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14. ABSTRACT: Epidemiological and experimental evidence supports the concept that chronic inflammation promotes the development and progression of cancers; however, the mechanisms underlying this relationship are poorly understood. We have demonstrated previously that secretion of the pro-inflammatory cytokine interleukin 1b (IL-1b) from 4T1 mammary carcinoma cells (4T1/IL-1b) promotes tumor progression and decreases the survival of tumor-bearing animals. Tumor progression in many patients and experimental animals with cancer is frequently associated with the expansion of a population of myeloid cells, termed Myeloid-derived Suppressor Cells (MDSC). These cells have potent immunosuppressive activity and inhibit both innate and adaptive immunity by inhibiting T cell activation, NK cell cytotoxicity, and reducing the number of mature dendritic cells. Chronic inflammation at the tumor site enhances and alters the quality of MDSC by inducing a phenotypically and functionally distinct population of MDSC, which are more potent suppressors of CD8+ T cells. Using mice deficient for the IL-1 receptor (IL-1R-/-), we demonstrate that reducing inflammation delays tumor growth, the development of lung metastases, and the expansion of MDSC. MDSC in an inflammation-deficient environment are phenotypically and functionally similar to MDSC in wild-type BALB/c mice. Here we demonstrate that inflammation-induced MDSC skew immunity towards a type-2 response by reducing IL-12 production by macrophages and producing elevated levels of IL-10. In addition to the delays in MDSC induction, reducing inflammation prevent the accumulation of a more potent population of MDSC induced by inflammation, as MDSC from IL-1R-/- mice produce similar levels of IL-10 as BALB/c mice. These data suggest that limiting tumor-associated inflammation may delay the onset of systemic immune suppression that accompanies breast cancer progression and may enhance the efficacy of current and future therapies.					
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INTRODUCTION

The concept that chronic inflammation promotes tumor progression was originally proposed by Virchow in the late 1800's (1). Epidemiological and experimental observations support the hypothesis that chronic inflammation contributes to the development and progression of cancers (1-4); however the mechanisms underlying the relationship between inflammation and breast cancer are poorly understood. Inflammatory components have been shown i) to induce DNA damage which contributes to genetic instability and transformed cell proliferation (1); ii) to promote angiogenesis and thereby enhance tumor growth and invasiveness (4); and iii) to impair myelopoiesis and hematopoiesis thereby causing immune dysfunction and inhibiting immune surveillance (5, 6). Although it is generally accepted that inflammation enhances tumor progression (1, 4, 7), the mechanisms by which inflammation mediates its effects are not well understood.

Tumor progression in many patients and experimental animals with cancer is frequently associated with the expansion of a population of cells of myeloid origin, termed Myeloid-derived Suppressor Cells (MDSC). MDSC accumulate in the spleen, blood and lymphoid organs as immature myeloid cells, characterized by the surface expression of CD11b and Gr1 markers. In tumor-free individuals these immature cells differentiate into granulocytes, macrophages and/or dendritic cells (Figure 1). However, in tumor-bearing individuals, differentiation is blocked by tumor-secreted factors that impair hematopoiesis and lead to the accumulation of MDSC (5). These MDSC have potent immunosuppressive activity and inhibit both adaptive and innate immunity by preventing the activation of CD4⁺ and CD8⁺ T cells, reducing the number of mature dendritic cells, suppressing NK cell cytotoxicity, and by skewing immunity towards a type-2 phenotype (6, 8, 9).

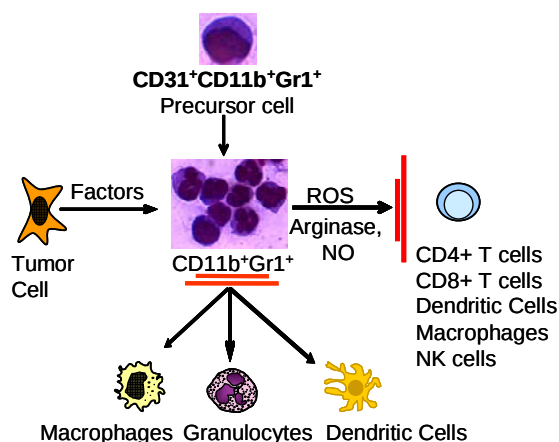


Figure1. Tumor-secreted factors promote the accumulation of myeloid-derived suppressor cells (MDSC) and inhibit myeloid cell development and differentiation.

These MDSC have potent immunosuppressive activity and inhibit both adaptive and innate immunity by preventing the activation of CD4⁺ and CD8⁺ T cells, reducing the number of mature dendritic cells, suppressing NK cell cytotoxicity, and by skewing immunity towards a type-2 phenotype (6, 8, 9).

We hypothesize that chronic inflammation causes an increase in MDSC which inhibit immune surveillance and anti-tumor immunity, thereby facilitating malignant cell transformation and proliferation. To study these mechanisms we have developed an experimental system in which tumors constitutively express the pro-inflammatory cytokine,

interleukin 1 β (IL-1 β), resulting in an inflammatory microenvironment at the tumor site (9). We have used IL-1 β because it is a key cytokine in mediating an inflammatory response (10) and has been previously shown to promote primary tumor growth (11-13) and to enhance metastatic disease (14, 15). The spontaneously metastatic BALB/c-derived 4T1 mammary carcinoma (16-18) was used because breast cancer is one of the cancers for which inflammation is associated with poor prognosis (6), and because we want to study the effects of inflammation on the progression of both primary and metastatic tumor.

In addition to confirming the concept that a pro-inflammatory microenvironment enhances tumor progression, our results suggest a novel mechanism by which inflammation facilitates tumor growth. We demonstrated previously that inflammation in the tumor microenvironment (4T1/IL-1 β) promotes tumor growth, decreases survival and enhances the accumulation of MDSC (9). These inflammation-induced MDSC are a phenotypically and functionally distinct subpopulation compared to MDSC induced by 4T1 tumor alone. Inflammation-induced MDSC are more potent suppressors of CD8⁺ T cells and suppress through an arginase independent mechanism (9). The finding that IL-1 β up-regulates MDSC accumulation in tumor-bearing mice has led us to propose the following causal relationship linking chronic inflammation with tumor progression: As tumor cells proliferate they induce an inflammatory microenvironment consisting of IL-1 β and other pro-inflammatory mediators. The persistence of these mediators causes the accumulation and retention of MDSC. The MDSC, in turn, initiate and maintain an immune suppressive state which blocks immune surveillance, thereby facilitating the survival and proliferation of transformed cells.

Immune dysfunction is a significant impediment to immunotherapy; therefore the goal of this project is to understand the role of inflammation in the mechanisms underlying MDSC accumulation and immune suppression, so that novel interventional strategies may be developed. We hypothesize that reducing inflammation will limit tumor growth and the accumulation of MDSC and inhibit the development of a more potent population of inflammation-induced MDSC. Reducing inflammation by blocking the IL-1 signaling pathway (IL-1R^{-/-}) reduces tumor growth and metastatic disease and delays the accumulation of MDSC. These data suggest that reducing inflammation limits tumor progression and immune suppression by delaying MDSC induction and preventing the accumulation of a more potent population of inflammation-induced MDSC.

