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Macrophages Are More Potent Immune Suppressors Ex Vivo Than Immature Myeloid-Derived Suppressor Cells Induced by Metastatic Murine Mammary Carcinomas

Melisa J. Hamilton,* Momir Bosiljic,* Nancy E. LePard,* Elizabeth C. Halvorsen,* Victor W. Ho,† Judit P. Banáth,* Gerald Krystal,† and Kevin L. Bennewith*

Myeloid-derived suppressor cells (MDSCs) are emerging as potential promoters of metastatic tumor growth, and there is interest in targeting immature MDSCs by inducing their differentiation into more mature myeloid cells. We used all-trans retinoic acid (ATRA) to differentiate MDSCs in mice bearing metastatic 4T1 or 4TO7 murine mammary tumors, and assessed the immune-suppressive mechanisms and potencies of different myeloid cell subpopulations. Metastatic mammary tumors induced the accumulation of distinct populations of immature CD11b+Gr1+Ly6CmidLy6G+ MDSCs (“Gr1+ cells”) and mature CD11b+Gr1–F4/80+ (“F4/80+ cells”) in metastatic target organs. ATRA triggered the differentiation of Gr1+ cells into F4/80+ cells in the lungs and, unexpectedly, enhanced pulmonary metastatic tumor growth. We found that F4/80+Ly6C+Ly6G+ mature macrophages (MΨs) were up to 30-fold more potent immune suppressors than Gr1+ cells on a per-cell basis, which we postulate may contribute to the increased metastatic growth observed with ATRA treatment. F4/80+ cells and Gr1+ cells used different reactive oxygen species (ROS)–mediated mechanisms of immunosuppression ex vivo, with F4/80+ cells producing higher levels of ROS, which is consistent with their superior immunosuppressive abilities. These data highlight the potent immunosuppressive functions of MΨs, reveal that MΨs can suppress T cell responses via ROS production, and suggest that ROS inhibitors may be useful in promoting antitumor immune responses. Our findings also caution against using ATRA to modulate myeloid cell differentiation and function to treat breast cancer metastases in the lung, and support the development of therapeutic strategies to enhance antitumor immunity by targeting myeloid cells as a collective group. The Journal of Immunology, 2014, 192: 000–000.
into mature myeloid cells, including Mφs (10). Tissue Mφs are derived from circulating blood monocytes and exhibit great functional diversity depending on their specific microenvironment. Murine Mφs express F4/80, M-CSFR (CD115), and CD11b (with the exception of alveolar Mφs, which express CD11c instead of CD11b because of their unique lung environment) (11). Although F4/80 is also expressed on some subsets of immature myeloid cells, murine Mφs lack expression of Gr1, and differential Gr1 and F4/80 cell-surface markers are thought to distinguish mature Mφs from other members of the myeloid lineage, including MDSCs (2). Consistent with the role that Mφs play in normal wound healing, tumor-associated Mφs aid tumor progression via multiple mechanisms similar to those used by MDSCs (3, 4), and the presence of extensive tumor-associated Mφ infiltration correlates with poor prognosis and metastasis in a variety of human cancers, including breast, cervix, and bladder cancers (3, 12). Moreover, it has been suggested that Mφs play a critical role in facilitating tumor cell migration out of the primary tumor, entry into blood or lymph vessels, and seeding into distant sites (13). Nevertheless, agents that induce the differentiation of immature MDSCs into mature myeloid cells have been used with some success to restrict tumor progression, both in murine tumor models and the clinic (14–17).

More than 90% of breast cancer–related deaths are associated with secondary tumor metastases (18), highlighting the need for new and effective therapeutic strategies. Although there is emerging evidence that CD11b⁺ myeloid cells accumulate in tissues in response to factors produced by primary mammary tumors (5, 19), the precise identity of these myeloid cells remains unclear, and the efficacy of ATRA treatment in metastatic breast cancer is unknown. We used orthotopic and spontaneous metastatic murine mammary tumor models to test whether inducing the differentiation of MDSCs would inhibit tumor growth and metastasis. We found that metastatic tumors induced the accumulation of CD11b⁺ host cells in metastatic target organs, and that these myeloid cells were largely composed of two phenotypically and functionally distinct populations: CD11b⁺Gr1⁺F4/80⁻ cells and CD11b⁺Gr1⁻F4/80⁺ cells (henceforth termed Gr1⁺ or F4/80⁺ cells, respectively). We found that ATRA treatment of mice bearing metastatic mammary tumors altered the balance of these myeloid cell populations, reducing the number of Gr1⁺ cells and increasing F4/80⁺ cells, and enhanced metastatic tumor growth. Further analysis of these myeloid cell subpopulations revealed that F4/80⁺ cells, and in particular a subgroup of F4/80⁻Ly6C⁻Ly6G⁻ Mφs, were more potent suppressors of immune responses than Gr1⁺ cells isolated from the same tissues. We also found that Mφs exerted their suppressive effects via production of reactive oxygen species (ROS), and that Mφs generated higher levels of ROS than MDSCs. Collectively, these studies caution against strategies that terminally differentiate myeloid cells to treat metastatic breast cancer, increase our understanding of the mechanisms used by different myeloid cell subpopulations to promote tumor progression, and support the development of therapeutic strategies to enhance antitumor immunity by targeting multiple myeloid cell types.

Materials and Methods

Mice and tumor models

Female BALB/c mice (8–12 wk old) were purchased from Taconic (Germantown, NY). BALB/c DO11.10 transgenic mice, which express a TCR specific for chicken OVA peptide (323–339) restricted to I-A⁺ (20), and MMTV-PyMT transgenic mice that spontaneously develop metastatic mammary tumors because of expression of the polyomavirus middle-T Ag driven by a murine mammary tumor viral promoter (21), were purchased from The Jackson Laboratory (Bar Harbor, ME). Metastatic murine mammary carcinoma cell lines 4T1 and 4T07 (kind gifts from Dr. Fred Miller, Karmanos Cancer Institutes, Detroit, MI) were maintained in RPMI 1640 medium + 10% FCS and used within 20 passages. These cell lines were derived from a spontaneous mammary tumor in a BALB/cxc3H mouse (22). Mice were orthotopically inoculated with 10⁴ 4T1 or 10⁴ 4T07 cells in the fourth mammary fat pad and typically sacrificed 3 wk after tumor implantation. Where indicated, mice were s.c. implanted with placebo, or 5- or 10-ng slow-release (21 d) ATRA pellets (Innovative Research of America, Sarasota, FL) at the base of the neck 1 wk after tumor implantation. Mice were housed in the Animal Resource Centre at the BC Cancer Agency Research Centre under specific pathogen-free conditions. All animal experiments were performed in accordance with institutional and Canadian Council on Animal Care guidelines.

