interest

The authors declare no conflict of interest.

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Disclosures

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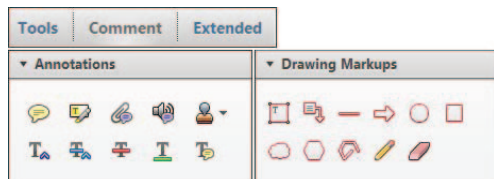
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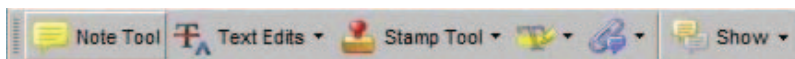
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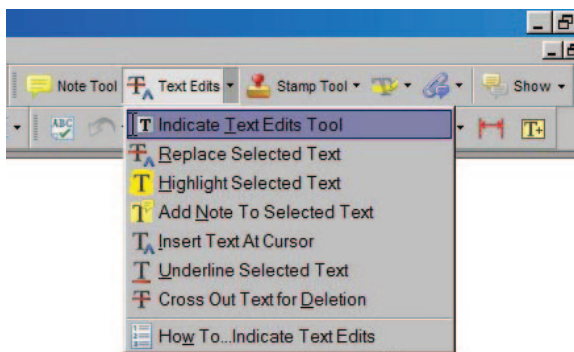
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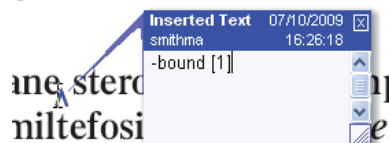
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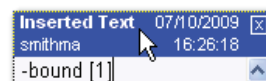
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REVIEW

Assessing the carcinogenic potential of low-dose exposures to chemical mixtures in the environment: the challenge ahead

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Abstract

Lifestyle factors are responsible for a considerable portion of cancer incidence worldwide, but credible estimates from the World Health Organization and the International Agency for Research on Cancer (IARC) suggest that the fraction of cancers attributable to toxic environmental exposures is between 7% and 19%. To explore the hypothesis that low-dose exposures to mixtures of chemicals in the environment may be combining to contribute to environmental carcinogenesis, we reviewed

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11 hallmark phenotypes of cancer, multiple priority target sites for disruption in each area and prototypical chemical disruptors for all targets; this included dose-response characterizations, evidence of low-dose effects and cross-hallmark effects for all targets and chemicals. In total, 85 examples of chemicals were reviewed for actions on key pathways/mechanisms related to carcinogenesis. Only 15% (13/85) were found to have evidence of a dose-response threshold, whereas 59% (50/85) exerted low-dose effects. No dose-response information was found for the remaining 26% (22/85). Our analysis suggests that the cumulative effects of individual (non-carcinogenic) chemicals acting on different pathways, and a variety of related systems, organs, tissues and cells could plausibly conspire to produce carcinogenic synergies. Additional basic research on carcinogenesis and research focused on low-dose effects of chemical mixtures needs to be rigorously pursued before the merits of this hypothesis can be further advanced. However, the structure of the World Health Organization International Programme on Chemical Safety 'Mode of Action' framework should be revisited as it has inherent weaknesses that are not fully aligned with our current understanding of cancer biology.

Abbreviations

AhR	aryl hydrocarbon receptor
BPA	bisphenol A
EPA	environmental protection agency
HTS	high-throughput screening
IARC	International Agency for Research on Cancer
IL	interleukin
LDE	low-dose effect
LOAEL	lowest-observed-adverse-effect level
LOEL	lowest observed effect level
miRNA	microRNAs
4-NP	nonylphenol
NF- κ B	nuclear factor- κ B
PBDE	polybrominated diphenyl ethers
PPAR	peroxisome proliferator-activated receptor
ROS	reactive oxygen species

Introduction

Cancer is a burden on humanity and among the leading causes of morbidity and mortality worldwide, with ~14 million new cases and 8.2 million cancer-related deaths in 2012 (1). In general, both genetic and environmental factors play a role in an individual's cancer susceptibility (2,3), so there has been a long-standing emphasis on avoidable 'lifestyle' factors (i.e. those that can be modified to reduce the incidence of the disease) and a parallel focus on exogenous chemical exposures (e.g. agricultural, occupational and so on) (4). But advances in our understanding of the complexity of cancer biology have resulted in serious critiques of current risk assessment practices related to exogenous exposures (5) along with calls for an expanded focus on research that will allow us to evaluate the (potentially carcinogenic) effects of *in-utero* exposures and low-level exposures to combinations of chemicals that occur throughout our lifetime (6,7).

The 2008–09 President's Cancer Panel Annual Report in the USA (8) opined that the 'true burden of environmentally induced cancer has been grossly underestimated' (7), whereas Parkin *et al.* (9) estimates in a British study that the fraction of cancer that can now be attributed to both lifestyle and environmental factors is only 43% (i.e. the underlying cause of 57% of all cancers is still unexplained). However, an expanded focus on research that will allow us to evaluate the (potentially carcinogenic) contribution of low-level exposures to combinations of chemicals that occur *in utero* and throughout our lifetime is not a trivial undertaking.

First of all, the number of chemicals to which we are exposed is substantial, and many have not been adequately tested.

Christiani (6) cited increased and persistently high incidence rates of various cancers and called on the National Institutes of Health to expand their investigation of environmental causes of cancer noting that 'Massive gaps exist in toxicologic data, even in the case of widely used synthetic chemicals. Only about 50% of chemicals classified by the Environmental Protection Agency as "high production volume" have undergone even minimal testing for carcinogenicity'. But even though the incidence of cancer attributable to environmental exposures has not been definitively established (3,6), it remains an important focus of our prevention efforts [with credible estimates from the World Health Organization [WHO] and the IARC suggesting that the fraction of cancers attributable to toxic environmental exposures is between 7% and 19%] (10,11).

The possibility that unanticipated low-dose effects (LDE) are also a factor in environmental carcinogenesis further complicates matters. Vandenberg *et al.* (12) recently reviewed the accumulating evidence that points to LDE that occur at levels that are well below those used for traditional toxicological studies. This review identified several hundred examples of non-monotonic dose-response relationships (i.e. examples where the relationship between dose and effect is complex and the slope of the curve changes sign—from positive to negative or *vice versa*—somewhere within the range of doses examined). Drawing on the known actions of natural hormones and selected environmental chemicals examined in cell cultures, animals and epidemiology, the authors emphasized that when non-monotonic dose-response curves occur, the effects of low doses cannot be predicted by the effects observed at high doses. However, endocrine disruption research to this point has been aimed primarily at chemicals that disrupt developmental processes through a relatively small subset of hormones (e.g. estrogen, androgen, thyroid and so on), and thus, many commonly encountered chemicals have not been tested at all for these effects (at environmentally relevant dose levels) and, to date, mechanisms that relate to carcinogenesis have typically not been the focus of these studies.

Generally for chemical risk assessments, toxicity studies are conducted with individual chemicals in animal models based on regulatory test guidelines [e.g. Organization for Economic Co-operation and Development (OECD) test guidelines (13)] with a key objective of providing a dose-response assessment that estimates a point of departure [traditionally the no-observed-adverse-effect level or the lowest-observed-adverse-effect level (LOAEL)], which is then used to extrapolate the quantity of substance above which adverse effects can be expected in humans. The no-observed-adverse-effect level, combined with uncertainty factors (which acknowledge gaps in the available data), is then used to establish safety criteria

for human exposure. However, in order to be able to detect adverse effects utilizing classical toxicological endpoints, dose selection has historically involved the use of high dose levels and appropriate dose level spacing to obtain the LOAEL or no-observed-adverse-effect level thresholds. Techniques such as linear extrapolation or benchmark dose modeling (14) are then employed to predict safety margins for low-dose exposures. This approach to risk assessment depends on the use of appropriate and sensitive endpoints, and on valid assumptions for extrapolation estimates (e.g. dose-response linearity) and calculations, and on the existence of thresholds of effects (15–17). So when the potential for non-linear dose-response relationships is combined with the possibility of synergism between and amongst low doses of mixtures of individual chemicals in the environment, it appears plausible that chemicals that are not individually carcinogenic may be capable of producing carcinogenic synergies that would be missed using current risk assessment practices.

The complex nature of the biology of cancer adds another layer of complexity for risk assessment. In a landmark paper in 1979, Ames (18) noted that damage to DNA appeared to be a major cause of most cancers and suggested that natural chemicals in the human diet and the tens of thousands of man-made chemicals that had been introduced into the environment in the preceding decades be tested for their ability to damage DNA. In doing so, he sketched out the difficulty of dealing with complex chemical mixtures and he proposed the use of rapid mutagenicity assays to identify environmental mutagens and carcinogens. The strategy was sound at the time, but it led to a scientific and regulatory emphasis on ‘mutagens as carcinogens’, whereas the issue of complex environmental mixtures, or carcinogens that are not mutagens, was never vigorously pursued. Instead, what followed was an international quest to find individual chemicals and a few well-defined mixtures (e.g. diesel exhaust) that could be shown to be ‘complete’ carcinogens (i.e. substances that could cause cancer on their own).

However, advances in cancer biology have revealed the limitations of this approach. Armitage and Doll first laid out a multistage theory of carcinogenesis in 1954 (19), and by 1990, initiation and promotion were well established as discrete steps in the evolution towards malignancy, along with the influence of ‘free radicals’, proto-oncogenes, oncogenes, epigenetic mechanisms and other synergistic or antagonistic factors (20). In 2000, Hanahan et al. (21) gave structure to this rapidly growing field of research with the proposal that ‘the vast catalog of cancer cell genotypes [could be organized into] a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth’. They called these alterations the Hallmarks of Cancer, defined as ‘... acquired capabilities’ common to most cancers that ‘... incipient cancer cells ... [must acquire to] enable them to become tumorigenic and ultimately malignant.’ The hallmarks delineated at the time were as follows:

- *Self-sufficiency in growth signals* (later renamed proliferative signaling)—cancer cells grow at a seemingly unlimited rate.
- *Insensitivity to antigrowth signals* (evading growth suppressors)—cancer cells are not subject to antigrowth signals or withdrawal of normal growth signals.
- *Evading apoptosis* (resisting cell death)—cancer cells avoid the usual process whereby abnormal or redundant cells trigger internal self-destroying (as opposed to cell death) mechanisms.
- *Limitless replicative potential* (enabling replicative immortality)—cancer cells do not senesce (or age) and die after a limited number of cell divisions.
- *Sustained angiogenesis* (inducing angiogenesis)—cancer cells elicit new blood vessels to sustain growth.
- *Tissue invasion and metastasis* (activating invasion and metastasis)—in situ or non-invasive cancers, e.g. ductal carcinoma in situ in the breast or carcinoma in situ in colon polyps, grow into pre-existing spaces but invasive tumors must create a space to expand into normal tissue.

From this perspective risk assessments based on limited ‘mode of action’ information, assumptions of linear dose-response relationships and a focus on individual chemicals (as complete carcinogens) appeared to be inadequate to estimate human cancer risks. So in 2005, a scientist at the United States Environmental Protection Agency (EPA) called for a shift in risk assessment practices that would move the field towards the development of biomarkers directly related to the pathways found within the Hallmarks of Cancer framework (22).

The Hallmarks of Cancer framework was subsequently revisited by Hanahan et al. (21) and expanded to encompass additional areas suggested by subsequent cancer research (23). This expansion included the following:

Two enabling characteristics:

- *Genome instability and mutation*, which allows changes in one cell to pass to daughter cells through mutation or epigenetic changes in the parent cell DNA.
- *Tumor-promoting inflammation*, which helps cancer cells grow via the same growth signals normal cells provide to each other during wound healing and embryonic growth; inflammation further contributes to the survival of malignant cells, angiogenesis, metastasis and the subversion of adaptive immunity (24).

Two ‘emerging’ hallmarks:

- *Avoiding immune destruction* whereby tumor cells avoid immune surveillance that would otherwise mark them for destruction.
- *Dysregulated metabolism*, one of the most recognizable features of cancer; its exclusion from the original list of hallmarks (21) probably represented a significant oversight, as it constitutes one of the earliest described hallmarks of cancer (25,26). It is needed to support the increased anabolic and catabolic demands of rapid proliferation and is likely an enabler of cancer development and its other associated hallmarks.

Unfortunately, risk assessment practices that are currently used to assess the carcinogenic potential of chemicals have changed very little (despite the vast literature that now underpins the main tenets of the Hallmarks of Cancer framework). For example, a chemical that disrupts DNA repair capacity might prove to be non-carcinogenic at any level of exposure (when tested on its own), but that very same chemical may have the potential to be an important contributor to carcinogenesis (e.g. in the presence of mutagens that cause DNA damage). Similarly, a chemical that has immuno-suppressive qualities may not be carcinogenic on its own, but if it acts to suppress the immune response, it may contribute to carcinogenesis (by dismantling an important layer of defense) in the presence of other disruptive chemicals. Considering the multistep nature of cancer and the acquired capabilities implied by each of these hallmarks, it is therefore a very small step to envision how a series of complementary exposures acting in concert might prove to be far more carcinogenic than predictions related to any single exposure might suggest (see Figure 1). Interacting contributors need not act simultaneously or continuously, they might act sequentially or discontinuously. So a sustained focus on the carcinogenicity of individual chemicals may miss the sorts of synergies that might reasonably be anticipated to occur when combinations of disruptive chemicals (i.e. those that can act in concert on

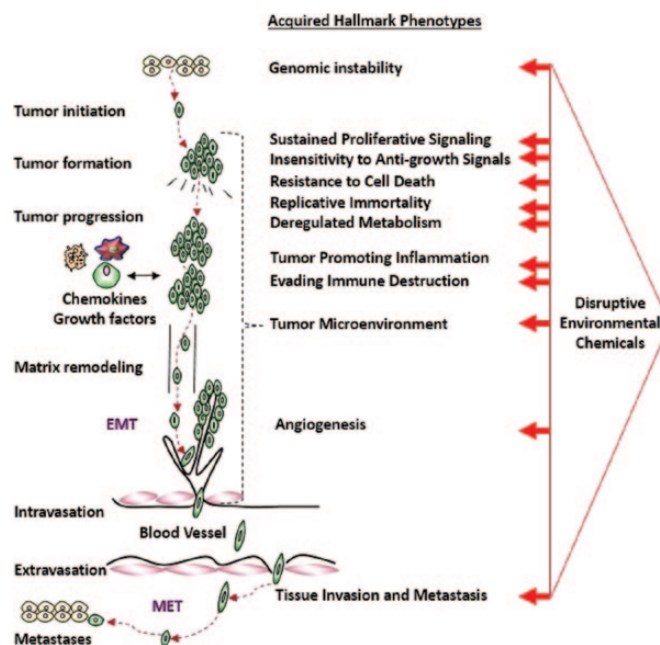


Figure 1. Disruptive potential of environmental exposures to mixtures of chemicals. Note that some of the acquired hallmark phenotypes are known to be involved in many stages of disease development, but the precise sequencing of the acquisition of these hallmarks and the degree of involvement that each has in carcinogenesis are factors that have not yet been fully elucidated/defined. This depiction is therefore only intended to illustrate the ways in which exogenous actions might contribute to the enablement of these phenotypes.

the key mechanisms/pathways related to these hallmarks) are encountered.

To address the biological complexity issue associated with chronic diseases, the EPA and other agencies have begun to pursue risk assessment models that incorporate biological information. This is the basis of the Adverse Outcome Pathway concept, a construct that is gaining momentum because it ties existing knowledge of disease pathology (i.e. concerning the linkage between a direct molecular initiating event and an adverse outcome at a biological level of organization) to risk assessment (27,28). This line of thinking inspired a recent initiative by the EPA, where the agency tested a proposal for characterizing the carcinogenic potential of chemicals in humans, using *in-vitro* high-throughput screening (HTS) assays. The selected HTS assays specifically matched key targets and pathways within the Hallmarks of Cancer framework. The authors tested 292 chemicals in 672 assays and were successfully able to correlate the most disruptive chemicals (i.e. those that were most active across the various hallmarks) with known levels of carcinogenicity. Chemicals were classified as 'possible'/'probable'/'likely' carcinogens or designated as 'not likely' or with 'evidence of non-carcinogenicity' and then compared with *in-vivo* rodent carcinogenicity data in the Toxicity Reference Database to evaluate their predictions. The model proved to be a good predictive tool, but it was developed only as a means to help the EPA prioritize many untested individual chemicals for their carcinogenic potential (i.e. in order to establish priorities for individual chemical testing (29)).

What is still needed, is an approach employing the Hallmarks of Cancer framework that can be used to identify priority mixtures (i.e. those with substantive carcinogenic potential). Without a way to anticipate the carcinogenicity of complex mixtures, an important gap in capability exists and it creates a significant weakness in current risk assessment practices. Countries around the globe have made a significant investment in the regulatory infrastructure and risk

assessment practices that protect us from unwanted exposures to harmful chemicals and carcinogens, so we wanted to review the biology of cancer to map out the challenges associated with the development of an approach that would help us assess the carcinogenic potential of low-dose exposures to chemical mixtures in the environment. Such an approach was seen as a reasonable step to provide impetus for progress in this area of research and ultimately to inform risk assessment practices worldwide.

Materials and methods

In 2012, the non-profit organization 'Getting to Know Cancer' instigated an initiative called 'The Halifax Project' to develop such an approach using the 'Hallmarks of Cancer' framework as a starting point. The aim of the project was to produce a series of overarching reviews of the cancer hallmarks that would collectively assess biologically disruptive chemicals (i.e. chemicals that are known to have the ability to act in an adverse manner on important cancer-related mechanisms, but not deemed to be carcinogenic to humans) that might be acting in concert with other seemingly innocuous chemicals and contributing to various aspects of carcinogenesis (i.e. at levels of exposure that have been deemed to be safe via the traditional risk assessment process). The reviews were to be written by 12 writing teams.

The writing teams were recruited by Getting to Know Cancer circulating an email in July 2012 to a large number of cancer researchers, asking about their interest in the project. Respondents were asked to submit personal details through a dedicated webpage that provided additional project information. A total of 703 scientists responded to the email, and from that group, 11 team leaders were selected to lead reviews of each hallmark (10 Hallmarks plus an 11th team to consider the tumor microenvironment as a whole) and one leader for the cross-validation team (see below). Writing group leaders were asked to form individual teams drawn from the pool of researchers who expressed interest in the project and from their own circles of collaborators. Leaders were encouraged to engage junior researchers as well. Team leaders received project participation guidelines and ongoing communication from the project leaders, L.Lowe and M.Gilbertson. Each team

included: a lead author with a published expertise in the hallmark area; domain experts who assisted in the production of the descriptive review of the biology; environmental health specialists (e.g. specialists in toxicology, endocrine disruption, or other related disciplines) and support researchers.

Each writing team was charged to describe the hallmark, its systemic and cellular dysfunctions and its relationships to other hallmarks. A priority list of relevant (i.e. prototypical) target sites for disruption was to be developed by the team and a list of corresponding chemicals in the environment that have been shown to have the potential to act on those targets was requested, along with a discussion of related issues and future research needed (in the context of project goals).

Selection of target sites for disruption

A 'target' was broadly defined as a procarcinogenic disruption at the system level (e.g. the hypothalamic-pituitary-gonadal axis), organ level, tissue level or cellular level. It was assumed from the outset that a project intended to develop an approach for the assessment of the carcinogenic potential of low-dose exposures to chemical mixtures in the environment would encounter a practical upper limit to the number of potential targets that any given team could realistically review. Therefore, each team was asked to identify up to 10 relevant targets for their domain (bearing in mind that each target would also serve as a starting point for the identification of a disruptive environmental chemical that had already shown a demonstrated ability to act on that target). In theory, it was understood that this could lead to as many as 110 targets for the entire project, and since the teams were also asked to select one disruptive chemical for each target, a maximum of 110 chemicals.

In this phase, teams were asked to focus on specific gene changes common to many cancers as identified by The Cancer Genome Project (30) in order to estimate how the function of specific genes might be altered, not by specific gene mutations, but rather either by direct action or by epigenetic changes that might lead to the same functional ends. Most of these pathways and processes are found within both the hallmarks of cancer and the genomic frameworks, so teams were asked to evaluate both models and consider non-mutagenic/epigenetic pathways of interference as well (given that epigenetic changes such as DNA methylation and histone acetylation are relevant for cancer and often inducible by chemicals and may be transmitted to daughter cells).

Selection of disruptive chemicals

Teams were then asked to identify 'prototypical' chemicals in the environment that had demonstrated an ability to act on the selected targets. During workshops in Halifax, the teams settled on the following criteria to guide their choices:

- Chemicals should be ubiquitous in the environment because we wanted the broadest possible relevance for the general population.
- Chemicals should selectively disrupt individual targets such as specific receptors, specific pathways or specific mechanisms. Hypothetically speaking, a chemical could affect more than one pathway, receptor and so on; indeed, we expected that most chemicals would likely exert a multitude of actions. However, we used the term 'selectively disruptive' to encourage teams to avoid choosing mutagens that are randomly destructive in their action (i.e. unpredictable and capable of producing varying types of damage across a wide range of pathways).
- Chemicals should not be 'lifestyle' related, such as those encountered from tobacco, poor diet choices (e.g. red meats, French fries, lack of fruit and vegetables and so on), alcohol consumption, obesity, infections (e.g. human papillomavirus) and so on.
- Chemicals should not be known as 'carcinogenic to humans' (i.e. not IARC Group 1, carcinogens).

The choice to focus on environmental pollutants in this project was intentionally restrictive. Countries around the globe have made significant investments in regulatory infrastructure and risk assessment

practices to protect us from unwanted exposures to harmful chemicals and carcinogens. Therefore, we focused on chemicals that are commonly encountered in the environment. Primarily, we wanted to generate insights that would be valuable for cancer researchers who are specifically interested in environmental chemical exposures to chemical mixtures and/or those who are focused on risk assessment practices in general.

Dose-response characterizations and LDE

Given that much of the evidence in the toxicological literature that documents the disruptive actions of various chemicals has been produced under a wide range of differing experimental circumstances, we wanted to assess the quality and relevance of data that were gathered for exposures discussed in this review. Specifically, for each chemical selected and each mechanism identified, teams were additionally tasked to identify any dose-response characterization results and/or relevant low-dose research evidence that might exist. The term 'low dose' was defined using the European Food Safety Authority definition (i.e. responses that occur at doses well below the traditional lowest dose of 1 mg/kg that is used in toxicology tests) and the definition for 'LDE' was based on the EPA definition (31)—as follows:

Any biological changes occurring

- (a) in the range of typical human exposures or
- (b) at doses lower than those typically used in standard testing protocols, i.e. doses below those tested in traditional toxicology assessments (32), or
- (c) at a dose below the lowest dose for a specific chemical that has been measured in the past, i.e. any dose below the lowest observed effect level (LOEL) or LOAEL (33)
- (d) occurring at a dose administered to an animal that produces blood concentrations of that chemical in the range of what has been measured in the general human population (i.e. not exposed occupationally, and often referred to as an environmentally relevant dose because it creates an internal dose relevant to concentrations of the chemical measured in humans) (34,35).

Each team was then asked to categorize each chemical by using one of five possible categories (to determine the relevance and relative strength of the underlying evidence for each of the chemicals being considered). The categories were as follows: (i) LDE (i.e. levels that are deemed relevant given the background levels of exposure that exist in the environment); (ii) linear dose-response with LDE; (iii) non-linear dose-response with LDE; (iv) threshold (i.e. this action on this mechanism/pathway does not occur at low-dose levels) and (v) unknown. Additional details of the descriptions for each of these categories are shown in Table 1.

Cross-hallmark relationships

In recognition of the network of signaling pathways involved and the degree of overlap/interconnection between the acquired capabilities described in each hallmark area, the project included a cross-validation step to create a more complete mapping of the actions that might be anticipated as the result of an action on the target sites identified or by the disruptive effects of the chemicals selected. Given the diversity of the targets involved in the 11 hallmark areas, it was anticipated that inhibiting or stimulating a target relevant to one hallmark may have an impact on other targets that are relevant, especially if both are linked via signaling pathways.

Accordingly, the cross-validation team conducted additional background peer-reviewed literature review of submitted targets and chemicals from each writing team, searching for evidence to identify cross-hallmark activity. Each potential target-hallmark or approach-hallmark interaction was assessed to determine whether the inhibition or activation of each target and the corresponding biological activity of each chemical might reasonably be expected to have either a procarcinogenic or anticarcinogenic effect on key pathways/processes in the various hallmark areas.

The cross-validation team also sought out controversial interactions (i.e. mixed indications of hallmark-like effects) and

AQ14 Table 1. Dose-response characterization

Review team	Chemical name	Disruptive action on key mechanism/pathway	Low-dose effect (LDE, LLDE, NLDE, threshold, unknown)
Angiogenesis	Diniconazole	Vascular cell adhesion molecule and cytokine signaling	Threshold (H-PC) (36)
	Ziram	Vascular cell adhesion molecule and cytokine signaling	Threshold (H-PC) (36,37)
	Chlorothalonil	Thrombomodulin, vascular proliferation and cytokine signaling	Unknown (H-PC) (36), NLDE (A-in vivo) (38)
	Biphenyl	Angiogenic cytokine signaling	Unknown (H-PC) (36)
	Tributyltin chloride	Vascular cell proliferation and adhesion molecule signaling	Unknown (H-PC) (36)
	Methylene bis(thiocyanate)	Plasminogen activating system and cytokine signaling	Unknown (H-PC) (36)
	HPTE	Vascular cell adhesion molecule and cytokine signaling	Unknown (H-PC) (36), threshold (A-I ^a) (39), LDE (A-I ^a) (40)
	PPOS	Angiogenic cytokine signaling	Threshold (H-PC) (36), LDE (H-CL) (41)
	Bisphenol AF	Matrix metalloproteinase expression and estrogen receptor signaling	Unknown (H-PC) (36)
	C.I. solvent yellow 14	AhR and hypoxic signaling	Unknown (H-PC) (36)
Deregulated metabolism	Cypermethrin	AR and ER expression, reduction of ATP and mitochondrial enzymes, mitochondrial membrane potential	LLDE (A-I) (42), NLDE (A-I) (42), NLDE (H-CL) (36,43,44)
	Acrolein	p53 activation, DNA repair inhibition, PERK phosphorylation, mitochondrial dysfunction, cell survival	LLDE (A-I, A-CL, H-PC, H-CL) (45-50), NLDE (49), threshold (46)
	Rotenone	Cell cycle, DNA damage response, proliferation, differentiation, mitochondria	LLDE (H-CL) (51-53), NLDE (H-CL) (51,53), unknown (H-CL, H-PC) (36)
	Copper	p53 activation, p21 up-regulation, cell viability	LLDE (H-CL) (54-56)
	Nickel	Neutrophil apoptosis, E-cadherin regulation, matrix metalloproteinase (MMP) production	LLDE (H-CL) (57), NLDE (H-CL) (58), Threshold (H-CL) (58)
	Cadmium	p53-dependent apoptosis, cell proliferation	LLDE (H-CL) (59), threshold (H-CL) (60)
	Diazinon	AChE activity, neuronal cytotoxicity	Unknown (A-PC) (61), LLDE (H-CL) (62), threshold (H-CL) (36)
	Iron	KRAS mutations	LLDE (A-I) (63)
	Malathion	Lymphocyte Mutations, Cytotoxicity	Unknown (H-PC, H-E) (36,64)
	BPA	MMP-2 and MMP-9 expression, increased migration, invasion, EMT, oxidative stress, ER signaling	LDE (H-CL) (65,66), threshold (H-CL, H-PC) (36)
Tissue invasion and metastasis	Hexachlorobenzene	Activation of c-Src, HER1, STAT5b and ERK1/2 signaling	LLDE (H-CL, A-I) (67)
	Sulfur dioxide	MMP-9 expression	Unknown (A-PC) (68)
	Phthalates	MMP-2 and MMP-9 expression	LDE (H-CL) (66), Unknown (H-CL, H-PC) (36)
	Iron	ROI generation, NF- κ B activation, uPA expression	Unknown (H-CL) (69)
	Biorhythms/melatonin	GSK3 β activation, EMT regulation	Unknown (H-CL, H-E) (70,71)
	BPA	Inhibition of GJIC, activation of mTOR pathway, down-regulation of p53, p21 and BAX, binding to ER- α , weakly binds to TH receptor and AR, activation of ERK1/2 and p38	NLDE(H-CL, A-CL) (72-74)
	Dibutyl phthalate	Activation of PPAR- α , inhibition of GJIC, expression of cyclin D and cdk-4, activation of AhR/HDAC6/c-Myc pathway	Threshold (H-CL, H-PC) (36)
	Chlorothalonil	Up-regulation of ErbB-2 tyrosine kinase and MAP kinase, aromatase inhibitor	NLDE (H-CL) (75), unknown (H-CL, H-PC) (36)
	Lindane	Induction of MAPK/ERK pathways	Threshold-based (i.e. non-linear) (A-I) (76), unknown (H-PC) (36), threshold (H-CL) (36)
	Dichlorvos	Expression of p16, Bcl-2 and c-myc	Threshold-based (i.e. non-linear) (A-I) (77), threshold (H-CL) (36)
Resistance to cell death	MXC	Binding to ER- α receptor, up-regulation of cyclin D1, down-regulation of p21	LLDE (H-CL, A-CL) (75,79), unknown (H-PC) (36), threshold (H-CL) (36)
	Oxyfluorfen	Expression of Cyp2b10 and Cyp4a10 transcripts (markers of PPAR- α activation)	Threshold (A-I) (80), unknown (H-CL, H-PC) (36)
	DEHP	Activation of PPAR- α , inhibition of GJIC	Threshold-based (i.e. non-linear) (A-I) (81)
	Linuron	Hypersecretion of LH, inhibition of GJIC	Unknown (H-CL) (82)

Table 1. Continued

Review team	Chemical name	Disruptive action on key mechanism/pathway	Low-dose effect (LDE, LLDE, NLDE, threshold, unknown)
Replicative immortality	Nickel-derived compounds, (e.g. nickel chloride)	Epigenetic silencing of p16	LLDE (H-CL, A-PC) (83)
	Diethylstilbestrol	Allelic loss and point mutation in ETRG-1 gene	LLDE (A-I) (84)
	Reserpine	Epigenetic modifications	Unknown (A-PC) (85), threshold (H-CL) (36)
	Phenobarbital	Reduces expression of the CDKN1A product p21, CAR activation	LLDE (A-I) (86,87)
	Acetaminophen	Cellular energy loss, mitochondrial damage, telomerase activation	LDE (H-CL, A-I, A-CL) (88-92)
	Cotinine	Telomerase activation	LLDE (H-PC) (93)
	Nitric oxide	p53 inactivation	LLDE (H-PC, H-CL, A-CL, A-I) (94)
	Na-selenite	p53 promoter methylation	LLDE (A-CL, A-I) (95,96)
	Lead	p53 inactivation	LLDE (H-PC, H-CL, A-CL, A-I) (94)
	BPA	Estrogen receptor activation, cell cycle/senescence	LLDE (A-I, H-CL, H-E) (12,97), NLDE (A-I) (98,99), threshold (H-CL) (36)
Sustained proliferative signaling	Cyprodinil	Increased proliferation signaling, AhR activation	Unknown (H-PC, H-CL) (36,100,101), threshold (H-CL) (36)
	Imazalil	AR signaling	NLDE (A-I) (102,103), threshold (H-CL, H-PC) (36)
	Maneb	Nitric oxide signaling	Unknown (A-CL, H-CL, H-PC) (36,104,105)
	Methoxychlor	ER signaling	Threshold (H-CL) (36), LDE (A-I) (106,107), NLDE (A-I) (108)
	PFOs	Nuclear hormone receptors	Threshold (H-CL) (36), LLDE (A-I) (109,110)
	Phthalates	CAR, ER signaling	Unknown (H-CL) (36), LDE (A-I) (111-113)
	Phosalone	Increased proliferation, PXR signaling	Unknown (H-PC, H-CL) (36,114,115)
	PBDEs	ER signaling	Unknown (H-PC, H-CL) (36,114,115)
	Prochloraz	ER signaling	LDE (A-I) (116,117)
	Trenbolone acetate	Insulin-like growth hormone-1 and AR signaling	LDE (A-I) (118,119)
Tumor-promoting inflammation	BPA	Immune cell proliferation, proinflammatory cytokine induction	Unknown, LDE (A-I, H-CL, H-E) (120,121)
	Phthalates	Immunomodulation of macrophages, lymphocytes, eosinophils and neutrophils	Threshold (H-PC) (36), LDE (A-I, H-CL, H-E) (122-126)
	PBDEs	Induction of pro-inflammatory cytokines (IL-6, IL8 and CRP), inhibition of anti-inflammatory cytokines (IL-10)	Unknown (H-PC, H-CL, H-E) (36,127)
	Atrazine	Immunomodulation of T cell and B cells, proinflammatory cytokines	Threshold (H-PC, H-CL) (128-131)
	Vinclozolin	Proinflammatory cytokine induction, NF-κB activation	Unknown (H-PC, A-I) (36,132,133)
	4-NP	Proinflammatory cytokine induction, NF-κB activation, iNOS induction	Unknown (H-PC, A-I) (36,134-136)
	Pyridaben	Chemokine signaling, TGF-β, FAK, HIF-1α, IL-1α pathways	Unknown (H-CL, H-PC, A-CL) (36,139,140), threshold (A-I) (141)
	Triclosan	Chemokine signaling, TGF-β, FAK, IL-1α pathways	Threshold (H-CL, H-PC, A-I) (36,142-144), LDE (A-I, H-CL) (145,146)
	Pyraclostrobin	Chemokine signaling, TGF-β, IL-1α pathways	Unknown (H-CL, H-PC) (36)
	Fluoxastrobin	Chemokine signaling, EGR, HIF-1α, IL-1α pathways	Unknown (H-CL, H-PC) (36)
Immune system evasion	BPA	Chemokine signaling, TGF-β pathway	Threshold (H-PC) (36), LDE (A-I) (12), NLDE (H-CL) (147), NLDE (A-CL) (148-151), NLDE (A-I) (152-155)
	Maneb	P13K/Akt signaling, chemokine signaling, TGF-β, FAK, IGF-1, IL-6, IL-1α pathways	Unknown (H-CL, H-PC) (36,139,156-158), LDE (A-I) (159), threshold (A-I) (139,160), threshold (A-CL, A-I) (161)

Table 1. Continued

Review team	Chemical name	Disruptive action on key mechanism/pathway	Low-dose effect (LDE, LLDE, NLDE, threshold, unknown)
Evasion of antitumor signaling	DDT	Induces MDM2, cyclin D1, E2F1 expression, disrupts gap junctions	NLDE (A-I, H-CL, A-CL) (162-164)
	Chlorpyrifos	Increases proliferation	LDE (H-CL, H-PC) (165,166)
	Folpet	Disrupts G ₁ -S checkpoint kinases, down-regulates p53, promotes proliferation	LDE(A-C) (167)
Tumor microenvironment	Atrazine	Induces estrogen production and proliferation	LDE(H-CL, A-I) (168-170)
	BPA	Reduced p53, reduced connexin 43 expression, increased proliferation	NLDE (H-CL, A-I) (171-174)
	Nickel	ROS and cellular stress	NLDE (A-I) (175)
Genome instability	BPA	IL-6 expression, improper DC maturation and polarization, ROS production	LLDE (A-I) (176), NLDE (A-I) (176)
	Butyltins (such as tributyltin)	NK cell inhibition	LDE (A-I) (177)
	MeHg	Chronic oxidative stress	LDE (H-PC, H-CL) (178,179)
	Paraquat	Chronic ROS production, cellular stress	Unknown (A-I) (180)
	Lead	Dysfunctional DNA repair, defect in telomere maintenance	Unknown (A-CL) (181-183), threshold (H-CL, H-E) (184,185)
	Acrylamide	Inactivation of DNA repair proteins/enzymes	Unknown (A-CL, A-I, H-CL) (186,187)
	Quinones	Affect free cysteine residues in catalytic center of DNA methyltransferases (DNMT)	Unknown (A-CL) (188)
	Nickel	Affect enzymes that modulate post-translational histone modification	LDE (H-E) (189,190), LDE (A-CL, H-CL) (191)
	BPA	Epigenetic changes via interactions with miRNA	Threshold (H-PC) (192)
	Alloy particles (tungsten/nickel/cobalt)	Disruption of DNA damage/redox signaling involving Nrf, NF- κ B, Egr, and so on	LDE (A-I) (193)
	Titanium dioxide NPs	Decreased NADH levels and impaired mitochondrial membrane potential and mitochondrial respiration, ROS generation	Unknown (A-PC) (194)
	Benomyl	Spindle defects leading to formation of micronuclei	Threshold (H-CL) (195), Threshold (A-CL) (196)
	Carbon nanotubes	Spindle defects leading to formation of micronuclei	LLDE (A-CL) (197,198), unknown (A-I) (198)

Each chemical in the table was categorized by using one of five possible categories (to determine the relevance and relative strength of the underlying evidence for each of the chemicals being considered)—as follows: (1) LDE (low-dose effect)—the ability of this chemical to exert this particular effect is not well characterized at a range of dose levels, but the evidence suggests that this chemical can exert this effect at low-dose levels (i.e. levels that are deemed relevant given the background levels of exposure that exist in the environment and as further defined below); (2) LLDE (linear dose-response with low-dose effects)—the ability of this chemical to exert this particular effect is well characterized at a range of dose levels and the evidence suggests that a linear dose-response relationship exists with effects at low-dose levels being evident (i.e. levels that are lower than the LOEL/LOAEL or threshold and deemed relevant given the background levels of exposure that exist in the environment). Note: a linear dose-response model implies no threshold. Effects at low doses are the same as at higher doses even if at a lesser extent. The effect is directly proportional to the dose; (3) NLDE (non-linear dose-response with low-dose effects)—the ability of this chemical to exert this particular effect is well characterized at a range of dose levels and the evidence suggests that a non-linear dose-response relationship exists with exaggerated effects at low-dose levels being evident (i.e. levels that are lower than the LOEL/LOAEL or threshold and deemed relevant given the background levels of exposure that exist in the environment). Note: a non-linear dose-response with low-dose effect implies that the effect does not vary according to the dose of the agent. The effect at low doses may be the same as at the higher doses or different. The non-linear dose-response may have or not have a threshold. It is represented by a sigmoid curve. The non-linear dose-response at low doses may be a non-monotonic dose-response; (4) Threshold—the ability of this chemical to exert this particular effect is well characterized at a range of dose levels, and a threshold has been established for this chemical that suggests that this action on this mechanism/pathway does not occur at low-dose levels (i.e. levels that are lower than the LOEL/LOAEL or threshold and deemed relevant given the background levels of exposure that exist in the environment); (5) Unknown—although the ability of this chemical to exert this particular effect has been shown at higher dose levels, this effect is not well characterized at a range of dose levels, so a LOEL/LOAEL or a threshold has not been determined for this chemical and there is no evidence showing that this chemical exerts this action at low-dose levels (i.e. levels that are lower than the LOEL/LOAEL or threshold and deemed relevant given the background levels of exposure that exist in the environment). A-I, *in-vivo* animal models; A-CL, animal cell lines; A-PC, animal primary cells; H-PC, human primary cells; H-CL, human cell lines; H-E, human epidemiological studies. With respect to the human primary cell (H-PC) data from ToxCast (36): unknown signifies that the compound was tested against a range of doses and showed statistically significant activity against the specified targets at the lowest test concentrations (~0.01 μ M), therefore, a threshold could not be established. Threshold in this data set signifies that there was no activity against the targets at one or more of the lowest concentrations tested.