BODY

Note: Text appearing in the original statement of work (SOW) is underlined.

Task 1: Phenotypic characterization of IL-1 β induced MDSC.

Final Result for Task 1 -- Completed (see previous annual report).

Task 2: Functional characterization of IL-1 β induced MDSC and determination of target cells which are suppressed by MDSC.

Final Result for Task 2 -- Completed (see previous annual report).

Task 3: Determine if IL-1 β directly stimulates the accumulation of MDSC.

Final Result for Task 3 – Completed (see previous annual report).

Task 4: Test cytokines downstream if IL-1 β for their ability to induce MDSC accumulation.

- Analyze the sera and dissociated tumor tissue from 4T1 and 4T1/IL-1 β inoculated mice, by ELISA, for expression of cytokines (IL-1 β , IL-6, IL-10, VEGF) (9-18 months).
- Determine expression of cytokine receptors (IL-1 β , IL-6, IL-10, and VEGF) on surface of MDSC from 4T1 and 4T1/IL-1 β inoculated mice by flow cytometry using antibodies to the cytokine receptors (9-18 months).

Results: Serum and dissociated tumor tissue from mice with 4T1 tumors, 4T1/IL-1 β tumors and IL-1R^{-/-} mice with 4T1 tumors, were analyzed for expression of inflammatory cytokines (IL-1 β , IL-6, TNF α , IL-10, IFN γ , IL-12, MCP-1, IL-5 and others) by multiplex at the Biopolymer Cytokine Core Facility, University of Maryland, Baltimore. Serum and dissociated tumor tissue were also analyzed for TGF β by ELISA. Cytokine analysis of the dissociated tumor tissue suggests an inflammatory tumor microenvironment is present during tumor growth. This inflammatory environment is exacerbated by the secretion of IL-1 β from 4T1/IL-1 β tumors, as the cytokine analysis reveals elevated levels of IL-13 and decreased levels of IL-12 with 4T1/IL-1 β tumors suggesting that inflammation drives a predominantly Th2 environment in the presence of tumor. Additionally, decreased levels of IL-6 and MCP-1 were observed in the dissociated tumor tissue of IL-1R^{-/-} mice with 4T1 tumors, suggesting that the loss of IL-1R may limit the inflammatory milieu and that the expression of these cytokines may be partly IL-1 dependent. The results of the serum analysis are still in progress.

MDSC do not express the IL-1R (9) which suggests an intermediate cell and intermediate mediator(s) which are triggered by the secretion of IL-1 β from the primary tumor (4T1/IL-1 β) and lead to the expression of other factors, which in turn act directly on the MDSC. A potential candidate is interleukin 6 (IL-6), since IL-1 β leads to the production of IL-6 (19), and the levels of IL-6 in the tumor microenvironment are reduced in IL-1R^{-/-} mice. To determine if MDSC can interact directly with IL-6, the expression of the IL-6R on MDSC was evaluated by flow cytometry (Figure 2). MDSC from 4T1 and 4T1/IL-1 β tumor-bearing mice express the IL-6R indicating that MDSC can interact directly with IL-6 and supporting the hypothesis that IL-6 may be an intermediate mediator.

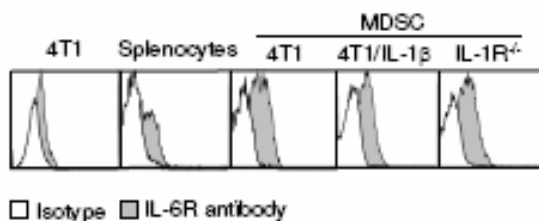


Figure 2. Blood CD11b⁺Gr1⁺ MDSC from tumor-bearing BALB/c or IL-1R^{-/-} mice with 4T1 or 4T1/IL-1 β were stained with IL-6R antibody or isotype control antibody. 4T1 tumor cells and BALB/c splenocytes were stained as negative and positive controls, respectively. All MDSC express the IL-6R. Data are pooled from 2-3 experiments.

The rest of Task 4 has not been attempted yet.

Task 5: Determine if macrophages are involved in MDSC accumulation.

Results: MDSC have been observed to suppress a variety of immune cells, such as CD4⁺ and CD8⁺ T cells, NK cells, and dendritic cells; however the suppressive function of MDSC on macrophages is unknown. Preliminary studies demonstrated that MDSC could skew macrophages responses towards a type 2 response by altering the cytokine profile of the macrophages. Additionally, we examined whether the MDSC induced by inflammation which were demonstrated to be more suppressive towards CD8⁺ T cells (9), were able to exacerbate the effect on MDSC on macrophages. To determine the suppressive effect of MDSC on peritoneal macrophages, macrophages were classically activated with LPS and IFN γ and co-cultured with purified MDSC from 4T1 or 4T1/IL-1 β tumor-bearing mice. Classically activated macrophages (M1 macrophages) are typically tumoricidal and produced elevated levels of IL-12 and do not secrete IL-10. Conversely, alternatively activated macrophages (M2 macrophages) support tumor progression, and produced elevated levels of IL-10, but no IL-12 (20). The presence of 4T1 MDSC in the co-culture of classically activated macrophages reduced the production of IL-12 by macrophages and increased the presence of IL-10. Co-culture with 4T1/IL-1 β MDSC significantly reduced the production of IL-12 by macrophages compared to 4T1 MDSC and significantly doubled the production of IL-10 (Figure 3A). Interestingly, addition of LPS and IFN γ to either population of MDSC in the absence of macrophages leads to the production of IL-10 implying that the MDSC can be activated to secrete IL-10, although 4T1/IL-1 β MDSC secrete significantly more IL-10 than 4T1 MDSC (Figure 3B). These results suggest that MDSC can skew immunity towards a type 2 response by limiting the production of IL-12 by macrophages and by secreting immune suppressive IL-10, and that this effect is enhanced by MDSC induced by chronic inflammation.

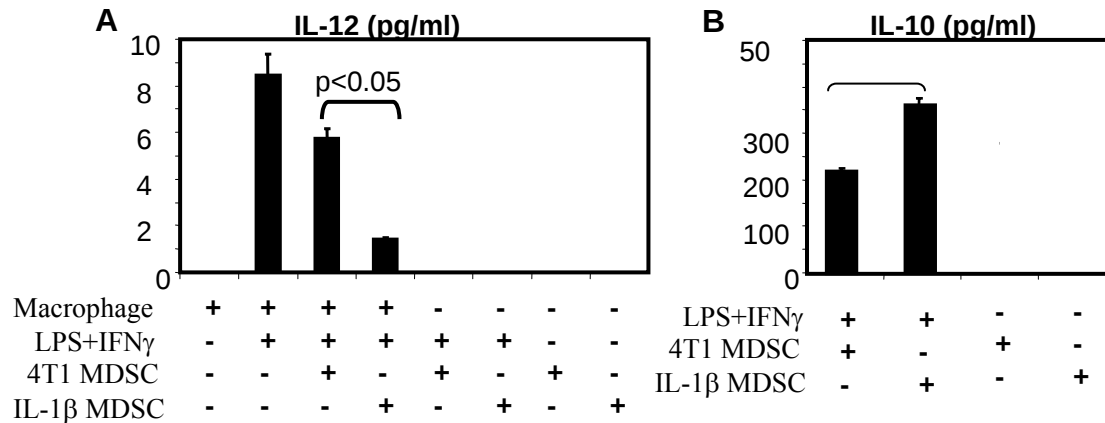


Figure 3. Inflammation-induced MDSC reduce IL-12 production by macrophages and secrete elevated levels of IL-10 upon LPS and IFN γ stimulation. Blood MDSC from BALB/c mice inoculated with 4T1 or 4T1/IL-1 β were co-cultured for 24 hours in the presence or absence of peritoneal macrophages, with or without LPS and IFN γ stimulation. Supernatants were tested for IL-12 (A) and IL-10 (B) by ELISA. Data are representative of 3 experiments.

