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Inflammation Induces Myeloid-Derived Suppressor Cells that Facilitate Tumor Progression[1]

Stephanie K. Bunt, Pratima Sinha, Virginia K. Clements, Jeff Leips, and Suzanne Ostrand-Rosenberg[2]

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 proinflammatory cytokine IL-1β to produce a chronic inflammatory microenvironment at the tumor site. Mice with 4T1/IL-1β tumors have a decreased survival time and elevated levels of immature splenic Gr1$^+$CD11b$^+$ myeloid-derived cells. These myeloid suppressor cells (MSC) are present in many patients with cancer and inhibit the activation of CD4$^+$ and CD8$^+$ T lymphocytes. 4T1/IL-1β-induced MSC do not express the IL-1R, suggesting that the cytokine does not directly activate MSC. Neither T or B cells nor NKT cells are involved in the IL-1β-induced increase of MSC because RAG2$^{-/-}$ mice and nude mice with 4T1/IL-1β tumors also have elevated MSC levels. MSC levels remain elevated in mice inoculated with 4T1/IL-1β even after the primary tumor is surgically removed, indicating that the IL-1β effect is long lived. Collectively, these findings suggest that inflammation promotes malignancy via proinflammatory cytokines, such as IL-1β, which enhance immune suppression through the induction of MSC, thereby counteracting immune surveillance and allowing the outgrowth and proliferation of malignant cells. *The Journal of Immunology,* **2006, 176: 284–290.**

The concept that chronic inflammation promotes tumor progression was originally proposed by Virchow in the late 1800s (1). Epidemiological studies offer strong support for this concept. For example, the risk of some organ-specific cancers, particularly colorectal cancers, is significantly higher in individuals with chronic inflammation of the target organ (1–4). Experimental findings also support the observation that inflammation facilitates malignant growth. Inflammatory components have been shown 1) to induce DNA damage, which contributes to genetic instability and transformed cell proliferation (1); 2) to promote angiogenesis and thereby enhance tumor growth and invasiveness (4); and 3) to impair myelopoiesis and hemopoiesis, which cause immune dysfunction and inhibit 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.

To study these mechanisms, we have developed an experimental system in which tumors constitutively express the proinflammatory cytokine, IL-1β, resulting in an inflammatory microenvironment at the tumor site. We have used IL-1β because it is a key cytokine in mediating an inflammatory response (8) and has been previously shown to promote primary tumor growth (8–11) and to enhance metastatic disease (11–13). The spontaneously metastatic BALB/c-derived 4T1 mammary carcinoma (14–16) 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 proinflammatory microenvironment enhances tumor progression, our results suggest a novel mechanism by which inflammation facilitates tumor growth. We find that IL-1β-producing tumor cells are potent inducers of a population of immature Gr1$^+$CD11b$^+$ myeloid-derived cells called myeloid suppressor cells (MSC)[3]. Elevated levels of MSC are frequently found in many cancer patients and cause a global and profound immune suppression (5, 6, 17–20). The IL-1β-induced MSC described in this study are effective suppressors of CD4$^+$ and CD8$^+$ T lymphocytes. The finding that IL-1β upregulates MSC 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-1β and other proinflammatory mediators. The persistence of these mediators causes the accumulation and retention of MSC. The MSC, in turn, initiate and maintain an immune suppressive state that block immune surveillance, thereby facilitating the survival and proliferation of transformed cells.

Materials and Methods

Mice

BALB/c and D011.10 transgenic mice (21) (both from The Jackson Laboratory), RAG2$^{-/-}$ mice (Taconic Farms), and clone 4 transgenic mice (22, 23) were bred in the University of Maryland Baltimore County (UMBC) animal facility (Baltimore, MD). BALB/c nude mice were obtained

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[2] Address correspondence and reprint requests to Dr. Suzanne Ostrand-Rosenberg, Department of Biological Sciences, University of Maryland Baltimore County, 1000 Hilltop Circle, Baltimore, MD 21250. E-mail address: srosenbe@umbc.edu

[3] Abbreviations used in this paper: MSC, myeloid suppressor cell; ROS, reactive oxygen species; hIL-1, human IL-1; D-NMMA, N^G-monomethyl-D-arginine; L-NMMA, N^G-monomethyl-L-arginine; Nor-NOHA, N^W-hydroxy-nor-L-arginine; ssIL-1β, signal sequence IL-1β; HA, influenza hemagglutinin.

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from the National Cancer Institute (Frederick, MD). All animal procedures have been approved by the UMBC Institutional Animal Care and Use Committee.

Cell lines

The 4T1 mammary carcinoma cell line was maintained as described (24). The LBRM TG6 mouse T cell lymphoma cell line (American Type Culture Collection) was cultured in IMDM (BioSource International) supplemented with 10% fetal clone 1 (FBP; HyClone Laboratories), 1% glutamax (Invitrogen Life Technologies), 1% gentamicin sulfate (BioSource International), 1% antibiotic-antimycotic solution (BioSource International), and 0.02 mM 2-ME (J. T. Baker Chemical). The 293T cells were maintained as described (25). 4T1 transductants were grown in the same medium as the parental cells supplemented with 400 or 600 μg/ml G418 (Sigma-Aldrich) for 4T1/RV and 4T1/IL-1β cells, respectively. 4T1/IL-1β cells cultured in vivo or grown as solid tumors in mice secreted similar amounts of IL-1β as measured by ELISA.