*Extrapolated from *in-vivo* data on the parent compound, MXC.

instances where no known relationship existed. It was our belief that target sites or chemicals that demonstrated a substantial number of 'anticarcinogenic' effects in other hallmark areas would be less suitable to serve as instigating constituents in the design of carcinogenic mixtures (where procarcinogenic synergy was being sought).

It is important to note that the cross-validation team was not given any restrictions for literature selection for this effort, and contributing authors were neither restricted to results from low-dose testing, nor to cancer-related research. This approach was taken because it was realized at the outset that this sort of breadth and homogeneity (of low-dose evidence) does not yet exist in the literature. As a result, the types and sources of data gathered in this effort varied considerably, resulting in an admixture of reviews and original studies. Moreover, many studies that were cited in this effort only considered a chemical's ability to instigate or promote an action that mimics a hallmark phenotype in a manner directionally consistent with changes that have been associated with cancer. So, although we have referred to these actions as procarcinogenic and anticarcinogenic, as these changes are frequently neither fixed nor specific for cancer, the specificity of these changes and implications for carcinogenesis cannot and should not be immediately inferred from this data set. Short-term toxicity and toxic responses—particularly in data from *in-vitro* HTS platforms—must be distinguished from truly 'carcinogenic' long-term changes. In other words, the tabularized results from this particular aspect of the project were only compiled to serve as a starting point for future research. Where cross-hallmark effects were reported (at any dose level and in any tissue type), we wanted samples of that evidence to share with researchers who might be trying to anticipate the types of effects that might be encountered in future research on mixtures of chemicals (in a wide range of possible research contexts).

Results

The results are presented roughly sequenced in a manner that captures the acquired capabilities found in many/most cancers. The section begins with two enabling characteristics found in most cancers, *Genetic instability* and *Tumor-promoting inflammation*, followed by *Sustained proliferative signaling* and *Insensitivity to antigrowth signals*, the two related hallmarks that ensure that proliferation is unabated in immortalized cells. These sections are followed by *Resistance to cell death* and *Replicative immortality*, two critical layers of defense that are believed to be bypassed in all cancers and then by *Dysregulated metabolism*. Sections on *Angiogenesis* and *Tissue invasion and metastasis* follow and speak to the progression of the disease, and finally, the *Tumor micro-environment* and *Avoiding immune destruction* sections offer summaries related to the very last lines of defense that are defeated in most cancers. Additionally, dose-response characterizations and evidence of LDE are then presented for all of these areas and the results from the cross-validation activity are summarized and reviewed.

Genetic instability

The phenotypic variations underlying cancer result from interactions among many different environmental and genetic factors, occurring over long time periods (199). One of the most important effects of these interactions is genome instability—loosely defined as an increased likelihood of the occurrence of potentially mutagenic and carcinogenic changes in the genome. The term is used to describe both the presence of markers of genetic change (such as DNA damage and aneuploidy) and intrinsic factors that permit or induce such change (such as specific gene polymorphisms, defective DNA repair or changes in epigenetic regulation).

DNA damage—which can be caused by exposure to external chemicals or radiation, or by endogenous agents such as reactive oxygen or faulty replication—is an event that can initiate the multistep process of carcinogenesis (200). Protection is afforded

at different levels; removal of damaging agents before they reach the DNA, by antioxidant defenses and the phase I/phase II xenobiotic metabolizing enzymes; a second line of defense, DNA repair, operating on the damage that occurs despite the primary protection; and as a last resort, apoptosis (programmed cell death), disposing of heavily damaged cells.

A clear sign of genome instability is aneuploidy—a deviation from the normal number of chromosomes (201). Aneuploidy is a very common feature of human cancers. Another hallmark of cancer is loss of the normal mechanism of telomere shortening, which allows abnormal cells to escape senescence, by avoiding the body's 'editing' processes that normally eliminate aging cells with their accumulated genome aberrations (202,203).

The genes of most significance for cancer are the (proto)-oncogenes which, if defective, or abnormally expressed, lead to uncontrolled cell proliferation; tumor suppressor genes, the normal products of which tend to switch off replication to allow repair, and promote cell death if damage is excessive; and genes such as those involved in DNA repair that can—if faulty—lead to a 'mutator phenotype'. Mutated proto-oncogenes and tumor suppressor genes are found in most if not all cancers and play key roles in cancer etiology (204–207). Rare mutations in DNA repair genes greatly increase the risk of cancer (208,209). However, the evidence for links between common variants of repair genes and cancer is generally inconclusive (210).

The term 'epigenetics' refers to covalent modifications of the DNA (methylation of cytosine in 'CpG islands' within regulatory regions of genes) or of the histones. These modifications can control gene expression and the pattern of modifications is altered in many cancers (211,212). For instance, hypomethylation of proto-oncogenes can lead to overexpression, which is undesirable. MicroRNAs (miRNAs) are responsible for specific down-regulation of gene expression at a post-transcriptional level, by preventing translation from messenger RNAs. miRNAs participate in DNA damage responses and some miRNAs are deregulated in many cancers (213–215).

Mutations in germ and stem cells are potentially more serious than those in other cells as they are passed to the cells' progeny within the developing embryo or regenerating tissue (216,217). There is a presumed survival benefit when stem cells tend to show a particularly stringent maintenance of genome integrity through cell cycle regulation and enhanced responses to DNA damage (218).

The selected 'chemical disruptors' that induce genome instability include chemicals that not only directly damage DNA or cause mutations, but act indirectly, via pathways such as DNA damage signaling, DNA repair, epigenetic regulation or mitochondrial function. They include the following:

Metals such as lead, nickel, cobalt and mercury (common water pollutants) are known to disrupt DNA repair (181,219), whereas nickel also affects epigenetic histone modification (189,191) and lead causes defective telomere maintenance (184,220). Alloy particles, containing tungsten, nickel and cobalt, can be inhaled and disrupt redox signaling (193,221). Titanium dioxide nanoparticles are also common in many consumer products and foods and have been reported to disrupt mitochondrial function and increase oxidative stress, as well as inhibit DNA repair and disrupt mitosis (194,222,223).

Acrylamide occurs in many fried and baked food products, and (apart from the well-known DNA adduct formation) can inactivate many critical proteins by binding sulphhydryl groups (186).

Bisphenol A (BPA) is a plasticizer used for manufacturing polycarbonate plastics and epoxy resins, and it can leach

from plastics into food and water. It is implicated in disruption of DNA methylation, histone acetylation and disturbance of miRNA binding (192,224,225), redox signaling (226) and induction of micronuclei through spindle defects in mitosis (227).

The fungicide benomyl is metabolized to carbendazim; both are classified as possible human carcinogens at present. The route of exposure is most likely ingestion via residues in crops. Benomyl disrupts the microtubules involved in the function of the spindle apparatus during cell division, leading to production of micronuclei (Frame, S.R. et al., unpublished report, Schneider, P.W. et al., unpublished report, (228)).

Halobenzoquinones are disinfection by-products in chlorinated drinking water (229). Quinones are electrophilic compounds, known to react with proteins and DNA to form adducts. These electrophilic chemicals can interact with functional thiol groups via Michaelis-Menton type addition, causing modification of enzymes involved in methylation and demethylation (188). This mechanism might be shared by other xenobiotics that increase reactive oxygen species (ROS).

Human exposure to nano-sized materials used in cosmetics, biomedical compounds, textiles, food, plastics and paints has increased not only in a conscious way but also passively by the leakage of nanomaterials from different objects. Nanoparticles can induce genome instability via mitochondrial-related apoptosis (230), decreased DNA repair (222,230,231), hypoacetylation of histones (232), disruption of DNA methylation (231), up-regulation of miRNA (233), reducing telomerase activity (220) and—more specifically for carbon nanotubes—interacting with components of the mitotic spindle during cell division, or with proteins directly or indirectly involved in chromosome segregation (197,234). Nano-sized materials can also produce inflammation and alteration of the antioxidant defenses that can lead to genome instability.

Tumor-promoting inflammation

One of the earliest hypothesized causes of tumors subsequently supported experimentally was the irritation hypothesis proposed by Virchow. Although it was recognized initially that injury alone was insufficient for carcinogenesis, it was also recognized that 'irritation may have an accessory or predisposing influence in tumor formation, and that it may be enough finally to upset the balance of a group of cells which for some other reason were already hovering on the brink of abnormal growth' (235). Indeed, it is now recognized that inflammatory responses, similar to those associated with wound healing or infection, support the development of invasive carcinomas by altering the microenvironment in favor of proliferation, cell survival, angiogenesis and tumor cell dissemination while also disrupting antitumor immune surveillance mechanisms. In other words, inflammation plays a critical role in tumorigenesis (23,24).

Inflammation is an immediate and necessary host defense mechanism in response to infection or tissue injury by noxious stimuli. In tumor-associated inflammation, both the epithelium and the immune cells express receptors that signal the activation and production of a wide array of biologically active proteins most analogous to an unhealed wound. The sustained or uncontrolled release of potent and reactive molecules such as prostaglandins, cytokines, ROS and chemokines from both the tumor cell and the microenvironment constituents leads to progressive genomic instability, alterations in the integrity and function of the microenvironment including alterations in the vasculature (e.g. vascular hyperpermeability, neovascularization and angiogenesis), as well as alterations in local immune dynamics. The cellular and molecular mechanisms include a

diverse array of immune- and tumor-cell-derived effector molecules such as the proinflammatory reactive oxygen and nitrogen species, a number of cytokines, chemokines as well as cyclooxygenase-2 and its product, prostaglandin E_2 .

In general, there is a paucity of experimentation, and when present, inconsistent findings for the role of environmental chemicals as proinflammatory molecules and more so for a pro-inflammatory action as a co-factors in carcinogenesis. However, some recent studies provide a credible mechanistic basis, particularly early life exposures that might act by disrupting the immune cell balance toward inflammation, and that manifest in adulthood. One example is BPA, one of the most abundant and best studied environmental endocrine disruptors, and its controversial role as an immune disruptor. Specifically, studies in male rats found that early life BPA exposure leads to the development of prostate intraepithelial neoplasia (a prostate cancer precursor lesion) through a pathological process that includes BPA-dependent epigenetic reprogramming of genes involved in the development of lateral prostate inflammation in adulthood (236,237).

This work in prostate is complemented by a much more extensive study of BPA effects on immune cell components, particularly the T-cell compartment, demonstrating that BPA acts as an immune disruptor by promoting 'immune' cell proliferation though the exact nature of the effect on specific cells of the immune system is poorly delineated. Most interesting is the work by Yan et al. (122), who reported findings suggesting that the timing of BPA exposure during development (prenatally, early life or adult) alters the effect of BPA on regulatory T cells. BPA actions also map over to the effects on the immune system including the promiscuity of BPA for a number of nuclear receptors relevant to immune cells such as the estrogen receptor and the aryl hydrocarbon receptor (AhR). As well, bulky BPA analogs may act as antagonists of members of the peroxisome proliferator-activated receptor (PPAR) family, an important family of nuclear receptors with potent anti-inflammatory function (238,239). Effects on the PPAR nuclear receptors may also explain inflammation-associated phenotypes observed with exposures to certain phthalates and nonylphenol (4-NP).

A second example is the reported immunotoxic effects of atrazine (6-chloro-*N*-ethyl-*N*-(1-methylethyl)-1,3,5-triazine-2,4-diamine) (240), a chemical that is the most commonly detected triazine herbicide in USA soil and water. Atrazine is banned by the European Union and drinking water exposures are supposed to be limited in the USA to <3 µg/l (although exposures exceed this limit regularly), but the use of this chemical is high and increasing in Asia and other countries. Thus, atrazine is an important pesticide to which humans are exposed. Atrazine exhibits weak mutagenicity and low oncogenic properties, but research by a number of authors is emerging that suggests that immune system disruption might be a concern (132,240,241).

Although the majority of work on atrazine has been focused on its endocrine disrupting properties, there is also evidence to support immunotoxicity including effects on T-lymphocytes composition with oral dosing (242,243), modulation of nitric oxide production (244) and potential generation of ROS (245,246). The local production of reactive nitrogen species and ROS by mast cells and macrophages are among the better studied immune modulatory molecules for which recent evidence supports important roles both in the tumor microenvironment and in the tumor progression (247–249). Notably, these reactive species trigger oxidative/nitrosative modifications, which can initiate redox signaling that tightly modulates the inflammatory

response in a manner that is highly relevant for carcinogenesis (250,251).

We also looked at polybrominated diphenyl ethers (PBDEs) and their effects on inflammatory cytokines. Peltier et al. (128) recently found that placental explants treated with a mixture of the congeners BDE-47, -99 and -100 and then exposed to *Escherichia coli* were 'reprogrammed' toward a proinflammatory response (increased IL-1 β and tumor necrosis factor α) and away from the expected anti-inflammatory response (decreased IL-10) compared with untreated placenta. Although these studies are preliminary, chronic PBDE exposure may lower the threshold for bacteria to stimulate a proinflammatory response, which has potential relevance given the established link between bacteria and certain cancers (e.g. *Helicobacter pylori* and gastric cancer), where tumor development is dependent on inflammation.

Vinclozolin was also of particular interest as an environmental chemical because transient early life exposures *in utero* have been linked to both adult-onset disease and transgenerational disease that involves inflammation (134,135). For example, transient vinclozolin exposure *in utero* has been shown to promote inflammation in the prostate (prostatitis) of postpubertal rats coupled with a down-regulation of the androgen receptor and increase in nuclear factor- κ B (NF- κ B). The late or delayed effect of exposure is hypothesized to reflect a mechanism whereby vinclozolin exposure during a critical development window imprints an irreversible alteration in DNA methyltransferase activity, leading to reprogramming of the androgen receptor (AR) gene(s), which manifests as inflammation in early adult life with adverse effects on spermatid number.

Similarly, 4-NP has been shown to increase progenitor white adipose levels, body weight and overall body size in rodents exposed prenatally. Like vinclozolin, 4-NP effects on adipogenesis in the perinatal period confer transgenerational inheritance of the obesogenic effects observable in F2 offspring, consistent with genome reprogramming through an epigenetic process (252) and others have reported immune and inflammation-related effects (137,138) making it relevant to carcinogenesis a deserving further investigation.

Sustained proliferative signaling

Sustained proliferative potential is an essential component of cancerous growth. Progressive conversion of normal cells into cancer cells requires a series of genetic alterations, where each alteration confers one or more types of growth advantage. One such alteration that affords the transformed cell a distinct growth advantage over its normal counterparts is the acquired capacity of the cancer cell to proliferate in a sustained manner, so as to crowd out and outnumber the normal cell population (23). One of the fundamental differences between a normal and a transformed cell is that normal cells halt proliferation when subjected to growth inhibitory signals or in the absence of growth stimulatory signals (253). But tumor cells act to sustain proliferative signaling in several different ways. They can activate specific genes to produce relevant growth factors, which in turn bind to signaling receptors giving rise to an autocrine loop (254). Growth factors produced by tumor cells can also stimulate the proliferation of stromal cells that in turn produce growth factors to sustain tumor cell proliferation (255). Sustained proliferation can additionally be maintained at the receptor level by truncation of signaling receptor proteins whereby the ligand-activated switch is missing (256). Alternatively, the number of high-affinity receptor proteins may be increased to levels that will sustain proliferative signaling in otherwise normal growth factor levels. Finally, sustained proliferative signaling may well

be the result of perpetual activation of the intracellular signaling chain independent of growth factors or receptors (e.g. mutated ras (257) or truncated src (258) are intermediaries of a normal proliferation signaling chain responsible for sustained proliferation).

We hypothesized that disruptive environmental chemicals acting in a procarcinogenic manner by inducing what is referred to as 'sustained cell proliferation' likely exert their action by interfering with some basic control mechanisms (23,253). For instance, they could achieve this by positively regulating targets within and outside the cell known to promote cell proliferation or negatively regulating targets within and outside the cell known to halt cell proliferation. In this way, such chemicals could confer proliferative advantage to a distinct cell population and contribute to that population's capability to successfully breach innate anticancer defense mechanisms and to become progressively autonomous.

Specifically, we identified a total of 15 ubiquitous chemical disruptors capable of producing sustained cell proliferation. The majority of these chemicals interacted with multiple targets, and we have tabled this information in our review. In summary, we identified several commonly used insecticides and fungicides capable of causing sustained proliferation. These included cyprodinil, etoxazole, imazalil, lactofen, maneb, methoxychlor (MXC), phosalone, prochloraz and pyridaben, all of which targeted estrogen receptor α and frequently other steroid hormone receptors such as androgen receptor (102,259–275). Most of these chemicals also targeted growth factors and their receptors (260,264,267,276–280) and induced cytokines and cytokine receptors (identified by ToxCast high-throughput assay). Top disrupting chemical fungicides and insecticides were cyprodinil and MXC, which each interacted with a total of six individual targets that further included the AhR (100), B-lymphocyte markers (ToxCast 2009 high-throughput assay, both chemicals), AP-1 proteins/transcription/translation regulators, downstream signaling molecules and cell cycle regulators (281,282). Other strong performers for sustained proliferation were BPA (activated all targets activated by the insecticides and fungicides above except growth factors and their receptors, B lymphocyte markers and PPAR, but included cell cycle regulators alongside AP-1 proteins/transcription/translation regulators and downstream signaling) (272,281,283–285) (also identified in ToxCast high-throughput assay, 2009), polyfluorinated octinoid sulfate and polybrominated diphenylethers (flame retardants) that either activated AhR (286,287) or up to five other targets that included steroid receptors, growth factors, cytokines and cell cycle regulators (109) (ToxCast high-throughput assay 2009). Three other contenders were phthalates (plasticizers that acted via three targets that included AhR, steroid hormone receptors and PPAR) (265,288–292), trenbolone acetate (a synthetic anabolic steroid that unsurprisingly acted through steroid hormone receptors) (120,293–297) and finally, edible oil adulterants (food contaminants produced during food processing that acted via downstream signaling) (298,299).

We have shown estrogen and androgen receptors to be important targets in relation to sustained proliferative signaling (300), and note that environmental estrogens and androgens are frequently recognized as prototypical disruptor(s) of this hallmark. Although this is a small sample, there are a great number of chemicals in the environment (both naturally occurring and man-made) are estrogenic, interact with estrogen receptor and produce estrogen metabolites (just as naturally derived ovarian estrogen does during metabolic breakdown). Catechol estrogens (hydroxyl derivatives of estrogens), which are formed during

estradiol metabolism, are also potentially important mediators of endogenous estradiol levels, and therefore of sustained proliferative signaling and oncogenesis (301).

Insensitivity to antigrowth signals

Cell cycle arrest is important for maintaining genomic integrity and for preventing genetic errors from being propagated. The normal cell cycle contains multiple checkpoints to safeguard against DNA-damaging agents. Specific proteins at these checkpoints are activated in response to harmful stimuli, ensuring that cellular proliferation, growth and/or division of cells with damaged DNA are blocked.

There are multiple key mediators of growth inhibition that may become compromised during carcinogenesis. Some, such as p53 and RB1, cause cells to arrest at the G₁-S phase transition when they are activated by DNA damage. Mutations in the p53 gene occur in ~50% of all cancers, although certain tumor types, such as lung and colon, show a higher than average incidence (302). Some, such as p53, RB1 and checkpoint kinases, cause cells to arrest at the G₁-S phase transition when they are activated by DNA damage. Similarly, pRb hyperphosphorylation (303), direct mutations (304), loss of heterozygosity (305) and disruption of the INK4-pRb pathway (INK4-CDK4/6-pRb-E2Fs) (306) are common events in the development of most types of cancer. Cancer cells may also evade the growth inhibitory signals of transforming growth factor- β (TGF- β) (307) and modulate the action of downstream effectors as well as crosstalk with other pathways.

Cells also receive growth inhibitory signals through intercellular communication via gap junctions. Gap junctions disperse and dilute growth-inhibiting signals, thereby suppressing cell proliferation. In contrast, loss of gap junctions increases intracellular signaling, leading to enhanced proliferation and tumor formation. The molecular components of gap junctions are the connexin proteins (308). Connexins are recognized as tumor suppressors and have been documented to reduce tumor cell growth. Numerous environmental stimuli have been reported to directly affect gap junction intercellular communication. Adherens junction machinery mediates contact inhibition of growth, and loss of contact inhibition is a mediator of tumor cell growth.

Chemicals that may contribute to insensitivity to antigrowth signals through multiple targets of this hallmark are BPA, a common constituent of everyday plastics, and pesticides such as DDT, folpet and atrazine. BPA promotes proliferation by disrupting the growth inhibitory signals of p53 and gap junction communication (171,309). DDT has also been shown to enhance proliferation by increasing the expression of Ccnd1 (cyclin D1)/E2f, inducing phosphorylation of pRb, increasing the expression of p53-degrading protein Mdm2 (a negative regulator of p53) (162) and disrupting gap-junctional intercellular communication (163,164). Folpet down-regulates the functions of p53 and ATM/ATR checkpoint kinases (167) and promotes proliferation. Atrazine shows genotoxic effects at subacute dose on Wistar rats, and the genotoxicity was also associated with increased transcription of connexin accompanied with increased oxidative stress (310).

Resistance to cell death

Cell death is an actively controlled and genetically regulated program of cell suicide that is essential for maintaining tissue homeostasis and for eliminating cells in the body that are irreparably damaged. Cell death programs include: apoptosis, necrosis, autophagy, senescence and mitotic catastrophe (21). Defects in these pathways are associated with initiation and

progression of tumorigenesis. Normally, cells accumulate from an imbalance of cell proliferation and cell death, permissive cell survival amidst antigrowth signals such as hypoxia and contact inhibition, resistance to the killing mechanisms of immune cell attack and anoikis resistance (311). Increased resistance to apoptotic cell death involves inhibition of both intrinsic and extrinsic apoptotic pathways.

The link between malignancy and apoptosis is exemplified by the ability of oncogenes, such as MYC and RAS, and tumor suppressor genes, such as TP53 and RB, to engage both apoptosis and the aberrant alterations of apoptosis regulatory proteins such as BCL-2 and c-FLIP in various solid tumors (312). This variety of signals driving tumor evolution provides the selective pressure to alter apoptotic programs during tumor development. Some chemical carcinogens and sources of radiation cause DNA damage and increase genetic and/or epigenetic alterations of oncogenes and tumor suppressor genes leading to loss of cellular homeostasis (313). Other signals include growth/survival factor depletion, hypoxia, oxidative stress, DNA damage, cell cycle checkpoint defects, telomere malfunction and oncogenic mutations, and exposure to chemotherapeutic agents and heavy metals (314,315).

Cancer cells resist apoptotic cell death by up-regulation of antiapoptotic molecules and the down-regulation, inactivation or alteration of pro-apoptotic molecules. Activation of p53 usually induces expression of pro-apoptotic proteins (Noxa and PUMA) and facilitates apoptotic cell death (316). Antiapoptotic Bcl-2 family proteins suppress pro-apoptotic Bax/Bak [which would otherwise inhibit mitochondrial outer membrane permeabilization]. Mitochondrial outer membrane permeabilization releases cytochrome c and triggers apoptosis through an intrinsic pathway (317). Thus, regulation of apoptosis can be achieved by inhibiting the antiapoptotic Bcl-2 family proteins and Bcl-X_L proteins as this restores a cell's ability to undergo apoptosis. During the process of, mitochondrial outer membrane permeabilization, mitochondrial proteins (Smac/DIABLO and Omi/HtrA2), which inhibit the X-linked inhibitor of the apoptosis protein, are leaked to trigger caspase activity in apoptosis (318,319).

Normal cellular metabolism is important for the survival of cells, whereas dysregulated metabolism in cells (see Dysregulated metabolism) can induce either apoptosis or resistance to apoptotic stimuli (320). In the liver, nearly every enzyme in glycolysis, in the tricarboxylic acid cycle, in the urea cycle, in gluconeogenesis and in fatty acid and glycogen metabolism is found to be acetylated, and this N- α -acetylation confers sensitivity to apoptotic stimuli (321). The antiapoptotic protein, Bcl-xL reduces the efflux of acetyl-CoA from the mitochondria to the cytosol in the form of citrate and decreases N- α -acetylation of apoptotic proteins, which enables cells less sensitive toward apoptotic stimuli to mediate cell proliferation, growth and survival. Thus, N- α -acetylation might be a major factor in overcoming apoptotic resistance in cancer cells (322,323).

Death receptor ligands such as TRAIL—which is bound to DR4/DR5—induce receptor oligomerization and recruitment of Fas-Associated protein with Death Domain (FADD) and caspase-8 to form death-inducing signaling complex, which leads to subsequent cell death via apoptosis. Thus, expression of death receptors and their decoy receptors (Dcr1/2) mediates apoptosis in tumor cells (324). When normal cells lose contact with their extracellular matrix or neighboring cells, they undergo an apoptotic cell death pathway known as 'anoikis' (311). During the metastatic process, cancerous cells acquire anoikis resistance and dissociate from primary sites, travel through the vascular system and proliferate in distant target organs.

A blockage of gap junction intracellular communication (GJIC) between normal and preneoplastic cells also creates an intra-tissue microenvironment in which tumor-initiated preneoplastic cells are isolated from growth controlling factors of normal surrounding cells resulting in clonal expansion (325). Gap junction channels and Cxs control cell apoptosis by facilitating the influx and flux of apoptotic signals between adjacent cells and hemi-channels between the intracellular and extracellular environments, and Cx proteins (in conjunction with their intracytoplasmic localization), may act as signaling effectors that are able to activate the canonical mitochondrial apoptotic pathway (326).

Several anthropogenic chemicals can affect resistance to cell death. For example, BPA has been shown to strikingly impair TP53 activity and its downstream targets, cell cycle regulators, p21WAF1 and RB, or pro-apoptotic BAX, thereby enhancing the threshold for apoptosis (172).

Chlorothalonil, a broad-spectrum fungicide that is used on vegetables, fruit trees and agricultural crops, is considered to be non-genotoxic but classified as 'likely' to be a human carcinogen by all routes of exposure (29). In a eukaryotic system, chlorothalonil reacted with proteins and decreased cell viability by formation of substituted chlorothalonil-reduced glutathione derivatives and inhibition of specific nicotinamide adenine dinucleotide thiol-dependent glycolytic and respiratory enzymes (327). Caspases (cysteine-dependent proteases) and transglutaminase are some of the thiol-dependent enzymes involved in apoptosis, so inhibition of these thiol-dependent enzymes in tumor-initiated cells may disrupt apoptotic cell death and aid in tumor survival.

Dibutyl phthalate and diethylhexyl phthalate (DEHP) are diesters of phthalic acid and commonly referred to as phthalates. In general, they mimic the function or activity of the endogenous estrogen 17 β -estradiol (E2) and bind to estrogen receptors. Interestingly, phthalates can mimic estrogen in the inhibition of TAM-induced apoptosis in human breast cancer cell lines by increasing intracellular Bcl-2/Bax ratio in breast cancer (328).

Lindane, an organochlorine pesticide, bioaccumulates in wildlife and humans. Exposure to lindane induces tumor formation in the mouse 42GPA9 Sertoli cell line by disrupting the autophagic pathway and sustained activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway (329).

MXC (1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane) is a DDT derivative that was developed after the ban of DDT and it exhibits antiandrogenic and estrogenic activity. MXC stimulates proliferation and human breast cancer cell growth by the up-regulation of genes that involve cell cycle (cyclin D1), and the down-regulation of genes p21 and Bax affecting G₁/S transition and apoptosis, respectively, through ER α signaling (330).

Replicative immortality

Cellular senescence is a state of irreversible arrest of cellular proliferation characterized by changes in transcription, chromatin conformation, cytoplasmic and nuclear morphology, DNA damage signaling and a strong increase in the secretion of proinflammatory cytokines (331). Senescence is the first line of defense against potentially transformed cells (332). Progression to malignancy correlates with a bypass of cellular senescence. Thus, senescence inhibits the activation of the tumorigenic process (332). Senescence has been observed *in vitro* and *in vivo* in response to various stimuli, including telomere shortening (replicative senescence),

oncogenic stress, oxidative stress and chemotherapeutic agents (333).

Cellular senescence exhibits several layers of redundant regulatory pathways. These pathways converge to arrest the cell cycle through the inhibition of CDKs. The best-known effector pathways are the p16INK4a/pRB, the p19ARF/p53/p21CIP1 and the PI3K/mammalian target of rapamycin (mTOR)/FOXO pathways (334–337), which show a high degree of interconnection. Additionally, the pRB and the mTOR pathways are two routes that have been proposed to be responsible for permanent arrest of the cell cycle (338). More pathways and genes are being discovered, increasing the complexity of our knowledge of this physiological process (336). Most, if not all of these genes have been related to human tumorigenesis.

Despite the relevance of senescence as a gatekeeper in the process of tumorigenesis, there is not a large body of information exploring the effect of chemicals on this safeguard. Little research has been undertaken on chemicals that alter gene expression regulating senescence and few genes have been identified (e.g. telomerase, p53, pRB, INK4a) (83,339,340). Traditional protocols for the assessment of the carcinogenic risk rely on the detection of tumors induced by agents that alter many different pathways at the same time (including senescence). These agents are mainly unspecific mutagens or epigenetic modifiers. The effect of some compounds is being explored including nickel-derived compounds (e.g. nickel chloride), diethylstilbestrol, reserpine or phenobarbital (83,341–344).

There may be environmental chemicals that are not mutagens or epigenetic modifiers, but that target specific proteins on the senescence pathways and may affect the initiation of tumorigenesis by other compounds allowing senescence bypass. The contribution of these compounds to the carcinogenesis process is largely unknown. A few compounds bypass senescence in this specific manner—acetaminophen, cotinine, nitric oxide, Na-selenite and lead. Other chemicals known to alter senescence only are mostly unknown (86,88–91,345–348).

Senescence has strong fail-safe mechanisms, and experimental attempts to bypass senescence are usually recognized as unwanted signals and trigger a senescence response anyway. However, these conclusions are based on the interpretations of experimental designs in which acute molecular or cellular alterations are produced. There are few experiments regarding the effects of chronic, low-dose alterations and even fewer studies that consider the different cellular and molecular contexts that can arise over the course of a lifetime.

