Pulmonary Alveolar Macrophages Contribute to the Premetastatic Niche by Suppressing Antitumor T Cell Responses in the Lungs

Sharad K. Sharma, Navin K. Chintala, Surya Kumari Vadrevu, Jalpa Patel, Magdalena Karbownikczek and Maciej M. Markiewski

J Immunol 2015; 194:5529-5538; Prepublished online 24 April 2015; doi: 10.4049/jimmunol.1403215
http://www.jimmunol.org/content/194/11/5529

References
This article cites 43 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/194/11/5529.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2015 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Pulmonary Alveolar Macrophages Contribute to the Premetastatic Niche by Suppressing Antitumor T Cell Responses in the Lungs

Sharad K. Sharma,1 Navin K. Chintala,1 Surya Kumari Vadrevu,1 Jalpa Patel, Magdalena Karbowniczek, and Maciej M. Markiewski

In contrast to tumor-associated macrophages, myeloid-derived suppressor cells, or inflammatory monocytes, functions of tissue resident macrophages, including alveolar macrophages (AM), in cancer were not well studied. Using a mouse model of breast cancer, we show that AM promote cancer metastasis to the lungs by suppressing antitumor T cells in this organ. AM accumulated in the premetastatic lungs through complement C5a receptor–mediated proliferation but not through recruitment from the circulation. AM preconditioned by breast tumors inhibited Th1 and favored generation of Th2 cells that had lower tumoricidal activity than Th1 cells. In addition, AM reduced the number and maturation of lung dendritic cells by regulating TGF-β in the lung environment. Depletion of AM reversed immunosuppression imposed by these cells and strengthened local Th1 responses, which significantly reduced lung metastatic burden. C5a receptor deficiency, which also lessens myeloid-derived suppressor cells in the premetastatic niche, synergized with the depletion of AM in preventing metastasis, leading to protection of mice from lung metastases. This study identifies AM as a new component of the premetastatic niche, which is harnessed by tumors to impose immunosuppression, and as a new target for cancer immunotherapies to eliminate or reduce metastasis. Because the lungs are the most common target for hematogenous metastasis, this research offers a plausible explanation for susceptibility of the lungs to cancer metastasis.  

The Journal of Immunology, 2015, 194: 5529–5538.

Received for publication December 24, 2014. Accepted for publication March 21, 2015.

This work was supported by Cancer Prevention and Research Institute of Texas Grant RP 120168 (to M.K.) and Department of Defense Breast Cancer Research Program Grant BC 111038 (to M.M.M.).

Views and opinions of and endorsements by the author(s) do not reflect those of the U.S. Army or the Department of Defense.

Address correspondence and reprint requests to Dr. Maciej M. Markiewski and Dr. Magdalena Karbowniczek, Texas Tech University Health Sciences Center, 1718 Pine Street, Abilene, TX 79601. E-mail addresses: maciej.markiewski@ttuhsc.edu (M.M.M.) and magdalena.karbowniczek@ttuhsc.edu (M.K.)

Abbreviations used in this article: AM, alveolar macrophage; BM, bone marrow; C5aR, complement C5a receptor; DC, dendritic cell; GzB, granzyme B; IM, interstitial macrophage; m. murine; MDSC, myeloid-derived suppressor cell; TAM, tumor-associated macrophages; TRM, tissue resident macrophage; WT, wild-type.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/S25.00

Department of Immunotherapeutics and Biotechnology, School of Pharmacy, Texas Tech University Health Science Center, Abilene, TX 79601

1S.K.S., N.K.C., and S.K.V. contributed equally to this work.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/S25.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1403215
Mice and cell lines

Balb/c, C57R-deficient (C5AR–/–), C57BL/6, Balb/cByJ (both C45.1 and CD45.2), OTII TCR, and FVB/Nj Her2-transgenic mice were from The Jackson Laboratory. C5AR+ mice were backcrossed to Balb/c for 10 generations. Mice were housed in the animal facility of Texas Tech University Health Sciences Center. All experiments were approved by the Committee for Institutional Animal Care of Texas Tech University Health Sciences Center. The 4T1 (CRL-2539, ATCC), B16-F10 (CRL-6475, ATCC), and chicken ovalbumin-expressing B16-F10 cells (a gift from Dr. Laurence Wood, Texas Tech University Health Sciences Center) were maintained in the cell culture media as recommended by the supplier and routinely tested for Mycoplasma.