Reagents and Abs

$OVA_{323-339}$ and $HA_{518-526}$ peptides were synthesized in the Biopolymer Core Facility at the University of Maryland Medical School (Baltimore, MD). mAbs Gr1-PE, CD11b-FITC, CD3-FITC, CD4-PE, CD8-PE, B220-PE, CD11c-PE, I-A/I-E-FITC, D^d-FITC, CD86-PE, CD80-FITC, CD40-PE, CD44-FITC, CD14-FITC, CD23-FITC, CD31-FITC, CD34-FITC, CD16/CD32-FITC, rat IgG2α-PE isotype control, and rat IgG2α-FITC isotype control were from BD Pharmingen. F4/80-FITC was from Caltag Laboratories; PDL2-PE from eBioscience; CD83-FITC from BioCarta; and DEC205-FITC from Cedarlane Laboratories. Rat anti-mouse Gr-1 (clone RB6-8C5) and goat anti-rat IgG microbeads for MSC sorting were from BD Pharmingen and Miltenyi Biotec, respectively. DCFDA (dichlorodihydrofluorescein diacetate) for ROS detection was from Molecular Probes.

Plasmids, retroviral vectors, and transductions

The pLXSN/ssIL-1β plasmid containing the signal sequence of the IL-1ra fused to the mature hIL-1β gene (26) was used to transduce 4T1 cells to create the 4T1/IL-1β cell line. The 4T1/RV cell line, a retroviral vector control, was created by transduction of 4T1 parental cells with the pLNCX2/*Avr*II plasmid (Clontech Laboratories). 4T1/RV cells were identical with 4T1 parental cells in their phenotype and in vivo growth; hence, the results for these lines are pooled and labeled as 4T1. Retroviral production and transduction were as previously described (25). Briefly, 293T cells were plated in six-well plates at 1×10^6 cells/well and transfected by the $CaCl_2$ method with the group-specific Ag, polymerase, and envelope genes, and pLXSN/ssIL-1β plasmid. After 48 h, viral supernatants were collected. To create 4T1/IL-1β cells, 4T1 cells were plated in six-well plates at 1×10^5 cells/well 15–18 h before the addition of polybrene (4 μg/ml) and viral ssIL-1β supernatants (500 μl/well). Cells were incubated at 37°C for 5–6 h, washed with sterile PBS, and cultured an additional 48 h before G418 selection was added.

Tumor inoculations, surgery, and metastasis (clonogenic) assay

Female 6- to 10-wk-old BALB/c mice were inoculated on day 0 in the mammary fat pad with 7×10^3 tumor cells in 50 μl of PBS. Primary tumors were measured as described (14). Survival time indicates the day after tumor inoculation when mice are moribund and are sacrificed. For metastasis assays, lungs were harvested at the indicated times and metastatic disease was quantified using the clonogenic assay by plating cells in 6-thioguanine supplemented medium (14).

Splenic MSC

$Gr1^+CD11b^+$ MSC were isolated from the spleen of tumor-bearing mice using MACS (Miltenyi Scientific) as described (27).

T cell proliferation assays

The $CD4^+$ and $CD8^+$ T cell proliferation assays were performed as described (27). Briefly, DO11.10 or clone 4 splenocytes were cocultured with OVA or influenza hemagglutinin (HA) peptide, respectively, and irradiated MACS purified splenic MSC from BALB/c mice inoculated with 4T1 or 4T1/IL-1β cells. Cultures were pulsed with [^{3}H]thymidine on day 3 and cells were harvested 24 h later. For the arginase and NO assays, the NO inhibitor N^G-monomethyl-L-arginine (L-NMMA), the enantiomer N^G-monomethyl-D-arginine (D-NMMA), or the arginase inhibitor N^W-hydroxy-nor-L-arginine (nor-NOHA) (Calbiochem) was added. Data are expressed as the average cpm of triplicate wells.

Arginase assay

Arginase was quantified by measuring the production of urea as described (27).

Flow cytometry

Cells were labeled for direct immunofluorescence as described (14) and analyzed using an Epics XL flow cytometer using the Expo32 ADC software (Beckman Coulter).

IL-1β Fluorokine assay

The IL-1R Fluorokine assay was performed according to the manufacturer's instructions (R&D Systems). Briefly, 1×10^5 purified MSC from tumor-bearing mice were incubated with either 10 μl of rIL-1β-biotin or negative control biotinylated soybean protein for 1 h at 4°C and then incubated with 10 μl of avidin-FITC for 30 min at 4°C in the dark. Cells were washed twice with 1XRDF1 buffer and analyzed by flow cytometry for IL-1R expression.

Reactive oxygen species (ROS)

ROS production was measured by staining with DCFDA as described (27). To block ROS production, MSC were incubated with nor-NOHA at 37°C for 10 min before DCFDA addition. Cells were washed with sterile PBS and assayed by flow cytometry (27).

IL-1β ELISA

Supernatants from 5×10^5 cells cultured in 3 ml of media for 24 h were frozen at −80°C until assayed using a hIL-1β ELISA kit according to the manufacturer's directions (Endogen). Plates were read at 420 nm on a Bio-Tek 311 microplate reader and quantified using a standard curve. Data are the mean ± SD of triplicate wells.

Statistical analyses

Student's two-tailed *t* test for unequal variance was performed for all figures using Microsoft Excel 2003. Differences in tumor diameter in the inoculated mice were tested using a repeated measures profile ANOVA (28, 29) using the procedure in SAS v.9.1 (SAS Institute) (see Fig. 4*A*).