Dysregulated metabolism

The highly glycolytic cancer phenotype described by Warburg *et al.* (25) in the early 20th century determined much of the initial direction in cancer research (26). Other characteristic metabolic abnormalities have also been described (25,26,349,350) and have recently garnered increased attention (351–355). These changes are neither fixed nor specific for cancer (356–358), but the universality of metabolic dysregulation suggests major roles in cancer genesis, maintenance and progression. Precise definitions of what constitutes cancer metabolism, and when such changes first occur during the course of cancer development, are lacking. From a teleological perspective, alterations in both intermediary metabolism and its control are not surprising insofar as highly proliferative cancer cells exhibit increased energy demands and expanded requirements for macromolecular precursors to support nucleic acid and protein biosynthesis, as well as membrane biogenesis, for increased biomass. Metabolic reprogramming ostensibly equips cancer cells to cope with these demands, as

well as accompanying cellular stresses. Although much of the attention on cancer metabolism has focused on enhanced glucose utilization via glycolytic and pentose phosphate pathways, cancer cells are also capable of the oxidative utilization of carbohydrates, lipids and peptides, and the metabolism of these individual substrate classes remains intimately intertwined as in normal cells (26,352,359).

Major control of glycolysis is traditionally ascribed to glucose transport, hexokinase, phosphofructokinase and pyruvate kinase (359). Glyceraldehyde-3-phosphate dehydrogenase also normally couples glycolytic flux to mitochondrial metabolism in the presence of oxygen and to lactate generation in its absence, but this relationship is fundamentally altered in cancer (26,352,360,361). Given the central importance of the pentose phosphate pathway to anabolic metabolism and redox homeostasis, glucose-6-phosphate dehydrogenase and its redox coupling partners represent similarly attractive carcinogenic targets (362). In addition, the enzymes of the tricarboxylic acid cycle, such as fumarate hydratase, succinate dehydrogenase and isocitrate dehydrogenase, play crucial roles in oxidative energy metabolism and the interconversion of metabolic intermediates, making them appealing candidates for study as well (363,364).

The central importance of the mitochondrial electron transport chain to oxidative energy metabolism and its established role in toxic responses and dysregulated mitochondrial function in cancer makes its assembly and function attractive topics for study (365–367). Despite well-established roles for lipid and amino acid metabolism in cancer development and progression, they have historically received less attention than carbohydrate metabolism (26). Lipogenic, lipolytic and lipophagic phenotypes are now widely recognized (351,368–370), so targets such as acetyl-CoA carboxylase, fatty acid synthase, cellular lipases and lipid transporters represent additional attractive targets for study. Amino acid metabolism—particularly glutamine and serine metabolism—also has well-established roles in cancer (371–373), providing additional potential targets for study that include 3-phosphoglycerate dehydrogenase (353,372,374,375) and cellular transaminase coupling mechanisms. Study of both lipid and protein metabolism must accommodate the fact that cancer cells exhibit substrate preferences, including well-described endogenous lipid- and protein-sparing effects of exogenous glucose availability in cancer cells.

The metabolic capacity of both normal cells and cancer cells generally exceeds their catabolic and anabolic requirements (371,376,377), and only a fraction of the available potential energy is ultimately required for cell survival (378,379). Moreover, very small changes in metabolic flux can have profound phenotypic consequences, and metabolic control analysis has suggested that the importance of increased cancer-associated glycolytic and glutaminolytic fluxes may lie not in their magnitudes, but in the maintenance and control of smaller branched pathway fluxes (371). For these reasons, rigorous functional validation is needed for all cancer-associated changes in gene expression or metabolite accumulation. Well-described moonlighting functions for many metabolic enzymes (380–382), including the novel antiapoptotic roles of mitochondrial hexokinases (383), cannot be simply extrapolated from our knowledge of classical roles in cellular metabolism.

These enzymes and their pathways constitute broad categories of potential targets for disruption that could serve to enable the observed metabolic phenotypes of cancer cells (384). Although metabolic control is broadly distributed over all individual steps for a given pathway (359,385), the most obvious

targets for conceptual and experimental scrutiny involve major rate-controlling elements of pathways capable of supporting the anabolic and catabolic needs of rapidly proliferating cancer cells.

Numerous studies have demonstrated cancer-associated changes in metabolism or related gene expression (26). We looked at acrolein, copper, cypermethrin, diazinon, hexythiazox, iron, malathion and rotenone as chemicals that had been reported to show relevant disruptive potential (51,386–390); however, the toxicological data that are available for many suspected or known environmental disruptors, generally lack mechanistic information regarding their potential roles as determinants of the observed metabolic hallmarks of cancer. Even prior metabolic screening platforms, including tetrazolium reduction assays, have limited specificity and can be profoundly influenced by experimental screening conditions. Unfortunately, standardized chemical screening has typically not been conducted under controlled or limiting substrate conditions that would directly inform our understanding of the functional relevance of observed changes. None have established unambiguous causal relationships between specific chemical exposures and the parallel or sequential development of dysregulated metabolism of cancer in the same model, and most observed changes in gene expression with potential relevance to cancer metabolism have not been accompanied by validating functional studies.

Angiogenesis

Angiogenesis, the process of formation of new blood vessels from existing blood vessels, is a critical process for normal organ function, tissue growth and regeneration (e.g. wound healing, female menstruation, ovulation and pregnancy) as well as for pathological conditions (e.g. cancer and numerous non-cancerous diseases, such as age-related macular degeneration, diabetic retinopathy, rheumatoid arthritis, endometriosis, diabetes and psoriasis) (391,392).

Tumor angiogenesis is an early critical event for tumor development: A tumor cannot grow beyond 1 mm³ (by estimate) without angiogenesis (393). Tumor growth, invasion and metastasis depend on blood vessels and neovascular development to provide nutrients, oxygen and removal of metabolic waste as tumors grow in primary sites, invade adjacent tissues and metastasize to distant organs (394,395). Inhibition or eradication of tumor angiogenesis by antiangiogenic inhibitors (396,397) or by antineovascular agents (such as vascular-disrupting agents (398–400) and fVII/IgG Fc (401), the latter also called ICON (402–404)) can treat pathological angiogenesis-dependent diseases, including cancer and many non-cancerous diseases.

Under physiological conditions, angiogenesis is well balanced and controlled by endogenous proangiogenic factors and antiangiogenic factors. Factors produced by cancer cells can shift the balance to favor tumor angiogenesis. Such factors include vascular endothelial growth factor (VEGF) and tissue factor (TF). VEGF, one of the most potent proangiogenic factors produced by cancer stem cells and cancer cells, binds to vascular endothelial cells via its receptor VEGFR, initiating VEGF/VEGFR intracellular signal transduction pathways and activating many gene transcriptions and translations toward angiogenesis. TF is a transmembrane receptor (405) not expressed on quiescent endothelial cells (406,407). Upon stimulation of VEGF, TF is selectively expressed by angiogenic endothelial cells, the inner layer of the tumor neovasculature. Thus, TF is a specific biomarker for tumor angiogenesis (408–410). Both of the membrane-bound receptors VEGFR and TF can mediate separate intracellular signaling pathways that contribute to tumor angiogenesis.

Environmental exposures can promote tumor development, but the role of chemicals in tumor angiogenesis, particularly the role of low-dose *non-carcinogens*, is largely unknown. Some food-use pesticides that are non-genotoxic act as tumor promoters, and other chemicals affect various hallmarks such as apoptosis, proliferative signaling, evading growth suppression, enabling replicative immortality, metastasis, avoiding immune destruction, tumor-promoting inflammation and deregulating cellular energetics—in addition to tumor angiogenesis.

Chemical disruptors that may promote tumor angiogenesis included diniconazole, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), methylene bis(thiocyanate), perfluorooctane sulfonate (PFOS), ziram, biphenyl, chlorothalonil, tributyltin chloride and bisphenol AF. Diniconazole (pesticide), for example, targets certain angiogenic molecules (CXCL9, CXCL10, MMP1, uPAR, VCAM1 and THBD) *in vitro* (29). MXC (the parent compound to HPTE) induces histological expression of angiogenic factors such as VEGF, VEGFR2 and ANG1 in rat pituitary and uterus (39), and exposure to PFOS induces actin filament remodeling, endothelial permeability changes and ROS production in human microvascular endothelial cells (41). ziram can induce angiogenesis through activation of MAPK and decreases cytolytic protein levels in human natural killer (NK) cells (411,412).

Tissue invasion and metastasis

Tissue invasion and metastasis are also key processes of tumor progression. In normal cells, E-cadherin holds the epithelial cells together as a society of cells that are well differentiated and otherwise quiescent (413). Carcinomas constitute almost 90% of cancers and upon oncogenic transformation, the process of tissue invasion and metastasis begins with the down-regulation of E-cadherin. Concomitant with this down-regulation of E-cadherin is the conversion of epithelial to mesenchymal cells (EMT) (414). The transcription factors that control EMT, such as Snail, Slug, Twist and Zeb1/2, are some of the best-characterized signaling molecules in biology (415,416). During the process of EMT, a number of inflammatory cells are attracted to the growing tumor mass (417). Upon attaining mesenchymal characteristics, tumor cells are able to move out of their natural environment, aided by cross talk between them and stromal cells, resulting in the secretion of matrix degrading enzymes such as matrix metalloproteinases (418). This process is accelerated by chronic inflammation mediated by NF- κ B (417). Other invasion mediating molecules include hepatocyte growth factor, secreted mainly by tumor-associated fibroblasts to signal metastatic cells to move upon their interactions with their cell surface receptor cMet (419).

Attracted by chemokines, metastatic cells move to the nearest blood vessel or lymphatic vessel, where they complete the process of intravasation, entering the capillaries and are then transported to the capillary bed in their colonized site or new environment (420). In this new location, tumor cells undergo extravasation where they come out of the capillaries or lymphatic vessels, most likely again following the cues emanating from the chemokines in their new microenvironments. To survive in their new home, they may have to revert back and assume the cuboidal morphology of epithelial cells—undergoing the reversal of EMT otherwise known as mesenchymal to epithelial transition (421). At this point, they may remain dormant for a very long time until conditions for their division and growth become favorable.

Mounting evidence supports the involvement of exosomes (nano-vesicles secreted by tumor or cancer-associated fibroblasts) in adhesion and motility of metastatic cells. The secretion

of exosomes is accelerated by increases in intracellular calcium ions, and low-dose environmental mixtures that increase intracellular calcium may promote the secretion of exosomes and the subsequent invasion and metastasis processes of the tumor cells.

Environmental chemicals, such as tetrabromobisphenol A and its metabolites, BPA and tetrabromobisphenol A dimethyl ether, which mediate the activation of EMT enzymes or drive their synthesis, may also contribute to the process of tissue invasion (422). Low-dose exposure to hexavalent chromium may accelerate the EMT transition (423). Other contributing factors may also be low-dose environmental contaminants, such as formaldehyde, or bacteria, e.g. *H. pylori*, that drive the transcription of NF- κ B and exacerbate the process (424,425).

Tumor microenvironment

The tumor microenvironment is a complex mix of cells in addition to tumor cells themselves; it is constructed of a complex balance of blood vessels that feed the tumor, the extracellular matrix that provides structural and biochemical support, signaling molecules that send messages, soluble factors such as cytokines and many other cell types. Tumors can influence the microenvironment and *vice versa*. The micro-environmental reaction to early tumor cells begins with the recruitment and activation of multipotent stromal cells/mesenchymal stem cells, fibroblasts, endothelial cell precursors, antigen-presenting cells such as dendritic cells (DCs) and other white blood cells. All of these tumor stromal cells secrete a variety of growth factors and chemokines that, together with the tumor cells and secreted factors, culminate in the generation of the tumor microenvironment (426–429).

The tumor microenvironment is important because any cell within this process has the potential to be affected by carcinogens, either alone or in mixtures, or by the inflammation that results from the carcinogenic insult (430). Although often associated with infection, chronic inflammation can be caused by exposure to carcinogens such as irradiation or environmental chemicals. Carcinogenesis can also be fostered via effects on the tissue context surrounding preneoplastic lesions. For example, transplantation experiments of preneoplastic cells have clearly documented that a growth-constrained tissue microenvironment can promote the growth and progression of preneoplastic cell populations (431).

Several compounds appear to influence the complex heterogeneity that forms the support network for cancer growth. The exposure to nickel chloride has been associated with the generation of ROS and inflammation (432). ROS are important because they can stimulate the induction of angiogenesis growth factors, such as VEGF, and can promote cell proliferation and immune evasion and play a role in cell survival (57,433–435). Prenatal exposure to BPA in experimental animals disrupts ER α and triggers angiogenesis, and other BPA exposure studies have demonstrated that BPA interplays with cell proliferation (226), genomic instability (436), inflammation (437) and cell immortalization (438). Butyltins, and specifically tributyltin, which is suspected to act as an endocrine disruptor, have been found to inhibit the cytotoxic activity of NK cells (439), affect inflammation (439) and disrupt membrane metalloproteinases (439). Cooperatively, disruption of these processes can lead to proliferation, migration and angiogenesis. Methylmercury (MeHg) is a neurotoxic compound deriving from metallic mercury through bacteria-supported metabolism in an aquatic environment. Bio-concentration in fish and shellfish poses a risk for sensitive population categories such as pregnant women and infants.

MeHg-induced ROS production may be involved in inflammation and apoptosis (440) as well as endothelial cell cytotoxicity (441). We also looked at paraquat, which may also have relevance for the tumor microenvironment via its role in oxidative stress (442,443).

Avoiding immune destruction

The concept of immune surveillance suggests that the host immune system could identify tumor cells and destroy them. If this is true, tumor cells need to be poor stimulators of or challenging targets for the host immune system. To provide an effective immune response, multiple types of the cells are involved within innate and adaptive immune 'arm' with some cells (e.g. DCs and the NK cells) 'bridging' these two types of immunity (444). To avoid a strong immune response of the host, the expression of tumor antigens may be down-regulated or altered (resulting in decreased or impossible recognition of malignant cells) (445) and various soluble factors and cytokines may be released resulting in subverted effectiveness of antitumor immune response (446–448). Tumor cells can also escape host immune response by inducing apoptosis in activated T cells (449).

Multiple genes are involved in immune evasion mechanisms and, therefore, can interfere with chemical exposures from anthropogenic environment: ADORA1 (adenosine A1 receptor), AKT1 (v-akt murine thymoma viral oncogene homolog 1), CCL2 (chemokine C-C motif ligand 2), CCL26 (chemokine C-C motif ligand 26), CD40, CD69, COL3A1 (type III collagen of extracellular matrix), CXCL10 (also called interferon-inducible protein-10), CXCL9 (monokine induced by interferon- γ), EGR1 (early growth response protein 1), HIF-1 α (hypoxia-inducible factor), IGF1R (insulin-like growth factor 1 receptor) and interleukins (IL) such as IL-1 α and IL-6. Based on available studies, several candidate signaling pathways that are related to the host immune response can be identified for further study; e.g. the pathways involving PI3K/Akt, chemokines, TGF- β , FAK, IGF-1, HIF-1 α , IL-6, IL-1 α , CTLA-4 and PD-1/PDL-1.

Biologically disruptive environmental chemicals can affect the host immune responses as follows: (i) if a certain chemical is immunotoxic, and, in particular, if it affects activity of DCs, T cells or NK cells, it is also likely to affect tumor immuno-surveillance and enable malignant growth to proceed; (ii) if a chemical targets the immune system, it can increase the cancer risk related to other factors/exposures; (iii) exposures to certain toxins or toxicants can dramatically increase the number of cancerous cells and impact immuno-regulatory signals suppressing the mechanisms of immune control. Collectively, these sorts of actions suppress the immune system, so it cannot be effectively stimulated and cannot eliminate tumor cells, thus allowing some tumor cells to escape and metastasize.

We looked at several groups of environmentally ubiquitous chemicals such as pesticides and personal care products that might potentially interrelate with mechanisms of tumor immuno-surveillance. Although none of them are recognized as human carcinogens (450–452), the research on these chemicals and their interactions with the immune response may be valuable. For example, the fungicide maneb is a cortisol disruptor (453) that has shown a wide spectrum of potential effects on multiple pathways, including some that are relevant to immune evasion (139,156–158,454). By comparison, pyraclostrobin and fluoxastrobin (455) interfere with a narrower spectrum of cancer hallmarks (36,456–459). Atrazine has also shown potential to impact immune system evasion by directly targeting maturation of DCs and decreasing the levels of major histocompatibility

complex class I molecules (243,460). The insecticides pyridaben and azamethiphos can also both be disruptive to immuno-surveillance (139,140,461,462).

Commonly used in personal care products, triclosan and BPA (463), are endocrine disruptors (464–466) that are often detected in waters downstream in urban areas (467,468). In addition to immune evasion mechanisms (36,142,145), they interfere with wide spectrum of cancer-related mechanisms (36,173,436,469–471). DEHP (472) is also an endocrine disruptor (473,474) that can impact multiple hallmarks such as immune evasion, resistance to cell death, evasion of antiproliferative signaling, sustained proliferative signaling and tumor-promoting inflammation (36,288,475,476).

Knowing whether or not cumulative low-dose exposures to these chemicals interfere with the host immune response can help to stimulate further studies (e.g. on screening of lesions at the pre-malignant stage of tumor development) to determine the influence of such exposures on host immunity and to evaluate their potential to increase the risk of tumor cell survival.

Dose-response characterizations and LDE

For all the chemicals selected and target sites for disruption that were identified, dose-response characterization results and/or relevant low-dose research evidence were reviewed and categorized using the criteria mentioned in the Materials and methods. Table 1 sets out these results and the supporting references.

In total, 85 examples of environmental chemicals were reviewed (for specific actions on key pathways/mechanisms that are important for carcinogenesis) and 59% of them (i.e. 50/85) were found to exert LDE (at levels that are deemed relevant given the background levels of exposure that exist in the environment) with 15 of the 50 demonstrating their LDE in a non-linear dose-response pattern. Indeed, all of the teams selected at least one or more disruptive chemicals that exerted their effects on the target sites at low-dose levels. In contrast, only 15% of the chemicals reviewed (i.e. 13/85) showed evidence of a threshold.

The remaining 26% of the chemicals reviewed (i.e. 22/85) were categorized as 'unknown'. Some of these chemicals (5 of the 22) had been tested using human primary cell data from ToxCast and had showed statistically significant activity across a full range of doses against the specified targets (i.e. they were active even at the lowest test concentrations of ~0.01 μ M). However, even though no threshold could be discerned for these chemicals, we did not characterize them as having LDE (because it was not clear that the lowest test concentrations were low enough to be equated to levels of exposure that are normally seen in the environment).

Evidence of cross-hallmark relationships

Teams then evaluated the chemicals selected and target sites for disruption for known effects on the other cancer hallmark pathways. Evidence in the literature that showed procarcinogenic actions or anticarcinogenic actions in other hallmark areas were reported, and in instances where no literature support was found, this was documented as well. The same approach was used for the chemicals that were reviewed. A sample of these cross-hallmark results is provided in Table 2—Sample of cross-hallmark relationships of target pathways/mechanisms and in Table 3—Cross-hallmark relationships of selected chemical disruptors.

Note that Tables 2 and 3 contain just a single set of unreferenced results from the review on the hallmark insensitivity to

Table 2. Sample of cross-hallmark relationships of target pathways/mechanisms

Insensitivity to antipgrowth signals (targets)	Antipgrowth	Dysreg metab	Gen instab	Angio	Cell death	Immun	Immort	Prolif	Metas	Inflamm	Tumor micro	PRO	ANTI	MIX
p53	n/a	+/-	-	+/-	-	+/-	-	-	-	+	+	2	5	3
pRB	n/a	+/-	-	-	-	0	-	-	-	+	+	2	6	1
TGF-β	n/a	+	-	+	-	+	-	+	+	+	+	7	3	0
LKB1	n/a	+	-	+	+/-	0	0	+	-	+	+	5	2	1
Connexins	n/a	-	-	0	0	0	0	-	+/-	+	+	2	3	1
Contact inhibition	n/a	+/-	-	0	0	+	0	-	-	+	-	2	4	1

One set of results (from the insensitivity to antipgrowth signals review) is shown here without references to support a discussion on the range of effects that have been reported for the selected targets in each article. Specific references supporting these effects for any given hallmark area can be found in the individual reviews within this special issue. Cross-hallmark relationships are reported in the first 11 columns of the table—table heading abbreviations are as follows:gen instab, genetic instability; dysreg metab, dysregulated metabolism; antipgrowth, insensitivity to antipgrowth signals; angio, angiogenesis; cell death, resistance to cell death; immun, avoiding immune destruction; immort, replicative immortality; prolifer, sustained proliferative signaling; metas, tissue invasion and metastasis; inflamm, tumor-promoting inflammation; tumor micro, tumor microenvironment. The number of procarcinogenic (PRO), anticarcinogenic (ANTI) and mixed (MIX) (i.e. procarcinogenic and anticarcinogenic reports) cross-hallmark relationships for each target have been summed and are reported in the last three columns of the table. Target pathways/mechanisms for each hallmark area were evaluated by each team for known effects in other cancer hallmark pathways. Targets that were found to have anticarcinogenic actions in another hallmark area were indicated with ‘-’, whereas targets that were found to have procarcinogenic actions in another hallmark area were indicated with ‘+’. In instances where reports on relevant actions in other hallmark areas were mixed (i.e. reports showing both procarcinogenic potential and anticarcinogenic potential), the symbol ‘+/-’ was used. Finally, in instances where no literature support was found to document the relevance of a target in a particular aspect of cancer’s biology, we documented this as ‘0’.

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Table 3. Cross-hallmark relationships of selected chemical disruptors

Insensitivity to antipgrowth signals (disruptors)	Antipgrowth	Dereg metab	Gen instab	Angio	Cell death	Immun	Immort	Prolif	Metas	Inflamm	Tumor micro	PRO	ANTI	MIX
BPA	n/a	+	+	+	+/-	0	+	+	+	+	0	7	0	1
DDT	n/a	0	+	+	+	+	+	+	0	+	0	7	0	0
Folpet	n/a	0	+	0	+	0	0	+	0	+	0	4	0	0
Atrazine	n/a	0	+	0	0	0	0	+	0	+	0	3	0	0

One set of results (from the insensitivity to antipgrowth signals review) is shown here without references to support a discussion on the range of effects that have been reported for the selected disruptors in each review. Specific references supporting these effects for any given hallmark area can be found in the individual reviews within this special issue. Cross-hallmark relationships are reported in the first 11 columns of the table—table heading abbreviations are as follows:gen instab, genetic instability; dereg metab, dysregulated metabolism; antipgrowth, insensitivity to antipgrowth signals; angio, angiogenesis; cell death, resistance to cell death; immun, avoiding immune destruction; immort, replicative immortality; prolifer, sustained proliferative signaling; metas, tissue invasion and metastasis; inflamm, tumor-promoting inflammation; tumor micro, tumor microenvironment. The number of procarcinogenic (PRO), anticarcinogenic (ANTI) and mixed (MIX) (i.e. procarcinogenic and anticarcinogenic reports) cross-hallmark relationships for each target have been summed and are reported in the last three columns of the table. Prototypical chemical disruptors selected by each team were evaluated for reported actions in other cancer hallmark pathways. Disruptors that were found to have anticarcinogenic actions in a particular hallmark area were indicated with ‘-’, whereas disruptors that were found to have procarcinogenic actions in a particular hallmark area were indicated with ‘+’. In instances where reports on relevant actions in other hallmarks were mixed (i.e. reports showing both procarcinogenic potential and anticarcinogenic potential), the symbol ‘+/-’ was used. Finally, in instances where no literature support was found to document the relevance of a chemical in a particular aspect of cancer’s biology, we documented this as ‘0’. Specific references supporting these effects for any given area can be found in the individual reviews in this special issue.

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antigrowth signals. This is intended only to illustrate the categories of cross-hallmark effects that were reviewed and to show how they were presented. Fully referenced results for each hallmark area can be found in each of the individual reviews within this special issue.

The decision to review target sites for disruption and prototypical disruptors for cross-hallmark effects was driven by the fact that many individual studies and reviews of chemical exposures fail to account systematically for the spectrum of incidental actions that can result from exposures to a single given chemical. It was our belief that this approach constitutes a better way to ensure that we had assembled a reasonably complete view of the literature (i.e. where any sort of evidence of cross-hallmark activity had been reported). Future research will likely involve empirical testing of mixtures, so we wanted to create a heuristic that could serve as a starting point for other researchers who might be considering such research.

For researchers focused on low-dose exposure research intended to produce carcinogenesis, we anticipated that there would be interest in chemicals that had been reported to exhibit a large number of procarcinogenic actions across a number of hallmarks and we anticipated that a lack of anticarcinogenic potential would be important to identify (as targets or approaches that exert anticarcinogenic actions would potentially represent a confounding influence/factor in empirical research aimed at the identification of carcinogenic synergies). To that end, [Table 4](#) provides a summary of the aggregated number of procarcinogenic actions, anticarcinogenic actions and instances where mixed actions (i.e. procarcinogenic and anticarcinogenic) have been found for each pathway/mechanism (across the full range of hallmark domains—i.e. from all of the areas covered by the reviews in this special issue). Similarly, [Table 5](#) provides a summary of the aggregated number of procarcinogenic actions, anticarcinogenic actions and mixed actions (i.e. procarcinogenic and anticarcinogenic), where cross-hallmark effects have been reported for each chemical (across the full range of hallmark domains—i.e. from all of the areas covered by the reviews in this special issue).

Note that, in some instances, the underlying evidence used to support the indication of cross-hallmark relationships was robust, consisting of multiple studies involving detailed *in-vitro* and *in-vivo* findings. In other instances, the underlying evidence that was used to report the existence of a cross-hallmark relationship was quite weak (e.g. consisting of only a single *in-vitro* study involving a single cell-type). The selected prototypical disruptors are likely biased towards agents that have been extensively studied, and not necessarily those that will prove to be the most important biologically. Finally, there are examples of chemicals that are known to exert different effects at different dose levels, but dose levels were not used to discriminate when gathering evidence of cross-hallmark effects. So, the referenced cross-validation results in the individual tables (reported in the many reviews within this special issue) should be seen only as a starting point for those who are pursuing mixtures research (e.g. references would need to be further scrutinized to determine whether or not the dose levels noted for specific results are suitable points of reference for the type of research that is being undertaken).

Particular attention should also be given to results related to the endocrine system due to mechanistic complexity. For example, xeno-estrogen compounds are typically compared with estradiol based on binding affinity strength. However, many xeno-estrogens that are 'weak' by this measure can alter the steroidogenic cascade (e.g. significantly up-regulate the activity of P450 aromatase, the enzyme that increases intracellular

estradiol synthesis within estrogen-sensitive cells (477–480) or alter levels of ER α or the ratio of ER α :ER β (260)). In other words, a weak xeno-estrogen can stimulate the production of estradiol, a potent endogenous carcinogen (481) or alter the receptors with which a cell will respond to estrogen.

Nonetheless, given that the overarching goal in this project was to create a foundation that would allow researchers to look systematically across the literature in each of these areas, the tables should serve as a useful starting point as long as they are approached with these caveats in mind. We believe that this heuristic will be useful to consider synergies that might be anticipated in testing that involves certain target sites for disruption and/or mixtures of chemical constituents that are being considered for procarcinogenic effects. Future research efforts to improve this approach could involve a large-scale collaborative effort to generate high-quality *in-vitro* data and low-dose *in-vivo* data in a range of predefined tissues.

Discussion

Getting to Know Cancer hosted the initial project meeting in Halifax, Nova Scotia giving participants an opportunity to have presentations, break-out sessions, and chances for conversation and debate among experts who came from a range of different disciplines. Cancer biologists with specialized expertise in areas related to individual hallmarks met with specialists from other areas such as environmental health, toxicology and endocrinology. Although some researchers in the field of environmental health are cancer scientists in their own right, many conference participants commented on the novelty of having an opportunity to work so closely with cancer biology specialists. As a result, many interdisciplinary barriers were removed and the discussions that ensued were challenging but productive.

At the outset, participants overwhelmingly agreed that the Hallmarks of Cancer provides a useful organizing heuristic for systematic review of ways that biologically disruptive chemicals might exert procarcinogenic and anticarcinogenic influences in biological systems. Most of the individual writing teams were then readily able to identify ubiquitous environmental contaminants with disruptive potential in their respective areas of study. The only teams that had significant challenges in this regard were the ones that focused on the bypassing of senescence (i.e. *replicative immortality*) and deregulated metabolism, both being areas of cancer research that have not yet received a lot of attention from researchers in the field of toxicology.

Considerable discussion was devoted to the criteria that were used to select prototypical disruptors from the long list of known potential contaminants. Indeed, it seems that much of the population is now exposed to a wide variety of exogenous chemicals that have some disruptive potential, but we did not have any intention of implicating any of the selected chemicals as being carcinogenic *per se*. It was simply agreed that chemicals would be chosen that met the basic criteria and that then could be used as 'prototypical' disruptors. In other words, the chemicals that were selected for this review were not deemed to be the most important, and they were not selected to somehow imply (based on current information) that they are endangering us. Rather, we simply wanted to illustrate that many non-carcinogenic chemicals (that are ubiquitous in the environment) have also been shown to exert effects at low doses, which are highly relevant to the process of carcinogenesis. We also wanted to lay out a heuristic framework that would be helpful for other researchers who are interested in considering these and other chemicals as potential constituents for low-dose mixtures research.

Table 4. Aggregated evidence of cross-hallmark effects for selected pathways/mechanisms

Key targets	Originating review	Procarcinogenic	Anticarcinogenic	Mixed
Adenosine A1 receptor (ADORA1)	ISE	3	1	0
AhR	Ang	7	0	2
	SPS	7	0	2
Bcl-2/p53	RCD	6	2	1
Cell cycle/cell division: spindle defect	GI	6	0	1
Checkpoint kinase 1 and checkpoint kinase 2 (Chk1/2)	EAS	4	3	1
Chemokine (C-C motif) ligand 2 (CCL2)	Ang	8	1	0
Chemokine (C-XC motif) ligand 2 (CXCL2)	Ang	4	1	1
Chemokine (C-XC motif) ligand 10 (CXCL10)	Ang	3	2	0
Chemokine (C-XC motif) ligand 9 (CXCL9)	ISE	6	1	2
Chemokine signaling pathway (CCL2, CCL26, CXCL9, CXCL10)	TM	6	1	1
Chronic oxidative stress	TIM	5	1	0
Clock-genes-mediated metastasis	Ang	3	0	0
Collagen type III (COL3)	EAS	4	3	0
Contact inhibition	TIM	3	1	1
cSrc/Her1/STAT5B/ERK1/2	SPS	4	0	2
Cyclin D, IL8, CXCL	TIM	8	1	0
Cyclooxygenase expression and stimulation calcium signaling in migration.	TPI	8	1	0
Cyclooxygenase-2	GI	8	1	0
DNA damage signaling: disturbed by Redox signaling (NF-κB, Nrf, EGR)	GI	6	0	2
DNA repair pathways	GI	7	0	1
Eck fatty acid metabolism	GI	6	1	1
Electron transport chain complexes II and IV	DM	6	1	2
Epidermal growth factor receptor	DM	3	2	0
Epigenetic pathways	SPS	6	0	1
Disturbed miRNA binding	GI	6	0	2
DNA methylation	GI	7	0	1
Histone acetylation	GI	6	1	1
EMT	TIM	5	0	1
EMT, catenin-Wnt pathway	TIM	6	1	1
ErbB-2/HER-2 tyrosine kinase	RCD	6	1	0
ERK/MAPK	RCD	8	2	0
Estrogen receptor	TPI	5	3	1
Estrogen receptor α (binding to)	RCD	5	1	1
Gap junction connexins	EAS	2	2	2
GJIC	RCD	2	1	1
Gluconeogenesis	DM	5	3	0
Glycolysis	DM	8	1	0
Hexokinase 2	DM	6	1	0
H-Ras	SPS	6	1	2
Hypersecretion of luteinizing hormone by gonadotroph cells in pituitary gland	RCD	2	1	0
HIF-1-α pathway	ISE	8	0	2
Inducible nitric oxide synthase	TPI	6	1	0
IGF-1 signaling pathway	ISE	6	2	1
Intercellular adhesion molecule 1 (ICAM1)	Ang	6	3	0

Table 4. Continued

Key targets	Originating review	Procarcinogenic	Anticarcinogenic	Mixed
IL-6	TPI	7	0	0
IL-6 expression, improper DC maturation and polarization	TM	5	2	0
Jun/Fos/AP1	SPS	4	1	3
Lipid metabolism/cholesterol metabolism	DM	4	2	1
Liver kinase B1 (Lkb1)	EAS	4	2	2
MMP 1	Ang	6	1	0
MMP-9 activation	TIM	5	1	1
Mitochondrial function	GI	5	2	2
MAPK	RCD	9	0	1
mTOR activation	DM	7	1	1
mTOR inactivation	RI	3	6	1
NK cell inhibition	TM	4	3	0
NF-κB	TPI	4	2	0
Oxidative stress and IL-6 production	TM	3	1	1
P16/p53	RCD	4	4	0
P53 inactivation	EAS	10	0	0
	RCD	10	0	0
	RI	10	0	0
PPAR	SPS	5	2	0
PPAR-α	RCD	3	3	1
PI3K/Akt signaling pathway	ISE	9	0	1
Pyruvate dehydrogenase (PDH)	DM	1	5	0
ROS (increase)	DM	6	0	4
ROS and cellular stress	TM	5	0	4
Retinoblastoma protein (pRb) inactivation	EAS	9	0	0
	RI	9	0	0
Steroid hormone receptors	SPS	5	0	1
Telomerase activation	RI	9	1	0
Telomere loss	GI	4	4	0
The tricarboxylic acid cycle	DM	5	4	0
Thrombomodulin	Ang	2	3	0
Transforming growth factor β	EAS	6	3	1
Tumor necrosis factor α	TPI	8	0	1
Urokinase receptor (uPAR)	Ang	6	2	0
Vascular cell adhesion molecule 1 (VCAM1)	Ang	6	0	0

Aggregated number of procarcinogenic actions, anticarcinogenic actions and instances where mixed actions (i.e. procarcinogenic and anticarcinogenic) where cross-hallmark effects have been reported (for each pathway/mechanism across the full range of hallmark domains—i.e. from all of the areas covered by the reviews in this special issue)—see samples of this data in Table 2. Note: fully referenced data for these cross-hallmark effects can be found in each of the reviews in this special issue. ANG, angiogenesis; DM, deregulated metabolism; EAS, evasion of antitumor growth signaling; GI, genetic instability; ISE, immune system evasion; RCD, resistance to cell death; RI, replicative immortality; SPS, sustained proliferative signaling; TIM, tissue invasion and metastasis; TM, tumor microenvironment; TPI, tumor-promoting inflammation.