Tumor model and AM depletion

In a syngeneic model of breast cancer, mice were injected with 1 × 10^5 4T1 cells in the mammary fat pad. This model recapitulates stage IV human breast cancer, because primary tumors metastasize to the vital organs, as in human malignancy (13). FVB/Nj Her2-Tg mice develop spontaneous breast cancer, because primary tumors metastasize to the vital organs, as described earlier (17). Dendritic cells (DC) were CD11c+MHCII+, interstitial macrophages (IM) were CD11b+F4/80+, myeloid-derived suppressor cells (MDSC) were identified as CD11b+Gr-1+, and AM were CD11bnegative. For AM depletion, mice were intranasally administered 60 g/ml LPS for 8–12 h in the presence of Golgi inhibitors. As negative controls, unstimulated cells were used. Cytokine production was assayed by standard intracellular cytokine staining procedure, as described in our earlier study (12). Staining for Ki-67 was performed after permeabilization with 70% chilled ethanol. For surface staining, cells were incubated with fluorescently labeled Abs. For examining direct tumor cell death by clodronate liposomes, 4T1 cells were incubated with clodronate or PBS liposomes for various time points (0–24 h), and 4T1 cells were harvested and stained for annexin V. All Abs were from Bio-legend unless otherwise specified; mouse BV605 CD45 (30-5-F1), AF488-conjugated CD45.1 (A20), BV605 CD45.2 (104), PerCPCy5.5 CD4 (RM4-5), PE-TR CD8a (53-6.7, RM4-5), PE-IFN-γ (XMG1.2), PE/Cy7 IL-4 (11b12), AF647 IL-17A (Tc11-18H10.1), PE CD11b (M1/70), PE/Cy7 F4/80 (BMD8), APC/Cy7 CD11c (N418), PerCPCy5.5 IFNγ (MHCI) (MH5/14.152), PE CD80 (16-10A1), AF647 CD86 (GL-1), PE/Cy7 C5aR (20/70), PE Ki-67 (16A8), FITC granzyme B (B911), PE/Cy5.5 TGFβ-1 (TW7-16B4), APC-PE CD11c+IL-23 p40 (C15.6), polyclonal rabbit caspase-3 IgG (R&D Systems), and FITC goat anti-rabbit (Santa Cruz Biotechnology). Live-dead stain was from eBioscience, and staining for live-dead marker was performed in the absence of other Abs according to the manufacturer’s instructions. Cells were incubated with CD16-CD32 Abs (Fc block; 2.4G2) to block Fc receptors prior to staining. Staining, cells were fixed in 1% paraformaldehyde stored at 4°C until data acquisition on an LSRFortessa equipped with 3 lasers (12 parameters), and analyzed using FlowJo (TreeStar). Cells were first gated based on forward- and side-scatter properties, followed by gating on CD45+ live cells before identifying the individual population of interest (Fig. 1C). Dendritic cells (DC) were CD11c+MHCI, interstitial macrophages (IM) were CD11b+F4/80+, myeloid-derived suppressor cells (MDSC) were identified as CD11b+Gr-1+, and AM were CD11bnegative. CD11c+F4/80+ (16). Tumor cells were identified as CD45+ cells in the lungs, as described earlier (17).

Immunofluorescence, immunohistochemistry, and quantititative PCR

Lungs were fixed in 10% (v/v) formalin or frozen in OCT medium. Formalin-fixed samples were processed for histological study, and frozen sections were sectioned with a cryostat (Leica) for immunofluorescence. AM were analyzed by immunofluorescence with rat anti-mouse F4/80 (BMS, BioLegend) and CD11b (M1/70; BD Biosciences) Abs at the recommended concentration. Stained sections were analyzed in a blinded fashion by confocal microscopy (Nikon) and Nikon Elements Advanced Research Image-Analysis software either by counting stained cells in a higher power field (×63) or by quantifying an area in a section occupied by AM. H&E-stained lung sections were scanned and digitalized (Aperio Technologies). Analysis was performed using ImageScope (Aperio). Quantitative real-time PCR for C5 was performed as described by us earlier (12). C5 (forward: 5’-CCTGTACCCGATGTAAGAGGCAG-3’; reverse: 5’-TCGTTTTAGTGTACCGGACGCTG-3’) primers were procured from Sigma-Aldrich (St. Louis, MO). Gene expression analysis was performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Gene expression was normalized to 18S using the ΔΔCT method. Interpretation of data was performed using QIAGEN RT2 Proiler PCR Array Data Analysis version 3.5.