Reagents

Neutralizing Abs to cytokines were purchased as follows: IL-4 (eBioscience, San Diego, CA); IL-10 (BD Biosciences, Mississauga, ON); and IL-13, IFN-γ, and TGF-β (R&D Systems, Minneapolis, MN). Reagents were purchased as follows: recombiant human latency-associated peptide (R&D Systems); N6-(1-iminoethyl)-l-lysine, a specific inhibitor of inducible NO synthase (iNOS; Calbiochem, San Diego, CA); carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, an NO scavenger (Cayman Chemicals, Ann Arbor, MI); soluble anti-cD3 and anti-cD28 (eBioscience); and OVA peptides (257–264 and 323–339; GenScript, Piscataway, NJ). (S)-2-boronoethyl)-l-cysteine, a competitive inhibitor of arginase (Arg) 1 and 2 that does not inhibit iNOS, was a kind gift from Dr. Jean-Luc Boucher (Université Paris Descartes, Paris, France). Unless otherwise stated, all tissue culture reagents were from StemCell Technologies (Vancouver, BC) and all other reagents were from Sigma-Aldrich (St. Louis, MO).

Isolation of myeloid cells

Spleens, livers, and LNs were passed through a 70-μm filter, to prepare single-cell suspensions, whereas lungs were finely minced before agitation for 40 min at 37°C with 0.5% trypsin (BD Biosciences) and 0.08% collagenase in PBS. After incubation, 0.06% DNsase was added, and the cell suspension was filtered through 30 μm nylon mesh. Tumors were minced and incubated for 30 min at 37°C with 125 μg Liberase (Roche Diagnostics, Laval, QC) in IMDM. Peripheral blood was harvested by cardiac puncture, and BM was flushed from femurs using PBS. Samples for flow cytometry were treated with ammonium chloride solution (NH₄Cl; 0.8% with 0.1 mM EDTA; 7 min on ice) for erythrocyte lysis and either fixed in 2% paraformaldehyde, or stained with appropriate Abs and analyzed immediately.

Gr1⁺ and F4/80⁺ cells were isolated from single-cell suspensions using magnetic-activated cell sorting (MACS) magnetic-activated cell sorting isolation system (StemCell Technologies). Gr1⁺ cells were isolated using Gr1-PE⁺ selection, whereas F4/80⁺ cells were isolated using F4/80-PE⁺ or F4/80-allophycocyanin⁺ selection, according to the manufacturer’s instructions. Purity was >95% as determined by flow cytometry based on CD11b and either Gr1 or F4/80 expression. Peritoneal Mφs (PMφs) were obtained by lavage of the peritoneal cavity with 3 × 5 ml HL-1 medium (BioWhittaker, Basel, Switzerland) in 1 μl EDTA. PMφs were resuspended in HL-1 medium without EDTA, plated, and allowed to adhere for at least 3 h at 37°C before the nonadherent cells were washed away. Analysis of the adherent cells revealed that >95% were PMφs, coexpressing F4/80 and CD11b, and exhibiting characteristic Mφ morphology.

Flow cytometry and ROS determinations

Cells were suspended in HBSS + 2% FCS + 0.05% NaN₃ and blocked with 1 μg rat anti-mouse CD16/CD32 Ab (2.4G2; BD Biosciences) for 10 min at 4°C. Cells were incubated for 30 min at 4°C with allophycocyanin, FITC-, or PE-conjugated Abs specific for mouse CD11b (eBioscience), CD11c (eBioscience), Gr1 (eBioscience), F4/80 (Innogenot), Ly6C (BD Biosciences), or Ly6G (BD Biosciences). Cells were washed 2× with PBS and stained with 20 μM dichlorofluorescein diacetate diacetate dye for 45 min at 37°C to measure ROS production. Cells were then washed 2× with cold PBS, and suspended for flow cytometric analysis of mean fluorescence intensity of oxidized dichlorofluorescein diacetate. Data were acquired using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). Absolute numbers of cells were calculated by multiplying the proportion of a particular cell type as determined by flow cytometry by the total number of cells recovered from disaggregated tissue. Cells for FACS were prepared in PBS and stained with cell-surface Abs and propidium iodide. Viable cells were sorted using a FACSAriaII cell sorter (BD Biosciences).
Immunofluorescence and morphology

Lungs from tumor-bearing mice were fixed in OCT medium (Sakura Finetek, Torrance, CA), and 8- to 10-μm serial sections were stained with unconjugated Abs against cytoketatin (Dako, Burlington, ON), Gr1, or F4/80 (eBioScience) with Alexa 488 or 594 secondary Abs (Invitrogen, Burlington, ON). To assess morphology, we stained cytoxin preparations of Gr1+ or F4/80+ lung cells with Giemsa-eosin. Images were captured with a Retiga EXI camera (QImaging, Surrey, BC) using an Axiosvert S100 microscope (Carl Zeiss Canada, Toronto, ON, Canada).