Table 5. Aggregated evidence of cross-hallmark effects for selected chemical disruptors

Chemicals	Originating review	Procarcinogenic	Anticarcinogenic	Mixed
12-O-Tetradecanoylphorbol-13-acetate	SPS	5	1	0
HPTE	ANG	4	0	0
Acetaminophen	RI	0	4	2
Acrolein	DM	3	3	3
Acrylamide	GI	3	1	1
Atrazine	ISE	3	0	1
	EAS	4	0	1
	TPI	3	0	1
Azamethiphos	ISE	1	0	0
Benomyl	GI	0	3	1
Benzo(a)pyrene	SPS	8	1	0
Biorhythms	TIM	3	2	0
Biphenyl	ANG	2	2	1
BPA	EAS	6	0	1
	GI	6	0	1
	ISE	7	0	1
	RCD	7	0	0
	SPS	6	0	1
	TIM	7	0	1
	TM	7	0	1
	TPI	6	0	1
Bisphenol AF	ANG	5	1	0
Butyltins (such as tributyltin)	TM	4	2	0
C.I. solvent yellow 14	ANG	4	0	0
Carbendazim	GI	0	2	1
Carbon black	GI	5	1	0
Chlorothalonil	ANG	5	1	0
	RCD	5	0	0
Cobalt	GI	5	2	0
Copper	DM	6	0	3
Cotinine	RI	4	1	0
Cypermethrin	DM	5	0	0
DDT	EAS	6	0	0
Diazinon	DM	2	3	0
Dibutyl phthalate	RCD	4	0	0
Dichlorvos	RCD	4	0	0
DEHP	ISE	4	0	1
	RCD	4	0	0
Diniconazole	ANG	2	0	0
Fluoxastrobin	ISE	2	1	0
Folpet	EAS	2	1	0
Hexachlorobenzene	TIM	5	2	0
Hexythiazox	DM	0	0	0
Imazalil	SPS	3	1	0
Iron	DM	5	1	3
	TIM	5	1	2
Lactofen	SPS	2	0	0
Lead	GI	3	1	0
	RI	3	1	0
Lindane	RCD	5	0	0
Linuron	RCD	2	0	0
Malathion	DM	5	0	0
Maneb	ISE	4	2	0
Mercury	GI	3	2	1
MXC	RCD	3	0	0
Methylene bis(thiocyanate)	ANG	2	1	0
MeHg	TM	5	2	0
Na-selenite	RI	0	4	2
Nickel	GI	6	1	1
	TM	6	1	1
Nickel chloride	RI	6	0	2
Nitric oxide	RI	5	2	2
4-NP	TPI	2	1	0
Oxyfluorfen	RCD	4	0	0

Table 5. Continued

Chemicals	Originating review	Procarcinogenic	Anticarcinogenic	Mixed
Paraquat	GI	4	2	0
	TM	4	2	0
PFOS	ANG	4	1	0
	SPS	4	1	0
Phosalone	SPS	1	1	0
Phthalates	TIM	6	0	1
	TPI	6	0	1
PBDEs	TPI	2	0	2
Pyraclostrobin	ISE	2	1	0
Pyridaben	ISE	1	3	1
Quinones	GI	1	6	1
Rotenone	DM	2	5	1
Sulfur dioxide	TIM	5	1	0
Titanium dioxide NPs	GI	3	1	1
Tributyltin chloride	ANG	3	1	0
Triclosan	GI	2	2	1
	ISE	3	2	1
Tungsten	GI	2	1	1
Vinclozolin	TPI	2	1	0
Ziram	ANG	3	1	1

Aggregated number of procarcinogenic actions, anticarcinogenic actions and mixed actions (i.e. procarcinogenic and anticarcinogenic) where cross-hallmark effects have been reported (for each chemical across the full range of hallmark domains—i.e. from all of the areas covered by the reviews in this special issue)—see samples of this how this data were reported in Table 3. Note: fully referenced data for these cross-hallmark effects can be found in each of the reviews in this special issue.

ANG, angiogenesis; DM, deregulated metabolism; EAS, evasion of antiproliferative signaling; GI, genetic instability; ISE, immune system evasion; RCD, resistance to cell death; RI, replicative immortality; SPS, sustained proliferative signaling; TIM, tissue invasion and metastasis; TM, tumor microenvironment; TPI, tumor-promoting inflammation.

LDE, chemical mixtures and carcinogenicity

Although we did not specifically ask the teams to focus on disruptive chemicals that were known to exert LDE, the summary of dose-response characterizations for the chemicals that were selected by these teams is dominated by chemicals (i.e. 50/85) that have been shown to produce LDE, and 15 of the 50 showed evidence of a non-linear dose-response. Surprisingly, only 15% of the chemicals reviewed (i.e. 13/85) showed evidence of a threshold. We believe that this helps to validate the idea that chemicals can act disruptively on key cancer-related mechanisms at environmentally relevant levels of exposure.

Historically, the axiom ‘the dose makes the poison’ has had some merit, so many people remain skeptical about the idea that adverse outcomes can result from minute exposures to commonly encountered chemicals. But we are now at a point in time where our knowledge of the biology of cancer has advanced considerably, and we know that carcinogenesis can begin when key events have occurred in a single cell, between cells or in the surrounding microenvironment. So the idea that LDE from many environmental chemicals (acting together) might serve to instigate, support or fully enable carcinogenesis, no longer appears to be an unreasonable assertion.

At this stage, we are not making any assumptions about whether or not future empirical research will find support for this hypothesis, nor are we assuming that this is a significant problem. We are simply impressed by the fact that we are now starting to see evidence of a wide range of LDE (that are directly related to carcinogenesis) that can be exerted by chemicals that are ubiquitous and unavoidable in the environment. As a result, we are compelled to explore and consider this possibility.

In-utero exposures and transgenerational effects

Additionally, a number of the teams cited *in-utero* exposure studies in their reviews and presented evidence on transgenerational

effects. Although this detail is not fully captured in the team summaries offered in this capstone paper (please see the individual reviews in this special issue for complete details), these effects are important to acknowledge. For example, the inflammation team noted that transient early life exposures *in utero* to vinclozolin have been linked to both adult-onset disease and transgenerational disease that involves inflammation. Similarly, the immune system evasion team reported that there is increasing evidence from animal studies that *in-utero* or neonatal exposures to BPA are associated with higher risk of immune system dysregulation that may develop later in life.

Taken together, these and other similar types of examples raise intriguing possibilities about vulnerabilities at the population level, and the contributions that *in utero* and early life exposures to mixtures of those chemicals might make towards cancer susceptibility. Single-generation experimental models are inadequate to detect this sort of disruptive activity (for exposures to a given chemical or to mixtures of chemicals), but these sorts of effects may increase cancer risks by promoting and/or enabling tumorigenesis.

The interplay between genetic factors and environmental factors

Given the number of key cancer-related mechanisms that can apparently be disrupted by chemicals that are commonly found in the environment, and the possibility that *in-utero* and/or early life exposures may also contribute to population vulnerability, the interplay between genetic factors and environmental factors should also be mentioned. For example, a hereditary genetic vulnerability (such as mutations to BRCA1/2 genes which greatly increase the lifetime risk of breast and ovarian cancer (482)) can predispose someone to a higher risk of cancer. But many hereditary genetic mutations and somatic mutations do not result in cancer, presumably because additional actions (e.g. sustained proliferative signaling) are needed or additional

biological safeguards still need to be suppressed or defeated (e.g. apoptosis, senescence, immuno-surveillance and so on) before a fully immortalized cellular phenotype can emerge. In these instances, cancer may not be assured, but it is easy to see how the disruptive effects of low-dose exposures to certain chemicals might act on key pathways/mechanisms and play a supporting role in the steps involved in carcinogenesis and/or increase the overall risk of getting cancer.

This same issue applies to other sensitive subpopulations who might be predisposed to higher levels of cancer risk. In some instances, vulnerabilities that exist are genetic in nature (e.g. cancer patients in remission), due to endogenous factors (e.g. due to obesity) or due to external influences (i.e. smoking). But in all cases, the enhanced risks in these subpopulations leave the affected individuals vulnerable to carcinogenesis. Although a detailed investigation of this type of interaction is beyond the scope of this project, it is important to consider that low dose, disruptive chemical effects on key pathways and mechanisms in these subpopulations may serve to further enhance cancer susceptibility, or even fully enable carcinogenesis.

The low-dose carcinogenesis hypothesis

It is important to reiterate that this group has no interest in implicating any of the chemicals that were reviewed in this project as individual carcinogens *per se*. We fully realized at the outset that much of the evidence in the toxicological literature that documented the disruptive actions of these chemicals had been produced under a wide range of differing experimental circumstances. So it was agreed at the beginning that we would not make leaps between different lines of evidence nor draw any specific conclusions about chemical mixtures that might prove to be carcinogenic. Nonetheless, we are intrigued by the number of chemicals that we reviewed that were found to be capable of disruptive LDE on key pathways/mechanisms across all of the areas that were reviewed. Many of the environmental chemicals that we chose are well known as environmental contaminants, but they represent only a small fraction of the thousands of chemicals that are now ubiquitous and unavoidable in the environment. So although we cannot draw any firm conclusions at this stage, we emerge from this effort with a better understanding of the evidence that is available to support the merits of our initial hypothesis (i.e. that low-dose exposures to disruptive chemicals that are not individually carcinogenic may be capable of instigating and/or enabling carcinogenesis).

Although the breadth and scope of this review effort was daunting, we now believe that we have enough supporting evidence to offer a holistic overview of this issue. At a minimum, we hope that the studies cited in this review, the gaps that we have identified and the framework that we have proposed for future research will be useful to researchers who are encouraged to explore this hypothesis in greater detail.

The implications for risk assessment

Thirty-five years ago, the work of Ames and others who followed set in motion a quest for individual chemicals as (complete) 'carcinogens' that became a dominant paradigm that has shaped our thinking for decades (226). So dominant has the focus been on single chemicals, that combinations of chemicals are rarely tested or even considered. For example, although IARC has focused on extensive monographs of the carcinogenic nature of individual chemicals, little has been done to evaluate the possibility of carcinogenic effects attributable to chemical mixtures except in a few instances where mixtures of concern are encountered during occupational exposures (e.g.

polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans) or as a result of personal and cultural habits (e.g. cigarette smoke, diesel and gasoline engine exhausts).

But the search for mutagenic carcinogens was never matched with a corresponding search for chemicals that might contribute to the promotion of carcinogenesis along with other chemicals. We now know that individual chemicals can produce unique disruptions of cellular biology and specific combinations of non-carcinogenic chemicals have been able to demonstrate potent carcinogenic effects. Yet, we have only scratched the surface of the biology of mixtures, and we need to look carefully at the synergistic effects.

In risk assessments, the risks associated with exposures to mixtures of chemicals are often estimated using relatively simple, component-based approaches (483). Risk analysts evaluate information regarding the mode of action associated with individual mixture components and then use either 'dose addition' or 'response addition' to predict effects. Dose addition is an appropriate approach to assess mixtures risks, when the chemicals of interest act through a common mode of action. Although response addition assumes that constituent agents act independently of each other (cause the same outcome via different modes of action). In general, a dose addition approach would be appropriate for mixtures risk assessment if we wanted to consider a series of chemicals that were carcinogenic in their own right, and if they all produced the cancer by the same mode of action. The Hallmarks of Cancer framework suggests that we should be equally, if not more, concerned about mixtures of chemicals that are not individually carcinogenic but disruptive in a manner that is collectively procarcinogenic (i.e. potentially capable of producing carcinogenic synergies when combined with other chemicals that are acting on the diverse series of mechanisms involved in carcinogenesis).

With this in mind, there should be concern that the World Health Organization International Programme on Chemical Safety (WHO IPCS) has spent the past decade developing a risk analysis agenda predicated mainly on a 'Mode of Action' framework (484–487), where 'mode of action' is defined as a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes and resulting in an adverse outcome, in this case, cancer formation. The OECD guidance on the conduct and design of chronic toxicity and carcinogenicity (which is followed by many nations) now also reflects this approach (487). This analysis of risks from cumulative effects of chemical exposures is restrictive because it suggests that regulators should only focus on groupings of individual chemicals that are as follows:

- (a) known to act via a common sequence of key events and processes;
- (b) known to act on a common target/tissue and
- (c) known to produce a common adverse outcome (e.g. cancer).

So, for example, in the USA, the Food Quality Protection Act provides legislated guidance on testing for cumulative effects by using the term 'common mechanism of toxicity' (488), which is interpreted to mean 'mode of action' or 'the major steps leading to an adverse health effect following interaction of a pesticide with biological targets'. Similarly, in Canada, the Pest Control Products Act requires the government to assess the cumulative effects of pest control products that have a 'common mechanism of toxicity'. In the USA, there has also been a tradition of employing an additional restriction requiring chemical structural similarity when selecting groups of chemicals to be subjected to mixtures risk assessment (other than a few instances

where whole mixtures have been assessed, e.g. diesel exhaust, combinations of chemicals that are not similar structurally have been largely ignored (489). In light of current knowledge of cancer biology, these criteria appear to be inappropriately restrictive, and thus demand a number of considerations—as follows:

Cumulative risk assessment should anticipate synergies of chemicals acting via dissimilar sequences/processes

From the Hallmarks of Cancer framework, it becomes evident that chemicals that act via dissimilar pathways/targets or that produce different sorts of key events and/or employ different processes could very well produce synergies within carcinogenesis that would be relevant for cumulative risk assessment purposes. For example, ethylenediaminetetraacetic acid is a ubiquitous, presumably non-carcinogenic chemical that disrupts DNA repair (490,491), and it is well established that ethylenediaminetetraacetic acid influences chromosome breakage by mutagenic agents. In particular, when applied in combination with chemical mutagens, ethylenediaminetetraacetic acid enhances mutagen-induced aberration frequencies and contributes to genetic instability (492). But within the mode of action framework, a chemical that is a mutagenic carcinogen, would not be assessed for the cumulative risks associated with an additional exposure to a chemical that disrupts DNA repair (a key layer of cancer defense) because it is not known to produce a common sequence of key events and processes.

A 2008 report on phthalates and cumulative risk assessment emphasized that the chemicals considered for cumulative risk assessment should be ones that cause the same health outcomes or the same types of health outcomes, not ones that cause the health outcomes only by a specific pathway (493). Similarly, The European Food Safety Authority Panel on Plant Protection Products and their Residues (PPR Panel) produced a scientific opinion on the relevance of dissimilar modes of action and their relevance for cumulative risk assessment of pesticides residues in food (489). The PPR Panel found good evidence that combination effects can arise from co-exposure to chemicals that produce common (adverse) outcomes through entirely different modes of action and recommended cumulative risk assessment methods to evaluate mixtures of pesticides in foods that have dissimilar modes of action (403).

Cumulative risk assessment should anticipate synergies of chemicals acting on different targets/tissues

The Hallmarks of Cancer framework suggest that spatiotemporal aspects of chemical exposures are likely important as well. For example, the many constituent parts of the immune system and its distributed nature (e.g. lymph vessels, thymus, bone marrow and so on), the hypothalamic–pituitary–adrenal axis and cortisol in circulation, which are used to suppress macrophage migration inhibitory factor and control inflammation (494–496) and the surrounding tissues of the tumor microenvironment, are all relevant targets that could be chemically disrupted to produce procarcinogenic contributions to carcinogenesis.

For example, as noted previously, maneb is a fungicide with a potentially disrupting effect on cortisol (453), which could impact the body's response to inflammation suppression, whereas atrazine affects the host immune response by directly targeting maturation of DCs and decreasing the levels of major histocompatibility complex class I molecules (243,460). Both are highly relevant forms of disruption for carcinogenesis, but within the mode of action framework, the cumulative effects of these chemicals (and other chemicals acting on these and

similarly distributed targets) would never be assessed together because they do not act on a common biological target.

The PPR Panel recently pointed out that there is no empirical evidence for the validity of independent action as a predictive concept for multicomponent mixtures in the mammalian toxicological literature. Further, they argued that although overlapping toxic effects in different organs/systems may exist, it is difficult to identify a combination effect. Thus, the panel specifically restricted their focus to chemicals that ultimately produce a common adverse outcome (e.g. cancer) in the same target organ/system (489). Although it may be difficult to identify this sort of an effect, that does not mean, however, that we should ignore this possibility (i.e. now that our understanding of the biology of cancer has improved).

Cumulative risk assessment should anticipate synergies of non-carcinogens

The WHO IPCS mode of action framework accepts the notion of a common toxic endpoint and therefore that chemicals need to first be carcinogens themselves before they can be considered as possible constituents of carcinogenic mixtures. However, it is now evident that not every procarcinogenic action resulting from a chemical exposure must be the result of a chemical that is a carcinogen itself. Continued focus on individual carcinogens reflects a lingering paradigm that overlooks the examples of synergies such as those highlighted in this project. Low-dose mechanistic effects may be very important so approaches are needed that take this into account. In chronic and complex diseases, establishing dose thresholds using the whole disease as the endpoint (e.g. cancer) may be inappropriate, especially when exposures to individual chemicals can produce relevant (but not disease causing) mechanistic effects at much lower dose levels.

Cumulative risk assessment should anticipate synergies of structurally dissimilar chemicals

The EPA's emphasis on structurally similar classes of chemicals for mixtures risk assessments is unnecessarily restrictive. The dissimilar chemicals reviewed within this special issue are testament to the fact that similar disruptive effects can be produced by a wide range of chemical structures and failure to adapt testing to this fact is no longer acceptable (493).

In sum, it is concerning that the WHO IPCS approach is so highly restrictive when it comes to the assessment of cumulative effects. The OECD guidelines acknowledge that cancers originating from at least some cell types may arise by a variety of independent pathways, but the guidance is fundamentally focused on the identification of individual carcinogens and cumulative effects of carcinogens, specifically noting that the approach is intended to 'avoid misidentification of non-tumorigenic compounds as possible human carcinogens' (487). But in practice, as *in-vitro* and *in-vivo* evidence for many chemicals is frequently not available (i.e. to prove that they individually act via a common sequence of key events or process a common target/tissue to produce cancer), it means that risk assessments of the cumulative effects of exposures to mixtures of chemicals on carcinogenesis are rarely conducted.

The International Life Sciences Institute, which is a non-profit organization with members comprised largely of major corporate interests from the food and beverage, agricultural, chemical and pharmaceutical industries, has worked closely with the WHO IPCS to support this approach. But while it may serve to ensure the avoidance of the misidentification of (non-tumorigenic) chemicals/compounds as possible human carcinogens, it simultaneously discourages regulatory agencies

from exploring the sorts of synergies that might plausibly be expected to occur. Indeed, the biology of cancer suggests that the cumulative effects of non-carcinogenic chemicals acting on different pathways that are relevant to cancer, and on a variety of cancer-relevant systems, organs, tissues and cells may very well conspire to produce carcinogenic synergies that will be overlooked entirely as long as the mode of action framework (and the restrictions that it imposes) remains in use.

As mentioned briefly previously, a considerable effort has been made by toxicologists to advance a new approach called the Adverse Outcome Pathway framework. This is an extension of the Mode of Action framework and is primarily being developed as an alternative solution to *in-vivo* toxicity testing. The framework is based on the idea that any adverse human health effect caused by exposure to an exogenous substance can be described by a series of causally linked biochemical or biological key events with measurable parameters (28,497). Although the Adverse Outcome Pathway framework anticipates the possibility that multiple pathways may need to be defined (i.e. different pathways that can produce the same adverse human health effect), the concept is currently aligned with the mode of action approach and focuses mainly on individual chemical effects that follow a well-described pathway to produce an adverse health outcome. So as it is currently conceived, it has some of the same limitations that apply to the mode of action framework.

Nonetheless, this focus at a mechanistic level is progressive in nature and some researchers in this area are starting to call for the adoption of practices within the framework that can account for epigenetic effects, transgenerational effects and chronic toxicity (detrimental effects arising in individuals or at the population level following long-term continuous or fluctuating exposure to chemicals at sublethal concentrations—i.e. concentrations not high enough to cause mortality or directly observable impairment following acute, short-term exposure, but able to induce specific effects potentially leading to adverse outcomes occurring at a later point in time) (28).

So this framework may be suitable for research that is focused on mixtures of chemicals and the pathways involved in carcinogenesis, so long as the adherents to this approach are open to the possibility that all relevant pathways need not have adverse health outcomes as endpoints, and that synergies between pathways may need to be anticipated. In other words, a series of seemingly benign actions on different pathways may be needed to conspire to produce the adverse health outcome that is of interest. This is the case in cancer. There are so many layers of redundancy and safeguards in place that individual disruptions of certain pathways may never cause disease on their own. Yet, when a number of these pathways are enabled, they can produce a discernable adverse health outcome (i.e. cancer). If the adverse outcome pathway is robust enough to anticipate this type of complexity, it may be a model that will allow us to move past the limitations imposed by the mode of action model.

Many regulatory agencies that conduct chemical risk assessments also have a mandate to ensure that adequate safety margins are in place to protect sensitive subpopulations. So they will need to place an increasing emphasis on the interplay between environmental factors and genetic factors and also consider *in-utero* exposures and the potential for transgenerational effects. Some progress has been made in tackling the gene-environment interaction problem using pathway analysis to demonstrate the role of genetic variants in exposure-related cancer susceptibility (c.f. Malhotra et al. (498)), but very little research has been done on *in-utero* exposures to mixtures of chemicals that act on cancer-related mechanisms. An approach that focuses on

defining mixtures of constituents that act disruptively on key mechanisms that are related to individual hallmarks may serve as a useful starting point to find evidence of relevant transgenerational effects (c.f. Singh et al. (499)). This is definitely an area where additional research and regulatory input is needed.

Research needs: cancer versus carcinogenesis

One of the main challenges in this project has been the need to better understand *carcinogenesis* as a process characterized by a long latency—and the corollary possibility of both direct and indirect effects—rather than cancer as a disease endpoint that must occur rapidly and in the majority of exposed persons to be relevant. This is further complicated by the fact that the Hallmarks of Cancer are frequently neither fixed nor specific for cancer (356–358). Numerous experimental models have been used in cancer research over the years, and Vineis et al. (500) summarized them into at least five separate classes of models—see below:

- (a) Mutational models
- (b) Genome instability
- (c) Models based on non-genotoxic mechanisms, clonal expansion and epigenetics
- (d) ‘Darwinian’ or ‘somatic cellular selection’, and
- (e) ‘Tissue organization’.

All of these models have had significant support in the scientific literature (based upon empirical evidence) and there is considerable overlap between them. But our collective understanding of carcinogenesis is still largely constrained by a historically monolithic toxicology-based approach that has been focused on the effects of mutagens and the disease itself. So although the Hallmarks of Cancer framework helps us to better conceptualize the many acquired capabilities of the disease, it leaves much to the imagination when it comes to advancing our understanding of carcinogenesis *per se*. This lacuna was recently highlighted by Brash et al. (501,502) in an article on what they called ‘the mysterious steps in carcinogenesis’.

Carcinogenesis appears to be an evolution of factors that ultimately conspire towards various acquired capabilities (i.e. those delineated within the Hallmarks of Cancer framework), but how much does the sequencing of these acquired capabilities matter and in what order are these capabilities acquired? Figure 1 implies a rough sequencing of these capabilities, but do we know for certain that all hallmarks for established cancer are important for carcinogenesis as well (i.e. which hallmarks are necessary for all tumors, and of those, which are sufficient or perhaps distinct for certain cancers?). Other important questions to ask relate to whether or not the individual hallmarks are a cause or a consequence of cancer development? Do the individual hallmarks need to be expressed simultaneously or sequentially along the continuum of carcinogenesis (from exposure to unambiguous cancer phenotype development)? More importantly, how does our understanding of this framework inform our general approach to the study of carcinogenesis?

We have partial answers to some of these questions, but some of these questions remain unanswered, and given the prolonged latency of many cancers, these are important questions. Our lack of knowledge in this regard makes it difficult to draw immediate conclusions about the effects that exposures to mixtures of disruptive chemicals might cause and the synergies they might produce. Public health protection is challenged by the combinatorial complexity posed, not only by multiple exposures to chemicals at environmentally relevant doses (either simultaneously or sequentially) but also through the different

mechanisms played out in temporospatial manners (including life stages of development, which are different from those applied in traditional toxicologic and carcinogenic screening).

We, therefore, need to consider an expanded research agenda to include the origins, determinants and temporospatial evolution of the various cancer hallmarks and their interrelatedness. The key questions of reversibility and of cause versus consequence must also be rigorously addressed at every step from initiating carcinogenic exposure to established cancer, recognizing that not all hallmarks are either fixed or specific for any given cancer type.

Research needs: the Hallmarks of Cancer

Current approaches to the study of chemical exposures and carcinogenesis have not been designed to address effects at low concentrations or in complex mixtures. Procarcinogenic agents may be directly genotoxic, indirectly genotoxic or non-genotoxic. In principle, not every disruptive effect resulting in a change that mimics a cancer hallmark is necessarily carcinogenic. Such associations, when observed, still require rigorous validation to ensure that exposures are unequivocally linked to the development of both cancer and accompanying phenotypic hallmarks. These complex interactional possibilities, coupled with the fact that low-dose combinatorial effects on cancer development and progression have not been rigorously or comprehensively addressed, speak to major gaps in our understanding of environmental cancer risk and the specific role that mixtures of environmental chemical exposures might play in the incidence of cancer at the population level.

Unfortunately, the known effects for chemicals examined in isolation and at higher concentrations cannot be readily extrapolated to effects at lower concentrations. Interactions within complex mixtures will also occur against the backdrop of complex interactions with other environmental, genetic and epigenetic factors, so there is a need for expanded or complementary conceptual and experimental frameworks to better understand the determinants and specific functional contributions of environmental exposures in cancer.

A considerable amount of energy is now being placed on the development of research and technologies that can support the 'exposome' (503), an emerging concept aimed at representing the totality of chemical exposures received by a person during a lifetime. This approach encompasses all sources of toxicants and is intended to help researchers discern some of the contributing factors that are driving chronic diseases such as cancer. Related projects are expected to involve extensive biomonitoring (e.g. blood and urine sampling) and other techniques to assess biomarkers that might be relevant, and this information should be extremely helpful. Longitudinal studies should also be carried out in animal models to assess the tissue distribution of mixtures of chemical metabolites. To truly make good use of this information, we are going to need a better mechanistic understanding of the process of carcinogenesis itself and better early markers of cancer development.

It therefore makes sense to pursue empirical research based on our current understandings of the disease to test the effects of real-world environmental mixtures at relevant dose levels. Basic studies should be designed to test joint toxic action (of carefully designed combinations of chemicals) to assess both dose additivity (via common mode of action) and response additivity (via disparate modes of action). Research designs should anticipate the many layers of inherent defense and incorporate chemical constituents specifically intended to demonstrate predictable synergies and mechanistic relevance. It would also be

useful to know whether or not the chemical induction of certain numbers/combinations of hallmarks is sufficient to consistently produce *in-vivo* carcinogenesis.

Mixtures research that focuses on the carcinogenic synergies of non-carcinogenic constituents would be particularly useful. In addition, compounds or classes of chemicals already considered to be (complete) carcinogens in the classical sense may also contribute to carcinogenesis in complex mixtures at concentrations not traditionally deemed carcinogenic. For this reason and for completeness, 'classic' carcinogens with an established environmental presence at levels that are presumed to be inconsequential may still have pathogenic relevance and should be routinely included in the analysis.

Target sites that are being manipulated and disruptive chemicals that are being selected to produce carcinogenic effects should be scrutinized for confounding effects. Table 4 contains aggregated evidence of cross-hallmark effects for selected pathways/mechanisms, and although some target sites for disruption may be compelling starting points for researchers focused on a given phenotype (e.g. genetic instability), cross-hallmark relationships should be explored. So, for example, telomere loss is seen as a disruptive (procarcinogenic) effect from the perspective of the genetic instability team (i.e. the group in this project who selected this target) and it has also been shown to exert procarcinogenic effects in four other hallmark areas. But evidence also exists that suggests that telomere loss can have anticarcinogenic effects in four other hallmark areas. The exact circumstances of the various studies that support these cross-hallmark relationships would need to be reviewed to better understand the implications/relevance of these reported effects. But checking planned disruptions of each target across all of the other hallmark areas is a way to ensure that confounding (i.e. anticarcinogenic) effects are not inadvertently introduced into experiments that are aimed at producing carcinogenesis, or phenotypes that can support/contribute to carcinogenesis. Similarly, Table 5 contains aggregated evidence of cross-hallmark effects for the chemical disruptors in this review, so this table can be used for the same purpose.

It may also be productive to identify 'reference compounds' (ideal and prototypical disruptors) for each hallmark pathway as a guide to predict different combinations of chemicals that might act in a procarcinogenic manner on any one of the hallmarks. This may involve different systems and organs that have relevance to cancer and this sort of research could also be combined with similar sorts of research on other reference compounds or mixtures that are shown to enable other hallmarks. In doing so, researchers should evaluate epigenetic changes in multiple samples/organs/tissues from exposed animals/other experimental models using gene array technology, 'omics' approaches, real-time imaging of tumors in 3D both *in-vitro* (primary cells) and *in-vivo* models combined with molecular biomarkers of disease progression, and cellular immune parameters. The combination of use of computational chemical genomics (504), system biology/pharmacology and high-quality imaging techniques, quantitative-structure-activity-relationship studies through ligand-, target-based virtual ligand screening and mathematical models should help in finding quantitative-structure-activity-relationship correlations between the chemical structure of dissimilar disruptors and experimental data on biological activity, physiological changes, *in-vivo* toxicity endpoints and 3D cellular protein dynamics.

It is also conceivable that the combined effects of hundreds of chemicals in the environment may be involved in the process of enabling carcinogenesis at the population level, so basic

empirical research that can demonstrate carcinogenic effects with minimalistic combinations may initially be needed to reveal the more granular aspects of carcinogenesis. For example, initial research might test our assumptions of the step-wise progression of carcinogenesis using targeted mixtures of chemicals that exert LDE to test combinations of two, three, four chemicals etc. against specific hallmarks and then adding additional targets to move through the various steps that are believed to be needed to fully enable the process. Experiments of this nature may reveal increases as well as decreases in cancer risk when different mechanisms are disrupted and corresponding hallmark phenotypes are enabled (depending on the timing of various disruptive exposures). Batteries of tests may ultimately be needed to evaluate whole mixtures and key components individually and in various combinations. HTS approaches will be particularly helpful here, and a tiered approach may make sense to look for disruptive combinations, which can then be applied *in vivo*. Exposure sequencing and dosage may also be important and should be evaluated based on our current understandings of the biology of cancer.

In terms of setting research priorities, tissue fate is also a matter for consideration. It has been known for many years that certain chemicals have affinities for certain tissues, and radiotracer labeling studies that have been conducted on chemicals for regulatory purposes illustrate how certain chemicals tend to accumulate in certain tissues (c.f. Nolan, R. *et al.*, unpublished report). Additionally, it is well known that some tissue types give rise to human cancers millions of times more often than other tissue types (505). So, researchers may want to focus their work on mixtures of disruptive chemicals that prove to be complementary at a mechanistic level and individually known to accumulate in the same types of tissues, while at the same time choosing tissue types that are known to produce cancers more rapidly.

The work that has been done by the WHO IPCS on mode of action has been very useful. Understanding when chemicals operate through the same mode of action is definitely good information for analytical purposes, but given that we now recognize that non-carcinogens acting at very low-dose levels on different targets and mechanisms can still activate carcinogenesis-related pathways, the combined (carcinogenic) potential of the many commonly encountered chemicals within the environment still needs to be evaluated.

Increasingly, our information is improving and there are several tools that researchers can use to improve their research designs. For example, ToxCast™ is an approach launched by the EPA in 2007 to develop ways to predict potential toxicity of chemicals and to develop a cost-effective approach for prioritizing the thousands of chemicals that need toxicity testing. The ToxCast™ database was used in this project by a number of the teams and an enormous amount of data are available on *in-vitro* tests (produced using HTS) for a wide range of chemicals. For example, there are many results that are direct measures of actions related to important mechanisms found within the Hallmarks of Cancer framework, which would be useful for research focused along these lines.

Although the hallmark phenotypes in this project represent areas of cancer research for which there is considerable agreement, one critique of this framework is that it ignores the ‘missing hallmark’ of dedifferentiation (358). As well, the complexity encompassed by each of these areas of research is humbling. Moreover, cancer is not a singular or fixed entity, which frequently limits the ability to generalize about cancer biology (356–358). In a recent reflection on his career, Weinberg *et al.*

(506) noted not only widespread acceptance of the ‘Hallmarks of Cancer’ heuristic but also that this attempt to simplify the disease is rapidly being eclipsed by calls from the next generation of researchers who are now focused on assembling and analyzing enormous data sets to gain an increasingly sophisticated understanding of cancer (e.g. genomes, transcriptomes, proteomes—including isoforms, post-translational modifications and proteoforms, epigenomes, kinomes, methylomes, glycomes and matrisomes—each one of which encompasses staggering amounts of accumulated information) (506).

Many researchers have called for an analytical use of systems biology to transcend the study of individual genes/proteins and to integrate this complexity into higher order phenotypes (507,508). Systems biology enables researchers to identify properties that emerge from complex chemical-biological systems by probing how changes in one part affect the others and the behavior of the whole system. The combined effects of tens, if not hundreds, of simultaneous exposures may need to be accounted for. The fundamental challenge is that such models require parameters that are driven by data, but there are very few good examples of research on mixtures at environmentally relevant dose levels (509) (c.f. Porter *et al.* (510)), and there are fewer still that are focused on cancer.

Nonetheless, in the near term, this basic framework should serve as a useful starting point for foundational research and government funding agencies should consider new ways to support large-scale, team-based holistic approaches to this problem.

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Regulatory priorities (in the face of combinatorial complexity)

It will take time before we fully understand the carcinogenic potential of low-dose exposures to chemical mixtures in the environment. Nonetheless, we cannot afford to lose sight of the fact that the incidence of cancer remains unacceptably high, and that the unavoidable (i.e. not lifestyle related) causative factors that are, in part, underpinning this trend are still not fully understood (9–11,511,512). Populations worldwide are continually exposed to a wide range of chemicals, so keeping the precautionary principle in mind (513), there is a need to take the risks related to the cumulative effects of these chemicals seriously (429). Of primary concern is the fact that WHO IPCS mode of action framework (484) and the OECD guidelines for risk assessment (487) are restrictive to the point that regulators could be underestimating the risks posed by exposures to low doses of mixtures of chemicals.

National regulatory agencies and cancer research foundations must proactively pursue empirical research programs to assess any basic relationships that can be discerned between exposures to mixtures of commonly encountered chemicals and carcinogenicity. For example, systematic exploratory research in appropriate rodent models exposed to ‘whole-mixtures’ that consist of multiple chemical constituents at environmentally relevant dose levels could demonstrate the carcinogenic potential of complex mixtures that are relevant to the population. There is also a compelling need for complementary basic research to address specific causal relationships between environmental exposures and the associated development of cancer and its characteristic hallmarks.