CD45.1 BM transfer

A single-cell suspension was prepared from femur BM of tumor-free CD45.1 (ly5.1) Balb/cByJ mice, and 1 × 10^6 BM cells were injected into the tail vein of tumor-bearing and tumor-free wild-type (WT) Balb/cByJ mice (CD45.2). 14 d after 4T1 tumor cell injection into the mammary fat pad. After 7 d, blood, lungs, and spleens were harvested and analyzed for the presence of CD45.1 (transplanted) cells. Further CD45.1 cells in the lungs were analyzed for their differentiation into various populations (MDSC, IM, and AM), using FACS.

In vitro CD4+ T cell polarization in the presence of AM

To study the impact of AM from tumor-bearing versus tumor-free mice on the polarization of T cell responses, AM were isolated by two-step magnetic sorting (F4/80+ cells followed by CD11b+ gated, Miltenyi Biotec) from the lungs of tumor-bearing (21 days after tumor cell injection into the mammary fat pad) and tumor-free control mice. naïve CD4+ T cells were enriched (Miltenyi Biotec) from splenocytes of tumor-free Balb/c mice. The purity of cells was verified by FACS and found to be >98%. CD4+ T cells were differentiated into effectors in the presence of AM from either tumor-bearing or tumor-free mice in a 24-well plate, coated with CD3 Abs in the presence of soluble CD28 Abs. CD4+ T cell cultures were harvested at day 6, and intracellular staining for IFN-γ, IL-4, and IL-17 was performed.

Generation of CD4+ T cell subsets and tumor cell killing assay

For generation of OVA-specific Th1 and Th2 cell lines, CD4+ T cells were isolated from OVA splenocytes using magnetic columns (Miltenyi Biotec). A total of 5 × 10^5 CD4+ T cells were stimulated with 10 μg OVA323-339 peptide-loaded C57BL/6 splenocytes (5 × 10^5). These spleen cells were treated with 50 μg/ml mitomycin B (Sigma-Aldrich) before coculturing with T cells. To generate Th1 cells, cultures were supplemented with murine (m) IL-2 (20 U/ml, eBiosciences), mIL-12 (10 ng/ml, eBiosciences), and neutralizing mIL-10 (1 μg/ml, Bio-legend). For Th2 generation, mIL-2 (20 U/ml), mIL-4 (10 ng/ml), neutralizing mIL-12 (1 μg/ml, Bio-legend), and IFN-γ (1 μg/ml, BioLegend) Abs were added (18). Cells were grown in RPMI 1640 medium containing L-glutamine, HEPES, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 1% NEAA, and 1 mM sodium pyruvate. Cultures were maintained for 7–10 days in 24-well plates. Differentiated Th1 and Th2 cells were stimulated with OVA323-39 peptide-loaded B16-F10 cells to test their ability to produce IFN-γ and IL-4, respectively. Differentiated Th1 and Th2 cells were cocultured with tumor cells for 3 d, counted using a dissection microscope and then verified by routine histological examination.

Tumor measurement and metastatic burden evaluation

Tumor measurements were obtained in two dimensions (length and width). The depth of the tumor was estimated based on the smaller (width) measurement, and the volume of the tumor was calculated using the following formula: volume = (length × width × width)/2. Lung metastases were evaluated using a dissection microscope and then verified by routine histological examination.

Statistical analysis

Data were analyzed with an unpaired t test or nonparametric Mann–Whitney test, depending on the results of the normality test (Kolmogorov–Smirnov). For data with the normal distribution, a two-tailed unpaired t test or nonparametric Mann–Whitney test, depending on the results of the normality test (Kolmogorov–Smirnov). For data with the normal distribution, a two-tailed unpaired t test
Results
Primary breast tumors affect the cellular composition of the lung environment