Results

Transduced 4T1 cells secrete mature functional IL-1β

To examine the role of inflammation and IL-1β on the development and progression of breast cancer, 4T1 mammary carcinoma cells were transduced with a construct containing the mature form of hIL-1β fused to the signal sequence from the IL-1ra (ssIL-1β construct; 4T1/IL-1β cells), allowing active hIL-1β to be secreted extracellularly (26). 4T1/IL-1β cells secreted 1000 pg/ml/5×10^5/24 h as quantified by ELISA. As a control for transduction with a retroviral vector, 4T1 cells were transduced with an empty vector (4T1/RV). To evaluate whether the transduction or the secretion of IL-1β altered growth rate, 4T1 and 4T1/IL-1β cells were plated at equivalent densities and counted every 24 h for 3 days. Both lines had the same growth kinetics, indicating that IL-1β transduction did not affect the in vitro growth rate. To further characterize the 4T1/IL-1β cells, MHC class I and class II surface expression was analyzed by flow cytometry. Transduction of 4T1 cells with ssIL-1β did not alter the amount of H-2D^d, and neither 4T1 nor 4T1/IL-1β cells express MHC class II. MHC class I and class II phenotype and in vivo growth of 4T1 and 4T1/RV cells were identical, so results of these controls were pooled and are labeled as 4T1 in these experiments.

IL-1β enhances primary tumor growth and decreases survival time

To examine the role of IL-1β on primary tumor onset and growth, 4T1 and 4T1/IL-1β cells were injected into BALB/c mice on day 0. For both groups, primary tumors were measured every 3–5 days and were palpable by day 10. 4T1/IL-1β primary tumors grew significantly faster than parental 4T1 primary tumors ($p < 0.01$). To determine whether IL-1β affects survival, 4T1- and 4T1/IL-1β-injected mice were followed until they were moribund. 4T1/

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IL-1β-injected mice had significantly reduced survival time ($p < 0.01$; 26–44 days with a mean of 37 days; $n = 13$), compared with 4T1-injected mice (33–60 days with a mean of 46 days; $n = 15$). These data indicate that IL-1β increases the primary tumor growth rate and suggest that the difference in survival may be due to enhanced tumor progression.

IL-1β leads to an earlier and faster accumulation of MSC

Because MSC frequently accumulate in tumor-bearing individuals and can reduce survival time, we examined MSC levels in mice with 4T1/IL-1β vs 4T1 tumors. BALB/c mice were inoculated with 4T1 or 4T1/IL-1β cells and the percentage of splenic MSC was measured as a function of time after tumor inoculation. Mice with 4T1/IL-1β tumors have higher levels of MSC than mice with 4T1 tumors (Fig. 1*A*). Because MSC accumulation is known to increase with increasing tumor burden, mice with 4T1/IL-1β tumors may have more MSC because their primary tumors are larger. To address this question, the percentage of splenic MSC was plotted relative to tumor diameter. As seen in Fig. 1*B*, MSC levels are consistently higher in 4T1/IL-1β vs 4T1 mice regardless of the size of their primary tumors. Additionally, increased levels of MSC in other organs, such as the lungs, were also elevated in 4T1/IL-1β tumor-bearing mice, compared with 4T1 tumor-bearing mice (data not shown), suggesting that IL-1β secretion by the primary tumor systemically enhances MSC accumulation. These data indicate that IL-1β induces the accumulation of elevated levels of MSC.

Mice with IL-1β tumors do not have more lung metastases

To determine whether IL-1β affects the progression of lung metastases, BALB/c mice were injected with 4T1 or 4T1/IL-1β cells and lungs were harvested 5 wk later or when the mice were moribund. The number of lung metastases was quantified using the clonogenic assay (14). Both 4T1- and 4T1/IL-1β-injected mice developed lung metastases (Fig. 2). There is no difference in the number of lung metastases between 4T1- and 4T1/IL-1β-injected mice at week 5; however, when the mice become moribund, 4T1/IL-1β-injected mice have fewer lung metastases ($p < 0.05$), compared with 4T1-injected mice. These results indicate that IL-1β does not increase the number of metastatic cells in the lungs and suggest that IL-1β is not reducing survival time by increasing metastatic disease.

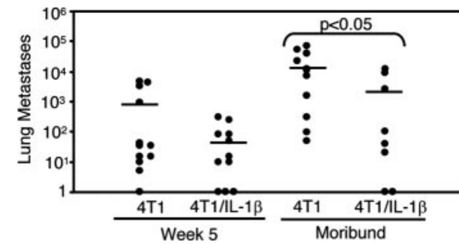

FIGURE 2. IL-1β does not alter the number of lung metastases. BALB/c mice were inoculated with 4T1 or 4T1/IL-1β tumor cells as in Fig. 1 and their lungs harvested at the indicated times. Metastatic cells were quantified using the clonogenic assay. Individual mice (●) are represented. Horizontal bars indicate the mean number of metastases. Data are pooled from four independent experiments.

4T1 and 4T1/IL-1β cells do not express the IL-1R type I

IL-1β may be mediating its effect by acting directly on 4T1 tumor cells or on host cells. To determine whether 4T1/IL-1β cells had the potential to respond to IL-1β, 4T1 and 4T1/IL-1β cells were labeled with fluorescent IL-1β protein and analyzed by flow cytometry. LBRM TG6 cells, which express the IL-1R type I, were used as positive control cells. Neither 4T1 nor 4T1/IL-1β cells express detectable IL-1R type I indicating that these cells cannot respond to IL-1β (Fig. 3). These results implicate host cells as the responders to the tumor-secreted IL-1β.