Hypothetically speaking, such a ‘whole mixture’ should be composed of non-carcinogens and potential carcinogens given that individual chemicals that are not carcinogenic could act on a range of different systems, tissues and/or cells and act synergistically with other chemicals to instigate carcinogenesis. The

goal of such investigations would not be to single out any given chemical as a carcinogen, but rather to determine whether or not unanticipated (procarcinogenic) synergies of many commonly encountered chemicals when combined are endangering public health.

In line with the 3Rs (Reduction, Replacement and Refinement) guiding principles for more ethical use of animals in scientific experiments, there has been a significant push for researchers and regulatory agencies to move away from *in-vivo* testing (e.g. European Union REACH legislation and in the USA, the NRC Toxicology for the 21st Century vision (514)) to take advantage of HTS and other new technologies. The EPA's effort to search for environmental chemicals that are most active in relevant assays across the various cancer hallmarks, and then to compare those results with *in-vivo* rodent carcinogenicity data for the same chemicals, was a definite step in this direction (29). However, HTS models of carcinogenicity will require validation, and significant hurdles remain before this sort of testing will be ready to replace *in-vivo* research (515). Therefore, in the near term, *in-vivo* testing still remains an important avenue for developing data sets to address cancer risks of complex mixtures.

Summary/Conclusions

For several decades, there has been a concerted effort to identify individual chemicals and other agents that are carcinogenic. At the same time, however, little has been done to determine whether or not chronic lifetime exposures to mixtures of non-carcinogenic chemicals in the environment (at low-dose levels) have carcinogenic potential. Many chemicals are known to accumulate in bodily tissues over time, but little is known about their combined effects at a mechanistic level and their impact on cancer-related mechanisms and carcinogenesis. In this project, teams of cancer biologists worked with researchers in the field of environmental health for the very first time to explore this possibility.

Teams that reviewed these cancer-related phenotypes (i.e. genetic instability, tumor-promoting inflammation, sustained proliferative signaling, insensitivity to antigrowth signals, resistance to cell death, angiogenesis, tissue invasion and metastasis, the tumor microenvironment and avoiding immune destruction) readily identified individual (non-carcinogenic) chemicals that are ubiquitous in the environment that have some potential to act on key/priority functional targets in each of these domains. In contrast, the teams focused on *replicative immortality* and *dysregulated metabolism* found examples of chemicals to consider but noted a significant lack of useful toxicological research in these areas.

In total, 85 examples of environmental chemicals were reviewed as prototypical disruptors (for specific actions on key pathways/mechanisms that are important for carcinogenesis) and 59% of them (i.e. 50/85) were found to exert LDE (at levels that are deemed relevant given the background levels of exposure that exist in the environment) with 15 of the 50 demonstrating their LDE in a non-linear dose-response pattern. Only 15% of the chemicals reviewed (i.e. 13/85) were found to have a dose-response threshold and the remaining 26% (i.e. 22/85) were categorized as 'unknown' due to a lack of dose-response information.

Cross-hallmark effects for all target sites for disruption and for all chemicals were found, but the evidence supporting these results varied considerably in strength and in context.

A number of the teams also cited relevant *in-utero* exposure studies in their reviews and presented data on transgenerational

effects related to different aspects of the disease (e.g. inflammation, immune evasion and so on). These examples raise intriguing possibilities about vulnerabilities at the population level, and the contributions that *in-utero* and early life exposures to mixtures of those chemicals might make towards cancer susceptibility.

Therefore, current regulations in many countries (that consider only the cumulative effects of exposures to individual carcinogens that act via a common sequence of key events and processes on a common target/tissue to produce cancer) should be revisited. Our current understanding of the biology of cancer suggests that the cumulative effects of (non-carcinogenic) chemicals acting on different pathways that are relevant to cancer, and on a variety of cancer-relevant systems, organs, tissues and cells could conspire to produce carcinogenic synergies that will be overlooked using current risk assessment methods. Cumulative risk assessment methods that are based on 'common mechanisms of toxicity' or common 'modes of action' may therefore be underestimating cancer-related risks. *In-utero* and early life exposures, transgenerational effects and the interplay between the low-dose mechanistic effects of chemical mixtures in the environment and the vulnerabilities of subpopulations who are predisposed to cancer (i.e. via genetics or other influences) must also be considered. Current policies and practices do not adequately address these issues and should therefore be revisited if regulatory agencies hope to better understand and assess these risks.

Finally, given the long latency period in most cancers, early detection of cancer is key so an improved understanding of the biology within originating tissues (during the latency period) would be very helpful. If we can use the heuristic presented in this review to better assess the combined effects of common exposures to chemical mixtures in the environment, it will help us improve our understanding of carcinogenesis and identify exogenous triggers and enabling factors (*in utero* and during this important latency period), all of which will be key for the development of effective strategies for prevention and early detection.

Contributions

The Halifax Project Task Force that worked on this manuscript involved nearly 200 people, many of whom contributed to, and signed on to this capstone article. The design of the Halifax Project was conceived by L.Lo. with scientific advice from M.G. Funding provided by the National Institute for Environmental Health Sciences was arranged by D.O.C., and this manuscript was first drafted by W.H.G. Starting with the Hallmarks of Cancer framework (Hanahan *et al.* (21)), 11 teams of international cancer biologists and toxicologists were established to review the literature on key cancer-related mechanisms/pathways in their respective domains and to also look at the disruptive potential of low-dose exposures to chemicals commonly encountered in the environment (i.e. as it relates to those same mechanisms/pathways). Each team had a leader and each team was responsible for contributing a section of related content within the capstone manuscript. The contributing authors from these teams are as follows: (1) Angiogenesis (Z.H., C-W.H., H-Y.H., L-T.L., M.X., N.K., S.A.B., T.M., V.D., W.K.R.); (2) Dysregulated metabolism (R.B.R., A.C.S., A.B., E.Ry., D.B., F.C., F.L.M., G.Wi., J.We., N.B.K., R.P.); (3) Evasion of antigrowth signaling (R.N., A.L., C.C.N., D.W.L., D.R., G.S.G., G.M.C., H.Kr., J.V., K.A.C-S., M.W., N.C., P.A.M., P.De., R.A-V., R.V., R.D.F., R.P-C., R.C.C., S.N.B.), (4) Genetic instability (S.A.S.L., A.L.d.C.S., A.Az., A.K.C., A.R.C., A-K.O., E.Ro., F.D., F.J.V.S., G.K.,

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G.B., L.Go., L.Le., L.Z., M.Val., M.K-V., N.v L., P.O-W., S.Pav., T.C.); (5) Immune system evasion (H.K.L., E.C., J.K., M.A.W., M.H.M., T.O., W.K.D.); (6) Replicative immortality (A.Ca., C.B-A., H.Y., H.Ko., J.P.W., J.F.M-L., M.L., S.S.W.); (7) Resistance to cell death (H.H.P., A.M.A., B.J.B., C.Y., E.R., K.B.N., L.S.D'A., L.Li., M.F.R., M.J.G., P.M.G., P.S.L., Q.(S.) C., R.K.S., R.D., S.Ro., S.L., T-J.L., Y.R.); (8) Sustained proliferative signaling (W.E., A.W., G.Wa., H.S., J.E.K., J.R., K.M., L.Gu., M.V.K., P.V., P.Da., R.M., S.Er., T.S., T.H.); (9) Tissue invasion and metastasis (J.O., B.P.Z., C.D., G.N., G.T.W., I.K., I.R.M., L.J.M., N.A., O.O., P.N-M., S.El., S.Pap., V.O-M., Y.L., Z.C.); (10) Tumor microenvironment (D.W.F., C.S.C., D.C.K., E.L., F.M., J.Ro., J.A.C., J.R.W., L.S., L.V., M.C., P.K.K., P.H., S.Ry., S.C.C., V.M-S.) and (11) Tumor-promoting inflammation (P.T., C.J.B., E-Y.M., J.S., L.J., M.K., S.H., T.G., V.S.).** Additionally, a special cross-functional team was established to investigate whether or not the chemicals that were identified by the teams as having disruptive potential for key mechanisms/pathways in a particular domain might also have been shown in other research to exert relevant effects on mechanisms/pathways in other domains. The results of the efforts from this team have been compiled and summarized in this article and can be found within Table 4. This team was comprised as follows: W.H.B., A.Am., A.I.S., A.Co., C.M., D.G.B., E.Ry., F.A-M., H.A.H., H.K.S., J.R., J.Wo., K.R.P., L.M., M.Vac., N.S., R.A-T., R.R., R.A.H. and S.F.** Note that team leaders are denoted by the first set of initials in each team list.

The first draft of this manuscript (prepared by W.H.G.) was distributed to all of the contributors within the task force for feedback and additional inputs. The many responses that followed were managed by W.H.G. (with the assistance of L.Lo., M.G. and D.O.C.). Then, multiple rounds of inputs were solicited from the entire task force with several subsequent rounds of revisions and refinements prior to submission.

In addition to the contributions mentioned above, The Halifax Project also benefited from the involvement of D.J.C. At the workshop in Halifax, Nova Scotia, Canada, she provided details related to NIEHS priorities and the agency's interest in unravelling the health effects of environmental mixtures. As well she provided inputs for the manuscript.

Finally, the journal's peer-review process was important, and resulted in the collection of additional evidence from the teams that related to thresholds, LDE and of non-monotonic dose-response relationships. The reviewer's critical analysis on these topics resulted in a substantial improvement to the data presented in this capstone document, which ultimately served to highlight the extent to which low-dose exposures to individual chemical constituents (within mixtures of environmental chemicals) might have relevance for the process of carcinogenesis. Dose-response characterization data and inputs were then submitted by all teams and subsequently reviewed and compiled by N.K., A.Co. and R.M.

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Appendix

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Immune Regulatory Function of Tregs

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GUEST EDITORS' INTRODUCTION

Immune Regulatory Function of Tregs

Masoud H. Manjili and Kyle K. Payne

Regulatory CD4⁺ T cells (Tregs) have become increasingly appreciated in their role of facilitating immunological tolerance during homeostasis and during the diseased state. A general understanding of the literature characterizes Tregs as key elements of the immune system involving in the suppression of immune responses against cancer or infectious diseases, as well as the prevention of autoimmune diseases. However, the immune regulatory role of Tregs goes beyond this simple suppressive function, since, for example, Tregs actively contribute to the maintenance of intestinal epithelial cell homeostasis by producing TGF- β , which in turn helps B cells produce IgA. Therefore, topics that were selected in this thematic issue cover the pleiotropic functions of Tregs during homeostasis and disease, which we hope will contextualize these cells within the broader immunological terrain. This thematic issue is meant to provide an overview of the contribution of Tregs during subclinical diseases or cancer dormancy as well as clinical diseases including cancer and autoimmunity. Authors were selected based on their research interests and accomplishments in the area of cellular immunology, with a focus on T cell biology. To this end, the contribution of Fabian Benencia of Ohio University, an expert on antigen presentation during the activation or suppression of T cells (Benencia et al., 2014), focuses on the role of Tregs in ovarian cancer (Singh et al., 2016), and outlines experimental approaches to impair their immunosuppressive function.

Paula Bos of Virginia Commonwealth University (VCU) is an expert on the interaction of Tregs with the tumor microenvironment. She investigated the role of Tregs during tumor progression during her postdoctoral training in the laboratory of Dr. Rudensky (Bos et al., 2013). In the review provided by Dr. Bos, we are introduced to non-classical functions of Tregs. Beyond the more classical role of Tregs as suppressors of immunity, these cells have also been shown to be contributors to tissue remodeling and repair, and have been demonstrated to exhibit immune-independent functions, such as angiogenesis. Dr. Bos discusses such alternative mechanisms by which Tregs may contribute to tumor progression (Bos, 2016).

Nejat Egilmez of the University of Louisville is an expert in the area of Th1 immune responses and modulation of Tregs during immunotherapy (Li et al., 2015). In their review, Li and Egilmez discuss the ontogeny of tumor-associated Tregs. The well-established and critical contribution of Tregs to immune suppression in the tumor microenvironment is discussed. The authors importantly consider that information regarding the origin and population dynamics of Tregs remains limited. The central question brought forward in this review is the relative contribution of thymic Tregs and peripheral Tregs to the total tumor Treg population, and the mechanisms underlying the prevalence of each population in tumors. Therefore, the ontogeny of tumor-associated Tregs is discussed in this review (Li and Egilmez, 2016).

B.J. Monzavi-Karbassi of the University of Arkansas for Medical Sciences is an expert in the area of cancer vaccines (Monzavi-Karbassi et al., 2007) whose work provides an insight into the role of Tregs during vaccination. In a contribution of original work, Monzavi-Karbassi et al. investigated the effect of modulation of the expression of tumor-associated antigens in influencing the immunogenicity of a cell-based vaccination strategy. Interestingly, they observed that crude tumor-secreted antigens activated Tregs and induced their suppressive potential. This suggests that tumor-associated antigens can be enriched using their glycan expression pattern to weaken immune suppression and to improve antitumor immune responses (Monzavi-Karbassi et al., 2016).

Masoud Manjili of VCU Massey Cancer Center is a tumor immunologist whose research program is focused on immunotherapy of breast cancer and targeting tumor dormancy while overcoming immune suppressor cells (Manjili, 2014; Payne et al., 2016). In their review, Manjili and Butler discuss the poorly understood concept of tumor cell dormancy in the context of immune-mediated maintenance, as well as escape and subsequent recurrence. Given this poorly defined nature of immune responses in the setting of tumor dormancy, the authors' contribution provides a well-timed review of the literature related to the role of Tregs to the maintenance of tumor dormancy and/or recurrence (Manjili and Butler, 2016).

Kyle Payne of the Wistar Institute is an expert in the cellular crosstalk of the tumor immuno-environment, and modulation of T cell responses as well as immune suppressor cells (Payne et al., 2016; Payne et al., 2013). In his review, Dr. Payne discusses the crosstalk Tregs establish with myeloid cells in the tumor microenvironment, and also discusses the emerging appreciation of $\gamma\delta$ -T cells as atypical regulators of antitumor immunity (Payne, 2016).

Qingguo Ruan is an expert in the field of immune regulation and the pathogenesis of autoimmune disease (Ruan et al., 2011). The original work by Wang and others from the laboratory of Dr. Ruan (Wang et al., 2016) investigates the requirement of the NF- κ B family transcription factor, c-Rel, in the *in vivo* generation of peripherally induced Tregs. The data presented by the authors suggest that c-Rel may play distinct roles in regulating the development of peripherally induced Tregs within diverse tissue microenvironments (Wang et al., 2016).

The original work by Sznurkowska et al. investigates regulatory T cells in children with inflammatory bowel disease (IBD). The authors hypothesized that defective immune regulation leads to pathological immune responses directed against gut flora at the onset of IBD, therefore they describe a study which quantified Tregs in these patients in order to identify possible correlations between the presence of regulatory T cell and the pathology of IBD (Sznurkowska et al., 2016).

Anthony Vella of the University of Connecticut is an expert in the area of dual co-stimulation of T cells, and modulation of Tregs, both CD4+ and CD8+ Tregs (St Rose et al., 2013). Wang and Vella summarize the current knowledge on the roles of Tregs during cancer development, as well as the underlying cellular and molecular mechanisms in their review. They discuss the dual role of Tregs in functioning for the development, progression, and treatment of cancers, in which evidence is cited for their suppressive function against antitumor immunity, as well as the ability of Tregs to act directly on transformed epithelial cells to exert opposing effects during cancer development (Wang and Vella, 2016).

Xingxing Zang of the Albert Einstein College of Medicine is an expert in T cell biology and the mechanisms by which co-stimulation and co-inhibition regulate T cells (Zang et al., 2016). The review contributed from Dr. Zang's laboratory by Liu et al. discusses the role of co-stimulatory and co-inhibitory signals in being key mechanistic contributors to the regulation of adaptive immunity, and, further, discusses the recent progress in delineating the roles of co-stimulatory and co-inhibitory signals in the context of Tregs (Liu et al., 2016).

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Tumor-reactive immune cells protect against metastatic tumor and induce immunoediting of indolent but not quiescent tumor cells

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ABSTRACT

Two major barriers to cancer immunotherapy include tumor-induced immune suppression mediated by myeloid-derived suppressor cells and poor immunogenicity of the tumor-expressing self-antigens. To overcome these barriers, we reprogrammed tumor-immune cell cross-talk by combined use of decitabine and adoptive immunotherapy, containing tumor-sensitized T cells and CD25⁺ NKT cells. Decitabine functioned to induce the expression of highly immunogenic cancer testis antigens in the tumor, while also reducing the frequency of myeloid-derived suppressor cells and the presence of CD25⁺ NKT cells rendered T cells, resistant to remaining myeloid-derived suppressor cells. This combinatorial therapy significantly prolonged survival of animals bearing metastatic tumor cells. Adoptive immunotherapy also induced tumor immunoediting, resulting in tumor escape and associated disease-related mortality. To identify a tumor target that is incapable of escape from the immune response, we used dormant tumor cells. We used Adriamycin chemotherapy or radiation therapy, which simultaneously induce tumor cell death and tumor dormancy. Resultant dormant cells became refractory to additional doses of Adriamycin or radiation therapy, but they remained sensitive to tumor-reactive immune cells. Importantly, we discovered that dormant tumor cells contained indolent cells that expressed low levels of Ki67 and quiescent cells that were Ki67 negative. Whereas the former were prone to tumor immunoediting and escape, the latter did not demonstrate immunoediting. Our results suggest that immunotherapy could be highly effective against

quiescent dormant tumor cells. The challenge is to develop combinatorial therapies that could establish a quiescent type of tumor dormancy, which would be the best target for immunotherapy. *J. Leukoc. Biol.* 100: 000-000; 2016.

Introduction

MDSCs are key cellular suppressors of anti-tumor immune responses in breast cancer patients. Tumor-derived factors drive the accumulation of MDSCs in the bone marrow and secondary lymphoid organs and at the site of the tumor, thereby inhibiting the efficacy of cellular immunotherapy against established tumors. A number of strategies have been used to enhance immunotherapy of cancer by overcoming MDSCs. These strategies fall into 3 major categories that include MDSC deactivation, depletion of MDSCs, or conversion of MDSCs to APCs [1, 2]. The latter approach identified NKT cells as a key facilitator in promoting MDSC maturation into mature myeloid cells with anti-tumor immune stimulatory function. Therefore, it was suggested that the term M_{regs} better represents the plasticity of these cells, rather than MDSCs [3]. With the use of PBMCs of patients with early-stage breast cancer, we demonstrated previously that an optimal frequency of CD25⁺ NKT cells within reprogrammed immune cells, cultured in the presence of MDSCs/M_{regs}, induced them to lose/down-regulate CD11b, which was associated with HLA-DR up-regulation. Such phenotypic modulation was shown to promote anti-human epidermal growth factor receptor 2/neu immune responses in vitro [4]. Therefore, inclusion of CD25⁺ NKT cells in AIT should enhance the anti-tumor efficacy of adoptively transferred T cells by modulating MDSCs/M_{regs} to become immunostimulatory instead of immunosuppressive.

Abbreviations: ADR = Adriamycin, AIT = adoptive immunotherapy, Aza = azacytidine, CTA = cancer testis antigen, CYP = cyclophosphamide, Dec = decitabine, FVS = fixable viability stain, FVS⁻ = FVS-negative, i.d. = intradermally, IHC = immunohistochemistry, i.p. = intraperitoneally, i.v. = intravenously, Ki67⁺ = Ki67-positive, Ki67⁻/low = low levels of Ki67, MDSC = myeloid-derived suppressor cell, MFI = mean fluorescence intensity, MMC = mouse mammary

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The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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Another barrier to successful cancer immunotherapy is that human cancers are usually poorly immunogenic, with the exception of melanoma. Therefore, the enhancement of the immunogenicity of tumor cells could make them better targets for immunotherapy. On the other hand, tumor immunoediting, such as loss of tumor antigens and engagement of the PD-1/PD-L1 pathway, is likely to occur in the face of robust anti-tumor immune responses. Therefore, the overcoming of tumor immunoediting and escape remains a major challenge for effective anti-cancer immunotherapies. To this end, it is critical to determine how tumors may or may not be prone to immunoediting and escape and how this tendency can be altered.

To address these challenges, we sought to modulate tumor-immune cross-talk by using reprogrammed T cells and NKT cells along with Dec. AIT, with reprogrammed, tumor-sensitized T cells and CD25⁺ NKT cells, is expected to overcome MDSCs and establish memory responses [5], whereas Dec is expected to render tumor cells highly immunogenic by the induction of the expression of CTAs [6, 7]. Dec is an epigenetic therapy for acute myeloid leukemia, which may also inhibit the suppressive function of MDSCs [8]. We evaluated this combinatorial therapy against established primary tumors and against experimental metastasis. Furthermore, we identified 2 types of tumor dormancy, which included indolent dormancy characterized by Ki67⁺/low and quiescent dormancy characterized by Ki67⁻. We demonstrated that quiescent, but not indolent, dormant tumor cells were resistant to immunoediting.

MATERIALS AND METHODS

Mouse model

FVBN202 transgenic female mice (The Jackson Laboratory, Bar Harbor, ME, USA) were used. These mice overexpress the nonmutated, nonactivated rat neu transgene under the regulation of the mouse mammary tumor virus promoter [9]. These mice develop premalignant mammary hyperplasia similar to ductal carcinoma in situ before the development of spontaneous carcinoma [10]. These studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Tumor cell lines

The neu-overexpressing MMC cell line was established from spontaneous mammary tumors harvested from FVBN202 mice [11]. Tumor cells were maintained in RPMI 1640, supplemented with 10% FBS.

Ex vivo reprogramming and expansion of splenocytes

The reprogramming of tumor-sensitized immune cells was performed as described previously by our group [5]. In brief, FVBN202 transgenic mice were inoculated in the mammary fat pad with 3×10^6 MMC cells. Tumor growth was monitored by digital caliper, and tumor volumes were calculated by $v = (L \times W^2)/2$, where v is volume, L is length, and W is width. As described previously [11], splenocytes were harvested 21–25 d after tumor challenge, when the tumor had reached ≥ 1000 mm³. Splenocytes were then

cultured in complete medium [RPMI 1640, supplemented with 10% FBS, L-glutamine (2 mM), 100 U/ml penicillin, and 100 µg/ml streptomycin] and were stimulated with Bryostatin 1 (2 nM; Sigma-Aldrich, St. Louis, MO, USA), ionomycin (1 µM; Calbiochem, EMD Millipore, Billerica, MA, USA), and 80 U/ml/10⁶ cells of IL-2 (PeproTech, Rocky Hill, NJ, USA) for 16–18 h. Lymphocytes were then washed thrice and cultured at 10⁶ cells/ml in complete medium with IL-7 and IL-15 (20 ng/ml each cytokine; PeproTech). After 24 h, 20 U/ml IL-2 was added to the complete medium. The following day, the cells were washed and cultured at 10⁶ cells/ml in complete medium with 40 U/ml IL-2. After 48 h, cells were washed and cultured at 10⁶ cells/ml in complete medium with 40 U/ml IL-2. Twenty-four hours later, lymphocytes were again washed and cultured at 10⁶ cells/ml in complete medium with 40 U/ml IL-2. Lymphocytes were harvested 24 h later on the sixth day and were then used for in vitro studies or in vivo for AIT.

Adoptive cellular immunotherapy

Twenty-four hours before AIT, FVBN202 mice were injected i.p. with CYP (100 mg/kg) to induce lymphopenia. Individual groups of mice were challenged i.d. in the mammary gland region, with 3×10^6 MMC cells, or i.v. with 10⁶ MMC. Individual groups of mice then received reprogrammed splenocytes i.v. at a dose of 70×10^6 /mouse, 3 d after tumor challenge when the tumor became palpable (50–70 mm³) or on the day of the i.v. tumor injection. Untreated tumor-bearing mice served as control.

In vitro and in vivo induction of CTA expression in MMC cells and cDNA synthesis

MMC cells (3×10^6 cells/3 ml) were cultured in the presence of 3 µM Dec (Sigma-Aldrich) for 72 h. Medium was then removed, and cells were washed with sterile PBS and then treated with TRIzol (Life Technologies, Thermo Fisher Scientific, Grand Island, NY, USA), per the manufacturer's instructions. In vivo, FVBN202 mice, bearing primary tumor ≥ 1000 mm³, were injected i.p. with a high-dose Dec (2.5 mg/kg), once daily for 5 d. Mice were euthanized, and tumors were harvested 3 d later, minced, and then treated with TRIzol, per the manufacturer's instructions. Contaminant DNA was then removed by DNase I digestion from the in vitro and in vivo specimens; RNA was then purified, followed by cDNA synthesis, as described previously by our group [12].

Real-time qRT-PCR for the detection of CTA expression

qRT-PCR was performed in triplicate wells using the SensiMix SYBR & Fluorescein Kit, according to the manufacturer's procedure (Bioline, Taunton, MA, USA), with the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). qRT-PCR was performed using primers specific for 6 murine CTAs and murine GAPDH. The reaction was initiated by a denaturing period of 10 min at 95°C, followed by 40 cycles of 95°C for 15 min, 60°C for 30 min, and 72°C for 15 min [6, 12]. Relative CTA expression was computed after normalization to GAPDH using the $\Delta\Delta$ quantification cycle method.

IFN-γ ELISA

Reprogrammed immune cells were cultured in complete medium with irradiated (140 Gy) MMC cells or irradiated CTA-expressing MMC, induced by Dec treatment in vitro at a 10:1 ratio for 20 h. Supernatants were then collected and stored at -80°C until assayed. IFN-γ was detected using a mouse IFN-γ ELISA kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol [5].

Characterization of splenocytes and tumor-infiltrating leukocytes

Spleens and metastatic tumor lesions of FVBN202 mice were harvested when the animals became moribund and were then separately homogenized

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carcinoma, M_{reg} = myeloid regulatory cell, PD-1 = programmed death 1, PD-L1 = programmed death ligand 1, PI = propidium iodide, qRT-PCR = quantitative RT-PCR, RT = radiation therapy, TIL = tumor-infiltrating lymphocyte

into a single cell suspension as described previously [11] and below. Splenocytes were then characterized using flow cytometry. Reagents used for flow cytometry include the following: anti-CD16/32 antibody (clone 93), FITC-CD3 (17A2); FITC-CD11b (M1/70); FITC-anti-mouse IgG (Poly4053); PE-GR-1 (RB6-8C5); PE-PD-1 (RMP1-30); PE-CD25 (3C7); PE-Ki67 (16A8); allophycocyanin-CD49b (DX5); allophycocyanin-CD62 ligand (MEL-14); allophycocyanin-Annexin V; PerCP/CY5.5-CD4 (GK1.5); PE/CY7-CD8 α (53-6.7); Brilliant Violet 421-PD-L1 (10F.9G2); Brilliant Violet 605-CD45 (30-F11); and PI, all of which were purchased from BioLegend (San Diego, CA, USA). BD Horizon V450-Annexin V and FITC-FVS were purchased from BD Biosciences. Anti-rat neu antibody (anti-c-ErbB2/c-Neu; 7.16.4), was purchased from Calbiochem. All reagents were used at the manufacturer's recommended concentration. Cellular staining was performed as described previously by our group [11] or as recommended by the manufacturer (Ki67, FVS). Multicolor data acquisition was performed using a LSRFortessa X-20 (BD Biosciences). Data were analyzed using FCS Express v4.07 (De Novo Software, Glendale, CA, USA).

Isolation and characterization of lung metastases

Lungs were harvested from the "Control" and "AIT" groups after animals became moribund. Metastatic lesions were excised individually from the residual lung tissue and minced and digested in Trypsin-EDTA (0.25%; Life Technologies, Thermo Fisher Scientific) overnight at 4°C. The following day, the suspension was incubated at 37°C for 30 min, followed by gentle tissue homogenization to create a cellular suspension. The cell suspension was then washed twice with RPMI supplemented with 10% FBS. Residual RBCs were then lysed using ammonium-chloride-potassium lysing buffer, followed by an additional wash with RPMI 10% FBS. The cell suspension was then placed in cell culture and cultured with RPMI 10% FBS. Adherent metastatic tumor cells were then characterized for the expression of rat neu and PD-L1 using flow cytometry.

Characterization of metastatic tumor-infiltrating leukocytes

Lungs from each group were harvested, and metastatic lesions were isolated as described above. After tissue digestion of the metastatic lesions and RBC lysis, 10⁶ cells of the suspension were placed in flow tubes and stained for surface molecules as described above. All analysis was performed by gating on viable leukocytes (Annexin V⁻ CD45⁺), thereby discriminating against apoptotic cells and tumor cells.

Establishment of ex vivo tumor cell dormancy

MMC cells were treated with 3 daily doses of ADR (doxorubicin hydrochloride, 1 μ M/d for 2 h; Sigma-Aldrich). Residual, dormant MMC cells remained adherent to tissue-culture flasks, whereas the MMC cells susceptible to ADR therapy became nonadherent and were removed from the culture periodically. Assessment of viability, Ki67 expression, and IFN- γ -induced PD-L1 up-regulation by flow cytometry occurred 3 wk after the final treatment. Likewise, 3 daily doses of RT (2 Gy/d) were also used to establish dormant MMC cells. ADR and RT-induced dormant MMC cells were used in the cytotoxicity assay, 8 d after the final treatment.

IHC

Sections of formalin-fixed paraffin-embedded tissue from each tumor were stained with H&E to examine the histomorphology. Additional sections are then subsequently immunolabeled using the standard IHC technique, using the avidin-biotin peroxidase system with a purified anti-mouse Ki67 (BioLegend). Sections of lymph nodes were used as the positive control. Nikon Eclipse 80i light microscope was used to examine the H&E and IHC. The most intense labeling regions (hot spots) away from the edge of the tissue were evaluated using IHC-positive tumor cells

as numerator and the overall tumor cells as the denominator. Representative images of the H&E and the corresponding hotspots were taken.

Statistical analysis

Statistical comparisons between groups were made using 1- and 2-tailed Student's *t* test. Time to death in the in vivo survival studies was calculated from baseline to the date of death. Mice were euthanized when they had a weight loss of $\geq 10\%$. Kaplan-Meier curves and log-rank tests are used to illustrate time to death and to test the difference between each group. $P \leq 0.05$ was considered statistically significant.

RESULTS

The reprogramming of tumor-immune cross-talk during immunotherapy fails to protect animals from an established primary mammary carcinoma

We have reported previously that AIT, using reprogrammed T cells and NKT cells in a prophylactic setting, protected animals against primary tumors and recall tumor challenge. This protection was associated with the presence of memory T cells, and CD25⁺ NKT cells that rendered T cells resistant to MDSC-mediated suppression [5]. Here, we sought to determine whether AIT as a single therapy can protect animals against established primary mammary carcinoma by overcoming MDSCs. FVBN202 mice bearing primary tumors received AIT using reprogrammed T cells and CD25⁺ NKT cells when the tumor had reached 50–70 mm³ or remained untreated. As shown in Fig. 1A and B, AIT alone did not slow the rate of tumor growth (Fig. 1A) or improve overall survival (Fig. 1B) in recipient mice compared with untreated control mice. Then, we combined AIT with epigenetic modulation of tumor cells in vivo to enhance immunogenicity of tumor cells, as well as eliminate MDSCs. To do so, tumor-bearing animals received Dec before AIT. Use of Dec induced the expression of a panel of CTAs in tumor cells (Fig. 1C and D) and resulted in the elimination of MDSCs (Fig. 1E; $P = 0.034$). However, AIT still failed to protect animals from established primary tumors when compared with Dec alone (Fig. 1F and G). This failure was observed in spite of successful reprogramming of tumor-sensitized immune cells (Supplemental Fig. 1) and their enhanced reactivity against CTA-expressing MMC (Fig. 1H; $P = 0.0001$).