Impact of primary tumors on the immune environment of the premetastatic niche requires clarification; therefore, we examined various immune cell populations with immunomodulatory roles in the lungs of tumor-bearing mice. Overall, primary breast malignancy induced the significant accumulation of hematopoietic cells (CD45<sup>+</sup>) in the lungs (Fig. 1A). Although recruited MDSC constituted the largest cell population (Fig. 1A, 1B), which is in concordance with previous reports (19), several other immune cells, such as IM, DC, CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells, were also increased in the lungs of tumor-bearing versus tumor-free mice. In addition to hematopoietic cells, we found a significant increase in the number of AM (CD11b<sup>+</sup>Gr-1<sup>−</sup>CD11c<sup>−</sup>F4/80<sup>+</sup>), defined by the set of previously described phenotypic markers (www.immgen.org; Ref. 16) (Fig. 1C). These cells could be distinguished from other myeloid populations, such as lung IM (CD11b<sup>+</sup>F4/80<sup>+</sup>), MDSC (CD11b<sup>+</sup>Gr-1<sup>−</sup>), or DC (CD11c<sup>+</sup>MHCII<sup>+</sup>) in both tumor-free and tumor-bearing mice (Fig. 1C). In addition to the alterations in cellular composition of the lung immune microenvironment, tumor-bearing mice demonstrated downregulation of MHC II (Fig. 1D, 1F) and CD86 (Fig. 1E, 1F) on lung DC, which is likely to contribute to the reduction of Ag-presenting functions of DC, as in the primary tumor microenvironment (20). Tumor-bearing mice also displayed reduction in Th1 function (% of IFN-γ–expressing/CD4<sup>+</sup> T cells) (Fig. 1G, 1H), corresponding to the reduced function of DC. Because Th1 cells have been shown to contribute to antitumor immunity (21), these changes suggest that primary tumors modify developing antitumor response at distant sites, which might facilitate metastasis. We did not observe differences in IL-17–producing cells between tumor-free and tumor-bearing mice (Fig. 1H). Altogether, these immune alterations represent an escape mechanism for metastasizing tumor cells.

To determine the time interval in which premetastatic changes occur, we tracked 4T1 cells that expressed GFP (4T1-GFP<sup>+</sup>) and determined that tumor cells arrived to the lungs at day 20, at the earliest, as a result of spontaneous metastasis of the implanted breast tumors (12). Therefore, we concluded that the accumulation of AM and other immune cells observed in this model, using the same 4T1 clone (Fig. 2A), occurs prior to metastasis and thus contributes to the premetastatic niche. To validate these findings in another model, we examined the lungs of FVB/N HER2/neu transgenic mice that develop spontaneous focal mammary adenocarcinomas metastasizing to the lungs (22), before and after the development of breast tumors, but before lung metastasis. We observed an increase in the number of AM in breast tumor-bearing mice prior to metastases, as determined by histological and immunohistochemical studies with AM marker F4/80 of the lung serial sections (Fig. 2B).

AM accumulate in the premetastatic niche through complement C5aR-mediated proliferation

In contrast to inflammatory macrophages and lung IM that originate from recruited blood monocytes, TRM, including AM, are maintained through adult life by self-renewal (5). Therefore, we hypothesized that an increase in the number of these cells in the lungs of tumor-bearing mice results from proliferation and sustained self-renewal capabilities rather than recruitment from the lungs of TF and TB mice (bar graph). ***p < 0.0001 (t test). (B) Absolute numbers of CD11b<sup>−</sup>Gr-1<sup>−</sup> MDSC, ***p < 0.0001 (t test); CD11b<sup>+</sup>F4/80<sup>+</sup> lung IM, ***p < 0.0001 (t test); CD11b<sup>+</sup>Gr-1<sup>−</sup>CD11c<sup>−</sup>F4/80<sup>+</sup> AM, *p = 0.00084 (t test); CD11c<sup>+</sup>MHCII<sup>−</sup> DC, ***p = 0.0001 (t test); CD8<sup>+</sup> T cells, ***p = 0.0001 (t test); and CD4<sup>+</sup> T cells, **p = 0.0110 (t test) determined by FACs in the lungs of TF and TB mice. (C) Gating strategy used to identify various immune cell populations in the lungs of TF and TB mice. Cells were first gated based on forward light scatter (FSC)/side light scatter (SSC), followed by gating on viable cells (live/dead staining), and then based on CD45 expression (middle plots). CD11b<sup>−</sup> negative cells were gated to identify AM (middle plots). Numbers in each plot depict the absolute counts of the cell population calculated from the total number of lung cells retrieved after single-cell preparation. (D) MHC II and (E) CD86 expression on lung DC from TF and TB mice. (F) Representative histograms with MFI. (G) The ratio of percentages of IFN-γ–expressing CD4<sup>+</sup> T cells (Th1 cells) to IL-4–expressing CD4<sup>+</sup> T cells (Th2 cells) determined by FACs in the lungs of TF and TB mice after ex vivo stimulation. **p = 0.0027 (t test). (H) Representative dot plots of IFN-γ– and IL-4–expressing CD4<sup>+</sup> T cells in the lungs of TF and TB mice. Data pertaining to TF mice are representative of two independent experiments with n = 10 mice, and data for TB mice are representative of three independent experiments with n<sub>1</sub> = 9, n<sub>2</sub> = 5, and n<sub>3</sub> = 10. MFI, median fluorescence intensity.
circulation. This hypothesis was supported by the high percentage of AM expressing proliferation marker Ki-67 (Fig. 2C), when compared with MDSC that are primarily recruited to the lungs from the circulation and, hence, have no proliferative capacity once recruited (Fig. 2C). These data were further confirmed by confocal microscopy that demonstrated nuclear expression of Ki-67 in AM (CD11b-negativeCD11c+F4/80+) in the lungs of WT and C5aR−/− mice (calculated based on FACS data). *p = 0.002. (F) Histogram shows C5aR expression on AM from WT and C5aR−/− (control) mice. (G) Absolute numbers of AM (CD11b-negativeCD11c+F4/80+) in the lungs of WT and C5aR−/− mice at the end point of study (day 30). *p = 0.001. (H) Mean relative expression of C5 in the lungs based on quantitative PCR. Data pertaining to TF mice were obtained from one experiment with n = 10 mice, and data for TB mice are representative of three independent experiments with n1 = 9, n2 = 5, and n3 = 10. Data for quantitative PCR were obtained from n = 4 mice per group.