Final Result for Task 5 – Completed

Task 6: Determine if blocking inflammatory responses prevents, delays, or minimizes tumor progression.

- Administer Celecoxib to BALB/c mice continuously, starting at time of 4T1 tumor inoculation. Monitor tumor onset, growth, survival, metastatic load, and MDSC accumulation (24-36 months).

Results: To determine whether blocking inflammation reduces tumor growth, metastases and the accumulation of MDSC, the selective COX-2 inhibitor, SC58236, was administered to tumor-bearing mice at the time of tumor inoculation. BALB/c mice were inoculated with either 4T1 or 4T1/IL-1 β tumors, SC58236 injections were given three times per week. Preliminary results demonstrate that treatment with a selective COX-2 inhibitor delays tumor growth and the accumulation of MDSC in both 4T1 and 4T1/IL-1 β tumor-bearing mice. These experiments are currently in progress.

Task 7: Determine if reducing inflammatory responses minimizes or delays tumor progression and immune suppression.

- Inoculate IL-1R-deficient BALB/c mice with 4T1 mammary carcinoma cells. Monitor tumor onset, growth, metastatic load and the accumulation of MDSC (12-24 months).

Results: To evaluate the tumor promoting effects of inflammation and because IL-1 β secretion enhances tumor growth, we examined tumor progression in mice deficient for the IL-1 receptor (IL-1R $^{-/-}$). BALB/c and IL-1R $^{-/-}$ BALB/c mice were inoculated with 4T1 cells on day 0. For both groups, tumors were palpable by day 10, and tumor

diameter was measured at the indicated time points (Figure 3A). Tumor onset did not differ between BALB/c and IL-1R^{-/-} mice, however, tumor growth was significantly delayed ($p=0.03$) in IL-1R^{-/-} mice as compared to BALB/c mice. These data imply that blocking signaling of the pro-inflammatory cytokine, IL-1, reduces the growth of 4T1 primary tumors, supporting a role for inflammation in tumor progression.

Since the loss of the IL-1 receptor limits tumor progression, we examined whether the delay in tumor growth was accompanied by a reduction in the development of lung metastases. BALB/c and IL-1R^{-/-} mice were inoculated on day 0 with 4T1 tumor cells and mice were sacrificed on day 36-40 when BALB/c mice were moribund. Lungs were harvested and the number of lung metastases was quantified using the clonogenic assay (18). Lung metastases were significantly reduced in IL-1R^{-/-} mice as compared to BALB/c mice (Figure 3B), indicating that eliminating IL-1 signaling during tumor progression limits metastatic dissemination to the lungs.

Tumor growth is accompanied by the induction of MDSC and the production of IL-1 β in the tumor microenvironment enhances this accumulation (9). We evaluated whether the reduction in tumor growth and lung metastases in the IL-1R^{-/-} mice was accompanied by a decrease in the systemic accumulation of tumor-associated MDSC by examining the levels of MDSC during tumor progression. BALB/c mice inoculated with 4T1 or 4T1/IL-1 β cells, and IL-1R^{-/-} mice inoculated with 4T1 cells at day 0, were followed as a function of tumor growth. At the indicated time points, mice were tail bled and the percent of blood CD11b⁺Gr1⁺ MDSC were measured by flow cytometry. No difference in the baseline levels of MDSC were observed between naïve BALB/c and naïve IL-1R^{-/-} mice, indicating that IL-1 is not required for normal levels of MDSC (Figure 4). MDSC levels in BALB/c mice with 4T1/IL-1 β tumors increase significantly by day 8-10 ($p<0.05$), compared to BALB/c mice with parental 4T1 tumors. In IL-1R^{-/-} mice with 4T1 tumor, MDSC accumulation was significantly delayed ($p<0.01$) on days 14-16, and did not increase until days 26-28 when MDSC levels were similar to those seen in BALB/c mice with either 4T1 or 4T1/IL-1 β tumors (Figure 4). These data suggest that the presence of inflammation in the tumor microenvironment promotes early MDSC induction, while MDSC accumulation is delayed in an IL-1-deficient environment. In addition, these data suggest that MDSC accumulation in the early stages of tumor growth is IL-1 dependent.

The delay in MDSC accumulation associated with the loss of IL-1 signaling may be due either to an effect of inflammatory mediators on the kinetics of MDSC expansion or to an accumulation of an altered subpopulation of MDSC. We examined whether MDSC from IL-1R^{-/-} mice were phenotypically similar to MDSC from BALB/c mice. Blood from BALB/c and IL-1R^{-/-} was harvested and a $>95\%$ CD11b⁺Gr1⁺ population was gated by flow cytometry. MDSC were stained with directly-coupled antibodies to the indicated surface markers and the expression levels of these markers were analyzed by flow cytometry. Expression of the indicated markers demonstrated that both BALB/c and IL-1R^{-/-} MDSC are phenotypically similar suggesting that loss of IL-1 signaling does not alter MDSC phenotype and that the delay in MDSC accumulation is not due to an altered population of suppressor cells.

We also examined whether the MDSC from IL-1R^{-/-} mice were as suppressive as MDSC from BALB/c mice. MDSC from BALB/c and IL-1R^{-/-} mice with 4T1 tumors were harvested from blood at day 40 after tumor inoculation and co-cultured with TS1

CD4⁺ TCR transgenic splenocytes in the presence or absence of HA peptide, with or without inhibitors to arginase (NOHA) or nitric oxide (NO). CD4⁺ T cell proliferation was measured by the incorporation of ³H-thymidine. Both BALB/c and IL-1R^{-/-} MDSC are equally suppressive towards CD4⁺ T cell, and this suppression is blocked by the addition of the arginase inhibitor, suggesting that suppression is mediated by arginase.