The reprogramming of tumor-immune cross-talk during immunotherapy prolongs survival of animals bearing metastatic tumor cells in their circulation

We have reported previously that administration of AIT along with another epigenetic modulator, Aza, was effective in prolonging survival of patients with multiple myeloma when treatment was delivered during minimal residual disease to prevent advanced stage disease [6]. Therefore, we sought to take a similar approach in our experimental model of breast cancer by administering AIT and Dec when tumor cells were present in the circulation and before establishing lung metastasis. AIT alone had a marginal impact on the survival of animals,

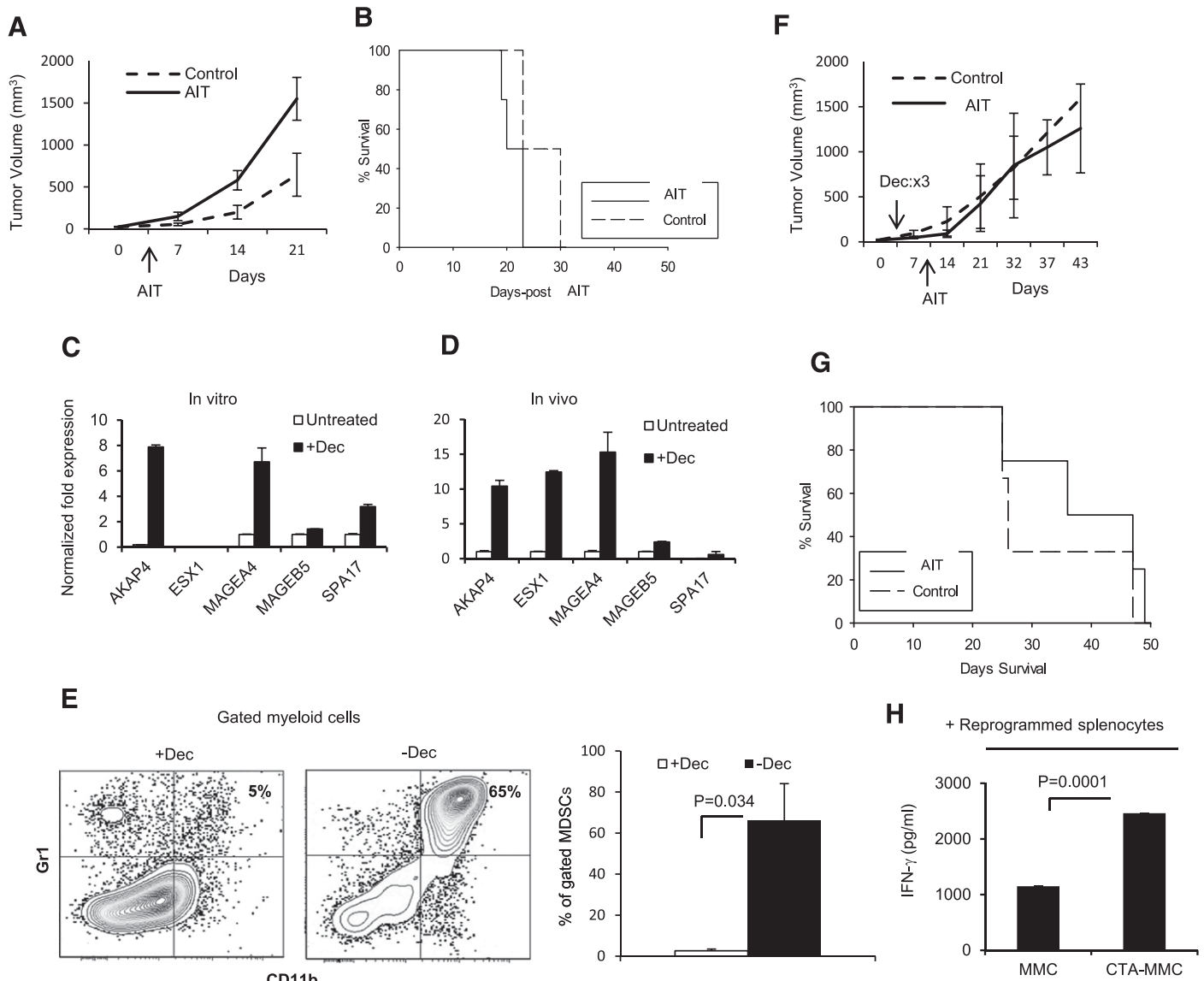


Figure 1. AIT, with or without Dec, fails to induce regression-established mammary carcinoma. (A and B) Animals were challenged with MMC (3×10^6) i.d. in the mammary gland region; upon the tumor reaching 50–70 mm³, animals were conditioned with CYP (100 mg/kg). The following day, mice remained untreated (Control; $n = 4$) or received AIT ($n = 4$). (C) MMC tumor cells were cultured with (+Dec) or without Dec (Untreated; 3 mM) for 72 h; RNA was then extracted and converted to cDNA, followed by qRT-PCR, using primers specific for 6 murine CTAs. (D) Tumor-bearing (~ 1000 mm³ i.d.) FVB/N202 mice received 5 injections of Dec, 1/d (+Dec; 2.5 mg/kg; $n = 1$) or remained untreated ($n = 1$); the tumors were harvested 3 d later, and cDNA was generated to quantify CTA expression, which was normalized to GAPDH. AKAP4, A-kinase anchor protein 4; ESX1, ; MAGEA4, melanoma-associated antigen 4; MAGEB5, melanoma-associated antigen B5; SPA17, ESX1, Extraembryonic, spermatogenesis, homeobox 1; SPA17, Sperm Autoantigenic Protein 17. (E) Animals were challenged i.d. with MMC (3×10^6) in the mammary gland region; after tumors reached 50–70 mm³, all animals were treated with Dec (every other day for 3 total injections; 2.5 mg/kg, i.p.; $n = 3$) or remained untreated ($n = 3$). Seven days later, mice were euthanized, and MDSCs were analyzed in the spleen. (F and G) Animals were challenged i.d. with MMC (3×10^6) in the mammary gland region; after tumors reached 50–70 mm³, all animals were treated with Dec [every day for 3 total injections ($\times 3$); 2.5 mg/kg, i.p.]. Two days later, animals were conditioned with CYP (100 mg/kg, i.p.). The following day, mice remained untreated (Control; $n = 3$) or received AIT ($n = 4$), derived from a CTA⁺ tumor-bearing donor. (H) MMC cells remained untreated (MMC) or were treated with Dec (3 mM; 72 h) to induce CTA expression (CTA-MMC). Tumor cells were then cocultured with reprogrammed splenocytes (1:10) for 20 h. IFN- γ was detected in the supernatant by ELISA. Data represent means $\pm$ SEM of duplicate wells.

whereas Dec alone resulted in prolonging the survival of animals (Fig. 2; $P = 0.013$). AIT + Dec was the most effective therapy that resulted in prolonging the survival of animals compared with the control group or Dec alone (Fig. 2; $P = 0.0001$ and $P = 0.037$, respectively). However, all animals eventually succumbed to metastatic tumors in the lungs.

Immunotherapy induces tumor immunoediting and escape in proliferating tumor cells and indolent dormant cells but not in quiescent dormant cells

To determine whether reprogrammed memory T cells were maintained in vivo, splenocytes of AIT recipients were collected when mice became moribund and cultured with MMC tumor

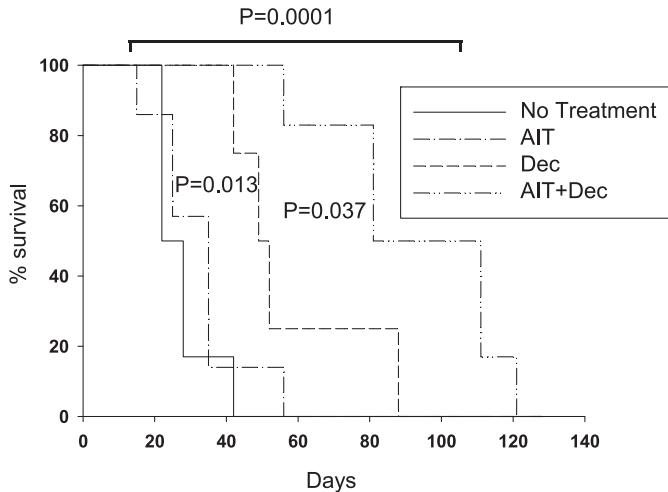


Figure 2. Combined use of Dec and AIT prolongs survival of animals and induces tumor immune editing. FVB/N202 mice were challenged with 1×10^6 MMC cells i.v. Mice then remained untreated (Control; $n = 4$), received AIT ($n = 7$) on the same day as tumor challenge, received Dec (Dec; $n = 4$; 5 daily doses beginning on d 3 after tumor challenge), or received Dec and AIT (AIT + Dec; $n = 6$; AIT on the day of tumor challenge, followed by Dec beginning on d 3).

cells. As shown in **Fig. 3A**, tumor-reactive IFN- γ production by endogenous splenic T cells from the AIT group was greater than that produced by T cells from the control group ($P = 0.003$). To determine the impact of treatments on tumor immunoediting and escape, T cells and tumor cells in the tumor-microenvironment of the lung were analyzed. Metastatic tumor lesions were isolated from the lung at the end of the trial and analyzed for the expression of the tumor antigen, neu, and PD-L1. The tumor lesions isolated from the AIT group showed down-regulation of neu antigen on the tumor cells compared with control MMC tumor cells and the lesions isolated from the control group (**Fig. 3B**, upper; $P = 0.00003$ and $P = 0.0008$, respectively). The AIT + Dec group showed similar trends as the AIT group. Additionally, 25% of MMC cells isolated from metastatic tumor lesions of the AIT group demonstrated total loss of neu expression compared with control MMC tumor cells and the lesions isolated from control group (**Fig. 3B**, lower; $P = 0.002$ and $P = 0.01$, respectively). Again, the AIT + Dec group showed similar trends as the AIT group. This suggests that metastatic MMC cells may eventually escape detection from neu-specific cellular immunity. Metastatic tumors of the control group that received no treatment did not show down-regulation or loss of neu antigen (**Fig. 3B**). As AIT was the major factor in neu loss/down-regulation, and the AIT-Dec group showed a similar trend, we looked at the expression of tumor PD-L1 expression in the AIT group. Interestingly, metastatic tumor cells from the control group had higher expression of PD-L1 in the tumor compared with the metastatic tumor cells from the AIT group or control MMC cells (**Fig. 3C**; MFI: 1360 vs. $390 \pm \text{SEM}$; $P = 0.011$). A similar trend was observed when MMC cells were cultured with IFN- γ or splenocytes of tumor-bearing animals (**Fig. 3D**).

The immune-suppressive function of PD-L1 requires engagement with PD-1, which renders immune cells unresponsive [13, 14]. Importantly, 40–50% of reprogrammed T cells and NKT cells that were used for AIT expressed PD-1 (**Fig. 3E**), but only CD8 $^+$ T cells were observed to up-regulate PD-1 as a result of reprogramming (**Fig. 3E**; $P = 0.01$). Therefore, we also analyzed tumor-infiltrating T cells for PD-1 expression to determine the potential for the PD-1/PD-L1 axis to mediate T cell suppression within the tumor site. Interestingly, as seen in **Fig. 3F**, tumor infiltration of CD8 $^+$ T cells into the tumor bed was greater in mice receiving AIT compared with untreated mice (14% vs. 3%, respectively; $P = 0.02$). However, expression of PD-1 on tumor-infiltrating CD8 $^+$ T cells did not significantly increase following AIT compared with the control group (**Fig. 3G**). We did not observe CD4 $^+$ T cell infiltration into the tumor lesions (data not shown). Splenic T cells and NKT cells that were isolated from the AIT and control groups when animals became moribund also expressed PD-1, although there was no statistical difference between the groups (**Fig. 3H**; 10% of T cells and 50% of NKT cells). Taken together, these data suggest that although AIT promotes the infiltration of CD8 $^+$ T cells, the highly proliferative nature of the metastatic tumors may evade such anti-tumor immune responses by emerging with reduced expression of the tumor antigen, neu. Metastatic tumor of the Dec group also showed down-regulation of neu antigen (**Supplemental Fig. 2A**, left; MFI: 442 vs. $202 \pm \text{SEM}$), as well as total loss of neu antigen in 36% of tumor cells compared with the control MMC cell line containing a residual 5% of neu-negative cells (**Supplemental Fig. 2A**, right). As the control group did not show neu loss or down-regulation, but the AIT group did, we sought to determine whether neu loss or down-regulation in animals who received Dec could result from the contribution of an endogenous T cell response induced by Dec, which induces the expression of CTAs and therefore, could function as an in situ vaccination by eliciting endogenous T cell responses. To determine the contribution of Dec in neu antigen loss or down-regulation, we performed in vitro studies by treatment of MMC with Dec alone, where the endogenous immune response did not have any contribution. Dec treatment resulted in the quantitative down-regulation of neu expression but did not induce total neu loss (**Supplemental Fig. 2B**; $P = 0.008$). IFN- γ induced down-regulation of neu (**Supplemental Fig. 2C**; $P = 0.002$), and Dec did not recover neu expression. Thus, we then began to question whether residual tumor cells that remain after conventional cytotoxic therapy, which are generally dormant, also use similar escape mechanisms or if they were perhaps more sensitive to immune-mediated elimination.

To determine whether dormant tumor cells were resistant to immunoediting and escape, MMC tumor cells were treated with ADR to establish tumor dormancy ex vivo. Dormant tumor cells were then treated with a product of anti-tumor T cell responses—IFN- γ —to determine sensitivity of different dormant cells, quiescent and indolent, to immunoediting. We looked at the expression of PD-L1, as this is the most immediate immunoediting change that occurs as a result of IFN- γ treatment. A clinically relevant proliferation marker, Ki67, along with a viability marker (FVS), was used to detect

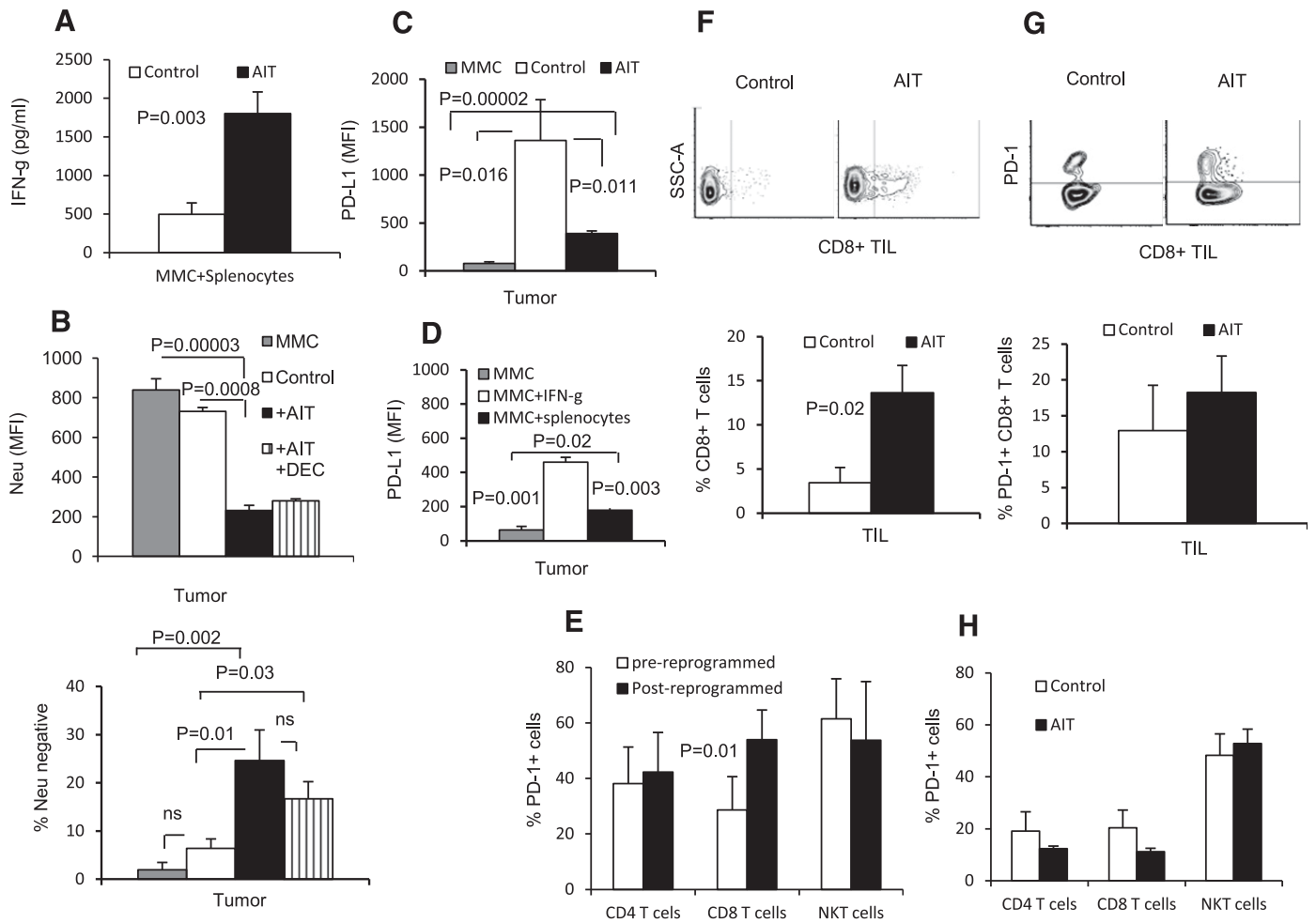


Figure 3. AIT promotes immunoeediting of lung metastatic lesions. (A) Splenocytes were harvested from untreated mice (Control; $n = 5$) and AIT recipients ($n = 3$) and cultured in the presence of MMC cells (10:1) for 20 h. Supernatants were collected and subjected to IFN- γ ELISA. (B) Metastatic lesions in the lung of FVB/N202 mice that remained untreated (Control; $n = 3$), AIT recipients ($n = 6$), and AIT + Dec recipients ($n = 4$) were harvested when mice became moribund. Tumor lesions were digested and analyzed. MFI of neu and frequency of neu loss (B) and MFI of PD-L1 (C) were then quantified using flow cytometry using MMC cell line (MMC) as an in vitro control. (D) MMC cells were cultured with IFN- γ or splenocytes of tumor-bearing mice, and PD-L1 was detected after 16–20 h ($n = 2$ –3). (E) Splens of FVB/N202 mice bearing primary mammary carcinoma ($n = 4$) were harvested after tumors were $\geq 1000 \text{ mm}^3$. PD-1 expression was then quantified on the splenocytes, pre- and postreprogramming. (F and G) Metastatic lesions in the lung of FVB/N202 mice that remained untreated (Control) and AIT recipients were harvested when mice became moribund. (F) The frequency of CD8 $^+$ T cell infiltration metastatic lesion in the lung of control mice and the AIT group ($n = 3$) was determined on gated CD3 $^+$ cells. SSC-A, Side-scatter-area. (G) Expression of PD-1 was determined on CD3 $^+$ CD8 $^+$ cells (Control, $n = 1$; AIT, $n = 3$) by gating on CD45 $^+$ viable leukocytes. (H) Splens of FVB/N202 mice that had received AIT ($n = 4$) or remained untreated ($n = 3$) and were i.v. challenged with MMC were analyzed by flow cytometry after tumor-bearing mice became moribund to quantify PD-1 expression. Data represent means $\pm$ SEM.

FVS $^-$ viable, indolent tumor cells (Ki67 $^+$ /low) and quiescent tumor cells (Ki67 $^-$). As shown in Fig. 4A, ADR induced apoptosis in the majority of MMC cells, such that by 3 wk after treatment, the number of FVS $^-$ viable MMC cells was reduced from 77% to 31% ($P = 0.005$). The remaining residual viable tumor cells that escaped ADR-induced apoptosis entered a state of dormancy, as there was no significant increase in the number of tumor cells between 1 and 3 wk after completion of ADR treatment (Fig. 4B). To determine if dormant tumor cells could exploit immune escape mechanisms, we established ADR-induced tumor dormancy, followed by treatment of dormant MMC cells with

IFN- γ , 3 wk after the completion of ADR treatment to provoke PD-L1 expression [15]. We evaluated the expression of PD-L1 on viable proliferating control MMC cells (Ki67 $^+$), without (Untreated) and with IFN- γ (Untreated $\rightarrow$ IFN- γ) treatment, as well as on viable dormant tumor cells without (+ADR) and with IFN- γ (+ADR $\rightarrow$ IFN- γ) treatment. As shown in Fig. 4C, left, ADR-treated MMC showed a significant shift from Ki67 $^+$ toward Ki67 $^+$ /low cells ($P = 0.026$), indicative of indolent tumor dormancy. ADR or ADR $\rightarrow$ IFN- γ treatment also resulted in a shift from Ki67 $^+$ toward Ki67 $^-$ quiescent cells, as shown by an increased frequency of Ki67 $^-$ MMC (Fig. 4C; $P = 0.02$ and $P = 0.001$, respectively) and a decreased

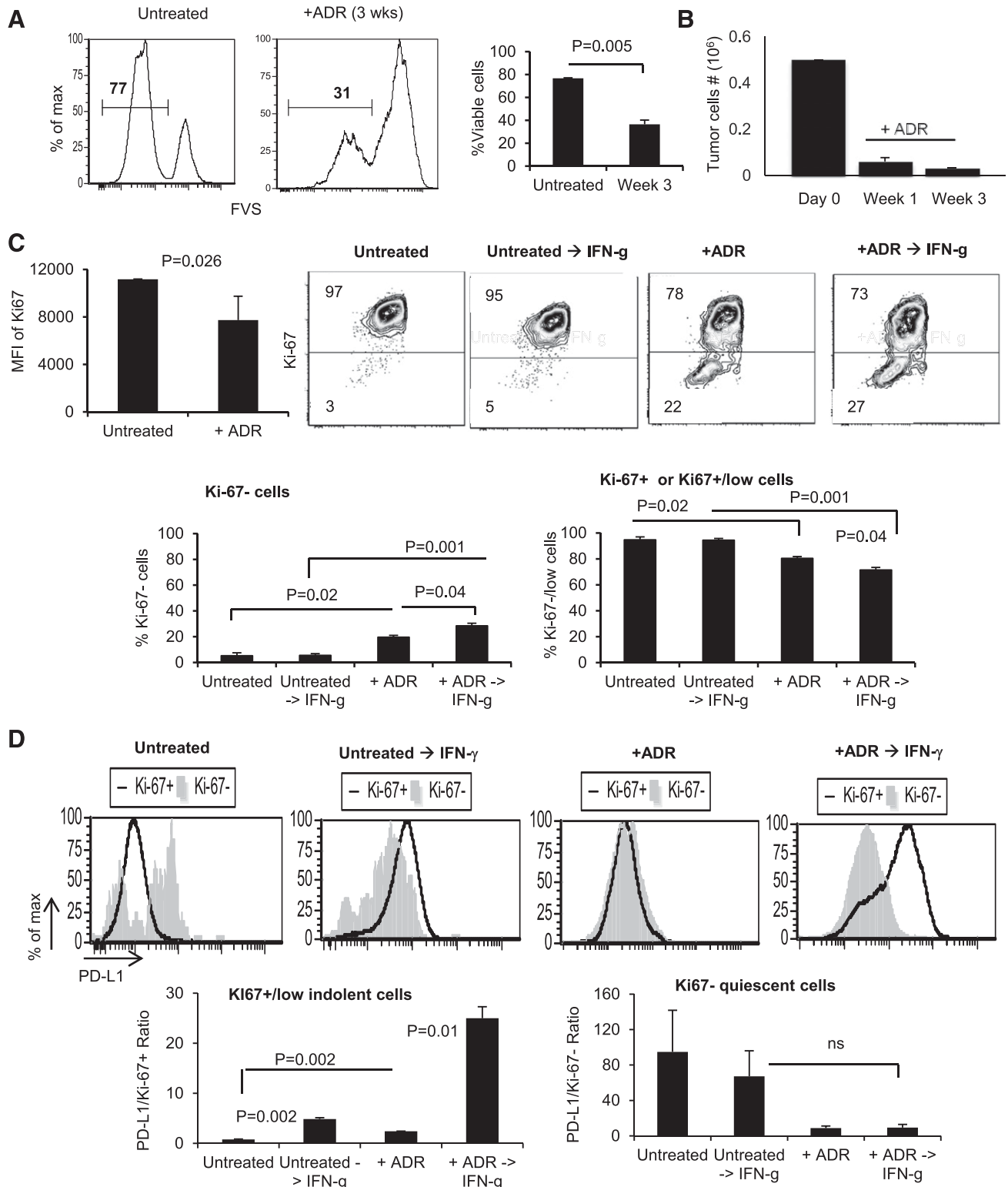
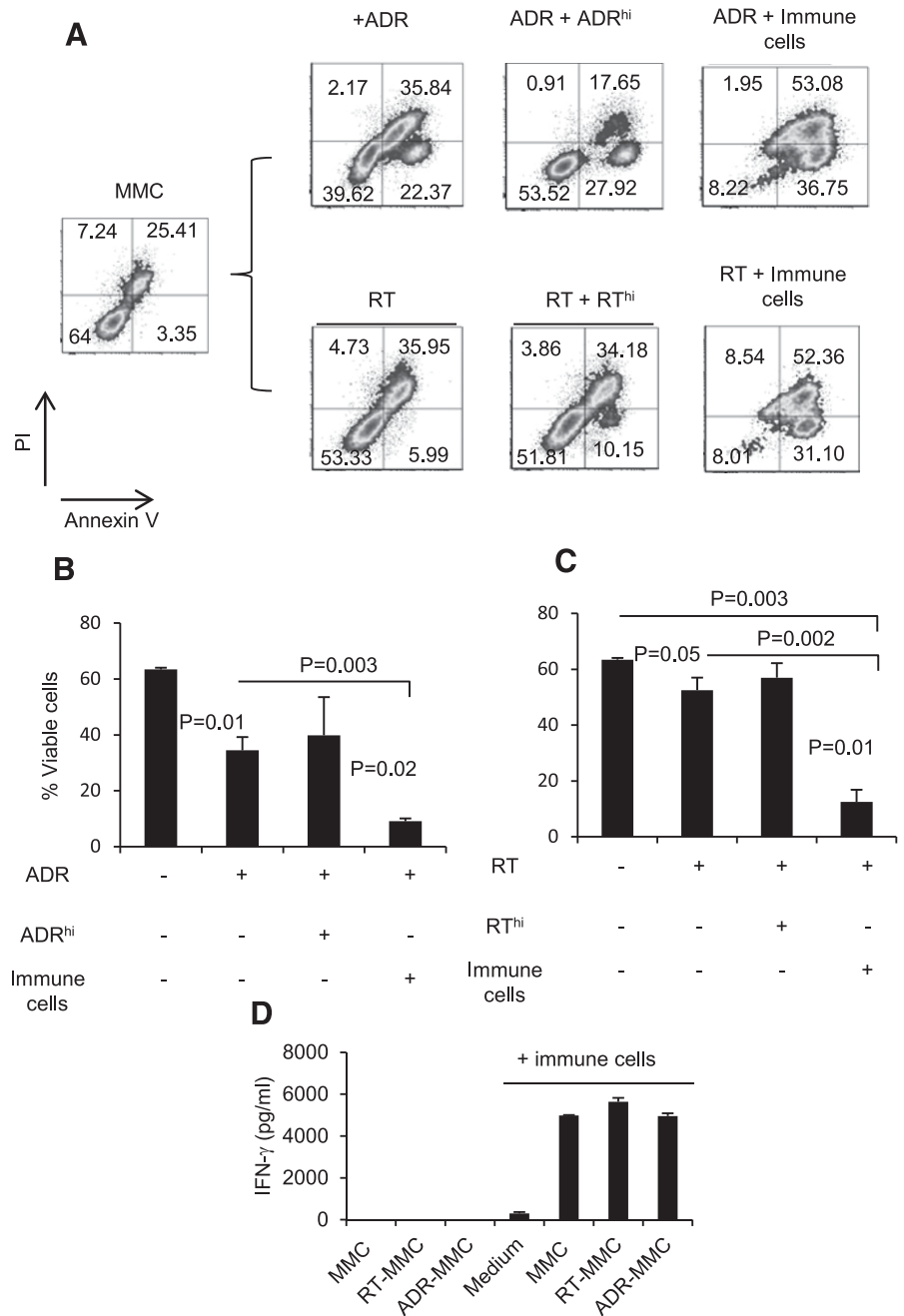


Figure 4. ADR treatment results in the emergence of indolent and quiescent tumor dormancy. (A) MMC tumor cells were treated with 3 daily doses of ADR (1 μ M for 2 h) and then remained untreated for 3 wk. The frequency of viable MMC cells was determined by quantifying FVS⁺ cells using flow cytometry. (B) At wk 1 and 3 post-treatment, adherent and viable tumor cells were counted by trypan blue exclusion. (C and D) MMC cells were treated for 3 consecutive d with ADR (1 μ M, 2 h) or left untreated. Three weeks later, ADR-treated and untreated MMC cells were stimulated with IFN- γ (50 ng/ml) for 12–16 h to induce the expression of PD-L1. (C) Emergence of Ki67 was determined in control MMC cells (Untreated), as well as ADR-treated cells (+ADR) $\pm$ IFN- γ stimulation. (D) The expression of PD-L1/cell was calculated by dividing PD-L1 MFI by the frequency of Ki67⁺ cells in ADR-treated and untreated MMC cells $\pm$ IFN- γ stimulation. Data represent 3 independent experiments and means $\pm$ SEM.

Figure 5. Immunotherapy displays cytotoxic function against treatment refractory dormant tumor cells in vitro. (A) MMC cells ($n = 3$) treated with ADR ($1 \mu\text{M}$, 2 h) or 2 Gy RT (RT-treated MMC) for 3 consecutive d and remained in culture for 8 d total to establish tumor cell dormancy in vitro. (B) On d 8, these dormant tumor cells were treated with a high-dose ADR ($1 \mu\text{M}$, 24 h; ADR-treated MMC + ADR^{hi}) or reprogrammed immune cells (ADR-treated MMC + immune cells; ADR-treated MMC + immune cells). Two days later, cells were stained with Annexin V/PI and analyzed by flow cytometry. Data represent 3 biologic repeats and means $\pm$ SEM. (C) On d 8, these dormant tumor cells were treated with 18 Gy RT (RT-treated MMC + RT^{hi}) or reprogrammed immune cells (RT-treated MMC + immune cells). Two days later, cells were stained with Annexin V/PI and analyzed by flow cytometry. (D) MMC tumor cells or dormant MMC cells (RT-MMC, ADR-MMC) were cultured in the absence or presence of the reprogrammed immune cells in a 10:1 ratio for 24 h. Control immune cells were cultured alone (Medium). IFN- γ release was detected in the supernatant using ELISA. Data represent 2 biologic repeats and means $\pm$ SEM.



frequency of Ki67⁺ MMC cells (Fig. 4C; $P = 0.02$ and $P = 0.001$, respectively). IFN- γ treatment induced up-regulation of PD-L1 on Ki67⁺/low indolent MMC (Fig. 4D, left; $P = 0.002$ and $P = 0.01$). Interestingly, Ki67⁻ control MMC cells and Ki67⁻ quiescent MMC cells did not up-regulate PD-L1 in the presence of IFN- γ (Fig. 4D, right).

Dormant MMC cells established by ADR or RT become resistant to higher doses of chemotherapy or RT but remain sensitive to immunotherapy

ADR chemotherapy usually induces tumor dormancy, which could lead to tumor recurrence. To determine the direct effect

of ADR on tumor dormancy, we performed ex vivo experiments. ADR treatment increased the proportion of Ki67⁻ tumor cells, which lasted for 3 wk. This trend was associated with reduced viability, 3 wk after treatment, which improved 6 wk after treatment (Supplemental Fig. 3; 77% > 31% > 50%). To determine whether dormant MMC cells established by ADR treatment remain sensitive to tumor-reactive immune cells, dormancy was established by treating MMC with 3 daily doses of ADR ($1 \mu\text{M}/\text{d}$ for 2 h; Supplemental Fig. 4A); 8 days after the final treatment, MMC cells received a high dose of ADR ($1 \mu\text{M}$ for 24 h) or were cultured with tumor-reactive immune cells for 48 h. ADR treatment induced apoptosis in MMC cells

(Fig. 5A and B; $P = 0.01$). Tumor cells that survived apoptosis became chemo refractory, such that additional ADR treatment at a higher dose (1 μM for 24 h) did not induce cell death (Fig. 5A and B; average 40% vs. 54%). However, they remained sensitive to tumor-reactive immune cells. In the presence of tumor-reactive immune cells, the frequency of viable ADR-treated dormant MMC dropped from 40% to 8% (Fig. 5A and B; $P = 0.003$). In fact, lymphocytes were more effective than a high dose of chemotherapy in inducing apoptosis in dormant MMC (Fig. 5A and B; $P = 0.02$). We also established dormant MMC by 3 daily doses of RT (2 Gy/d); again, surviving dormant cells became refractory to RT. An additional RT at a higher dose (18 Gy) did not markedly decrease the frequency of viable tumor cells (Fig. 5B and C; 53% vs. 52%). However, RT refractory MMC cells remained sensitive to tumor-reactive lymphocytes as the viability dropped from 53% to 8% (Fig. 5B and C; $P = 0.002$). In recapitulating our results with chemotherapy-induced tumor cell dormancy, tumor-reactive immune cells were more effective than high-dose RT at inducing apoptosis in dormant MMC (Fig. 5B and C; $P = 0.01$). To determine whether higher levels of apoptosis in dormant tumor cells were a result of their greater sensitivity to immune cells rather than a higher reactivity of the immune cells, IFN- γ ELISA was performed using reprogrammed immune cells cultured with MMC tumor cells or ADR- and RT-induced dormant MMC cells. As shown in Fig. 5D, tumor-reactive immune cells produced comparable levels of IFN- γ upon stimulation with MMC or dormant MMC (RT-MMC, ADR-MMC). To determine the establishment of Ki67[−] quiescent and Ki67⁺/low indolent tumor cells, experimental animals bearing primary MMC were treated with ADR or remained untreated. Animals treated with ADR exhibited suppression of tumor growth (Supplemental Fig. 4B). Tumors of the ADR group showed a shift from Ki67⁺ toward Ki67⁺/low indolent and Ki67[−] quiescent cells (Supplemental Fig. 4C).

DISCUSSION

We developed an experimental metastatic mouse model by i.v. injection of highly proliferative MMC cells to FVB/N202 mice. Animals in this model became moribund within 20–40 d and presented with lung metastases upon macroscopic inspection. This model represents the onset of advanced stage disease. We demonstrated that concurrent use of Dec with AIT using reprogrammed CD25⁺ NKT and T cells prolonged survival of the experimental animals but failed to eliminate the tumor, as all mice eventually succumbed to metastatic disease in the lungs. Failure of tumor elimination was associated with down-regulation of the tumor antigen, neu, on metastatic tumor cells. Studies involving AIT without Dec treatment in vivo or Dec alone without immune response in vitro confirmed that total neu antigen loss and down-regulation were mediated by anti-tumor immune responses, whereas Dec alone only had the capacity to induce down-regulation of neu antigen. We have reported previously that treatment of neu-negative tumor cells (antigen-negative variant) with Dec resulted in the induction of neu expression at mRNA but not at protein levels [11].

Likewise, Dec treatment did not overcome IFN- γ -induced down-regulation of neu protein in MMC. Here, we also showed that a high dose of Dec induced down-regulation of the neu protein in vivo. These data suggest that high-dose Dec might have different effects on neu expression during mRNA transcription and protein translation. It was reported that a low dose of Dec could have only a hypomethylating effect when incorporated into DNA, whereas a high-dose Dec could also incorporate into RNA and show different effects [16]. Although Dec or Aza render tumor cells highly immunogenic by inducing the expression of highly immunogenic CTAs, this effect is usually transient in that tumor cells lose CTAs after the cessation of Aza therapy [6].

We hypothesized that targeting dormant but not highly proliferating tumor cells might overcome tumor immunoediting and escape. Therefore, we conducted studies to determine the sensitivity of dormant tumor cells to immunoediting and escape. We demonstrated that ADR treatment induced 2 types of tumor dormancy: 1) an indolent type of dormancy, characterized by the positive/low expression of Ki67; this type of dormancy is maintained through balanced proliferation and death, as these cells keep producing dead cells, whereas the total number of viable cells remains unchanged; and 2) a quiescent type of tumor dormancy that is characterized by lack of Ki67 expression (Ki67[−]); this type of dormancy is maintained through total cessation of proliferation. We demonstrated that proliferating tumor cells, either untreated tumor cells (Ki67⁺) or indolent tumor cells (Ki67⁺/low), were susceptible to immunoediting and escape during cell division, but quiescent tumor cells (Ki67[−]) failed to undergo immunoediting; in fact, they failed to up-regulate PD-L1 in the presence of IFN- γ stimulation. These results suggest that quiescent dormant cells could be the best target for immunotherapy.

It was reported that innate IFN- γ is essential for up-regulation of PD-L1 expression [17]. Intriguingly, an adaptive immune response following AIT resulted in a >3-fold inhibition in the induction of PD-L1 expression on tumor cells compared with the control group, although it was still significantly higher than MMC tumor cells in vitro before challenge. Similar results were obtained when tumor cells were cultured with IFN- γ or lymphocytes of tumor-bearing mice. These data suggest that a T cell-independent inflammatory response, which involves IFN- γ , has a greater impact than T cells on up-regulation of PD-L1. In addition, AIT was associated with a significant inhibition in the induction of tumor PD-L1 compared with no AIT control group, suggesting that the PD-1/PD-L1 axis is more active in tumor-bearing animals in the absence of AIT. To test this, we cocultured reprogrammed tumor-reactive T cells with MMC in the presence or absence of PD-1 blocking antibody; the anti-tumor function of reprogrammed T cells was not affected by PD-1 blockade in vitro (data not shown).