Although the complement anaphylatoxin C5a is considered a potent chemotractant for leukocytes and MDSC (12, 23), C5a/C5aR signaling has a significant role in regeneration of the liver and, importantly, in proliferation of other types of TRM Kupffer cells (24). Therefore, we examined whether the accumulation of AM was dependent on C5aR. We found a significant reduction in the number of Ki-67-expressing AM in C5aR-deficient (C5aR−/−) mice compared with WT tumor-bearing mice, prior to metastasis, at day 14 after tumor cell injection into the mammary fat pad (Fig. 2E). Together with the high expression of C5aR on AM (Fig. 2F) and the reduced number of AM in C5aR−/− lungs at the end point of study, these data suggest that C5a/C5aR signaling contributes to the accumulation of AM in the premetastatic lungs. These findings are also concordant with our previous report indicating complement activation and generation of C5a from C5 in the premetastatic lungs (12). Although liver is a major site for complement protein synthesis and large quantities of C5 circulate in plasma, macrophages are also known to produce C5 (25); therefore, we examined the expression of C5 in the lungs of tumor-bearing mice. We found that C5 was upregulated at several time points after tumor cell inoculation (Fig. 2H), indicating that the local production of C5 contributes to the C5a/C5aR signaling in AM proliferation.

To evaluate the contribution of recruited myeloid cells to the increase in AM, we adoptively transferred 1 × 10^6 BM cells from tumor-free mice carrying the Ptprca allele (CD45.1) to tumor-free and breast tumor-bearing WT mice that carry the Ptprcb allele (CD45.2), at day 14 after tumor cell inoculation into the mammary fat pad. With this approach, it is possible to distinguish CD45.1+ donor-derived cells that are recruited from the circulation from CD45.2+ cells of the recipient mouse, using FACS staining. We detected transplanted CD45.1 cells in the blood, lung, and spleen of both tumor-free and tumor-bearing mice at day 7 after the transplant. However, in all these locations, CD45.1 cells were significantly higher in tumor-bearing mice (Fig. 3A), indicating that the environment of the tumor-bearing host was more favorable for survival of transferred cells. Notably, transplanted CD45.1 cells in the lungs of tumor-bearing mice (CD45.2) lacked the AM phenotype (CD11b-negativeF4/80+CD11c+) (Fig. 3B, the left dot plot and two next upper plots) in contrast to CD45.2 originating from recipient mice (Fig. 3B, the left dot plot and two next lower plots). The majority of the transplanted cells in the lungs expressed CD11b and Gr-1 typical markers for MDSC (Fig. 3B, the upper second from the right plot, 3C) similar to CD45.2 cells (Fig. 3B, the lower second from the right plot, 3C). Small fractions of CD45.1 express markers of IM also defined as inflammatory macrophages (Fig. 3B, the right plots, 3C). These observations remain in agreement with studies demonstrating that MDSC and inflammatory monocytes (converted later to IM) are recruited from the circulation to the lungs of the tumor-bearing host (2, 19) and exclude the recruitment of BM-derived cells as a source of AM. As with the lungs, trans-
planted CD45.1 cells expressed markers of MDSC in peripheral blood and spleen (Fig. 3C). In addition, these cells expressed markers of macrophages in spleen but not in the circulation (Fig. 3C). To determine if the source of transferred cells has an impact on results, we also adoptively transferred CD11b+ splenocytes that were CD45.1 from tumor-free mice to tumor-free and breast tumor–bearing WT mice (CD45.2). As with experiments involving BM cells, we did not detect expression of AM markers on transplanted cells in the lungs (data not shown).