T cell proliferation assay and cytokine assays

Unless otherwise stated, assays were performed using HL-1 serum-free medium supplemented with 1% penicillin, 1% streptomycin, 1% Glutamax, and 5 × 10⁻⁵ M 2-ME (23). For some experiments, Low Protein Medium (Biological Industries, Beit Haemek, Israel), a serum-free and albumin-free medium supplemented with 1% penicillin, 1% streptomycin, 1% Glutamax, and 5 × 10⁻³ M 2-ME, was used. For selected experiments, 10% FCS (Hyclone, Logan, UT) was added to supplemented HL-1 medium. Erythrocyte-depleted splenocytes were cultured at 2 × 10⁵ cells/well (T cell proliferation) or 5 × 10⁵ cells/well (cytokine assay) ± irradiated (2000 rad) myeloid cells. BALB/c splenocytes were stimulated with 1 μg/ml anti-CD3 + 5 μg/ml anti-CD28 (polyclonal), and DO11.10 splenocytes were stimulated with 10 μg/ml OVA peptide (323-339; Ag specific). Inhibitors were added as indicated. Cells were incubated at 37°C for 72 h, and 1 μCi/well [3H]Thymidine (2 Ci/mM; Perkin Elmer, Woodbridge, ON) was added for the last 18 h. Cells were harvested onto filtermats, and radioactivity was measured using a Betaplate liquid scintillation counter (Wallac, Waltham, MA). Data are expressed as cpm (mean ± SEM) of triplicate cultures. Responder control (RC) indicates stimulated splenocytes alone. The relative percentage of RC proliferation was calculated as:

\[ \frac{\text{Proliferation of stimulated splenocytes with test cells}}{\text{Proliferation of stimulated splenocytes (RC) alone}} \times 100\% \]

For cytokine assays, cell-free supernatants were collected after 72 h, and IL-10 and IFN-γ production assayed using cytokine ELISA kits (BD Biosciences), according to manufacturer’s instructions. For cell contact studies, test cells were added to the lower chamber of Transwell plates (0.4-μm pores, polycarbonate membrane; Corning Life Sciences, Corning, NY), and splenocytes were added to the upper chamber.

Resazurin assay

4T1 tumor cells were plated at 10⁴ cells/well in 500 μl RPMI + 10% FCS. The next day, 0.5, 1, or 2 μM ATRA or 2 μM DMSO vehicle was added. The metabolic activity of cells was quantified at different time points by adding 0.22 mM resazurin to cells and measuring relative fluorescence units 3 h later using an Infinite microplate reader (Tecan, Männedorf, Switzerland).

Clonogenic assays

Monodispersed lung cells (derived by enzymatic disaggregation of lung tissue as outlined earlier) were washed by centrifugation before NH4Cl erythrocyte lysis. Cells were washed in PBS, resuspended in medium, and aliquots of 3 × 10⁴ to 1 × 10⁵ cells were plated in clonogenic assays containing 60 or 30 μM 6-thioguanine (to specifically allow growth of 4T1 and 4TO7 cells, respectively). Cells were incubated for 9–12 d (37°C, 5% CO₂) before staining colonies with malachite green for enumeration. The total number of clonogenic tumor cells in the lungs was calculated by multiplying the proportion of colony-forming tumor cells by the total number of cells recovered from the lungs.

SDS-PAGE and Western blot analysis

Cells were washed with PBS and lysed with 1× SDS sample buffer. All samples were boiled for 2 min, loaded onto 10% polyacrylamide gels, and subjected to SDS-PAGE and Western blot analysis as described previously (23). The following Abs were used: anti-INOS (N-20; Santa Cruz Bio-technology, Santa Cruz, CA), anti-Arg1 (BD Biosciences), and anti-tubulin.

Arg and NO assays

Arg activity was assessed indirectly by measuring the concentration of urea generated by the arginase-dependent hydrolysis of l-arginine, as described previously (24). NO production was determined indirectly by measuring the accumulation of the stable end product, NO−. In cell-free culture supernatants using the Griess assay, as described previously (25).

Statistical analysis

Unless otherwise stated, data are mean ± SEM of triplicate determinations and are representative of three independent experiments. Student t tests or ANOVA were performed using Microsoft Office Excel 2007. The p values ≥ 0.05 were considered NS; *p < 0.05, **p < 0.01, ***p < 0.001.

Results

Metastatic mammary carcinomas induce the development of two distinct myeloid cell populations

Myeloid cells are greatly expanded in the majority of mouse tumor models (8, 26) and in cancer patients (10). Consistent with these data, mice bearing 4T1 tumors exhibited a dramatic expansion of CD11b+ cells in their lungs 3 wk after orthotopic tumor implantation (Fig. 1A). Flow cytometric analysis revealed that these tumor-induced CD11b+ cells were largely composed of two distinct, nonoverlapping populations (Fig. 1B). The majority of CD11b+ cells coexpressed Gr1+ and lacked F4/80, indicative of an immature myeloid cell phenotype (10), whereas the second CD11b+ population coexpressed F4/80 and lacked Gr1, consistent with the phenotype of more mature myeloid cells, including Mδs (2, 27). We examined the induction of these two myeloid cell subpopulations by metastatic mammary tumors and found that mice bearing 4T1 tumors exhibited significantly higher proportions of both CD11b+Gr1+ (“Gr1+”) and CD11b+Gr1− F4/80+ (“F4/80+”) cells in their lungs and spleens compared with naïve control mice (Fig. 1C, left panel). Similarly, the absolute number of both myeloid cell types was considerably higher in the lungs and spleens of tumor-bearing mice; 4T1 tumor-bearing mice displayed up to 600-fold more Gr1+ cells and 230-fold more F4/80+ cells in lung tissue than control animals (Fig. 1C, right panel), consistent with a large influx of myeloid cells into the lungs. Furthermore, the expansion of myeloid populations in the spleen was consistent with the dramatic splenomegaly and myelopoiesis that characterizes the 4T1 tumor model (28). Mice bearing 4T07 tumors also had significantly elevated proportions (Fig. 1D, left panel) and absolute numbers (Fig. 1D, right panel) of pulmonary and splenic Gr1+ cells and F4/80+ cells 3 wk after tumor implantation. In addition, we found that the proportions of Gr1+ cells and F4/80+ cells were increased up to 7-fold in the spleens and lungs of mice with spontaneously developed MMTV-PyVmT tumors (harvested when tumors reached ∼800 mm³) compared with FVB/NJ control mice (data not shown).