IL-1β-induced tumor growth is not dependent on host T cells, B cells, or NKT cells

Immunodeficient and knockout mice were used to determine whether host immune cells are required for the IL-1β effect. Wild-type BALB/c mice and $RAG2^{-/-}$ BALB/c mice, which are deficient for T cells, B cells, and NKT cells, were inoculated with 4T1 or 4T1/IL-1β cells and primary tumor growth was measured every 3–5 days (Fig. 4*A*). 4T1/IL-1β primary tumors grew significantly more rapidly in both BALB/c and $RAG2^{-/-}$ mice ($p < 0.01$) and mice with 4T1/IL-1β tumors became moribund sooner than mice with 4T1 tumors. Therefore the increased tumorigenicity of 4T1/IL-1β tumor cells is not dependent on host lymphoid cells.

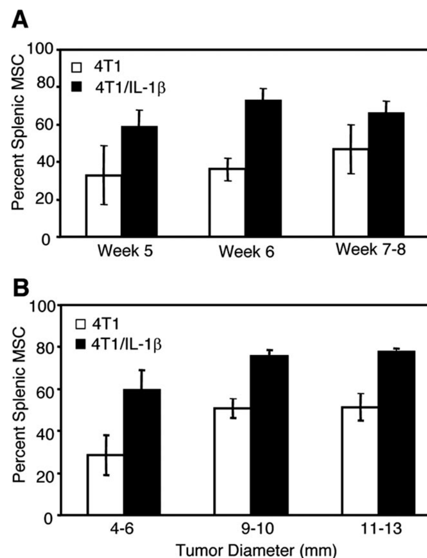

FIGURE 1. IL-1β increases the accumulation of splenic MSC. BALB/c mice were inoculated with 7000 4T1 or 4T1/IL-1β tumor cells in the abdominal mammary gland on day 0. Spleens were harvested at the indicated time points (*A*) or tumor diameters (*B*), and $Gr1^+CD11b^+$ MSC were quantified by flow cytometry. Values are the average ± SD of 5–18 and 5–19 mice per group for *A* and *B*, respectively. Data are pooled from five experiments. 4T1 and 4T1/IL-1β groups are statistically significantly different ($p < 0.05$) from each other for all time periods and tumor diameters.

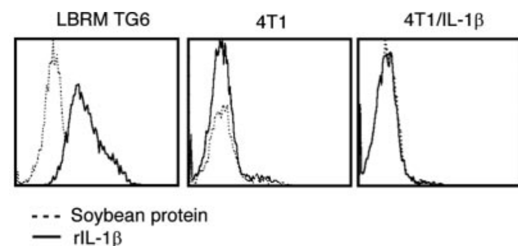

FIGURE 3. 4T1 and 4T1/IL-1β tumor cells do not express the IL-1R type I. 4T1 and 4T1/IL-1β tumor cells were stained with avidin-FITC and either biotinylated rIL-1β protein (solid line histogram) or biotinylated soybean protein (dashed histogram) and analyzed by flow cytometry. Soybean protein expression levels were identical on 4T1 and 4T1/IL-1β tumor cells and are shown as a single peak. Data are from one of three independent experiments.

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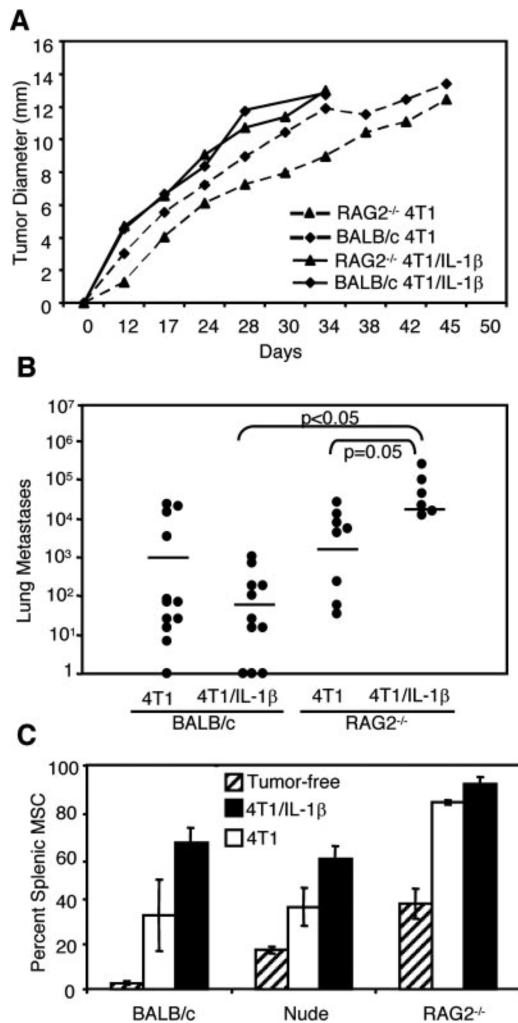

FIGURE 4. T cells, B cells, and NKT cells are not required for the IL-1β-induced accumulation of MSC. BALB/c and RAG2$^{-/-}$ mice were inoculated with 4T1 or 4T1/IL-1β tumor cells and monitored for primary tumor growth and survival. *A*, Primary tumor diameter plotted as a function of time after tumor inoculation. Each curve represents an average of 3–8 mice. Growth of primary 4T1/IL-1β tumors is more rapid than growth of primary 4T1 tumors in both strains ($p < 0.01$). *B*, Lung metastases were quantified from the mice in *A* using the clonogenic assay. Individual mice are represented (●) and horizontal bars indicate the mean number of lung metastasis. *C*, BALB/c, nude, and RAG2$^{-/-}$ mice bearing 4T1 or 4T1/IL-1β tumors were euthanized at week 5 and the percentage of splenic Gr1$^+$CD11b$^+$ MSC was quantified by flow cytometry. All columns are statistically significantly different ($p < 0.01$) from other columns in the same strain and in other strains (including RAG2$^{-/-}$ with 4T1 vs RAG2$^{-/-}$ with 4T1/IL-1β) except BALB/c with 4T1 vs nude with 4T1 and BALB/c with 4T1/IL-1β vs nude with 4T1/IL-1β.