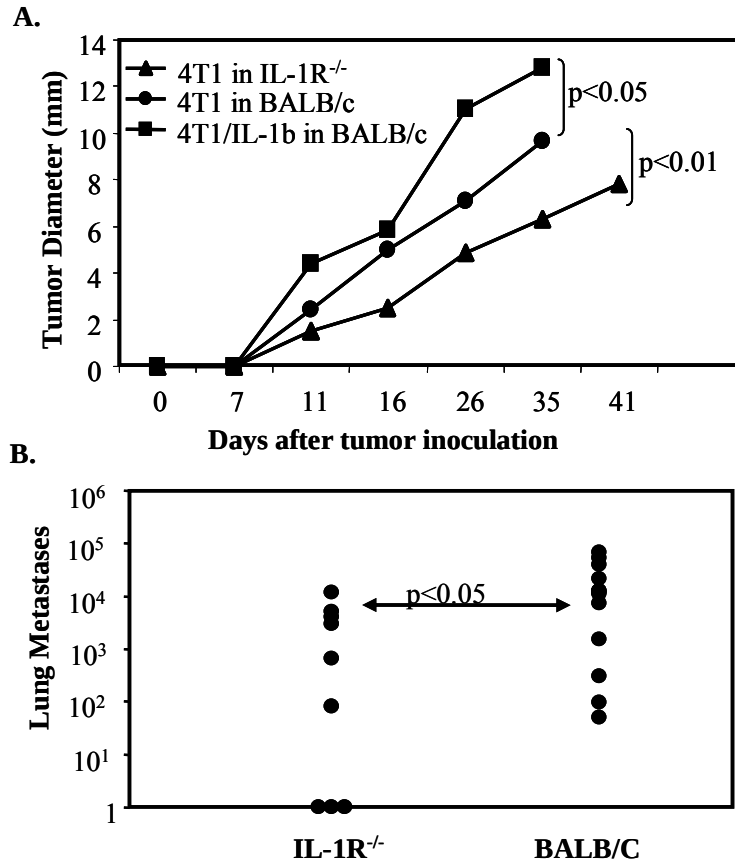
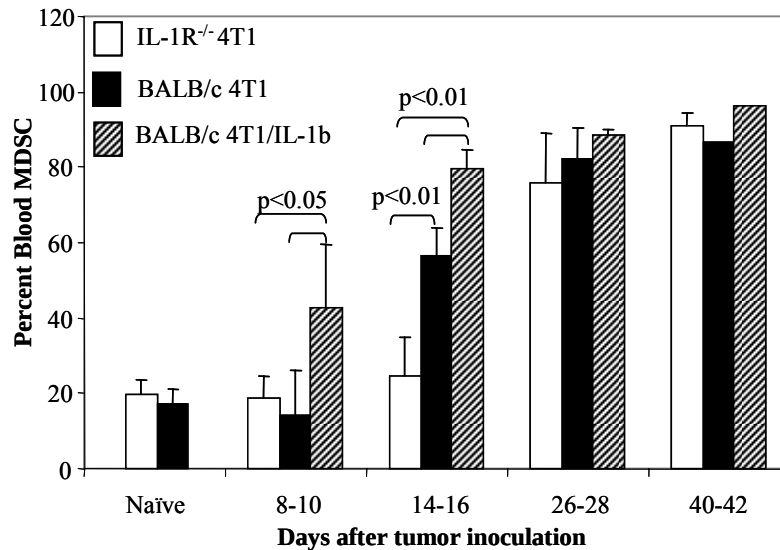


Figure 4. Loss of the IL-1 receptor delays tumor progression and the development of lung metastases. BALB/c mice and IL-1R^{-/-} mice were inoculated with 4T1 on day 0. (A) Tumor diameter was measured every week with a caliper. (B) Mice were euthanized and lungs were harvested when mice were moribund. Lung metastases were quantified using a clonogenic assay. Data shown are pooled results from 2-3 experiments.

Figure 5. MDSC expansion is IL-1 dependent in the early stages of tumor growth. BALB/c mice were inoculated with 4T1 or 4T1/IL-1 β and IL-1R^{-/-} mice were inoculated with 4T1 on day 0. Mice were tail bled at the indicated time points after tumor inoculation and the percentage of CD11b+Gr1+ MDSC was quantified by flow cytometry. Data shown are pooled results from 2-3 experiments.



Final Result for Task 7 – Completed.

Task 8: Determine whether hematopoietic or non-hematopoietic cells respond to tumor-derived IL-1beta.

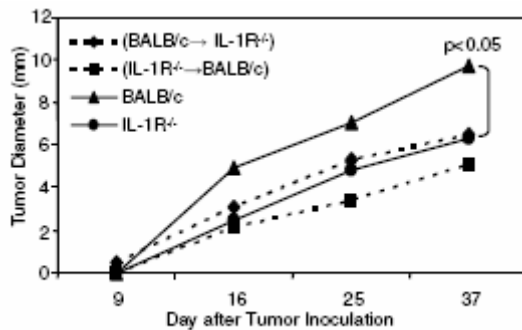
- Create chimeric mice using BALB/c and IL-1R-deficient mice. Reconstitute BALB/c mice with bone marrow from IL-1R-deficient mice and IL-1R-deficient mice with bone marrow from BALB/c mice. Inoculate chimeric mice with 4T1 mammary carcinoma cells. Monitor tumor growth, metastatic load, and MDSC accumulation (12-24 months).

Results: The IL-1R^{-/-} mice were used to identify the origin of the intermediate cells which respond to tumor-secreted IL-1β and induce increased tumor progression and MDSC accumulation. 4T1/IL-1β MDSC are unable to respond to IL-1β, thus implicating an intermediate cell which responds to IL-1β and in response acts upon the MDSC population. To determine if this effector cell is of hematopoietic or non-hematopoietic origin, we created bone marrow chimeras using BALB/c mice reconstituted with IL-1R^{-/-} bone marrow. The reciprocal chimeras were created using IL-1R^{-/-} mice reconstituted with BALB/c bone marrow. These chimeras were inoculated with 4T1 cells and tumor growth, metastases and MDSC levels were measured.

To further determine whether the delay in tumor progression in IL-1R^{-/-} mice is mediated by the hosts' hematopoietic or non-hematopoietic cells, bone marrow chimeras were created. IL-1R^{-/-} mice were lethally irradiated and reconstituted with BALB/c bone marrow (BALB/c→IL-1R^{-/-}), and the reciprocal chimeras of BALB/c mice reconstituted with IL-1R^{-/-} bone marrow (IL-1R^{-/-}→BALB/c) were inoculated with 4T1 cells and primary

Figure 6. IL-1 nonresponsiveness in either hematopoietic or non-hematopoietic compartments mediate a delays in tumor growth. BALB/c, IL-1R^{-/-} and BALB/c→IL-1R^{-/-} and IL-1R^{-/-}→BALB/c bone marrow chimeras were inoculated with 4T1 tumor cells and monitored for primary tumor growth. Tumor progression in both chimeras was delayed compared to BALB/c mice (p<0.05). Values are the average ± SD of 7-15 mice per group. Data are pooled from 2-3 experiments.

tumor growth was measured once a week following tumor inoculations. 4T1 tumor growth was significantly delayed in both BALB/c $\rightarrow$ IL-1R $^{-/-}$ chimeras and IL-1R $^{-/-}$ $\rightarrow$ BALB/c chimeras compared to 4T1 tumor growth in wild-type BALB/c mice (Figure 6). Tumor progression in both chimeras was not significantly different from tumor growth in IL-1R $^{-/-}$ mice, suggesting that IL-1 nonresponsiveness in either the hosts' hematopoietic or non-hematopoietic compartment can mediate the delay in tumor growth observed in these mice.