Lung tissue is a key site of 4T1 metastasis and a primary location of pretumoral niches induced by metastatic breast tumors (5). Interestingly, we saw a marked difference in the location of different myeloid cell types in 4T1 tumor-bearing mice with large pulmonary metastases; Gr1+ cells were located throughout the lung tissue and around the periphery of large metastatic tumor nodules (Fig. 1E, left panel), whereas F4/80+ cells were present throughout the interior of metastatic tumor nodules (Fig. 1E, right panel). Thus, both immature CD11b+Gr1+ F4/80+ cells and more mature CD11b−Gr1− F4/80− cells dramatically increase in the lungs of mice with metastatic tumors, and these cells aggregate in different areas of lungs that contain tumor metastases.

Gr1+ cells induced by metastatic tumors are MDSCs

One of the major mechanisms by which myeloid cells promote tumor growth is by inhibiting antitumor immune responses (29). Therefore, we assessed the effect of metastatic mammary tumors on the immunosuppressive functions of myeloid cells and observed a pronounced difference in the ability of Gr1+ cells to suppress T cell responses depending on whether they were isolated from control or tumor-bearing mice. To assess immunosuppressive function, we
cocultured splenic Gr1+ cells from control or tumor-bearing mice with activated splenocytes (an abundant source of T cells), and quantified T cell proliferation. Whereas 4T1 Gr1+ cells suppressed T cell proliferation, Gr1+ cells from naive mice exhibited minimal suppressive activity (Fig. 2A), even at higher ratios of Gr1+ cells to responder splenocytes (Fig. 2B). Gr1+ splenocytes from both 4T1 and 4TO7 tumor-bearing mice suppressed T cell proliferation in a dose-dependent manner and to an equal extent (Fig. 2C). Because tumor-induced myeloid cells are known to accumulate in multiple organs and tissues (1, 28), we investigated the immunosuppressive abilities of Gr1+ cells harvested from the spleens and lungs (Fig. 2D), and from tumors, kidneys, and livers (data not shown) of 4T1 mice 3 wk postimplantation. (E) Lung sections from 4T1 mice analyzed for cytokeratin (tumor cells; red) and anti-Gr1 (green; left) or anti-F4/80 (green; right). Areas in dotted lines are metastatic tumor nodules. Original magnification ×10. *p < 0.05, **p < 0.01, ***p < 0.001, relative to control.

Differentiation of MDSCs with ATRA enhances lung metastasis

MDSCs have been shown to contribute to primary tumor growth and metastasis in both animal models and human cancers (10, 29). Consequently, there is a great deal of interest in targeting these immature myeloid cells by inducing their differentiation into mature myeloid cells with agents such as ATRA, a member of the retinoid family (30). ATRA induces the differentiation of MDSCs into Mφs and DCs (14, 31), and is used clinically to treat some
forms of acute myeloid leukemia (30). The use of ATRA as a possible cancer therapy has been proposed in the literature (14–16), but the efficacy of ATRA in metastatic breast cancer remains unknown. To determine whether the differentiation of immature myeloid cells could be used to inhibit primary or metastatic mammary tumor growth, we treated 4T1 and 4TO7 tumor-bearing mice with ATRA. ATRA treatment did not alter primary tumor size (Fig. 3A) or tumor cell proliferation in vitro (Supplemental Fig. 1A) at doses known to induce myeloid cell differentiation (14), indicating that ATRA did not have a direct effect on tumor cells in this model. Although 4T1 tumors typically induce extensive myelopoiesis and splenomegaly (28), mice treated with 10 mg ATRA did not experience the tumor-associated splenomegaly exhibited by control or placebo-treated mice (Fig. 3B), suggesting that ATRA treatment decreases tumor-induced myelopoiesis in the spleen.

We next examined the effect of ATRA treatment on metastatic growth. Because the lungs are a principal site of metastasis in the 4T1 and 4TO7 tumor models, we assayed the number of metastatic tumor cells in the lungs of these mice after ATRA treatment. We found that ATRA treatment increased the number of clonogenic 4T1 tumor cells in the lungs in a dose-dependent manner (Fig. 3C). Moreover, ATRA treatment enhanced pulmonary metastatic growth of 4TO7 tumor cells to an even greater extent (Fig. 3D). Consistent with the ability of ATRA to differentiate immature myeloid cells, we observed a reduction in the number and proportion of CD11b+Gr1+ cells isolated from 4T1 tumor-bearing mice treated with ATRA for 3 wk. We also assessed the effect of ATRA on levels of Gr1+ cells, F4/80+ cells, and CD11b+CD11c+ DCs in different tissues, including the spleen, liver, BM, LNs, and peripheral blood, and found that ATRA generally promotes the differentiation of myeloid cells (Supplemental Fig. 1B), consistent with previous reports of the effect of ATRA on myeloid cell differentiation (14). We also noted that ATRA greatly reduced the numbers of all myeloid cell types (i.e., Gr1+ cells, F4/80+ cells, and CD11b+CD11c+ DCs) in the spleen (Supplemental Fig. 1B) because of the absence of splenomegaly in ATRA-treated tumor-bearing mice (Fig. 3B). Collectively, these results suggest that inducing the differentiation of immature myeloid cells is not an effective therapeutic strategy for metastatic mammary carcinoma because ATRA treatment enhances, rather than reduces, metastatic growth.