To determine whether lymphoid cells influence metastatic disease, wild-type BALB/c and RAG2$^{-/-}$ mice were inoculated with 4T1 and 4T1/IL-1β cells on day 0 and their lungs were harvested between days 25 and 35. Metastases were quantified using the clonogenic assay. IL-1β expression did not affect the average number of lung metastases in BALB/c mice; however, IL-1β induced a significant increase ($p = 0.05$) in the number of lung metastases in RAG2$^{-/-}$ mice (Fig. 4*B*). RAG2$^{-/-}$ mice with 4T1/IL-1β tumors have a statistically higher number of metastases, compared with BALB/c mice with 4T1/IL-1β tumors. Therefore, the presence of B cell, T cell, and NKT cells naturally limits the increase in lung metastases by IL-1β.

Enhanced splenic MSC accumulation by IL-1β does not require T cells, B cells, or NKT cells

To determine whether host lymphoid cells are required for the IL-1β-induced increase in MSC, BALB/c, T cell-deficient nude, and RAG2$^{-/-}$ mice were inoculated with 4T1 or 4T1/IL-1β cells and their splenocytes harvested on days 25–35 and assayed by flow cytometry for Gr1$^+$CD11b$^+$ cells. All strains with 4T1/IL-1β tumors had significantly more splenic MSC than mice with 4T1 tumors (including RAG2$^{-/-}$ with 4T1 vs RAG2$^{-/-}$ with 4T1/IL-1β; $p < 0.01$) (Fig. 4*C*), confirming the findings of Fig. 1*A* and demonstrating that the ability of IL-1β to elevate MSC does not require lymphoid cells. Interestingly, BALB/c and nude mice with 4T1/IL-1β tumors had similar MSC levels, whereas RAG2$^{-/-}$ mice with 4T1/IL-1β tumors had significantly higher numbers of MSC. Therefore, although lymphoid cells are not required for the IL-1β-induced effect, deletion of $\gamma\delta$ T cells and/or B cells appears to facilitate an even larger accumulation of MSC.

IL-1β-induced MSC are phenotypically distinct from 4T1 MSC

IL-1β may be augmenting an existing population of MSC, or it may be inducing a novel population. To distinguish between these two possibilities, BALB/c mice were injected on day 0 with 4T1 or 4T1/IL-1β cells, and splenic MSC were sorted by MACS for Gr1$^+$ cells on day 35. Sorted cells were >90% Gr1$^+$CD11b$^+$ as measured by flow cytometry (Fig. 5*A*). Fig. 5*B* shows the phenotype of the purified Gr1$^+$CD11b$^+$ cells for various cell surface markers that have been used to characterize MSC. 4T1/IL-1β MSC expressed higher levels of CD8, CD80, CD83, and CD14 and lower levels of CD44 and B220 relative to 4T1 MSC. To evaluate whether these differences were a result of direct action of IL-1β the MSC were stained for the IL-1R type I. Both 4T1- and 4T1/IL-1β-induced MSC were negative for the IL-1R type I indicating that MSC cannot respond directly to IL-1β. Therefore, 4T1 and 4T1/IL-1β MSC share some common markers; however, they also have phenotypic differences.

IL-1β-induced MSC suppress CD4$^+$ and CD8$^+$ T cells

Previous data demonstrate that 4T1-induced MSC suppress both CD4$^+$ and CD8$^+$ T cells via an arginase-dependent mechanism (27). Because IL-1β-induced MSC phenotypically differ from 4T1-induced MSC, it is possible that the 4T1/IL-1β MSC are also functionally distinct. To determine whether 4T1/IL-1β MSC have different functional activity from 4T1 MSC, BALB/c mice were injected with 4T1 and 4T1/IL-1β cells on day 0, and splenic MSC were harvested and purified by MACS on day 35, and tested for their ability to inhibit T cell proliferation. To test for suppressive activity against CD4$^+$ T cells, I-A^d-restricted OVA$_{323–339}$ peptide-specific DO11.10 transgenic splenocytes were stimulated with OVA$_{323–339}$ peptide in the presence or absence of purified MSC from 4T1- or 4T1/IL-1β-inoculated mice (Fig. 6*A*). Both 4T1 and 4T1/IL-1β MSC suppressed D011.10 proliferation by >95% as measured by [^{3}H]thymidine uptake. To identify the molecule mediating this suppression, L-NMMA or nor-NOHA, inhibitors of NO and arginase, respectively, were added to the D011.10 cultures. Nor-NOHA treatment partially reversed suppression by both 4T1 and 4T1/IL-1β MSC, whereas L-NMMA treatment had no effect. Arginase levels in 4T1- and 4T1/IL-1β-induced MSC were equivalent as measured enzymatically (data not shown). Therefore, 4T1/IL-1β- and 4T1-induced MSC are equally suppressive for CD4$^+$ T cells, and they both at least partially mediate their effect via arginase.