Because the loss of IL-1 signaling in either hematopoietic or non-hematopoietic cells can mediate the delay in tumor progression, we examined whether the inhibition of IL-1 in either compartment could lead to a reduction in the number of lung metastases. IL-1R $^{-/-}$ $\rightarrow$ BALB/c chimeras and BALB/c $\rightarrow$ IL-1R $^{-/-}$ chimeras were inoculated with 4T1 tumors and the lungs were harvested at day 40 after tumor inoculation. Lung metastases were determined using the clonogenic assay (18). Although lung metastases are significantly reduced in IL-1R $^{-/-}$ mice compared to BALB/c mice, both chimeras did not have a significant reduction in the number of lung metastases compared to BALB/c mice, suggesting that both IL-1 nonresponsiveness in both hematopoietic and non-hematopoietic cells is required to mediate a reduction in lung metastases.

To determine whether host non-hematopoietic cells respond to IL-1 to mediate a delay in MDSC accumulation in the early stages of tumor growth when a reduction in MDSC is observed in IL-1R $^{-/-}$ mice, we examined whether inhibition of IL-1 signaling in either cellular compartment using chimera mice would limit MDSC accumulation. IL-1R $^{-/-}$ $\rightarrow$ BALB/c chimeras and BALB/c $\rightarrow$ IL-1R $^{-/-}$ chimeras were inoculated with 4T1 tumors and the percentage of blood MDSC were quantified by flow cytometry at the indicated time points. At day 16, when MDSC in IL-1R $^{-/-}$ mice are significantly reduced ($p < 0.01$) compared to MDSC in BALB/c mice, we observe that MDSC in the BALB/c $\rightarrow$ IL-1R $^{-/-}$ chimeras are not significantly different than the IL-1R $^{-/-}$ mice, suggesting that the hosts' non-hematopoietic cells mediate MDSC accumulation. However, both IL-1R $^{-/-}$ $\rightarrow$ BALB/c chimeras and BALB/c $\rightarrow$ IL-1R $^{-/-}$ chimeras have significantly reduced levels of MDSC compared to BALB/c mice. These data suggest that although an IL-1R $^{-/-}$ background is sufficient to delay MDSC accumulation, an IL-1R deficiency in the hematopoietic compartment also leads to a significant reduction in MDSC accumulation.

Final Result for Task 8 – Completed

Task 9: Determine if interleukin 6 (IL-6) is a mediator of the tumor promoting effects IL-1 β .

- a. Create stable 4T1 cell lines which secrete IL-6 (4T1/IL-6), by transfection with an IL-6 plasmid containing IL-6 gene (12-24 months).
- b. Quantify IL-6 secretion of 4T1 stable transfectants by ELISA (12-24 months).
- c. Inoculate BALB/c and IL-1R-deficient mice with 4T1/IL-6 tumor cells. Monitor tumor onset, growth, metastatic load, and MDSC accumulation (12-24 months).

Results: The tumor promoting and immune suppressive effects of IL-1 β are not direct and involve an intermediate mediator, as neither the tumor cells nor the MDSC express the IL-1R. This suggests an intermediate mediator, such as interleukin 6 (IL-6), as the secretion of IL-1 β leads to the production of IL-6. Preliminary studies demonstrate that MDSC express the IL-6R, supporting the idea that IL-1 β secretion from the primary tumor acts on a host intermediate cell, leading the production and secretion of IL-6, which acts directly on the MDSC. To evaluate the effects of IL-6, as a potential candidate mediator of the effects of IL-1 β on tumor progression and immune suppression, 4T1 mammary carcinoma cells were engineered to secrete human IL-6 by stable transfection (4T1/IL-6). 4T1/IL-6 tumor cells stably secrete 1.5ng/mL/5x10⁵/24h as measured by ELISA.

BALB/c and IL-1R^{-/-} mice were inoculated with 4T1/IL-6 tumor cells, and tumor growth, the development of lung metastases, and the accumulation of MDSC was measured. No differences in tumor growth, metastatic dissemination to the lungs or the accumulation of MDSC were observed in BALB/c mice inoculated with 4T1/IL-6 compared to BALB/c mice inoculated with 4T1, suggesting that IL-6 in the quantities produced does not alter tumor progression. Interestingly, IL-1R-deficient mice inoculated with 4T1/IL-6 tumor cells had enhanced tumor growth (Figure 7A), elevated lung metastases (Figure 7B), and increased MDSC accumulation (Figure 7C) compared to IL-1R-deficient mice inoculated with 4T1. The production of IL-6 in mice deficient for IL-1R eliminated the reduction in tumor progression and immune suppression due to the absence of the IL-1 system, demonstrating that IL-6 can compensate for the loss of IL-1 and is a down-stream mediator of IL-1 mediated tumor progression and MDSC expansion.