**Mφs are more potent immune suppressors than MDSCs**

We hypothesized that the prometastatic effects of ATRA treatment were related to the ability of ATRA to alter the balance of myeloid cell subsets. Although the protumor functions of both immature and mature myeloid cells have been well documented (1, 2), less is known about the relative immunosuppressive potencies of different myeloid cell subpopulations. Because ATRA treatment enhances metastatic mammary tumor growth and induces the differentiation of Gr1+ cells into F4/80+ cells, we assessed the relative immunosuppressive properties of these two myeloid cell populations. We directly compared the abilities of Gr1+ and F4/80+ cells to inhibit T cell proliferation and found that F4/80+ cells were significantly more suppressive than Gr1+ cells on a per-cell basis in three separate metastatic mammary carcinoma models. The magnitude of this difference was striking; F4/80+ cells were >32-fold (polyclonal) or >10-fold (Ag-specific) more immunosuppressive than Gr1+ cells isolated from the same 4T1 tumor-bearing BALB/c mouse (Fig. 4A). F4/80+ cells were also 26-fold more potent immune suppressors than Gr1+ cells in BALB/c mice bearing 4TO7 tumors (Fig. 4B). In FVB/NJ mice that had spontaneously developed MMTV-PyVmT tumors, F4/80+ cells were 15-fold and 2-fold

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**FIGURE 2.** Gr1+ cells isolated from metastatic tumor-bearing mice are immunosuppressive MDSCs. (A) Splenic Gr1+ cells from non-tumor-bearing (control) or 4T1 mice were cocultured with polyclonal-stimulated splenocytes (1 Gr1+ cell: 2 splenocytes) and T cell proliferation measured by [3H] thymidine incorporation (RC; stimulated splenocytes in the absence of Gr1+ cells). (B and C) Splenic Gr1+ cells were isolated from 4T1 (●) and (B) control (○) or (C) 4TO7 (○) mice and cocultured with polyclonal-stimulated splenocytes at different Gr1+/cell/splenocyte ratios. Data are T cell proliferation relative to RC (i.e., stimulated splenocytes alone = 100%). (D) Gr1+ cells were isolated from the spleens or lungs of 4T1 mice and cocultured with polyclonal-stimulated splenocytes (1 Gr1+ cell: 2 splenocytes). (E) Splenic or pulmonary 4T1 Gr1+ cells were cocultured with polyclonal-stimulated splenocytes (1 Gr1+ cell: 2 splenocytes), and IFN-γ (left panel) and IL-10 (right panel) were measured in the culture supernatants by ELISA. Data are representative of three independent experiments performed in duplicate. **p < 0.01, ***p < 0.001, relative to RC.
more immunosuppressive than Gr1 + cells from the lungs and spleen, respectively (Fig. 4C).

We previously showed that serum, and specifically albumin, inhibits the immunosuppressive abilities of tumor-induced myeloid cells, and that it is therefore important to use albumin/serum-free medium to assess immunosuppressive function (23). Our present finding that F4/80 + cells are more potently immunosuppressive than Gr1 + cells was consistent using two different serum/albumin-free media, whereas the addition of serum abolished immunosuppressive functions of both myeloid cell types (Supplemental Fig. 2A), consistent with our previous work (23).

We isolated myeloid cells from different tissues of 4T1 tumor-bearing mice and found that F4/80 + cells suppressed T cell proliferation (Fig. 4D) and cytokine production (i.e., IFN-γ and IL-10; Fig. 4E) to a greater extent than Gr1 + cells for all tissues examined. Splenic F4/80 + cells were not as potently immunosuppressive as Gr1 + cells was consistent using two different serum/albumin-free media, whereas the addition of serum abolished immunosuppressive functions of both myeloid cell types (Supplemental Fig. 2A), consistent with our previous work (23).

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Next, we further characterized the Gr1 + and F4/80 + subsets of tumor-induced CD11b + myeloid cells (Supplemental Fig. 3A, 3B) and found they exhibited different activation phenotypes; F4/80 + cells isolated from the lungs of 4T1 tumor-bearing mice expressed Arg1 protein (Fig. 5A) and displayed Arg1 activity (Fig. 5B), indicative of an alternatively activated (M2) phenotype (33), whereas pulmonary Gr1 + cells exhibited a naive (nonactivated) phenotype, lacking expression and activity of both Arg1 and iNOS (Fig. 5A–C), the expression of which is characteristic of classically activated (M1) myeloid cells (33). In addition, Gr1 + and F4/80 + cell populations displayed different cell-surface phenotype profiles. Consistent with our previous results (Fig. 1B), we found that >99% of Gr1 + cells isolated from lungs and spleens of 4T1 mice were negative for F4/80; these cells also expressed Ly6G and moderate levels of Ly6C (Fig. 5D, Supplemental Fig. 3C), and exhibited a granulocytic morphology (Supplemental Fig. 3E, left panel), consistent with granulocytic-MDSCs (G-MDSCs). F4/80 + cells were a more heterogeneous population than Gr1 + cells and were composed of three different subpopulations (Fig. 5E, Supplemental Fig. 3D), containing cells that morphologically resem-
bled granulocytes, monocytes, or Mφs (Supplemental Fig. 3E, right). We isolated each of these F4/80+ subpopulations based on differential Ly6C/Ly6G expression (as defined in Fig. 5E, right panel) and found that almost all of the immunosuppressive activity of bulk pulmonary F4/80+ cells was contained in the Ly6C<sup>+</sup>/Ly6G<sup>+</sup> subpopulation (Fig. 5F, red). These F4/80+Ly6C<sup>+</sup>Ly6G<sup>+</sup> cells exhibited morphology characteristic of Mφs (Fig. 5G). In contrast, granulocytic Ly6C<sup>+</sup>Ly6G<sup>+</sup> cells and monocytes Ly6C<sup>+</sup>Ly6G<sup>+</sup> cells possessed modest immunosuppressive potencies (Fig. 5F, 5G), consistent with previous reports of G-MDSCs and monocytic-MDSCs (M-MDSCs), respectively (7). These results indicate that CD11b<sup>+</sup>F4/80<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>+</sup> Mφs are more potent immune suppressors than other populations of tumor-induced myeloid cells, including Gr1<sup>+</sup>, Ly6C<sup>+</sup>, and/or Ly6G<sup>+</sup> MDSCs.

**MDSCs and Mφs suppress T cell responses by different ROS-mediated mechanisms**

After finding that Gr1<sup>+</sup> MDSCs and F4/80<sup>+</sup> Mφs induced by metastatic mammary carcinomas differed in their immunosuppressive potencies, we asked whether MDSCs and Mφs also used different mechanisms to exert their immunosuppressive functions. Using a Transwell system, we first investigated whether direct cell–cell contact between the suppressive myeloid cells and activated T cells was required for suppression of T cell proliferation. We found that Gr1<sup>+</sup> cells isolated from either the spleen or lungs of 4T1 mice could suppress T cell proliferation whether or not they were separated from T cells by a semipermeable membrane (Fig. 6A), suggesting that although cell–cell contact increases MDSC-mediated immunosuppression, it is not required. In contrast, 4T1-induced Mφs were only immunosuppressive when in direct contact with T cells and slightly promoted T cell proliferation when contact was prevented (Fig. 6A). This finding underscores the dual role of Mφs in being able to both inhibit and activate immune responses depending on the specific context (34, 35).