To determine whether 4T1 IL-1β MSC also block CD8$^+$ T cell activation, HA$_{518–526}$ peptide-specific, K^d-restricted clone 4 transgenic splenocytes were stimulated with HA$_{518–526}$ peptide and

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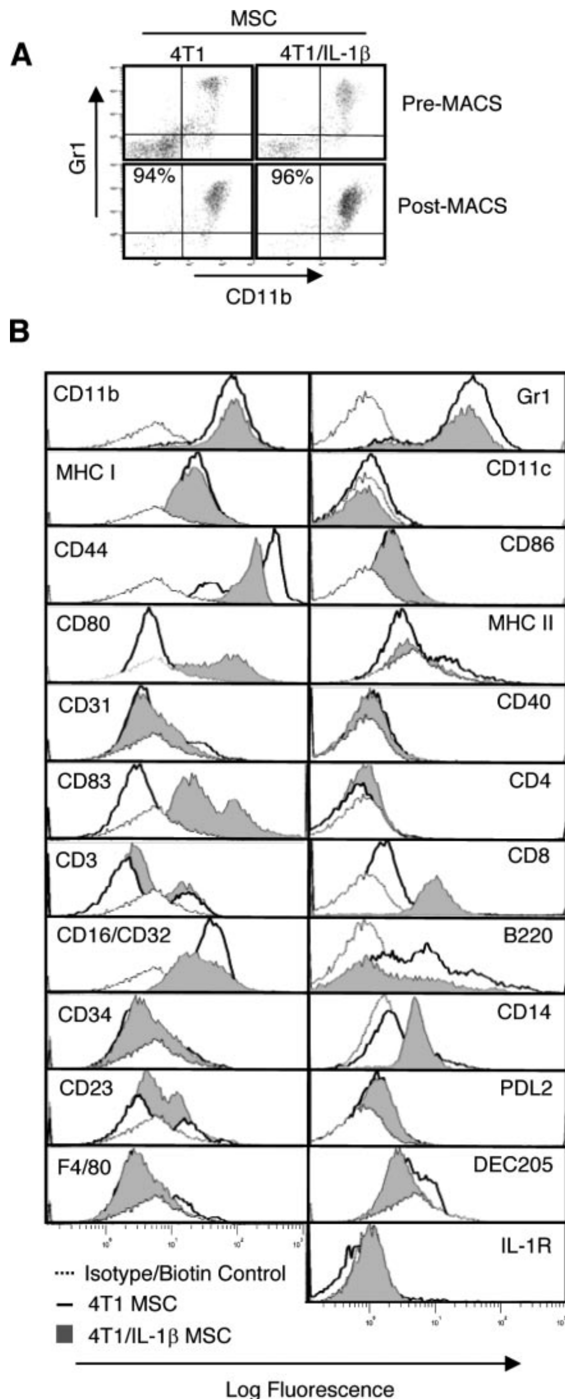

FIGURE 5. MSC from mice with 4T1/IL-1β tumors and MSC from mice with 4T1 tumors have phenotypic differences. BALB/c mice were inoculated with 4T1 or 4T1/IL-1β tumor cells, and spleens were harvested when tumor diameters reached 8–10 mm. *A*, MACS-purified MSC were analyzed by flow cytometry for Gr1 and CD11b expression. *B*, MACS-purified MSC were phenotyped with mAbs to the indicated markers. Dotted histograms are controls (fluorescently labeled soybean protein for IL-1R sample or anti-rat isotype-FITC and anti-rat isotype-PE Abs for all other samples; 4T1 and 4T1/IL-1β MSC had the same fluorescent staining for controls, hence only one peak is shown). Black solid line histograms and gray-filled histograms are MSC from 4T1- and 4T1/IL-1β-inoculated mice, respectively. Data are from one of three independent experiments.