**AM suppress Th1 responses in the lungs**

Because AM are the key players in quenching T cell responses to environmental Ags (10), we hypothesize that primary tumors harness this property of AM to suppress developing antitumor immune response in the lungs. To address this hypothesis, we selectively depleted AM by intranasal administration of clodronate liposomes (Fig. 4A, 4B). Intratracheally or intranasally administered clodronate liposomes do not affect other phagocytic cells in the lungs and other locations (14). Accordingly, we did not observe the impact of this treatment on lung IM (Fig. 4C). Of note, because MDSC have weak phagocytic properties (26) and intranasally administered clodronate liposomes are avidly taken up by highly phagocytic AM (14), clodronate liposomes did not reduce the percentage of MDSC in the lungs of tumor-bearing mice (Fig. 4D). The depletion of AM increased the percentage of lung DC in breast tumor–bearing mice (Fig. 4E, 4F) and upregulated the expression of CD86, CD80 (Fig. 4G, 4H), and MHC II on metastasizing (CD45negative) tumor cells (Fig. 4D). Therefore, we conclude that AM depleting them would enhance the Th1 cell response.

**AM facilitate lung metastasis**

The enhancement of immunity in the lungs resulting from AM depletion was associated with reduction in lung metastasis...
reversed this trend. Therefore, we investigated tumor cell killing by Th1 versus Th2 cells. To use identical Ag-specific T cells with different polarization conditions, we obtained naïve CD4+ T cells from OTII TCR transgenic mice. This system enables us to study cytotoxicity imposed by both Th subsets on tumor cells expressing OVA (OVA"). In vitro differentiated Th1 and Th2 cells were cocultured with OVA-expressing B16-F10 cells, and apoptosis represented by activation of caspase-3 was evaluated in tumor cells. We found that the percentages of OVA" tumor cells expressing caspase-3 were moderately, although significantly, increased in the cultures with Th1 cells compared with Th2 (Fig. 6E, 6F). Although less efficient, Th2 cells were also cytotoxic to OVA" tumor cells (Fig. 6E, 6F). In contrast, killing of WT B16-F10 cells (no OVA expression) by both Th1 and Th2 was minimal, showing the importance of Ag specificity in CD4+ T cell–mediated tumor cell apoptosis. In addition, we found a higher percentage of Th1 cells than Th2 cells expressing GzB upon stimulation with B16-F10–OVA, indicating the better potential of Th1 cells for Ag-specific cytotoxicity toward tumor cells (Fig. 6G, 6H, upper panels). In contrast, control B16-F10 cells without OVA expression did not induce granzyme expression (lower panels).

**Discussion**

**Immune privileged status of the lungs favors metastasis**

Cancer metastasis is a hallmark of tumor malignancy and a cause of ~90% of cancer-associated deaths. However, paradoxically, this process is the most poorly understood aspect of cancer pathophysiology (6). One of the key unanswered questions pertaining to metastasis is why certain organs are more often affected by metastases whereas other sites are spared or seldom affected. The hemodynamic features of the circulation and specific properties of the vasculature in metastases-targeted organs, together with the distinct molecular signatures of metastasizing tumor cells, do not seem to fully explain the receptiveness of the vital organs, such as lungs for tumor cells (6). Given the role of the immune system in cancer surveillance (30), the immune-privileged status of the lungs (10), and growing evidence for the role of immunosuppression in distant sites in facilitating metastasis (12, 19), we hypothesize that alterations in antitumor immunity in the lungs play a significant role in facilitating metastasis. The special immune status of the lungs is dictated by their positions as a barrier between the external environment and the body. The lung immune system must be controlled to avoid unwanted immune reactions to harmless environmental Ags (10). We hypothesize that these immunosuppressive homeostatic mechanisms that operate in physiology to protect the lungs from autoimmunity are harnessed by tumors to suppress antitumor immune responses.