We then investigated the mechanism(s) of 4T1-induced MDSCs and Mφ immune suppression. Myeloid cells have been reported to exert their immunosuppressive effects via a variety of mechanisms, including production of inhibitory cytokines, expression of inhibitory receptors, induction of regulatory T cells, l-arginine depletion, and/or formation of reactive oxygen species (ROS) or...
reactive nitrogen species (1, 4). We found that addition of exogenous IL-2 or inhibitors of immunosuppressive cytokines (i.e., IL-4, IL-10, IL-13, or TGF-β), Arg1, membrane-bound inhibitory molecules (i.e., CTLA4 or TGF-β), or the NO pathway did not restore the proliferation of T cells cocultured with either Gr1+ cells (Supplemental Fig. 4A) or F4/80+ cells (Supplemental Fig. 4B). However, we found that both 4T1-induced Gr1+ and F4/80+ cells produced ROS, and that F4/80+ cells produced 2-fold more ROS than Gr1+ cells, on a per-cell basis (Fig. 6B), consistent with the more potent immunosuppressive properties of F4/80+ cells. Because we recently found that ROS-mediated immune suppression by MDSCs was abrogated by albumin in FCS (23), we tested the effect of serum on ROS production by tumor-induced Gr1+ and F4/80+ cells. Whereas Gr1+ and F4/80+ cells produced ROS in two different serum/albumin-free media, the addition of 10% serum greatly reduced ROS production by both Gr1+ and F4/80+ cells (Supplemental Fig. 4C), consistent with our previous findings (23).

Next, we tested the effect of ROS inhibitors in our assay system to determine whether ROS production by Gr1+ or F4/80+ cells contributed to their immunosuppressive functions. N-acetyl-L-cysteine (NAC), a derivative of cysteine, has both indirect and direct antioxidant functions, acting as a scavenger of free radicals and serving as a precursor in the formation of the antioxidant glutathione (36). Catalase catalyzes the conversion of H2O2 to H2O and O2, whereas superoxide dismutase catalyzes the dismutation of O2 into O2 and H2O2. Catalase, but not NAC or superoxide dismutase, significantly blunted the suppressive effects of 4T1 Gr1+ cells (Fig. 6C and data not shown). These results were true for both pulmonary and splenic Gr1+ cells (Fig. 6D), and suggest that 4T1-induced MDSCs exert their immunosuppressive effects via H2O2 production. Conversely, NAC reversed immunosuppression mediated by both lung F4/80+ cells (Fig. 6E) and PMφs (Supplemental Fig. 4D) from 4T1 mice, suggesting that 4T1 Mφs suppress T cell activity via a ROS-mediated, H2O2-independent mechanism.

Myeloid cells have been reported to suppress T cell responses via cysteine depletion (37); therefore, abundant levels of cysteine can reverse the immunosuppressive function of myeloid cells isolated from some model systems. Given that NAC can act as a cysteine source, we wanted to ensure that NAC was acting by modification of ROS rather than by providing cysteine to the cocultures. We supplemented Mφ/T cell cocultures with L-cysteine and found that L-cysteine did not mimic the effect of NAC on Mφ immune suppression (data not shown).
Gr1+ and F4/80+ myeloid cells suppress T cell responses via different ROS-mediated mechanisms. (A) Gr1+ cells (splenic and pulmonary) and PMφs were harvested from 4T1 mice and cocultured with polyclonal-stimulated splenocytes (1 Gr1+ cell: 2 splenocytes or 1 PMφ: 8 splenocytes) in control (black bars) or Transwell (white bars) wells. (B) ROS production by 4T1-induced Gr1+ and F4/80+ pulmonary cells. Unstimulated PMφs were included as a negative control. (C) Pulmonary 4T1-induced Gr1+ cells were cocultured with polyclonal-stimulated splenocytes (1 Gr1+ cell: 1 splenocyte) ± 10 mM NAC or 1 mg/ml catalase. (D) Pulmonary or splenic Gr1+ cells from 4T1 mice were cocultured with polyclonal-stimulated splenocytes (1 Gr1+ cell: 1 splenocyte) ± 1 mg/ml catalase. (E) Pulmonary 4T1-induced F4/80+ cells were cocultured with polyclonal-stimulated splenocytes (1 F4/80+ cell: 1 splenocyte) ± 10 mM NAC or 1 mg/ml catalase. (F) 4T1 MDSCs suppress T cell responses via a contact-independent mechanism that is inhibited by treatment with catalase, whereas 4T1 Mφs suppress T cell proliferation to a greater extent via contact-dependent ROS production that is inhibited by treatment with NAC. *p < 0.05, **p < 0.01, relative to untreated cells.

These studies reveal that both 4T1-induced MDSCs and Mφs suppress T cell responses via ROS-dependent mechanisms: 4T1-induced MDSCs suppress T cells via contact-independent H2O2 production, consistent with MDSC immunosuppression found in other tumor models (1), whereas 4T1-induced Mφs exert their more potent immunosuppressive effects by production of contact-dependent ROS (Fig. 6F), which is a novel mechanism of immune suppression used by tumor-induced Mφs.