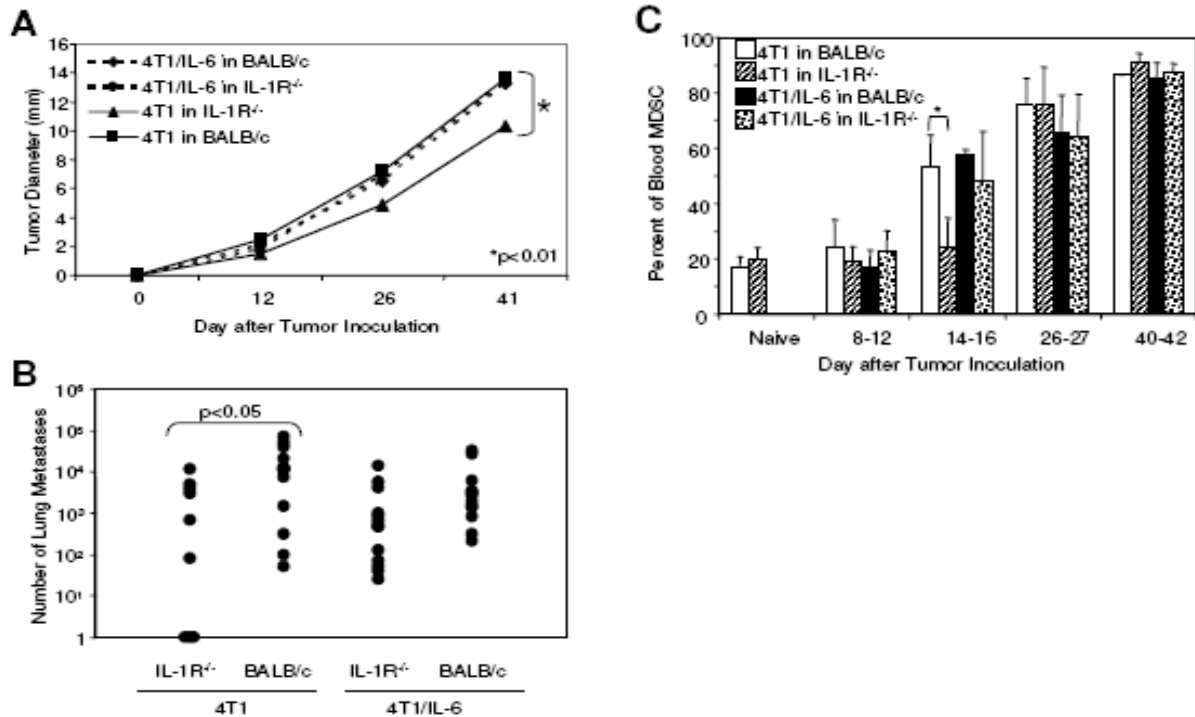


Figure 7. IL-6 compensates for tumor progression and MDSC accumulation in IL-1R^{-/-} mice. BALB/c and IL-1R^{-/-} mice were inoculated with 7000 4T1 or 4T1/IL-6 tumor cells in the abdominal mammary gland on day 0 and monitored for primary tumor growth, lung metastases, and the percentage of blood MDSC. (A) Tumor diameter was measured at the indicated time points. Values are the average $\pm$ SD of 15-17 mice per group. (B) Mice were sacrificed and lungs were harvested when BALB/c mice were moribund (days 35-40). Metastatic cells in individual mice ($\bullet$) were quantified using the clonogenic assay. (C) The percentage of blood CD11b⁺Gr1⁺ MDSC was measured by flow cytometry at the indicated time points. Values are the average $\pm$ SD of 15-17 mice per group. Data are pooled from 2-3 experiments.

Final Result for Task 9 – Completed

Task 10: Determine if inflammation-induced MDSC enhance macrophage type-2 responses.

- a. Co-culture LPS and IFN γ -activated peritoneal macrophages from BALB/c mice with MDSC from BALB/c mice inoculated with either 4T1 or 4T1/IL-1 β mammary carcinoma cells. Quantify IL-12 and IL-10 production by ELISA (24-30 months).

This task has not yet been attempted.

Task 11: Determine if cross-talk between MDSC and macrophages is TLR4-dependent.

- a. Determine the surface expression of TLR4, CD14 and CD11b on MDSC from BALB/c mice and TLR4-deficient mice inoculated with 4T1 or 4T1/IL-1 β carcinoma cells (24-30 months).
- b. Quantify the expression of activated NF κ B by Western Blot (24-36 months).
- c. Co-culture peritoneal macrophages from BALB/c mice with MDSC from TLR4-deficient BALB/c mice inoculated with 4T1 mammary carcinoma cells. Co-culture peritoneal macrophages from TLR4-deficient BALB/c mice with MDSC from BALB/c mice inoculated with 4T1 mammary carcinoma cells. Quantify IL-12 and IL-10 production by ELISA (24-36 months).

This task has not yet been attempted.

FUTURE DIRECTIONS

We wish to extend these experiments to IL-1 receptor antagonist deficient (IL-1Ra^{-/-}) BALB/c mice which we have recently acquired to further evaluate the role of inflammation in tumor progression and immune suppression. Our previous studies demonstrate that excessive inflammation through the production of IL-1 β promotes tumor growth and immune suppression, while these processes are delayed in the absence of IL-1 signaling as observed in the IL-1R^{-/-} mouse experiments. The IL-1Ra^{-/-} mice are deficient in the receptor antagonist and have a reduced ability to limit inflammatory responses (21). These mice will be inoculated with 4T1 tumor and tumor growth, metastases and MDSC accumulation will be measured. Because inflammation enhances tumor growth and immune suppression we hypothesize that the IL-1Ra^{-/-} mice, which are deficient in their ability to suppress inflammatory responses, will have enhanced tumor growth and elevated levels of MDSC.

Additionally, we previously demonstrated that inflammation-induced MDSC are more suppressive towards CD8⁺ T cells and have an increased ability to skew immunity towards a type 2 response. The MDSC from tumor-bearing IL-1Ra^{-/-} mice will be evaluated for their suppressive functions towards CD4⁺ and CD8⁺ T cells, as well as their effect on macrophage cytokine production. We anticipate that IL-1Ra^{-/-} MDSC will be more suppressive than MDSC from BALB/c mice because of the inflammatory environment present during their induction.

Key Research Accomplishments

- Serum and tumor tissue of 4T1, 4T1/IL-1 β , and IL-1R^{-/-} tumor bearing mice were analyzed for a variety of inflammatory and immunosuppressive cytokines.
- The expression of the IL-6 receptor was measured on MDSC: MDSC from 4T1 and 4T1/IL-1 β tumor bearing mice express the IL-6 receptor.
- The effect of MDSC from 4T1 and 4T1/IL-1 β tumor bearing mice on macrophages was determined: both types of MDSC reduce IL-12 production by macrophages, although 4T1/IL-1 β MDSC are more potent suppressors of IL-12 production on a per cell basis.

- MDSC were demonstrated to skew immunity towards a type 2 response by the secretion of IL-10 upon LPS stimulation, although 4T1/IL-1 β MDSC secreted significantly more IL-10 than 4T1 MDSC on a per cell basis.
- Tumor growth in the IL-1R^{-/-} mice was evaluated: a significant delay in tumor growth was observed in IL-1R^{-/-} mice with 4T1 tumors compared to BALB/c mice with 4T1 tumors.
- Tumor growth in the IL-1R^{-/-} bone marrow chimeras was evaluated to determine whether host hematopoietic or non-hematopoietic cells respond to IL-1: IL-1 nonresponsiveness in either hematopoietic or non-hematopoietic cells is sufficient to delay tumor progression.
- The development of lung metastases in the IL-1R^{-/-} mice was evaluated: a significant reduction of lung metastases was observed in the IL-1R^{-/-} mice with 4T1 tumors compared to BALB/c mice with 4T1 tumors.
- The development of lung metastases in the IL-1R^{-/-} bone marrow chimeras was evaluated: IL-1 nonresponsiveness in both hematopoietic and non-hematopoietic cells is required for a reduction in the number of lung metastases.
- The induction of MDSC in the IL-1R^{-/-} mice was measured: a significant delay in the accumulation of blood MDSC was observed in the IL-1R^{-/-} mice.
- The phenotypic and functional similarity between MDSC in BALB/c and IL-1R^{-/-} mice with 4T1 tumors was evaluated: both populations of MDSC are phenotypically and functionally similar.
- MDSC induction in the IL-1R^{-/-} bone marrow chimeras was measured: non-hematopoietic and hematopoietic cells respond to IL-1 and mediate MDSC accumulation.
- 4T1 cells were engineered to secrete IL-6 (4T1/IL-6).
- The effects of IL-6 on tumor progression and immune suppression were evaluated: IL-6 compensates for the loss of IL-1 in tumor growth, lung metastases and MDSC accumulation.

REPORTABLE OUTCOMES

The reported data has been presented as posters at the following meetings:

- Keystone Symposia Meeting on Inflammation and Cancer in February 2007 in Santa Fe, NM. The poster presented at the Keystone Symposia Meeting was awarded an NCI Scholar Travel Award for \$1000.
- Mechanism and Therapeutic reversal of Immune Suppression in Cancer meeting in January 2007 in Clearwater, FL.

A short talk entitled “Inflammation Induces Myeloid-Derived Suppressor Cells that Facilitate Tumor Progression” was presented at the American Association of Cancer Research (AACR) Annual Meeting in April 2006 in Washington D.C.

The following paper was published:

- Bunt, S.K., et al. "Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression". *J Immunol*. 2006 Jan 1;**176**(1): 284-90.