This is important, as reprogrammed T cells and NKT cells that were used for AIT expressed PD-1, and PD-1 expression was sustained after AIT. However, reprogrammed T cells also produce perforin and granzyme B [5], allowing them to induce apoptosis in tumor cells before they begin to up-regulate PD-L1 mediated by IFN- γ . These data suggest that AIT results in a

significant inhibition of tumor PD-L1 induction to the levels that might not be suppressive. Blockade of PD-1/PD-L1 by anti-PD-1 antibody did not have any effects on anti-tumor immune responses against MMC in vitro (data not shown). Therefore, prolonged survival in the AIT group could be associated with lower expression of PD-L1 in MMC when tumor cells were present in the circulation compared with the control group when tumors were established in the lungs. Given the high levels of PD-L1 expression on established tumors, administration of AIT as a single agent in a therapeutic setting is likely to fail in curing cancer, as it was evident in our therapeutic protocol (Fig. 1). Administration of AIT in a preventive setting, when tumor cells were in the circulation but before they establish lung metastasis, was highly effective, although animals succumbed to metastatic tumor, as their tumors begin to undergo neo antigen loss. Our data suggest that tumors use numerous mechanisms to change during cell division and escape from immunotherapy. These mechanisms were shown to overcome tumor immune surveillance and reduce the efficacy of immunotherapy [18, 19]. However, and intriguingly, dormant tumor cells that were established by chemotherapy or RT and that became chemo resistant or RT resistant remained sensitive to tumor-reactive immune cells. Our findings are consistent with the reports on the efficacy of AIT in patients with metastatic melanoma using TILs grown in IL-2. AIT, using IL-2-expanded TIL, resulted in tumor regression in 49% of patients [20]. When AIT was combined with total body irradiation, which was implemented to induce lymphopenia, objective responses increased to 72%. Among treated groups, 20% had complete tumor regression and >10 y relapse-free survival [21]. Thus far, of the 34 complete responders in the National Cancer Institute trials, 1 has recurred [22].

The results of this study suggest that administration of immunotherapy in a setting of advanced stage prophylaxis, i.e., after the completion of conventional cancer therapies, when tumor dormancy is established but before distant recurrence of the disease, could effectively target dormant tumor cells and prevent advanced stage disease. On the other hand, the application of immunotherapy to highly proliferative tumors renders the tumors prone to immunoeediting and subsequent immunologic escape during cell division [23]. The challenge is to develop combinatorial therapies, i.e., AIT, following the administration of epigenetic modulators or small molecules that could induce cell-cycle arrest and establish a quiescent type of tumor dormancy so as to render dormant tumor cells resistant to immunoeediting and escape from immunotherapy. The extension of knowledge gained from our preclinical studies to the clinical setting remains to be determined in patients with early-stage breast cancer or patients with minimal residual disease.

AUTHORSHIP

K.K.P. and M.H.M. contributed to the study's conception, design, experimental and analytical performance, and writing of the manuscript. R.C.K., L.G., M.O.I., and W.W. contributed to the

study's experimental and analytical performance and writing of the manuscript. A.A.T., X.-Y.W., and H.D.B. contributed to the study's conception, analytical performance, and writing of the manuscript.

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DISCLOSURES

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Department of Defense. The authors declare no conflicts of interest.

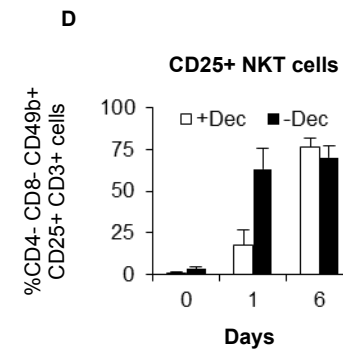
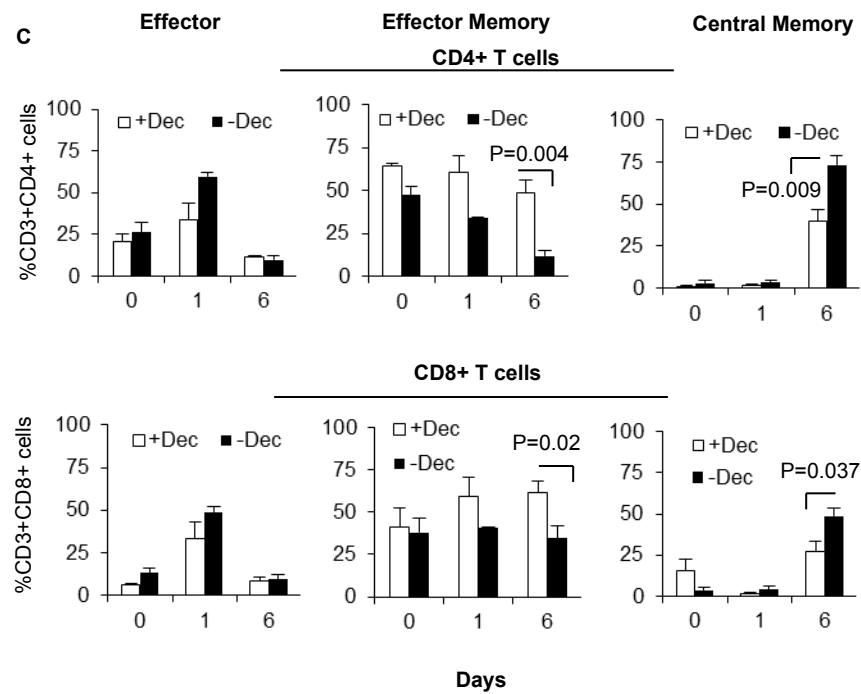
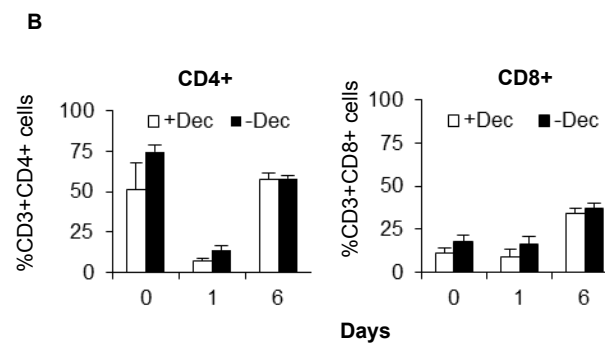
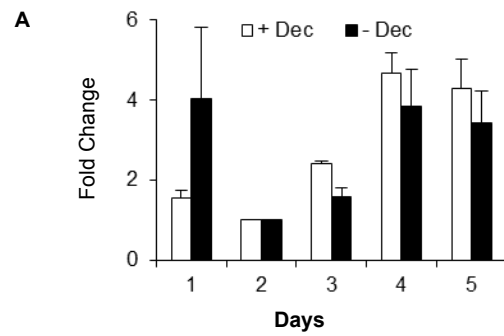
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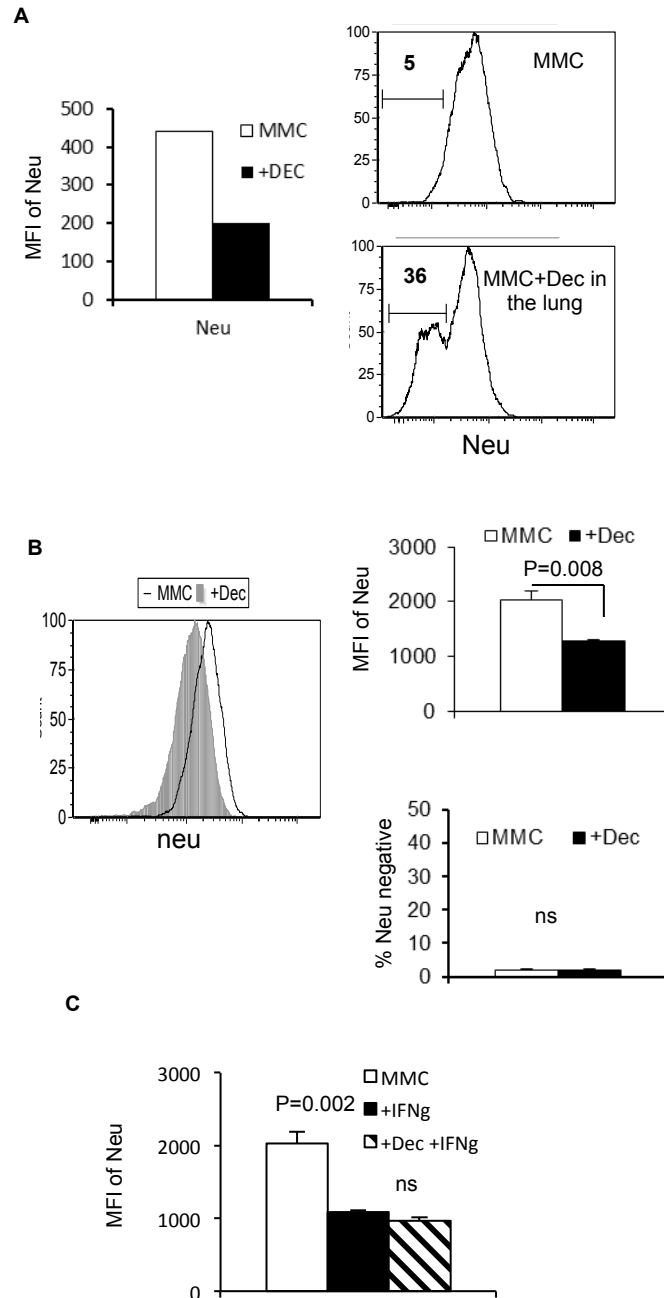
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KEY WORDS:

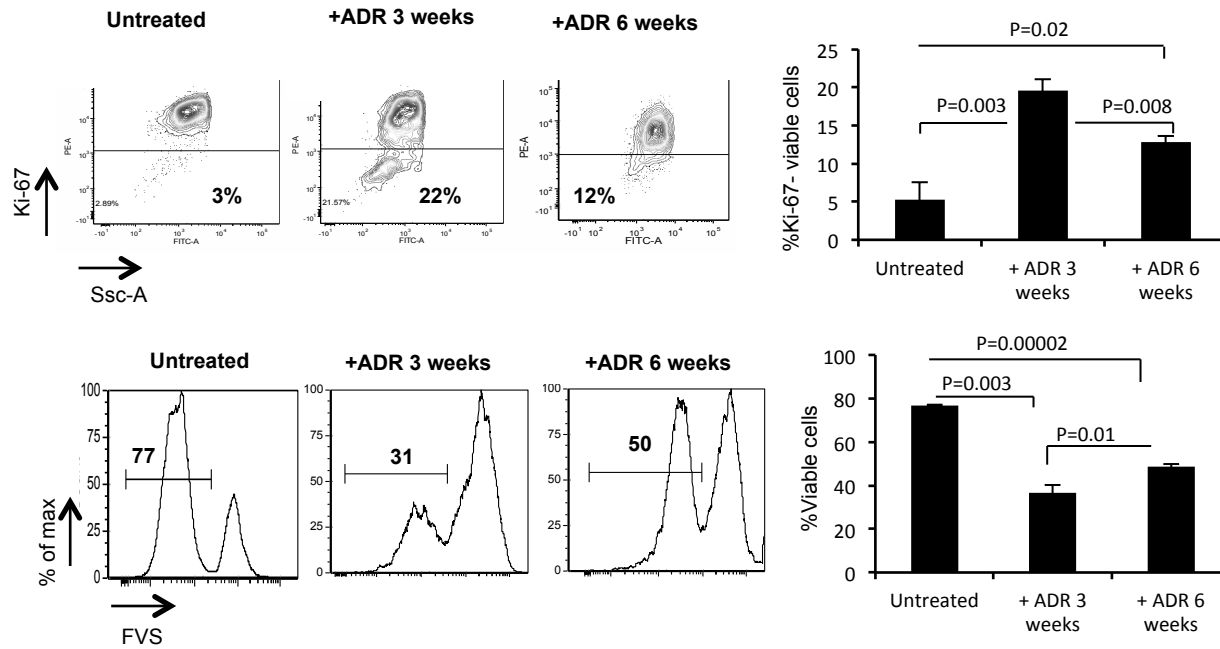
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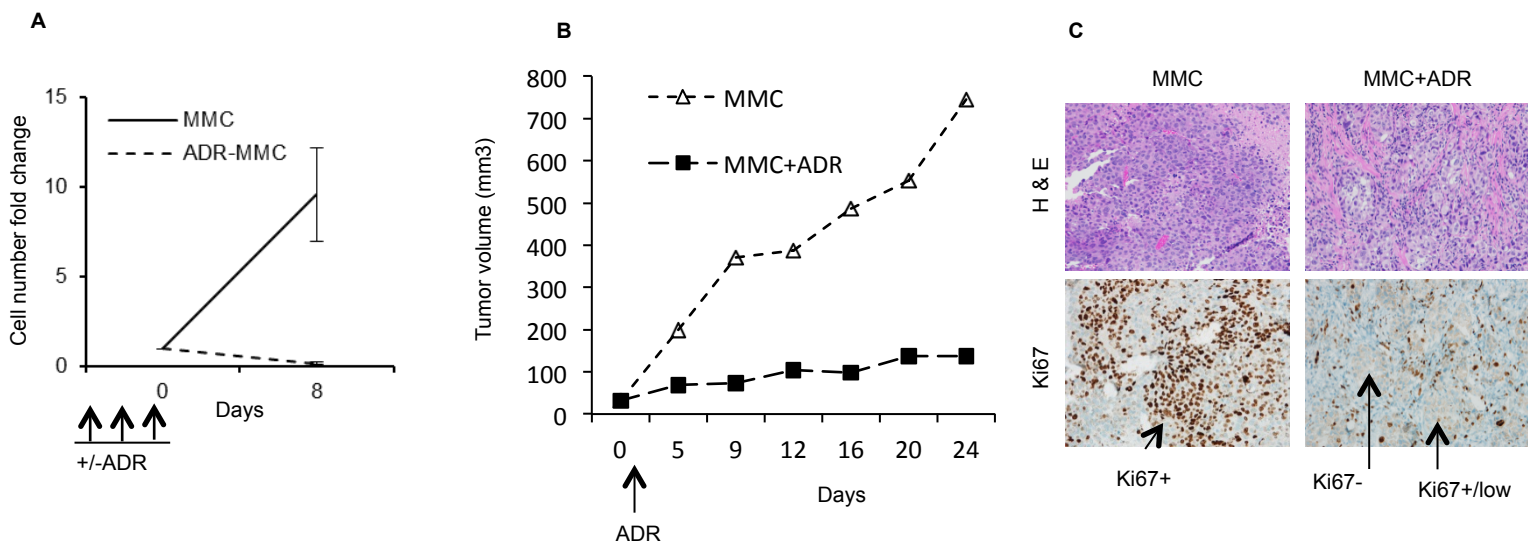
Supplemental Figure 1. Expansion and phenotypic reprogramming of tumor-reactive splenocytes is similar between animals bearing primary cancer with and without Decitabine preconditioning. FVBN202 mice were challenged with 3×10^6 MMC cells intradermally. A portion of the mice went on to receive five sequential injections of Dec (2.5mg/kg) once tumors reached 1000mm³ (+Dec), while the remaining mice were untreated (-Dec). Mice were euthanized and spleens were harvested 7 days after the final injection of Dec, and were then treated with B/I and g-c cytokines *ex vivo*. A) Cell counts of viable tumor-reactive immune cells was determined by trypan blue exclusion; fold change was calculated by normalizing the cell count of each day to the number of cells present on day 1. Flow cytometry was used to determine the frequency of total CD4⁺ and CD8⁺ T cells (B), phenotype of CD4⁺ and CD8⁺ T cells (C), and the frequency of CD25⁺ NKT cells (D). Data represent four biological repeats for each group and mean $\pm$ SEM.



Supplemental Figure 2. AIT promotes immunoediting of lung metastatic lesions. A) FVBN202 mice were challenged i.v. with MMC cells (1×10^6); 3 days later they were injected with Dec (2.5mg/kg) once daily for 5 days or remained untreated (MMC). After the mice became moribund, metastases were excised from the lung and established *in vitro*. Neu median fluorescence intensity (MFI) and percentage of neu negative cells were quantified using flow cytometry 10-14 days after the animals had been euthanized. B) MMC cells were treated with Dec (Dec; 3uM) or remained untreated (MMC), *in vitro*. After 10 days of culture, neu expression was quantified using flow cytometry. C) MMC cells remained untreated or were treated with one dose of IFN- γ (50ng/ml) or Dec+IFN- γ , *in vitro*. Expression of neu was determined 7 days after the final treatment. Data represent mean MFI $\pm$ SEM of triplicates.



Supplemental Figure 3. Dormant tumor cells recover proliferative capacity as a function of time. MMC tumor cells were treated with 3 daily doses of ADR (1uM for 2 hs), then remained untreated for 3 weeks and 6 weeks, *in vitro*. At weeks 3 and 6 post-treatment, Ki-67 expression and viability were quantified within the population of adherent tumor cells. Data represent 3 independent experiments and mean $\pm$ SEM.



Supplemental Figure 4. ADR treatment induces tumor dormancy. A) MMC cells (n=3) treated with ADR (1uM, 2 hs) for 3 consecutive days and remained in culture for 8 days total, in order to establish tumor cell dormancy, *in vitro*. B) FVBN202 mice were challenged with MMC (3x10⁶) in the mammary gland region; after tumors reached 30-50 mm³ animals were treated with ADR (MMC+ADR; 20 mg/kg; i.v.), or remained untreated (MMC). Tumor growth was monitored for four weeks, C) Animals were sacrificed and tumor specimens were collected and subjected to H & E staining as well as IHC for Ki67. Arrows show Ki-67+ proliferating tumor cells (dark brown), Ki-67- quiescent tumor cells (blue color like background) and Ki-67+/low indolent tumor cells (weak brown). Figures show a 200X magnification.

Tumor Dormancy and Relapse: From a Natural Byproduct of Evolution to a Disease State

Masoud H. Manjili



Abstract

Species evolve by mutations and epigenetic changes acting on individuals in a population; tumors evolve by similar mechanisms at a cellular level in a tissue. This article reviews growing evidence about tumor dormancy and suggests that (i) cellular malignancy is a natural byproduct of evolutionary mechanisms, such as gene mutations and epigenetic modifications, which is manifested in the form of tumor dormancy in healthy individuals as well as in cancer survivors; (ii) cancer metastasis could be an early dissemination event that could occur during malignant dormancy even before primary cancer

is clinically detectable; and (iii) chronic inflammation is a key factor in awakening dormant malignant cells at the primary site, leading to primary cancer development, and at distant sites, leading to advanced stage diseases. On the basis of this evidence, it is reasonable to propose that we are all cancer survivors rather than cancer-free individuals because of harboring dormant malignant cells in our organs. A better understanding of local and metastatic tumor dormancy could lead to novel cancer therapeutics for the prevention of cancer. *Cancer Res*; 77(10); 2564–9. ©2017 AACR.

Malignancy Is a Byproduct of Evolutionary Mechanisms of Cell Survival

DNA is a dynamic and adaptable molecule that is constantly changing through the process of mutation and epigenetic modification. These are evolutionary mechanisms that allow survival of an individual against environmental insults. DNA mutation could spontaneously occur during DNA replication or could be accidental as a result of environmental exposure to certain chemicals, UV radiation, or other external factors that impact DNA replication. Spontaneous somatic mutations lead to genotypic and phenotypic heterogeneity within and between tissues, generating genetic mosaicism in the body and the risk of cancer that could arise from those mutations (1). Randomness of DNA mutations and epigenetic modifications during cell division results in different outcomes in the host. Spontaneous mutations could be harmless, beneficial, or deleterious to human cells, whereas accidental mutations are often harmful. Dynamics of DNA mutation and epigenetic modification mechanisms make cellular transformation an inevitable event.

Harmless somatic mutations have been reported in healthy hematopoietic stem cells of women with a constant mutation rate of four mutations per year or three mutations per cell division. These mutations were found in regions that were not evolutionarily conserved (2). Another example of harmless somatic mutations includes somatic mutations in the hypoxanthine-guanine

phosphoribosyltransferase (hprt) gene in T cells of normal children. This is a V(D)J recombinase-mediated recombination event that is found in 30% to 35% of children under 5 years of age (3). The frequency of these specific changes is dramatically decreased in older children.

Beneficial somatic mutations constantly occur in cells of the immune system to maintain their effector function. For instance, somatic hypermutation in the variable regions of immunoglobulin genes is a major component of the process of affinity maturation, allowing diversification of B-cell receptors in recognizing numerous antigens and distinguishing self-antigens from foreign antigens (4). Lactose tolerance is also the result of beneficial mutations that create evolutionary polymorphism in lactase-phlorizin hydrolase, the enzyme responsible for hydrolysis of milk lactose into glucose and galactose. Lactose tolerance is found in around 35% of adults living in the world, mostly people with European ancestry (5). This enzyme is expressed during infancy, but after the weaning period is over, lactase production usually declines. However, 35% of human population continues to express lactase throughout adult life. Another beneficial mutation was reported in the *CCR5-delta32* gene, which can block the entry of human immunodeficiency virus (HIV) into CD4⁺ T cells and protect the mutant carrier from AIDS (6). Beneficial mutations in a gene may progress to a harmful mutation. For instance, a point mutation in just one copy of the hemoglobin gene can protect the host from malaria (7), whereas two copies of the mutated hemoglobin gene cause sickle cell anemia. T-cell differentiation is also regulated through beneficial epigenetic modifications. Analysis of Th0, Th1, and Th2 cells indicated that the IFN γ and Th2 cytokine loci were not modified in Th0. In fact, active or repressive histone modifications in the cytokine locus determine Th1/Th2 differentiation (8).

Deleterious somatic mutations or epigenetic alterations result in cellular malignancy and cancer. Changes in methylation patterns or histone deacetylation are hallmarks of epigenetic modulation, which can alter gene expression. As methylation and

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histone deacetylation rates are faster than genetic mutation rates, epigenetic alterations could occur very quickly during the lifetime of an individual. DNA hypermethylation can inactivate the genes that are responsible for DNA damage response and repair, facilitating the establishment of cancer. It was reported that each environmental insult alters a specific checkpoint that it triggers and increases risk of certain cancers (9). For instance, bisphenol A, a plasticizer used for manufacturing polycarbonate plastics, can leach from plastics into food and water, disrupts mitotic progression, and increases risk of prostate and breast cancers (10, 11). Environmental insults often result in deleterious mutations, although subsequent induction of tumor suppressor genes inhibits growth of malignant cells and prevents cancer development. For instance, p53 is activated in response to DNA damage, hypoxia, and nucleotide deprivation. Activation of p53 leads to cell-cycle arrest, apoptosis, or DNA repair to restore the integrity of cells. However, loss of p53 function through mutations could lead to survival of malignant cells. Once the process of malignancy is completed, transformed cells cannot necessarily form cancer. Fortunately, metastasis suppressor genes could still limit invasiveness of malignant cells, and mutant protein antigens expressed by malignant cells can be specifically recognized and attacked by the immune system, resulting in the maintenance of malignant dormancy. However, tumor cells that arise from normal cells have adapted similar evolutionary mechanisms of survival that enable them to escape immune surveillance. These mechanisms have been well explained by the tumor immunoeediting theory (12). In fact, humans have evolved two major mechanisms of survival from tumor-inducing environmental insults. These include (i) tumor-intrinsic mechanisms regulated by metastasis suppressor genes and cell-cycle checkpoint molecules, which could inhibit proliferation of malignant cells and establish tumor dormancy; and (ii) tumor-extrinsic mechanisms regulated by the immune surveillance, which could either eliminate or inhibit nascent transformed cells. Inhibition of transformed cells could, in turn, facilitate the establishment of immunogenic tumor dormancy. In fact, Th1 cells have been reported to inhibit HER2-positive tumor growth such that loss of anti-HER2 or anti-HER3 Th1 response was found to be associated with tumor recurrence (13, 14).

Cellular Dormancy Is an Evolutionary Conserved Mechanism of Survival

In a thorough review of the evidence of cancer dormancy, Aguirre-Ghiso suggested that cellular dormancy is an evolutionary conserved mechanism among organisms to help them adapt to stress and survive a hostile environment (15). In *Caenorhabditis elegans*, pathways that sense stress will induce cellular dormancy or growth arrest and result in resistance of larvae to nutritional deprivation (16). *Mycobacterium tuberculosis* and HIV survive in human cells by entering into a dormant, latent state (17, 18). Mammalian adult stem cells are also in a state of quiescent dormancy until they receive specific signals, such as tissue injury, to exit from dormancy and proliferate (19, 20). It was also reported that in the absence of antigen, memory T cells enter a state of dormancy associated with low energy utilization and proliferation to survive until they receive stimulatory signals during a subsequent infection (21). In fact, cellular dormancy is the mechanism by which memory T cells survive nearly throughout the lifetime to protect an individual from recall infections.

Memory T cells could escape from dormancy during recall infection and generate effector T cells with the ability to proliferate (21). Although the mechanisms of cellular dormancy are not fully understood, stress-induced autophagy could lead to cellular dormancy. In T cells, macroautophagy is upregulated just before the contraction phase, when T cells stop dividing and the pathogen has been cleared (22). Autophagy-deficient CD8⁺ T cells were found to be defective in generating memory phenotypes that are usually in the state of dormancy (22). Given that malignant cells arise from normal cells, it is reasonable to suggest that tumor dormancy recapitulates evolutionarily conserved mechanism of adaptation, that is, cellular dormancy to survive hostile microenvironment. This property facilitates the establishment of treatment-induced tumor dormancy following conventional cancer therapies or immunotherapy (23–25). In fact, IFN γ produced by tumor-reactive T cells induces tumor cell apoptosis as well as tumor cell dormancy, and relapse associated with tumor immunoeediting, simultaneously (26, 27). Such a paradoxical response by tumor cells to the immune response was shown to be due to the inherent heterogeneity of mammary tumor cells for the expression of IFN γ R α (28).

Local or Metastatic Tumor Dormancy Is Present Prior to Cancer

Patients with early-stage cancer do not die from primary cancer, which tends to be responsive to therapy, but rather as a result of distant recurrence of the tumor in the form of advanced stage diseases. Twenty percent to 45% of patients with breast or prostate cancer end up with distant recurrence of the disease years or decades after successful treatment of their primary cancer (29, 30). This phenomenon can be explained by cancer dormancy, a stage in which residual disease is present but remains asymptomatic, and most often, undetectable. Tumor dormancy is present in almost all cancers, particularly breast cancer. Emerging evidence suggests that local and metastatic tumor dormancy precede primary cancer and distant tumor metastasis, respectively.

Local tumor dormancy prior to establishment of primary cancer

The concept that local malignant dormancy precedes primary cancer is supported by the existence of "cancer without disease" (31), tissue-specific control of malignant dormancy (32), as well as clinical evidence in support of the existence of natural tumor dormancy in healthy individuals highlighted in the recent review articles (32, 33). For instance, postmortem examination of random sections of autopsied prostate tissues from men who did not have cancer revealed frequent "small carcinomata" in 14% of prostate specimens (34, 35). More recent studies revealed the presence of *in situ* carcinoma in 9%, 27%, and 34% of cancer-free men in their 20s, 30s, and 40s, respectively (35). Postmortem examination of women in their 40s showed a similar frequency (39%) of histologic breast cancers (36), although only 1% of women in this age range get breast cancer. Interestingly, all autopsied individuals ages 50 to 70 had *in situ* carcinomas in the thyroid gland (37), whereas the incidence of thyroid cancer in this age group is only 0.1% (31). Frequency of dormant lung cancer was lower, accounting for 1% of autopsied specimens from individuals who were cancer free (38). Pancreatic intraepithelial neoplasia being in a dormant state is remarkably common, particularly in cancer-free elderly (39). They contain mutations in the same genes that are mutated in invasive pancreatic cancer

(40, 41), suggesting the state of malignant dormancy. These data suggest that local tumor dormancy precedes primary cancer development and that tumor cells could remain dormant for the lifetime of an individual without ever causing cancer. Very recently, circulating tumor DNA carrying P53 mutations has been reported in healthy individuals (42), again suggesting that malignancy is present prior to the development of primary cancer.

Metastatic tumor dormancy prior to establishment of primary cancer

For the past century, it has been assumed that tumor metastasis follows a stepwise process from primary tumor to the regional lymph nodes and then distant organs. This classical understanding of tumor metastasis has guided removal of the draining lymph nodes during conventional therapies. Recent evidence from patients with solid malignancies indicates that metastasis is a very early event such that even small tumors (<5 mm) can establish metastasis long before they become detectable at the primary site. This phenomenon is defined as early dissemination but late metastasis, because metastatic cells could lie dormant for even a decade and then reemerge as metastatic disease (43, 44). More recently, the observations made in two groups of cancer patients have further challenged the classical view of tumor metastasis. The first group of patients comprises those with metastatic lesions either before the primary tumor became clinically detectable, or when harboring primary cancer at a very early stage without local invasion. For instance, patients with stage M0 breast cancer could relapse after complete resection of their primary tumor, and their metastatic tumor had significantly fewer genetic abnormalities than the primary tumor (45). Studies in melanoma model demonstrated that tumor cells were disseminated throughout the body even before primary tumor became clinically detectable (46). Mechanistic studies revealed that in early lesions prior to establishment of breast cancer, there was a subpopulation of early cancer cells that spread to distant organs. Further studies demonstrated that progesterone-induced signaling induces dissemination of malignant cells from early lesions shortly after HER2 activation and prior to breast cancer development (43). Another group of cancer patients comprises those with cancer of unknown primary. Up to 5% of all cancer diagnoses are classified as cancer of unknown primary (47). In these patients, primary cancer could not be identified after histopathologic review of biopsy material and CT scan, but full-body imaging identified metastatic lesions that were confirmed by biopsy. Even a postmortem examination of a small group of patients with cancer of unknown primary revealed only 55% to 85% of the primaries, which were very small asymptomatic tumors in the lung, gut, and kidney. The remaining were autopsy-negative primary sites with detectable metastatic lesions (48). Metastatic cancers of unknown primary were reported in cervical carcinoma, renal cancer, breast cancer, colorectal cancer, lung cancer, liver cancer, pancreatic cancer, and ovarian cancer. Cancer of unknown primary is a clinical puzzle for oncologists and could be explained by the notion that circulating tumor cells must be present very early during the process of malignancy and reside in distant organs in a dormant state prior to the establishment of primary tumor. These dormant cells can then establish metastatic cancer prior to the detection of primary cancer (cancer of unknown primary) or relapse at distant organs after successful treatment of the primary cancer. Perhaps, metastasis suppressor genes are involved in maintaining tumor dormancy at distant sites.

Although both metastasis suppressor genes and tumor suppressor genes are tumor cell-intrinsic mechanisms of survival, the former is distinct from the latter in that metastasis suppressor genes maintain metastatic cells in a dormant state without affecting the growth of the primary tumor (49–52). On the other hand, tumor suppressor genes undergo mutation or epigenetic alterations during tumorigenesis or latency. Each cancer type appears to have distinct metastasis suppressor genes. For instance, *Nm23* and *BRMS1* are involved in breast cancer, *KAI1*, *MKK4*, *Rkip*, *RHOCDI2*, and *Drg-1* are involved in prostate cancer, and *TXNIP*, *CRSP3*, and *KISS1* are involved in melanoma (50). Failure of tumor cell-intrinsic mechanisms of survival, including metastasis suppressor genes, tumor suppressor genes, and cell-cycle checkpoint molecules, does not immediately result in cancer because cell-extrinsic mechanisms mediated by the immunosurveillance could still support tumor dormancy by inhibiting the growth of nascent transformed cells (12). This mechanism has been demonstrated by the equilibrium phase of tumor immunoediting (53). However, escape from immune-mediated tumor dormancy could lead to distant recurrence of cancer (33).

Chronic Inflammation Awakens Dormant Malignant Cells and Results in Cancer

A substantial body of evidence supports the role of chronic inflammation in cancer development. For instance, colon carcinoma is associated with inflammatory bowel disease, esophageal cancer is associated with acid reflux esophagitis, liver cancer is associated with fatty liver disease and hepatitis, bladder cancer is associated with cystitis and schistosomiasis, and stomach cancer is associated with chronic *Helicobacter* infection. It has long been thought that chronic inflammation facilitates cell transformation and malignancy by increasing free radicals. During inflammation, there are high levels of reactive oxygen and nitrogen species (RONS), which can induce mutagenic DNA lesions. RONS also induce DNA double-strand breaks, which can also be potentially mutagenic if not accurately repaired. However, detection of malignant cells in postmortem autopsy specimens of individuals in the absence of any chronic inflammation outcasts a cause–effect relationship between chronic inflammation and cancer (31, 33, 39, 42). In addition, not all individuals with chronic inflammatory diseases end up with cancer. Tumorigenic manifestation of chronic inflammation could be due to its role in awakening dormant malignant cells rather than causing malignancy. To this end, the incidence and the type of cancer in individuals could be determined by the presence of malignant dormancy that each organ might carry to communicate with chronic inflammatory environment. In fact, chronic inflammation supports angiogenesis, which is an important factor in the promotion of growth of dormant micrometastasis (54). For instance, there is a strong correlation between inflammation and recurrence of endometrial cancer (55), oral cancer (56), breast cancer (57, 58), and tumor escape from dormancy induced by the inflammatory cytokine IFN γ (27, 28, 59, 60). In addition, data from patients with tumor recurrence after successful treatment of their primary cancer support this hypothesis. For instance, in a multisite study of 734 breast cancer survivors, high levels of circulating acute phase proteins (APP) were associated with distant recurrence of cancer (61). Therefore, posttreatment monitoring of serum inflammatory markers, such as APP, C-reactive

protein, and IL6, could be of prognostic value for predicting risk of breast cancer recurrence.