Discussion
Myeloid cells are key promoters of tumorigenesis and have consequently been identified as attractive therapeutic targets. In this study, we demonstrate that the CD11b+ myeloid cells that accumulate in peripheral tissues in response to metastatic mammary tumors are composed of two related, but phenotypically distinct, subpopulations. Both immature CD11b+Gr1+F4/80+ MDSCs and more mature CD11b+Gr1−F4/80− cells, including Mφs, are induced by tumors but are concentrated in different locations within the lungs of mice with metastatic tumor foci. Although there is much interest in targeting MDSCs by inducing their differentiation with therapeutics such as ATRA, we found that ATRA treatment enhanced metastatic growth in multiple mammary tumor models. Related to this, although 4T1-induced CD11b+Gr1+F4/80− cells were a relatively homogenous population of Ly6CmidLy6G+ G-MDSCs, we found that CD11b+Gr1−F4/80− cells were a heterogeneous population of cells composed of morphologically distinct Ly6Cmid-Ly6G+ G-MDSCs, Ly6C+Ly6G− M-MDSCs, and Ly6C−Ly6G+ Mφs (Fig. 5F). By independently isolating each of these F4/80+ subpopulations, we found that F4/80+ M-MDSCs and G-MDSCs possessed modest immunosuppressive function (i.e., comparable with Gr1+Ly6G− M-MDSCs), whereas F4/80+Ly6C−Ly6G+ Mφs were strikingly potent immunosuppressive cells (Fig. 5G). Importantly, isolated F4/80+Ly6C−Ly6G+ Mφs did not require the presence of F4/80+ MDSCs to exert their suppressive functions. Thus, the potent suppressive function of bulk F4/80+ cells is due to the F4/80+Ly6C−Ly6G+ Mφ subpopulation, and assaying the immunosuppressive function of the bulk F4/80+ cell population actually underestimates the immunosuppressive potency of F4/80+ Mφs (Fig. 5F).

Although there are reports that different intensities of F4/80 expression can be used to discriminate MDSCs (F4/80mid) from Mφs (F4/80high) (27), it remains difficult to accurately distinguish therapies that induce MDSC differentiation and/or Mφ accumulation, and suggest that strategies targeting multiple myeloid cell populations may be more effective at restricting mammary tumor metastasis.

There is both overlap and diversity in the phenotypes of different myeloid cell populations, which has led to some confusion about how to define and identify these cells. For example, populations of cells coexpressing CD11b, Gr1, and F4/80 have been described and termed either MDSCs or Mφs depending on the research group (29). Related to this, although 4T1-induced CD11b+Gr1+F4/80− cells were a relatively homogenous population of Ly6CmidLy6G+ G-MDSCs, we found that CD11b+Gr1−F4/80− cells were a heterogeneous population of cells composed of morphologically distinct Ly6Cmid-Ly6G+ G-MDSCs, Ly6C+Ly6G− M-MDSCs, and Ly6C−Ly6G+ Mφs (Fig. 5E, 5G). In addition, we found that MDSCs and Mφs suppressed T cell responses via different ROS-mediated mechanisms. Consequently, these data advise against...
myeloid cell types based on cell-surface marker expression. Moreover, we have found that CD11b<sup>+</sup>Gr1<sup>-</sup> immature myeloid cells from naive, tumor-free mice are not immunosuppressive (Fig. 2A) and, therefore, are nor MDSCs, consistent with previous reports (8). The current lack of consistent, unambiguous cell-surface phenotypes that correlate with immunosuppressive function underscores the importance of complementing flow cytometric–based assessments of cell-surface marker expression with ex vivo assessments of the immunosuppressive function of myeloid cells.

Although there is overlap in the phenotypes of different myeloid cell subpopulations, there are still key functional distinctions that may be important for the design of novel cancer treatments. In secondary pulmonary metastases, we found Gr1<sup>-</sup> cells primarily around the periphery of large metastatic tumor nodules, whereas F4/80<sup>+</sup> cells were within the interior of the tumor nodules (Fig. 1E). When taken with previous work identifying CD11b<sup>+</sup> myeloid cells in premetastatic niches in the lungs of mice bearing metastatic breast tumors (5), these data suggest that Gr1<sup>-</sup> MDSCs may promote early metastatic tumor development, whereas F4/80<sup>+</sup> Mφs may be involved primarily in supporting the growth of larger tumor metastases.

Our studies demonstrate that tumor-induced Mφs and MDSCs exert their immunosuppressive functions via different mechanisms. We found that both Mφs and MDSCs suppress T cell proliferation by ROS production, but the suppressive effects of MDSCs are contact independent and inhibited by catalase (Fig. 6F), suggesting that MDSCs inhibit T cell activity by production of extracellular H<sub>2</sub>O<sub>2</sub>. In contrast, Mφ-induced suppression of T cells is contact dependent and is inhibited by NAC (Fig. 6F), which serves as a proton donor via its thiol group (36) and functions via its cysteine group as the rate-limiting factor in the synthesis of the potent intracellular antioxidant glutathione (38). Although it is well established that MDSCs can suppress antitumor immunity via ROS production (1), this represents a novel mechanism of immunosuppression for tumor-induced Mφs, which have not been previously reported to produce ROS. Our data reveal that tumor-induced Mφs generate ROS and do so in greater quantities than MDSCs (Fig. 6B), consistent with the superior immunosuppressive abilities of Mφs.

Our finding that Mφs are significantly more potent immune suppressors than MDSCs is consistent with the ability of ATRA to enhance metastatic growth. Numerous studies have demonstrated that ATRA-mediated differentiation of MDSCs into mature myeloid cells potently decreases MDSC numbers in vitro (39, 40) and in vivo, both in tumor-bearing mice and in cancer patients (14–16). ATRA increases levels of glutathione synthase in MDSCs, which, in turn, increases glutathione levels and decreases ROS production (31), consistent with the idea that ROS mediates the suppressive effects of MDSCs and also contributes to the inability of MDSCs to differentiate into mature cells (41, 42). Although there is evidence that ATRA increases cancer treatment efficacy in combination with cancer vaccines or chemotherapy (14, 16, 43), little is known about the effect of ATRA on primary or metastatic tumor growth in the absence of other treatments. Our studies reveal that treating murine mammary tumor-bearing mice with ATRA reduces the number of MDSCs, increases the number of F4/80<sup>+</sup> cells (Fig. 3F), and increases metastatic growth in the lungs (Fig. 3C, 3D). Although our data demonstrate the potent immunosuppressive function of Mφs and the influence of ATRA treatment on MDSCs and Mφs in vivo, it is likely that other host immune cells may also be involved in promoting metastatic growth with ATRA treatment. ATRA treatment increases the differentiation of DCs (Supplemental Fig. 1B) and T regulatory cells (44), which may also contribute to immune suppression and tumor metastasis either directly or by inducing the development of other tolerogenic immune cells (29). Nevertheless, our data argue against the notion that inducing MDSC differentiation leads to increased antitumor immune responses and decreased tumorogenesis, and instead suggest that, at least in some cancers, inducing MDSC differentiation can promote metastasis.