CONCLUSIONS

The purpose of this project is to determine the mechanisms by which inflammatory factors, such as IL-1 β , promote tumor-associated immune suppression in breast cancer. We propose a novel hypothesis by which inflammation promotes tumor progression by enhancing immune suppression, thereby inhibiting natural immune surveillance and allowing for the outgrowth and proliferation of transformed cells. Delineating the mechanisms by which inflammation enhances tumor-associated immune suppression and the factors that induce dysfunction will lead to the circumvention of suppression and will increase vaccine effectiveness and facilitate immunotherapy. We have demonstrated that tumor growth associated with inflammation leads to significantly elevated levels of MDSC and that the inflammation-induced MDSC are a distinct and more potent subpopulation of suppressor cells. We have demonstrated that elimination of the IL-1 signal, in the IL-1R^{-/-} mice, reduces tumor growth, metastases, and delays MDSC induction, and that IL-6 can compensate for this reduction demonstrating that IL-6 is a downstream mediator of IL-1 promoted tumor progression and immune suppression. These results support the hypothesis that inflammation promotes tumor progression and immune suppression.

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APPENDIX A

Revised Statement of Work (SOW)

Immune Suppression and Inflammation in the Progression of Breast Cancer

TASK 1: Phenotypic characterization of IL-1beta induced myeloid-derived suppressor cells (MDSC).

[Note: Animal experiments will be conducted 2-3 times using 5 animals/experiment. BALB/c female mice, 6-20 weeks of age, will be used for these experiments]

- a. Create stable 4T1 cell lines which secrete mature IL-1beta(4T1/IL-1beta), by transduction with IL-1beta retrovirus containing mature IL-1beta gene (completed).
- b. Quantitate IL-1beta secretion of 4T1 stable transductants by ELISA (completed).
- c. Phenotype population of MDSC from 4T1 and 4T1/IL-1beta inoculated mice by flow cytometry using antibodies to surface markers (1-3 months).

TASK 2: Functional characterization of IL-1beta induced MDSC and determination of target cells which are suppressed by MDSC.

[Note: Animal experiments will be conducted 3-5 times using 5 animals/experiment. BALB/c female mice, 6-20 weeks of age; DO11.10 transgenic mice and HA transgenic mice will be used for these experiments]

- a. Perform proliferation assays to determine whether MDSC suppress CD4⁺ and/or CD8⁺ T cells. OVA-specific DO11.10 transgenic T cells or HA-specific transgenic T cells will be plated with specific peptide and MDSC from 4T1 and 4T1/IL-1beta inoculated mice. T cell proliferation will be measured with ³H thymidine (1-6 months).
- b. Perform proliferation assays (Task 2a) with inhibitors of reactive species, Nor-NOHA and L-NMMA, to identify the functional molecule responsible for suppression (6-9 months).
- c. Measure ROS expression in MDSC from 4T1 and 4T1/IL-1beta inoculated mice by flow cytometry using a fluorescent dye (DCFDA) (6-8 months).

TASK 3: Determine if IL-1beta directly stimulates the accumulation of MDSC.

[Note: Animal experiments will be conducted 2-3 times using 5 animals/experiment. BALB/c female mice, 6-20 weeks of age, will be used for these experiments]

- a. Determine the expression of IL-1beta receptor on the surface of MDSC by flow cytometry using an antibody to the IL-1beta type 1 receptor (9-12 months).

TASK 4: Test cytokines downstream of IL-1beta for their ability to induce MDSC accumulation.

[Note: Animal experiments will be conducted 2-3 times using 5-10 animals/experiment. BALB/c female mice, 6-20 weeks of age, will be used for these experiments]

- c. Analyze the sera and dissociated tumor tissue from 4T1 and 4T1/IL-1beta inoculated mice, by ELISA, for expression of cytokines (IL-1beta, IL-6, IL-10, VEGF) (9-18 months).

- d. Determine expression of cytokine receptors (IL-1 β , IL-6, IL-10, VEGF) on surface of MDSC from 4T1 and 4T1/IL-1 β inoculated mice by flow cytometry using antibodies to the cytokine receptors (9-18 months).

TASK 5: Determine if macrophages are involved in MDSC accumulation.

[Note: Animal experiments will be conducted 2-3 times using 5 animals/experiment. BALB/c female mice, 6-20 weeks of age, will be used for these experiments]

- a. Perform proliferation assay (Task 2b) to evaluate if bone-marrow-derived macrophages (BMDM) produce arginase and NO (18-24 months).

TASK 6: Determine if blocking inflammatory responses prevents, delays, or minimizes tumor progression.

[Note: Animal experiments will be conducted 3-5 times using 10 animals/experiment. BALB/c female mice, 6-20 weeks of age, will be used for these experiments]

- b. Administer Celecoxib to BALB/c mice continuously, starting at time of 4T1 tumor inoculation. Monitor tumor onset, growth, survival, metastatic load, and MDSC accumulation. Measure intratumor and serum production of inflammatory cytokines (Task 4a), by ELISA (24-36 months).

TASK 7: Determine if reducing inflammatory responses minimizes or delays tumor progression and immune suppression.

[Note: Animal experiments will be conducted 3-5 times using 10 animals/experiment. IL-1R-deficient BALB/c female mice, 6-20 weeks of age, will be used for these experiments]

- a. Inoculate IL-1R-deficient BALB/c mice with 4T1 mammary carcinoma cells. Monitor tumor onset, growth, metastatic load and the accumulation of MDSC (12-24 months).

TASK 8: Determine whether hematopoietic or non-hematopoietic cells respond to tumor-derived IL-1 β .

[Note: Animal experiments will be conducted 2-3 times using 10-20 animals/experiment. BALB/c female mice and IL-1R-deficient BALB/c female mice, 6-8 weeks of age, will be used for these experiments]

- b. Create chimeric mice using BALB/c and IL-1R-deficient mice. Reconstitute BALB/c mice with bone marrow from IL-1R-deficient mice and IL-1R-deficient mice with bone marrow from BALB/c mice. Inoculate chimeric mice with 4T1 mammary carcinoma cells. Monitor tumor growth, metastatic load, and MDSC accumulation (12-24 months).

TASK 9: Determine if interleukin 6 (IL-6) is a mediator of the tumor promoting effects IL-1 β .

[Note: Animal experiments will be conducted 3-5 times using 10 animals/experiment. BALB/c female mice and IL-1R-deficient BALB/c female mice, 6-20 weeks of age, will be used for these experiments]

- d. Create stable 4T1 cell lines which secrete IL-6(4T1/IL-6), by transfection with an IL-6 plasmid containing IL-6 gene (12-24 months).
- e. Quantify IL-6 secretion of 4T1 stable transfectants by ELISA (12-24 months).
- f. Inoculate BALB/c and IL-1R-deficient mice with 4T1/IL-6 tumor cells. Monitor tumor onset, growth, metastatic load, and MDSC accumulation (12-24 months).

TASK 10: Determine if inflammation-induced MDSC enhance macrophage type-2 responses.