Escape from Cell-Intrinsic and Cell-Extrinsic Mechanisms of Tumor Dormancy Results in Distant Recurrence of Cancer

Like normal cells, malignant cells that lie dormant could evolve and escape from dormancy. Such evolutionary mechanisms could be facilitated by chronic inflammation that induces mutations and epigenetic alterations in metastasis suppressor genes. This, in turn, abolishes tumor cell-intrinsic mechanisms of metastatic dormancy, resulting in distant recurrence of the disease in the form of advanced stage cancer. Fortunately, mutant protein antigens expressed by malignant cells can be specifically recognized and attacked by the immune system, thereby providing tumor cell-extrinsic mechanisms for the maintenance of metastatic dormancy. In fact, immunogenic tumor dormancy has been suggested to be a key mechanism of tumor dormancy (33, 62). For instance, tumor cells that were disseminated prior to the formation of primary cancer were in the state of dormancy in the lung as a result of the cytostatic function of CD8⁺ T cells (46). Depletion of CD8⁺ T cells resulted in the outgrowth and relapse of metastatic dormant cells (46). Studies in an animal model of pancreatic cancer demonstrated that circulating pancreatic cells underwent epithelial-to-mesenchymal transition (EMT) and seeded the liver. EMT and invasiveness were most abundant at inflammatory sites such that treatment with the immunosuppressive drug, dexamethasone, abrogated tumor invasiveness. The authors suggested that inflammation enhances cancer progression in part by facilitating EMT (63). It was also reported that localized inflammation in the lungs triggers escape from dormancy, which develop into macroscopic metastases (64). However, dormant tumor cells that arise from normal cells possess similar evolutionary mechanisms of survival that could result in escaping from immunosurveillance. Thus far, two types of tumor dormancy have been reported; these include Ki67[−] quiescent dormancy and Ki67^{low} indolent dormancy (27). The latter is maintained through a balance between sluggish cell proliferation and cell death. Interestingly, an indolent, but not a quiescent, type of tumor dormancy was found to be able to evolve through immunoeediting and escape from the immune response. The inflammatory cytokine, IFN γ , was a key factor in facilitating tumor immunoeediting (27). In fact, IFN γ -producing Th1 cells can induce apoptosis and HER2 loss in murine and human breast cancer (60). Immune

escape mechanisms include, but are not limited to, tumor antigen loss, expression of PD-L1, loss or downregulation of MHC class I, and induction of MDSCs and/or Tregs. Therefore, distant recurrence of cancer in some but not all cancer survivors could depend on the state of dormancy, that is, quiescent or indolent.

In summary, (i) cellular transformation is unavoidable in biological systems; (ii) malignant cells often enter the state of dormancy to survive environmental insults; (iii) malignant dormant cells are best targets for the prevention of metastasis, as suggested in a recent review of by Ghajar (65); and (iv) malignant dormant cells could evolve, escape from the immune surveillance or other cancer therapies, and relapse. Therefore, attempts to destroy and eliminate cancer without any risk of relapse would be unfruitful. Rather, we need to develop new therapeutic strategies to control malignant cells through retaining them in the state of residual dormancy and preventing distant recurrence of the disease. This could be achieved by immunotherapeutic targeting of dormant cells, because all other currently available cancer therapies are toxic with off-target effects, whereas immune cells could establish memory against dormant tumor antigens such as mutated tumor antigens, and keep them dormant for the lifetime of an individual.

Disclosure of Potential Conflicts of Interest

M.H. Manjili is a consultant/advisory board member for Getting To Know Cancer.

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Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Department of Defense.

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Masoud H. Manjili

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Role of Tregs in Cancer Dormancy or Recurrence

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ABSTRACT

The immunogenic tumor dormancy has been demonstrated in animal models of cancer, which can explain clinical observations such as an increased incidence of cancer following organ transplantation. The role of immune cell populations in the maintenance of, or escape from, tumor dormancy and subsequent recurrence is poorly understood. Here, we provide a review of literature related to the contribution of Tregs in tumor dormancy or recurrence. Based on clinical results, we suggest that anecdotal reports on the association of human Tregs with poor prognosis are circumstantial rather than implying a cause–effect direction. This could be due to a disparity among patients in harboring multiple factors associated with tumor immunoediting and immune evasion mechanisms.

KEYWORDS

Regulatory T cells; tumor dormancy; tumor immunoediting

Introduction

Patients with cancer are always at risk of developing distant recurrence of the disease years or decades after successful treatment of their primary cancer. This phenomenon can be explained by cancer dormancy, a stage that residual disease is present but remains asymptomatic, and most often, undetectable. Mechanisms that establish and maintain tumor dormancy or result in tumor recurrence by escaping from dormancy are poorly understood. Cancer dormancy can be explained by two distinct but interrelated mechanisms. These include (i) immunogenic tumor dormancy controlled by the immune system, and (ii) non-immunogenic or cellular tumor dormancy controlled by a balanced proliferation and death or by cellular quiescence (Manjili 2014; Manjili and Payne 2015). There is also another mechanism of tumor dormancy named angiogenic dormancy (Ghajar et al. 2013), which can be explained in the context of immunogenic or non-immunogenic dormancy. Tumor dormancy is present in almost all cancers, particularly breast cancer. Up to 30% of early-stage breast cancers with no evidence of metastasis will relapse in distant organs less than a decade after the treatment of primary cancer (Aguirre-Ghiso 2007). Therefore, adjuvant chemotherapy that kills cycling tumor cells is an option after successful resection of the tumor or lumpectomy. Yet, chemotherapy has been met with limited success as it reduces metastatic recurrence by only 30% at 10 years (Demicheli et al. 2005). While many tumor clones undergo apoptosis upon chemotherapy, some other

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tumor clones escape from apoptosis, become quiescent, and lie dormant (Aguirre-Ghiso 2007; Demicheli et al. 2005). Dormant breast cancer cells have been detected as disseminated tumor cells (DTC) that reside in distant organs, as well as circulating tumor cells (CTC) that can be detected in the bloodstream. In our recent review, we have discussed clinical and preclinical evidence that support the existence of tumor dormancy in healthy individuals, that is, natural dormancy as well as in cancer survivors, that is, treatment-induced dormancy (Manjili 2014; Manjili and Payne 2015). Presence of CTC or DTC in healthy individuals suggests that dormant tumor cells are not always derived from the primary tumors. It is yet to be determined whether CTCs in cancer survivors are derived from their primary tumors in response to conventional therapies, or they are secondary dormant cells that did not respond to conventional therapies. However, preclinical studies suggest that dormant tumor cells that establish distant metastasis are derived from the primary tumor (Aceto et al. 2014). Here, we review clinical and experimental evidence to understand the role of Tregs in tumor dormancy or recurrence.

T cells and immunogenic tumor dormancy

Immunogenic tumor dormancy and recurrence can be explained in the context of tumor immunoediting proposed by Dr. Schreiber (Dunn et al. 2002). According to the tumor immunoediting theory, immunogenic tumor clones can either be eliminated, elimination phase; or lie dormant and remain in check, equilibrium phase, or tumor dormancy, by tumor-specific immune responses. Dormant tumor cells could eventually relapse because of tumor escape such as antigen loss or immune evasion. This process is called three *Es* (elimination, equilibrium, and escape) of tumor immunoediting (Dunn, Old, and Schreiber 2004). Immunogenic tumor dormancy is also supported by spontaneous regression of highly immunogenic cancers associated with infiltrating CD4⁺ T cells (Halliday et al. 1995), and development of melanoma or cancers with a viral etiology in organ transplant recipients. In patients with basal cell carcinoma who participated in clinical trial of IFN- α , 20% of patients on the placebo arm of the trial experienced spontaneous regression of the tumor (Printz 2001), suggesting that about 20% of basal cell carcinomas undergo complete spontaneous regression. However, they did not investigate infiltrating T cell subsets to determine whether the ratio of effector T cells to Tregs was greater in 20% of patients with spontaneous tumor regression compared with those who failed to reject their tumor. The first report on spontaneous regression of breast cancer associated with extensive infiltration of T cells was published in 2014 (Tokunaga et al. 2014), again infiltrating T cell subsets were not characterized. Very recently, Dickerson et al. reported three cases that had spontaneous regression of renal cell carcinoma without any therapeutic interventions (Dickerson, Davenport, and Liu 2015). A patient with AIDS who had non-small cell lung cancer (NSCLC) experienced spontaneous regression of the tumor after immune reconstitution (Menon and Eaton 2015), suggesting the involvement of the immune system in tumor regression. These observations suggest that T cells can induce regression of immunogenic tumors.

IFN- γ producing CD8⁺ T cells have been shown to have a dual function, inducing tumor cell apoptosis – elimination – and inhibiting tumor cell growth – equilibrium (Kmieciak et al. 2011, 2013; Farrar et al. 1999). The latter can establish tumor dormancy, which could lead to tumor antigen loss and recurrence (Kmieciak et al. 2007; Payne et al. 2016). IFN- γ appears to be a key cytokine for the establishment of tumor dormancy, as well as the induction of epigenetic changes in tumor cells, leading to tumor antigen loss

and upregulation of PD-L1 in tumor cells (Payne et al. 2016; Kmiecik et al. 2013). We have recently reported two types of tumor dormancy, which include an indolent dormancy characterized by a balanced cell proliferation and death, and a quiescent dormancy characterized by lack of cell proliferation (Payne et al. 2016). We also showed that an indolent, but not a quiescent, tumor dormancy could eventually escape from immunogenic dormancy, and relapse (Payne et al. 2016). Escape from immunologic tumor dormancy and subsequent cancer development could also occur following immune suppression. For instance, organ recipients from healthy donors developed tumor in the organ following immunosuppression (Ali and Lear 2012). The US Scientific Registry of Transplant Recipients, which included 175,732 patients with solid organ transplantation (1987–2008) revealed 381 cases of melanoma in the recipients and an increased risk of 2.6 times higher than that of the general population (Engels et al. 2011). Similar results were obtained from a large combined Australasian registry-based prospective cohort study, which included 28,855 patients with up to 42 years of follow-up (Vajdic et al. 2006). These tumors generally had a viral etiology such as liver and cervical cancers, or were immunogenic tumors such as melanoma (Buell, Gross, and Woodle 2005; Penn 1988). These data suggest that highly immunogenic tumors that are in the state of immunogenic dormancy in the donor organ can establish cancer in the recipients because of the immune suppression to accept the graft. A meta-analysis of five population-based studies showed that the incidence of weakly immunogenic cancers, including breast, prostate, ovarian, and testicular cancers did not increase in transplant recipients (Vajdic and van Leeuwen 2009).

Tregs and tumor recurrence

Recent data have suggested that Tregs may be involved in the escape from immunogenic dormancy, and consequent recurrence. However, data related to the association of Tregs with poor outcome are controversial. Immunohistochemical analysis of FOXP3+Tregs in tumor specimens of 72 patients with early stage (I–III) breast cancer showed a significant correlation with a poor overall survival. Upon comparing paraffin-embedded tumors of multiple subsets, it was found that more aggressive subsets (lymph node metastases, immunopositivity for p53 and Ki-67) had higher numbers of FOXP3+ Tregs and lower numbers of CD8+ T cells. Further analysis indicated that an increase in FOXP3+Treg/CD4+ T-cell ratio was positively correlated with lymph node metastasis (Kim et al. 2013). Another group examined tumor specimens of 39 patients with glioblastoma (GBM) and demonstrated that a high ratio of CD8+ or CD3+ cells to FOXP3+ cells in primary tumor was associated with improved survival. There was no correlation between survival and a higher CD4 to FOXP3+ ratio (Sayour et al. 2015). These groups did not perform multi-color staining of tumor-infiltrating lymphocytes (TIL) to determine whether FOXP3+ cells were positive for CD4 and CD25. To this end, Suzuki et al. (2013) analyzed tumor specimens of 88 patients with colorectal cancer, and demonstrated that relatively low number of FOXP3+VEGFR2+ cells was significantly correlated with improved disease-free survival and overall survival. However, number of intratumoral FOXP3+ cells or FOXP3+VEGFR2– cells did not show significant correlation with disease-free survival and overall survival (Suzuki et al. 2013). FOXP3 is critical for the development and function of murine CD4+CD25+ Tregs (Haiqi, Yong, and Yi 2011). However, FOXP3 is also expressed in activated T cells upon stimulation of human CD4+CD25– T cells without conferring a

regulatory function (Kmieciak et al. 2009; Wang et al. 2007). Therefore, functional analyses as well as additional markers are needed to identify human Tregs. Although FOXP3+ T cells are hyporesponsive, they do not necessarily exhibit suppressor function (Ziegler 2007). Very recently, expression of the transcription factor Helios was proposed as a functional marker for naturally occurring Tregs. Muto et al. (2015) investigated the clinical significance of Helios expression in Tregs of 64 patients with NSCLC. They showed that patients with low levels of Helios expression in Tregs among their TILs had significantly poorer survival. Due to the variations in the markers used for the detection of human Tregs as well as different cancer types and contribution of other immune cells, it is difficult to determine the definitive role of human Tregs in tumor dormancy or recurrence.

In preclinical studies, Goding et al. (2013) reported that CD4+Foxp3+TRP-1 Tregs and chronically exhausted tumor-specific CD4+ T cells were increased during recurrence of B16 melanoma in mice. In order to determine if TRP-1 Foxp3+ tumor-specific CD4+ T cells cause tumor recurrence, TRP-1 transgenic mice were crossed with FoxP3-DTR (diphtheria toxin (DT) reporter) transgenic mice allowing for cell-specific ablation of Foxp3+ Tregs and cell-specific tracking. Tregs were depleted with DT either during recurrence or immediately following successful primary treatment of B16 melanoma. Selective depletion was confirmed by flow cytometric analysis showing that effector T cells remained present during depletion and that GFP expression faithfully marked Foxp3-DTR TRP-1 Tregs. Depletion of Tregs alone failed to prevent recurrence, however, when PD-L1 was blocked in combination with Treg depletion, there was a significant regression in recurrence of B16 melanoma. Neither depletion of Tregs or blocking PD-L1 alone decreased recurrence, suggesting that neither is solely involved in mediating recurrence. It was also found that the number of CD8+ T cells remained unchanged in primary and recurrent tumors. In addition, high levels of IFN- γ and TNF- α were seen in non-relapsing mice and low levels in relapsing mice. In a mouse model of melanoma, it was also shown that Tregs suppress anti-tumor function of effector T cells (Jensen et al. 2012).

Tregs are dispensable during tumor dormancy or recurrence

The main function of Tregs has been shown to be maintaining immunological tolerance and protecting the host from excessive immune responses (Sakaguchi et al. 2008). In the gut, they produce IL-10 and TGF- β . IL-10 maintains intestinal homeostasis (Roers et al. 2004). TGF- β not only repairs mucosal injury but also preserves the integrity of the intestinal mucosa (Dignass and Podolsky 1993; Planchon et al. 1994) thereby protecting intestinal mucosa from inflammatory Th1 cells rather than just suppressing Th1 cells (Howe et al. 2005). TGF- β signaling is also critical for mucosal IgA production to protect the gut from pathogens (Borsutzky et al. 2004). Therefore, IL-10 and TGF- β producing Tregs play an active role in protecting the gut from injury, rather than acting passively by suppressing effector cells. In other words, they tend to exhibit a regulatory function rather than a suppressive function. Contribution of Tregs in tumor microenvironment can also be attributed to their regulatory function. For instance induced (i) Tregs enhance anti-tumor function of NK cells by increasing Fas ligand and perforin production while reducing IL-2 production in the absence of target cells (Bergmann et al. 2011). Although they appear to counteract cytotoxic function of T cells at the tumor site, they

do not tend to suppress cytostatic function of T cells, as anti-tumor CD8⁺ T cells can establish and maintain tumor dormancy even in the presence of Tregs (Gerber et al. 2013). Despite high levels of CD25 expression on Tregs, systemic administration of IL-2 has been shown to enhance anti-tumor responses (Whiteside et al. 1993; Atkins et al. 1999). In a mouse model of melanoma, the expression of IL-2 in the tumor microenvironment inhibits tumor growth despite enhancing Tregs and anti-inflammatory cytokines such as IL-10 (Gerber et al. 2013). These data suggest that Tregs are dispensable in suppressing anti-tumor immune responses that lead to escape from immunogenic dormancy and results in tumor relapse. An occasional correlation of Tregs with poor prognosis could

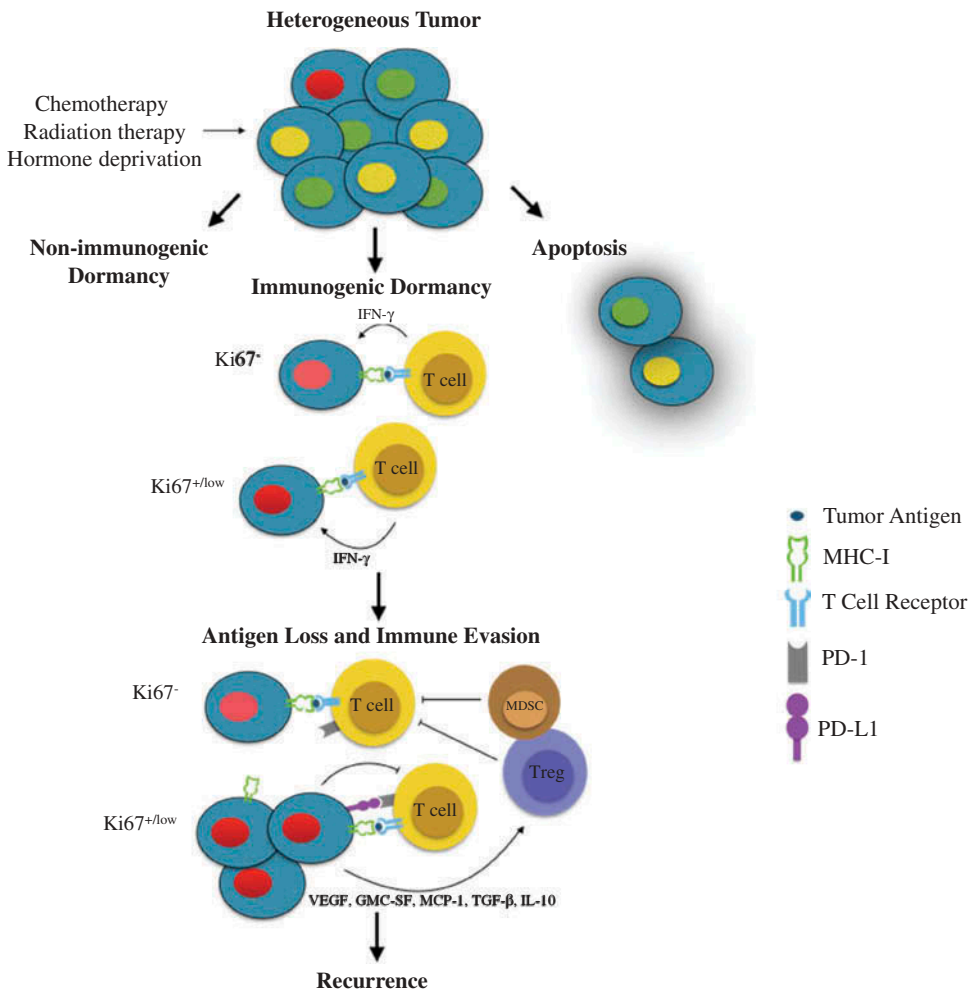


Figure 1. Tumor escape and immune evasion during immunogenic tumor dormancy. Dormant tumor cells could escape from dormancy and establish recurrence by undergoing immunoediting. Unlike quiescent dormant cells (Ki-67⁻), indolent dormant cells (Ki-67^{+/low}) can be changed by IFN- γ producing T cells, and lose their tumor antigen and/or upregulate PD-L1 (tumor escape). Indolent dormant cells could also produce cytokines/chemokines that support Tregs and/or MDSCs, thereby suppressing anti-tumor immune responses (immune evasions). These events promote tumor recurrence.

be circumstantial due to the contribution of other factors such as MDSCs and immune checkpoint pathways.

In conclusion, escape from immunogenic tumor dormancy and subsequent relapse is due to tumor escape and immune evasion ([Figure 1](#)). Tumor escape is characterized by epigenetic changes in the indolent dormant cells mediated by IFN- γ producing T cells that induce tumor antigen loss (Kmieciak et al. [2007](#), [2011](#), [2013](#); Beatty and Paterson [2000](#)). Immune evasion is characterized by: (i) increases of MDSCs, M2 macrophages, and/or Tregs mediated by cytokines and chemokines such as MCP1, VEGF, IL-6, IL-10 secreted from the indolent dormant cells, and (ii) the engagement of immune checkpoint molecules such as a PD-1/PD-L1 pathway. The IFN- γ released from tumor-reactive T cells is a major factor that induces/upregulates the expression of PD-L1 on indolent tumor cells, and result in the engagement of the immune checkpoint pathway.

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Conflicts of interest

The authors report no conflicts of interest.

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Prospects in Cancer Immunotherapy: Treating Advanced Stage Disease or Preventing Tumor Recurrence?

MASOUD H. MANJILI AND KYLE K. PAYNE

Abstract: Human vaccines against infectious agents are often effective in a prophylactic setting. However, they are usually not effective when used post-exposure. Rabies vaccine is one of the exceptions, which can be used post-exposure, but is effective only when used in combination with other treatments. Similar results have been obtained with cancer vaccines and immunotherapies. Cancer immunotherapies generally prolong patients' survival when they are used during advanced stage disease. The potential of immunotherapy to cure cancer could be revealed when it is applied in a prophylactic setting. This article provides a brief overview of cancer immunotherapeutics and suggests that immunotherapy can cure cancer if used at the right time against the right target; we suggest that targeting cancer during dormancy in order to prevent tumor recurrence as advanced stage disease is potentially curative. [*Discovery Medicine* 19(107):427-431, June 2015]

Cancer Immunotherapies: Premises and Challenges

Recently, there have been dramatic advances in the field of cancer immunotherapy. However, these advances generally have been limited to increasing patients' survival for a limited period of time when administered in a therapeutic setting against advanced stage disease. In April 2010, the U.S. Food and Drug Administration (FDA) approved the first therapeutic cancer vaccine. This vaccine, sipuleucel-T (Provenge, manufactured by Dendreon) is designed to stimulate an immune response to a prostate tumor antigen, prostatic acid phosphatase (PAP). In a clinical trial, sipuleucel-T

extended survival of patients with metastatic prostate cancer by a median of 4.1 months (Kantoff *et al.*, 2010). Blockade of immune checkpoint molecules has also prolonged survival of patients with advanced cancer. For instance, anti-CTLA-4 ipilimumab therapy resulted in a 3.5-month gain in overall survival in patients with stage III or IV metastatic cutaneous melanoma (Hodi *et al.*, 2010). Cumulative response rates for anti-PD-1 antibody among patients with non-small-cell lung cancer, melanoma, and renal-cell cancer were 18%, 28%, and 27%, respectively. Responses were durable such that 20 of 31 responses lasted 1 year or more in patients with 1 year or more of follow-up (Topalian *et al.*, 2012).

Ex vivo expansion of tumor reactive T cells administered therapeutically has shown promise in some patients with advanced tumors. In 2006, adoptive immunotherapy (AIT) utilizing normal circulating lymphocytes transduced with a retrovirus encoding a MART-1-specific T cell receptor (TCR) resulted in objective regression of melanoma lesions in 2 of 15 patients (Morgan *et al.*, 2006). In one patient, AIT resulted in 89% reduction of the liver tumor mass, at which time it was removed by surgery, and the patient remained disease free 21 months later. In another patient, AIT resulted in the regression of the hilar mass, and the patient remained disease free 20 months later (Morgan *et al.*, 2006). AIT utilizing lymphocytes genetically engineered to express a chimeric antigen receptor (CAR) against the B cell antigen CD19 also mediated regression of an advanced B cell lymphoma in one patient with progressive lymphoma. No information was provided as to whether the patient remained relapse-free (Kochenderfer *et al.*, 2010). The use of AIT in patients with metastatic melanoma utilizing tumor-infiltrating lymphocytes (TILs) grown in IL-2 resulted in tumor regression in 49% of patients (Dudley *et al.*, 2002). When AIT was combined with total body irradiation (TBI) objective responses increased to 72%. Among treated groups, 20% had complete tumor regression and over 10 years relapse-free survival (Rosenberg *et al.*, 2011). Thus far, of the 34 complete responders in the National Cancer Institute (NCI) trials, one has recurred (Rosenberg and Restifo, 2015).

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Despite the remarkable recent advances in cancer immunotherapy, the ability of immunotherapy to treat common carcinomas, which account for a majority of all cancer deaths, is limited. The application of immunotherapy to highly proliferative tumors renders the tumors prone to immunoediting and subsequent immunological escape during cell division. An important point to consider is that human vaccines against infectious diseases also are not effective in a setting of established disease. The rabies vaccine is an exception; however, it is ineffective as a single agent or at the onset of clinical illness.

Successful human vaccines against infectious diseases suggest that vaccines and administration of immunotherapy can be effective in a prophylactic setting either prior to exposure to infectious agents including pathogen-associated cancers or during the incubation period or dormancy after the exposure. For instance, the rabies vaccine can be used as post-exposure prophylaxis because the incubation period or dormancy for rabies is 1-3 months which provides a window for vaccination. Yet, it should be combined with anti-rabies immunoglobulin injections into the wound in order to control the infection and allow for the vaccine to work. Prophylactic cancer vaccines have also been successful. The FDA has approved two vaccines, Gardasil and Cervarix, that protect against HPV infection which is the leading cause of cervical cancer worldwide (Doorbar, 2006). HPV infection is also responsible for some vaginal, vulvar, anal, penile, and oropharyngeal cancers (Lowy and Schiller, 2006). The FDA has also approved a prophylactic cancer vaccine against HBV infection, which is a cause of liver cancer. Today, most children in the United States are vaccinated against HBV shortly after birth (Mast *et al.*, 2005).

Cancer Therapies and Tumor Recurrence

Tumor dormancy in the form of residual disease is evident in almost all cancers, particularly breast cancer (Manjili, 2014). Up to 30% of patients with early stage breast cancers who have no evidence of metastasis will end up with distant recurrence of disease less than a decade after the treatment of primary cancer (Almog, 2010). Therefore, adjuvant chemotherapy is an option after surgery in order to kill cycling residual tumor cells. Yet, chemotherapy has shown limited success as it reduces metastatic recurrence by only 30% at 10 years (Demicheli *et al.*, 2005). This is because while many tumor clones undergo apoptosis in the presence of chemotherapy other tumor clones escape from apoptosis, become indolent, and lie dormant (Almog, 2010; Demicheli *et al.*, 2005; Manjili, 2014). Similar escape mechanisms were reported as a result of immunotherapy. These include tumor antigen loss (Kmieciak *et al.*,

2007; Kmieciak *et al.*, 2013), HLA loss, tumor-induced immune suppressive mechanisms mediated by a suppressive type of myeloid regulatory cells (Mregs) namely myeloid-derived suppressor cells (MDSCs) and/or regulatory T cells (Tregs) as well as engagement of immune checkpoint pathways. Although reprogramming of tumor-sensitized immune cells can render them resistant to immune suppressor cells, their success in preclinical studies has been limited to a prophylactic setting (Kmieciak *et al.*, 2011; Manjili and Payne, 2012; Payne *et al.*, 2013). These escape mechanisms limit therapeutic application of immunotherapy as well as conventional therapies against cancer. Therefore, a major challenge in the treatment of cancer is to target and eliminate dormant tumor cells in order to prevent tumor recurrence as advanced stage disease.

Dormant breast cancer cells have been detected as disseminated tumor cells (DTC) that reside in distant organs, as well as circulating tumor cells (CTC) that can be detected in the bloodstream. In humans, DTC were isolated from bone marrow after removal of the primary lesion (Pantel *et al.*, 1993; Suzuki *et al.*, 2006). DTC that can resume proliferation and establish distant metastasis have been recently reported by Dr. Bissell's group (Ghajar *et al.*, 2013). They showed that DTC reside on the endothelium of the microvasculature in the lung, bone marrow, and brain, which are common metastatic destinations of breast cancer. In addition, CTC have been detected in the bloodstream of breast cancer survivors several years after successful treatment of primary breast cancer (Sinha, 2012). Detection of CTC in cancer patients is not limited to those with metastatic disease, as patients with non-metastatic cancer or early stage breast cancer also show CTC (Lucci *et al.*, 2012; Meng *et al.*, 2004; Sinha, 2012). Detection of CTC in breast cancer patients even 22 years after the completion of conventional cancer therapies suggest that: i) even recurrence-free cancer patients are at risk of tumor recurrence at any time, or ii) establishment of permanent tumor dormancy is feasible as a means to prevent tumor recurrence. Both forms of tumor dormancy, DTC and CTC, are different from metastasis. In fact, tumor dormancy is a step between treatment of primary cancer and recurrence as advanced stage disease. During dormancy, tumor cells maintain homeostasis and cellular integrity over prolonged periods of non-division, which is likely due to indolent growth defined by a balanced proliferation and death, and/or quiescent dormancy, defined by cell cycle arrest.

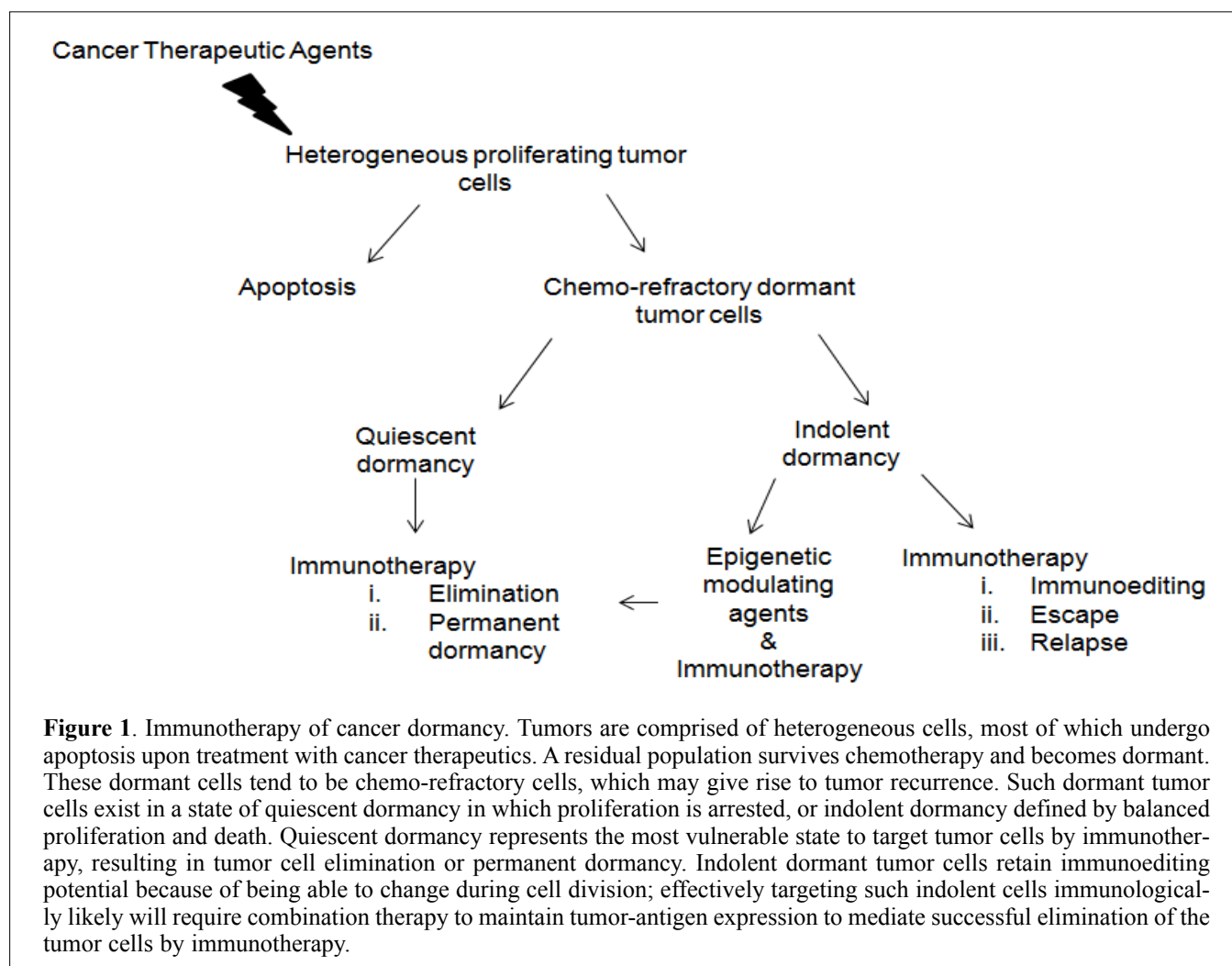
Immunotherapy of Dormant Tumor Cells for the Prevention of Tumor Recurrence

Primary cancers or advanced stage diseases harbor

highly proliferative tumor cells that can outnumber tumor-reactive T cells, and also secrete immune suppressive factors that dismantle immunotherapy of cancer. Therefore, cancer immunotherapy used against primary or advanced cancer likely will achieve prolonged patient survival, at best, rather than eliminating the tumor. Unlike highly proliferating tumor cells, dormant tumor cells are resistant to chemotherapy or radiation therapy due to their indolent nature, permitting their prolonged persistence *in situ*. Intriguingly, dormant tumor cells likely represent the best targets for immunotherapy as their secretion profile of immunosuppressive factors is dampened compared with proliferating tumors. Dormant tumor cells also represent a static target for immune cells, in contrast to proliferating tumors which may grow to skew the effector-to-target ratio in favor of the tumor. Therefore, the application of immunotherapy immediately after successful completion of chemotherapy, when tumor dormancy is likely established, can result in the prevention of tumor relapse by eliminating immune-vulnerable dormant

tumor cells or facilitating permanent dormancy. Lessons learned from the application of the rabies vaccine during clinical latency suggest that cancer immunotherapy can be successful during tumor dormancy. Allogeneic stem cell transplantation against hematological malignancies is also effective during minimal residual disease or semi-dormancy, which is established as a result of prior therapies, rather than against active and advanced stage disease. This is because indolent tumor cells, which become chemorefractory, remain sensitive to immunotherapy.

Mechanisms for cancer dormancy range from cell cycle arrest to immunoediting and angiogenic insufficiency (Teng *et al.*, 2008; Uhr and Pantel, 2011; Yu *et al.*, 1997). Fundamentally, these mechanisms vary between cellular quiescence and balanced proliferation and death (mitogenesis equally offset by apoptosis). Whereas cancer immunotherapy can control quiescent dormancy, it may induce immunoediting and result in the escape of dormant cells that are indolent due to their



ability to undergo cellular division. Therefore, a major challenge in immunotherapy of tumor dormancy is to elucidate the mechanisms which induce quiescent dormancy and reduce indolent dormancy.

Conclusions

Heterogeneity of tumor cells results in a range of responses to therapeutic agents from apoptosis to inhibition of tumor cell proliferation. The latter establishes tumor dormancy (Figure 1). These dormant cells usually become resistant to conventional cancer therapies. However, they are the best targets for immunotherapy. Therefore, administration of immunotherapy after successful completion of conventional therapies, when tumors enter the dormant state, could prevent distant recurrence of the tumor in the form of advanced stage disease. There are two types of dormancy which include quiescent dormancy or cell cycle arrest, and indolent dormancy or balanced proliferation and death. Whereas the former is resistant to immunoediting, the latter can change during cell division under immune pressure and escape immunotherapy. Implementing strategies to promote the acquisition of quiescent dormancy in combination with immunotherapy, or the application of epigenetic modulating agents to overcome immunoediting of indolent dormant cells, could reduce the risk of relapse and associated mortality of cancer patients.

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Disclosure

Authors have no potential conflicts of interest to disclose.

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