Despite the functional differences that exist between different myeloid cell subtypes, it is becoming clear that myeloid cells represent an intricate network of immature and differentiated cell types, each of which can contribute to tumor progression (29). Therefore, therapeutic strategies that deplete MDSCs without inducing their differentiation (i.e., sunitinib) (45) or that decrease MDSC immunosuppressive function (i.e., sildenafil) (46) may be more effective in promoting antitumor immunity. In addition, agents that target multiple populations of myeloid cells may be especially effective in combination with immunotherapy. Treatments such as clodronate have been used previously to target phagocytic myeloid cells, including both MDSCs and Mφs (47, 48), and have been shown to reduce tumor growth and metastasis (49). Similarly, depletion of myeloid cells via Abs common to multiple myeloid cell subsets (i.e., CD11b, Gr1, or F4/80) may also aid in reducing tumor growth and/or metastasis (50). Further, although ROS production is a known mechanism of T cell suppression by MDSCs (1), the involvement of ROS in immune suppression mediated by tumor-induced Mφs has important therapeutic relevance. Our data indicating that both tumor-induced Mφs and MDSCs inhibit T cell responses via ROS suggest that therapies targeting ROS production may be beneficial to enhance antitumor immunity, and thereby reduce tumor growth and metastasis.

Myeloid cells are one of the main contributors to the immunosuppressive environment induced by cancer and are attractive therapeutic targets. Our finding that the differentiation of immature, immunosuppressive myeloid cell populations can enhance metastatic mammary carcinoma growth emphasizes the need to consider the importance of distinct myeloid cell types in tumor progression. Effective therapeutic strategies will likely target multiple immunosuppressive myeloid cell populations, including both MDSCs and Mφs. Overall, the results presented in this article highlight the importance of MDSCs and Mφs in immune suppression and tumor metastasis, and will facilitate the development of novel, effective, immune-modulatory treatment strategies for cancer patients.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1. ATRA alters the proportions and numbers of different myeloid cell populations in a variety of tissues. A, 4T1 tumor cells were treated with different doses of ATRA or 2 μM DMSO vehicle for 24, 48, 72, or 96 hours and number of metabolically active cells measured by resazurin assay. B, Lungs, spleens, livers, bone marrow (BM), lymph nodes (LN), and peripheral blood (PB) were harvested from 4T1 mice implanted with 10 mg ATRA or placebo pellets for 3 weeks and the proportions and numbers of CD11b^+Gr1^+F4/80^- cells, CD11b^+Gr1^+F4/80^+ cells, and CD11b^-CD11c^+ cells quantified by flow cytometry. *,p<0.05; **,p<0.01; ***,p<0.001, relative to placebo.

Supplemental Figure 2. Effect of serum or ATRA treatment on the immunosuppressive abilities of 4T1 myeloid cells. A, Pulmonary Gr1^+ or F4/80^+ cells were isolated from 4T1 mice and co-cultured with polyclonal-stimulated splenocytes (1 myeloid cell: 1 splenocyte) in HL-1 (serum/albumin-free), LPM (serum/albumin-free), or HL-1 + 10% FCS medium. B, Splenic F4/80^+ cells isolated from control (o) or 4T1 (■) mice and co-cultured with polyclonal-stimulated splenocytes at different ratios were only mildly immune suppressive. C, ATRA treatment did not alter the immunosuppressive abilities of pulmonary Gr1^+ or F4/80^+ cells isolated from 4T1 tumor-bearing mice 0, 1, 2, or 3 weeks after placebo or ATRA pellet implant (co-culture with polyclonal-stimulated splenocytes; myeloid cell: 1 splenocyte).

Supplemental Figure 3. Gr1^+ and F4/80^+ cells exhibit different cell surface marker expression and morphology. Proportion of A, Gr1^+ and B, F4/80^+ lung cells in 4T1 mice. C, Gr1^+ and D, F4/80^+ spleen cells from 4T1 tumor-bearing mice were stained for antibodies against CD11b, Gr1, F4/80, Ly6C, and Ly6G and analyzed by flow cytometry. E, Morphology of positively-selected Gr1^+ (left) and F4/80^+ (right) cells isolated from the lungs of 4T1 mice. Arrows indicate Mφs. Images are 200x magnification.
Supplemental Figure 4. The suppressive properties of 4T1-induced myeloid cells are mediated by ROS production. Polyclonal-stimulated splenocytes were cultured with A, 4T1 pulmonary Gr1⁺ cells (1 Gr1⁺ cell: 2 splenocytes) or B, 4T1 PMφs (1 PMφ: 8 splenocytes) ± 10 μg/ml rat IgG, 10 μg/ml anti-IL-4, 2 μg/ml anti-IL-10, 10 μg/ml anti-IL-13, 10 μg/ml anti-TGF-β, 250 ng/ml LAP, 100 U/well mIL-2, 2 mM L-Arg, 200 μM BEC, 10 μg/ml anti-CTLA4, 0.5 mM L-NMMA, 1 mM L-NIL, and/or 10 μg/ml anti-IFN-γ. Concentrations of all agents are known to be biologically active in our hands using a variety of in vitro cell culture assays (25). C, ROS production by Gr1⁺ (left) and F4/80⁺ (right) pulmonary 4T1 cells cultured O/N in HL-1, LPM, or HL-1 + 10% FCS medium. D, 4T1 PMφs were co-cultured with polyclonal-stimulated splenocytes (1 PMφ: 8 splenocytes) ± 10 mM NAC, 1 mg/ml catalase, or 200 U/ml SOD. *, p<0.05; ***, p<0.001 relative to untreated cells.