**AM in metastasis**

The important feature of lung immune homeostasis is the abundance of AM that appear to have the critical role in physiological immunosuppression (31, 32). Therefore, we propose that AM-mediated suppression of lung immunity promotes metastasis independently of immunoregulatory mechanisms operating in primary tumors. C5aR deficiency or C5aR blockade lessens lung metastasis by reducing recruitment of MDSC to the lungs and inhibiting their suppressive function (12). Therefore, we hypothesize that inhibition of C5aR will synergize with AM depletion in reducing lung metastasis. Indeed, in the independent experiment the depletion of AM in C5aR−/− mice entirely prevented lung metastasis (Fig. 6C), indicating increased therapeutic efficacy of combined treatment versus depletion of AM or blocking C5aR alone. Bisphosphonates, including clodronate, have been reported to cause apoptosis of human breast cancer cells in vitro (29); therefore, we tested whether clodronate liposomes, used to deplete AM, induce apoptosis of 4T1 cells. No impact of clodronate on 4T1 cell apoptosis was observed (Fig. 6D), ruling out this possibility.

We conclude that AM facilitate metastases by suppressing Th1 T cell responses, respectively. The tumoricidal activity of CD8+ T cells is well established; however, a direct killing of tumor cells by Th1 cells has been less studied. We found that breast malignancy reduced the Th1/Th2 ratio in the lungs and AM depletion

**FIGURE 4.** AM inhibit DC in the lungs of tumor-bearing mice. (A) Percentages of AM (CD11b+Gr-1+CD11c+ F4/80+) in PBS (AM+, non-depleted) and clodronate liposome (AM−, depleted)–treated tumor-bearing (TB) mice. *p < 0.0001 (t test with Welch’s correction). (B) Representative FACS plots depicting AM in the lungs of TB AM+ and AM− mice with percentages of AM depicted. (C) Percentages of lung IM (CD11b+F4/80+) and (D) MDSC (CD11b+Gr-1+) in the lungs of TB AM+ and AM− mice. *p = 0.0023 (t test). (E) Representative FACS plots showing lung DC from TB AM+ and AM− TB mice, where numbers depict their percentages in the lungs. (G) CD86 and CD80 expression on lung DC from TB AM+ and AM− mice. *p = 0.024 (t test), **p < 0.0001 (t test). (H) Representative histograms. (I) MHC II expression on lung DC from TB AM+ and AM− mice. *p < 0.0001 (t test). (J) Representative histograms. Data are representative of at least three independent experiments with n = 10. MFI, median fluorescence intensity.
to reduce metastases of fibrosarcoma cells injected i.v. (35). These studies were intriguing but were devoid of modern-day understanding and technical advancement to finely characterize or identify the precise role of AM. Recent studies have demonstrated a role in facilitating metastasis for a distinct subpopulation of macrophages (CD11b+F4/80+), known as metastasis-associated macrophages, recruited to the lungs from the circulation (2). However, on the basis of phenotypic characteristics, these cells were different from AM that do not express CD11b and that do express CD11c and F4/80 (5) (www.immgen.org). In addition, depletion of AM by clodronate liposomes that were administered intratracheally did not affect lung metastasis when tumor cells were injected i.v. (2). Our data differ from those of previous studies demonstrating that AM depletion had no impact on lung metastasis. Of note, these studies used experimental metastasis (i.v. injected tumor cells). This approach differs from models of spontaneous metastasis used in the current work, as the presence of primary tumors preconditions the lungs and, consequently AM, to receive metastasizing tumor cells. This part of the metastatic process is omitted when tumor cells are injected into the circulation to otherwise tumor-free mice. Therefore, AM from tumor-free mice might be functionally different from the cells of tumor-bearing mice that develop spontaneous metastasis. However, it remains to be established how primary tumors that are distant from the lungs alter AM. A plausible scenario involves several recently described tumor-derived factors that facilitate the formation of the premetastatic niche, such as vascular endothelial growth factor (36), placental growth factor (36), TNF, and TGF-β (9). Alternatively, tumor-derived exosomes that have recently emerged as inducers of the premetastatic niche in mouse models and melanoma patients may take part in this process (37). Another possibility is that AM are altered through interactions with the BM-derived cells, including MDSC that are recruited to the premetastatic niche, similar to the interactions described for MDSC with TAM in the primary tumor microenvironment (1). MDSC are known to skew the polarization of TAM macrophages toward the M2 phenotype through various mechanisms (38); therefore, our data indicating that AM isolated from tumor-bearing mice were more efficient in favoring Th2 responses compared with AM from tumor-free mice support this concept.
AM arrive to the lungs during the embryonic stage and self-renew through proliferation thereafter (5, 7). We found that the number of AM gradually increased in tumor-bearing mice via proliferation of liver TRM Kupffer cells (24, 39), this scenario is plausible, although further mechanistic studies are required to elucidate this mechanism. Furthermore, adoptive transfer studies excluded contributions of circulating monocytes to the increase in number of AM, which further underscores the self-renewal capabilities of AM (5).