[Note: Animal experiments will be conducted 3-5 times using 5 animals/experiment. BALB/c female mice, 6-20 weeks of age, will be used for these experiments]

- b. Co-culture LPS and IFN γ -activated peritoneal macrophages from BALB/c mice with MDSC from BALB/c mice inoculated with either 4T1 or 4T1/IL-1 β mammary carcinoma cells. Quantify IL-12 and IL-10 production by ELISA (24-30 months).

TASK 11: Determine if cross-talk between MDSC and macrophages is TLR4-dependent.

[Note: Animal experiments will be conducted 3-5 times using 5 animals/experiment. BALB/c female mice and TLR4-deficient BALB/c female mice, 6-20 weeks of age, will be used for these experiments]

- a. Determine the surface expression of TLR4, CD14 and CD11b on MDSC from BALB/c mice and TLR4-deficient mice inoculated with 4T1 or 4T1/IL-1 β carcinoma cells (24-30 months).
- b. Quantify the expression of activated NF κ B by Western Blot (24-36 months).
- b. Co-culture peritoneal macrophages from BALB/c mice with MDSC from TLR4-deficient BALB/c mice inoculated with 4T1 mammary carcinoma cells. Co-culture peritoneal macrophages from TLR4-deficient BALB/c mice with MDSC from BALB/c mice inoculated with 4T1 mammary carcinoma cells. Quantify IL-12 and IL-10 production by ELISA (24-36 months).

[Note: For all tasks, statistics will be used to analyze all data.]

APPENDIX B

Abstract for poster presented at:

- Mechanism and Therapeutic reversal of Immune Suppression in Cancer meeting in January 2007 in Clearwater, FL.

REDUCING INFLAMMATION IN BREAST CARCINOMA LIMITS THE DEVELOPMENT AND ACCUMULATION OF A MORE SUPPRESSIVE SUBPOPULATION OF MYELOID-DERIVED SUPPRESSOR CELLS

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Epidemiological and experimental evidence supports the concept that chronic inflammation promotes the development and progression of cancers; however, the mechanisms underlying this relationship are poorly understood. We have demonstrated previously that secretion of the pro-inflammatory cytokine interleukin 1 β (IL-1 β) from 4T1 mammary carcinoma cells (4T1/IL-1 β) promotes tumor progression and decreases the survival of tumor-bearing animals. Tumor progression in many patients and experimental animals with cancer is frequently associated with the expansion of a population of myeloid cells, termed Myeloid-derived Suppressor Cells (MDSC). These cells have potent immunosuppressive activity and inhibit both innate and adaptive immunity by inhibiting T cell activation, NK cell cytotoxicity, and reducing the number of mature dendritic cells. Chronic inflammation at the tumor site enhances and alters the quality of MDSC by inducing a phenotypically and functionally distinct population of MDSC, which are more potent suppressors of CD8⁺ T cells. Here we demonstrate that inflammation-induced MDSC can be activated by LPS and IFN γ to secrete elevated levels of IL-10, thus skewing immunity towards a type-2 response, suggesting another mechanism by which MDSC promote immune suppression. Because inflammation enhances MDSC accumulation and suppression, we examined the effect of MDSC induction and accumulation in mice deficient for the IL-1 receptor (IL-1R^{-/-}). MDSC expansion is delayed in IL-1R^{-/-} tumor-bearing mice, and is accompanied by a reduction in tumor growth, and metastasis, and an increase in survival. These data suggest that limiting tumor-associated inflammation may delay the onset of systemic immune suppression that accompanies breast cancer progression and may enhance the efficacy of current and future therapies. (DOD W81XWH-05-1-0276, R01 CA84232, R01CA115880 and R01 CA52527)

APPENDIX C

Abstract for poster presented at:

- Keystone Symposia Meeting on Inflammation and Cancer in February 2007 in Santa Fe, NM. The poster presented at the Keystone Symposia Meeting was awarded an NCI Scholar Travel Award for \$1000.

Reducing Inflammation Limits the Induction and Development of a Potent Subpopulation of Tumor-associated Myeloid-derived Suppressor Cells

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Epidemiological and experimental evidence supports the concept that chronic inflammation promotes the development and progression of cancers; however, the mechanisms underlying this relationship are poorly understood. We have demonstrated previously that secretion of the pro-inflammatory cytokine interleukin 1 β (IL-1 β) from 4T1 mammary carcinoma cells (4T1/IL-1 β) promotes tumor progression. Tumor progression in many patients and experimental animals is associated with the expansion of a population of myeloid cells, termed Myeloid-derived Suppressor Cells (MDSC). These cells have potent immunosuppressive activity and inhibit both innate and adaptive immunity by inhibiting T cell activation, NK cell cytotoxicity, and reducing the number of mature dendritic cells. Chronic inflammation at the tumor site enhances and alters the quality of MDSC by inducing a phenotypically and functionally distinct population of MDSC, which are more potent suppressors of CD8⁺ T cells. Here we demonstrate that inflammation-induced MDSC can be activated to secrete elevated levels of IL-10, thus skewing immunity towards a type-2 response, suggesting another mechanism by which MDSC promote immune suppression. Because inflammation enhances MDSC accumulation and suppression, we examined the effect of MDSC induction and accumulation in mice deficient for the IL-1 receptor (IL-1R^{-/-}). MDSC expansion is delayed in IL-1R^{-/-} tumor-bearing mice, and is accompanied by a reduction in tumor growth, and metastasis, and an increase in survival. These data suggest that limiting tumor-associated inflammation may delay the onset of systemic immune suppression that accompanies breast cancer progression and may enhance the efficacy of current and future therapies. (DOD W81XWH-05-1-0276, R01 CA84232, R01CA115880 and R01 CA52527)

APPENDIX D

Abstract for published paper:

- Bunt, S.K., et al. "Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression". J Immunol. 2006 Jan 1;176(1): 284-90.

Epidemiological and experimental observations support the hypothesis that chronic inflammation contributes to cancer development and progression; however the mechanisms underlying the relationship between inflammation and cancer are poorly understood. To study these mechanisms we have transfected the mouse 4T1 mammary carcinoma with the pro-inflammatory cytokine, IL-1 β , to produce a chronic inflammatory microenvironment at the tumor site. Mice with 4T1/IL-1 β tumors have a decreased survival time, and elevated levels of immature splenic Gr1⁺CD11b⁺ myeloid-derived cells. These cells, termed myeloid suppressor cells (MSC), are present in many patients with cancer and inhibit the activation of CD4⁺ and CD8⁺ T lymphocytes. 4T1/IL-1 β -induced MSC do not express the IL-1 receptor, suggesting that the cytokine does not directly activate MSC. Neither T, B, nor NKT cells are involved in the IL-1 β -induced increase of MSC because RAG2^{-/-} and nude mice with 4T1/IL-1 β tumors also have elevated MSC levels. MSC levels remain elevated in mice inoculated with 4T1/IL-1 β even after the primary tumor is surgically removed, indicating that the IL-1 β effect is long-lived. Collectively, these findings suggest that inflammation promotes malignancy via pro-inflammatory cytokines, such as IL-1 β , that enhance immune suppression through the induction of MSC, thereby counteracting immune surveillance and allowing the outgrowth and proliferation of malignant cells.