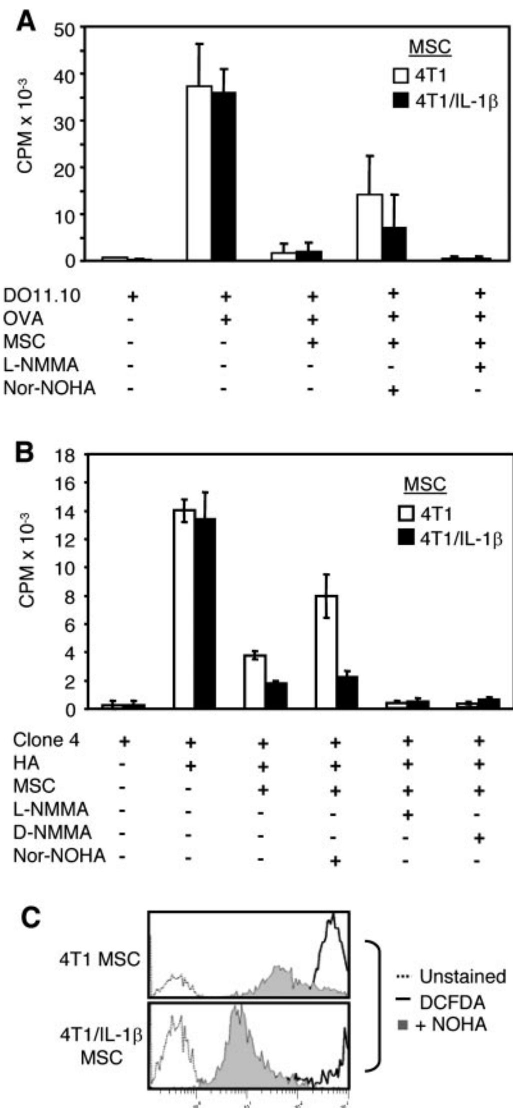

FIGURE 6. MSC from mice with 4T1/IL-1β tumors express increased levels of ROS and suppress CD4$^+$ and CD8$^+$ T cells. CD4$^+$ DO11.10 (*A*) or CD8$^+$ clone 4 (*B*) transgenic splenocytes were stimulated with $OVA_{323-339}$ or $HA_{518-526}$ peptide, respectively, and cocultured in the presence or absence of MACS-purified MSC from mice with 4T1 or 4T1/IL-1β tumors. Inhibitors of arginase (nor-NOHA), NO (L-NMMA), or the inactive enantiomer (D-NMMA) were added to some wells. T cell proliferation was measured as cpm of [^{3}H]thymidine. Data are from one of four independent experiments. *A* and *B*, Nor-NOHA-treated groups are statistically significantly different ($p < 0.05$) from their untreated counterparts. *B*, Nor-NOHA-treated 4T1 MSC are also statistically significantly different ($p < 0.05$) from nor-NOHA-treated 4T1/IL-1β MSC. *C*, MACS-purified MSC from mice with 4T1 or 4T1/IL-1β tumors were incubated with DCFDA in the presence or absence of the arginase inhibitor nor-NOHA and ROS production was measured by flow cytometry. Data are from one of three independent experiments.

cocultured in the presence or absence of purified 4T1 or 4T1/IL-1β MSC. Although both 4T1 and 4T1/IL-1β MSC suppress CD8$^+$ T cell activation, 4T1/IL-1β MSC are more suppressive on a per cell basis than 4T1 MSC (Fig. 6*B*; $p < 0.01$). However, in contrast to results of Fig. 6*A*, addition of the arginase inhibitor nor-NOHA does not reverse the suppression by 4T1/IL-1β MSC. Likewise, no reversal of suppression was observed when the NO inhibitor L-NMMA or its inactive enantiomer D-NMMA was added. Therefore, 4T1/IL-1β MSC are more effective suppressors of CD8$^+$ T cells than are 4T1 MSC, and they suppress by an arginase-independent mechanism.

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IL-1β-induced MSC express higher levels of ROS

To determine whether 4T1 and 4T1/IL-1β MSC differ in other potential effector mechanisms, ROS expression was analyzed in the two populations. MACS purified MSC from 4T1- and 4T1/IL-1β-injected BALB/c mice were stained with DCFDA and analyzed by flow cytometry. Both 4T1 and 4T1/IL-1β MSC have high levels of ROS; however, 4T1/IL-1β MSC contain more ROS (Fig. 6*C*). The addition of nor-NOHA differentially decreases but does not eliminate ROS levels. Therefore, 4T1/IL-1β MSC express more ROS on a per cell basis than 4T1 MSC, and a significant amount of this ROS production is affected by arginase production.

4T1/IL-1β MSC remain elevated even when primary tumor is removed

Surgical excision of primary 4T1 tumor from BALB/c mice leads to a partial regression of splenic MSC (27). To determine whether IL-1β affects this regression, BALB/c mice were inoculated with 4T1 or 4T1/IL-1β cells on day 0, and primary tumors were either left in place (nonsurgery group) or removed (surgery group) on day 25. Both groups were sacrificed on day 35, and the percentages of $Gr1^{+}CD11b^{+}$ splenic MSC were compared. As observed previously (27), surgical excision of the 4T1 tumor caused a significant decrease in MSC (Fig. 7*A*; $p < 0.01$). In contrast, there was no significant decrease in MSC in mice with resected 4T1/IL-1β tumors. These results imply that secretion of IL-1β by the primary tumor either irreversibly enhances MSC accumulation or the presence of residual IL-1β prevents the regression of MSC.

To evaluate the effect of IL-1β on metastatic disease after primary tumor removal, the number of metastatic cells in the lungs of the mice in Fig. 7*A* was quantified using the clonogenic assay. No differences in lung metastases were seen (Fig. 7*B*), in agreement with the results of Fig. 2 that IL-1β does not impact metastatic disease.

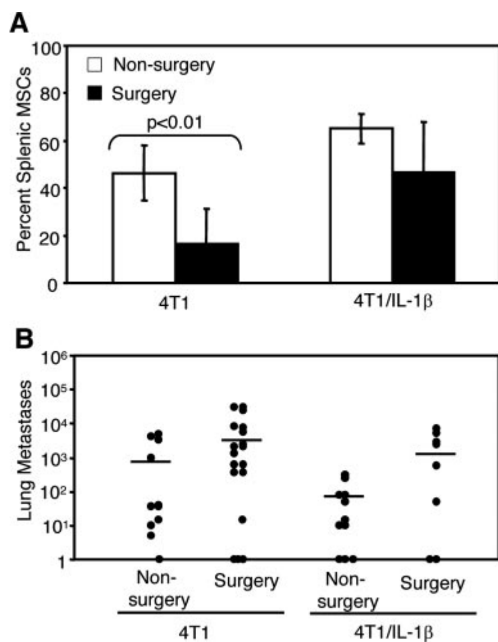

FIGURE 7. MSC do not regress after surgical resection of primary 4T1/IL-1β tumors. BALB/c mice were inoculated with 4T1 or 4T1/IL-1β tumor cells as in Fig. 1 and the primary tumors were either left intact (nonsurgery groups) or surgically excised on day 25 (surgery groups). Lungs and spleens were harvested on day 35. *A*, The percentage of $Gr1^{+}CD11b^{+}$ splenic MSC was quantified by flow cytometry. Values are the average of 7–19 animals per group and are pooled from two independent experiments. *B*, Lung metastases were quantified using the clonogenic assay. Individual mice (●) are represented, and horizontal bars indicate the mean number of metastases.

Discussion

Although the concept that chronic inflammation in the tumor microenvironment leads to increased malignant growth and tumor progression is broadly accepted, the mechanisms behind this interaction have remained elusive. The results presented in this study suggest that the induction of MSC by proinflammatory cytokines directly contributes to tumor progression by inhibiting tumor immunity. Cell-mediated immunity is known to protect against tumor onset and progression (30, 31), and MSC are known to block this process. Therefore, it is likely that the MSC induced during an inflammatory response facilitate tumor growth by inhibiting the activation and/or function of tumor-specific lymphocytes. This novel mechanism is consistent with the findings that MSC are found in many cancer patients, particularly patients who have head and neck cancers, lung carcinomas, or breast cancers (5, 6, 17–19), and that chronic inflammation in some of these malignancies, such as oral, esophageal, and lung cancers, may predispose to or enhance malignant growth (4). Chronic inflammation associated with infectious agents, such as schistosomiasis and *Helicobacter pylori*, is also thought to predispose to malignancy (1, 3, 32, 33), and some of these agents are associated with MSC accumulation (6). Collectively, these published observations plus our reported results support the hypothesis that chronic inflammation facilitates tumor growth by inducing MSC that down-regulate immune surveillance, thereby providing an environment favorable for tumor progression.

Previous studies have examined the effects of IL-1β on tumorigenicity by transfecting/transducing tumor cells with this cytokine. Most investigators find that IL-1β enhances tumor progression (9, 10), although a minority of reports indicates that it inhibits tumor growth (34, 35). These contradictory results are likely due to the different tumor systems studied and/or to the different amounts of IL-1β secreted by the various transfectants (as suggested by Ref. 8). IL-1β acts on many cell types, inducing a variety of effects including increased production of PGE_2, vascular endothelial growth factor, IL-6, and circulating levels of GM-CSF (36). Because all of these molecules have been suggested as inducers of MSC (5, 6), one or more of them may be the vehicle for IL-1β-induced immune suppression.

IL-1β was selected for these experiments because it is a pleiotropic cytokine that produces its effect through a wide array of mechanisms, cell types, and molecules (36). Because neither MSC nor 4T1 tumor cells contain a receptor for IL-1β, it is improbable that IL-1β is the direct inducer of MSC. More likely, IL-1β regulates MSC levels by activating a signal transduction pathway that stimulates cells, which then secrete factors that directly induce MSC accumulation. Many cell types express the IL-1R type I allowing them to respond to the cytokine (8, 36), and hence are potential candidates. Alternatively, IL-1β may regulate MSC accumulation by down-regulating cells and/or factors that normally minimize MSC expansion. This possibility is supported by the findings that tumor-bearing $RAG2^{-/-}$ mice have higher levels of MSC than wild-type BALB/c or nude mice, implying that γδ T cells and/or B cells normally limit the tumor-associated expansion of splenic MSC.

Although MSC are present in many tumor-bearing patients and experimental animals, there is significant phenotypic variation between MSC from different individuals (19, 27, 37, 38), suggesting there are distinct subpopulations of MSC (27). The observation that 4T1/IL-1β-induced MSC differentially express some cell surface markers relative to 4T1-induced MSC further supports the

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concept of MSC heterogeneity. However, this finding also raises the question of whether overexpression of IL-1β induces a distinct MSC subpopulation or whether it expands and modifies the MSC induced by wild-type 4T1 tumor. Compared with 4T1 MSC, 4T1/IL-1β MSC expressed higher levels of CD8, CD80, and CD83, a marker expressed by mature dendritic cells and activated lymphocytes. Additionally, expression of the LPS receptor CD14, usually expressed by macrophages and/or monocytes, was increased on 4T1/IL-1β MSC. 4T1/IL-1β MSC also expressed lower levels of the B cell marker, B220, and the cell adhesion marker, CD44. Although, the 4T1/IL-1β MSC phenotype suggests a partially activated cell, these surface markers do not classify the MSC into any known cellular category. It is interesting that CD14 is elevated only on 4T1/IL-1β MSC, suggesting that IL-1β production stimulates CD14 expression. CD14 has been shown to couple with TLR4, which can also complex with IL-1R, and leads to the activation of NF-κB, cyclooxygenase-2, and PGE_2 (39, 40). This pathway may be one of the mechanisms responsible for IL-1β-induced MSC accumulation and survival and deserves further study.

Although these phenotypic differences exist, it is unclear whether they are biologically significant, because 4T1- and 4T1/IL-1β-induced MSC are both highly immunosuppressive for $CD4^+$ and $CD8^+$ T cells. Experiments with wild-type 4T1 tumor have shown that surgical removal of primary tumor reduces MSC levels (27) and restores host immunocompetence (41). In contrast, mice with resected 4T1/IL-1β tumors retain very high levels of MSC, suggesting that inflammation-induced MSC are likely to be a significant obstacle to effective immunotherapy.

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Disclosures

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