**AM modulate antitumor immunity in the lungs of tumor-bearing mice**

We found that AM depletion resulted in an increase in IFN-γ-producing CD4+ T cells (Th1). This finding is in agreement with the key immunoregulatory functions of AM in the absence of malignancy (10). AM have been shown to inhibit adaptive immune responses, as confirmed by the induction of T cell responses to inhaled Ags in AM-depleted mice (32). However, in contrast to our previous work on the role of C5aR in the lung premetastatic niche (12), we did not observe a significant increase in IFN-γ-producing CD8+ T cells (data not shown). Therefore, the striking reduction in lung metastases in AM-depleted mice, we concluded that Th1 cells protect the lungs from metastasis. Although the cytotoxic function of Th1 cells is documented, it has been less frequently studied than cytotoxicity exerted by CD8+ T cells (27, 40). Therefore, we further verified the killing capacity of tumor-specific Th1 cells, using the OTII transgenic system. Of note, the increased expression of IFN-γ in the lungs of AM-depleted tumor-bearing mice led to the upregulation of MHC II on metastasizing tumor cells, rendering them more susceptible to Th1-mediated killing (41). The results of coculture experiments involving AM and T cells demonstrated that AM directly affected

**FIGURE 6.** AM promote lung metastasis. (A) Number of lung metastases in tumor-bearing (TB) mice in the presence of AM (AM+) or after their depletion (AM−). *p = 0.0013, t test. (B) Primary tumor volumes in AM+ and AM− mice. (C) Number of lung metastases in TB WT or C5aR−/− mice in the presence of AM (WT-AM+, C5aR−/−/AM+) or in AM-depleted lungs (WT-AM−, C5aR−/−/AM−). *p = 0.0325, Mann–Whitney test. **p = 0.0294, Mann–Whitney test. (D) Annexin V staining in 4T1 cells treated with PBS or clodronate liposomes. (E) Percentages of B16 and B16-OVA tumor cells expressing caspase-3 after incubation with in vitro generated OVA-specific Th1 and Th2 cells. *p = 0.0275, t test, ***p < 0.0001, t test. ****p < 0.0001, t test. (F) Representative histograms. (G) Percentages of OVA-specific Th1 and Th2 cells expressing GzB. *p = 0.0010, t test. (H) Representative FACS plots (upper panels); granzyme expression in T cells incubated with B-16 cells that did not express OVA (lower panel, control). Data are representative of three independent experiments with n1 = 5, n2 ≥ 8, n3 ≥ 9 mice for (A); one experiment with n = 10 mice per cohort for (B), (C), and (D); and one experiment with n ≥ 3 biological replicates for (E)-(H).

**FIGURE 7.** The role of AM in the premetastatic niche. C5a/C5aR-regulated proliferation of AM leads to an increase in the number of AM in the premetastatic lungs. Preconditioned by primary breast tumor, AM suppress antitumor T cell immunity in the lungs. AM suppress IFN-γ production in CD4+ T cells (Th1) that are directly cytotoxic to tumor cells in the context of MHC II by regulating TGF-β in lung cells or by inhibiting lung DC. AM also downregulate MHC II expression on tumor cells (TC), thereby preventing their interactions with tumor-specific cytotoxic CD4+ T cells. The alterations in antitumor immunity mediated by AM predispose lungs to metastasis.
the generation of effector Th1 cells, which supports the concept that AM can directly suppress T cells (10). However, AM depletion also increased the number and maturation of lung DC, which corresponds to previous studies in non tumor conditions demonstrating enhanced DC function and migration of these cells to alveolar spaces in AM-depleted animals (42). The impact of AM depletion on the function of DC also corresponds to our findings that AM regulate Th1/Th2 polarization. Furthermore, we observed that AM increased the production of TGF-β in hematopoietic lung cells. Because TGF-β suppresses production of inflammatory cytokines by AM (43), this mechanism could be responsible for the paracrine regulation of immunosuppressive functions of AM in the tumor-bearing host. Although AM represent the largest population of macrophages in the lungs, blood monocytes are recruited to the lungs by CCL2 and differentiate into macrophages during inflammation or in the tumor-bearing host (2, 44). However, the functional and phenotypic characteristics of these macrophages are different from those of AM (2, 10). Importantly, we found that clodronate liposomes administered intranasally efficiently depleted AM but did not affect monocyte-derived macrophages. Therefore, the consequences of clodronate liposome treatment on lung immunity and metastasis should be attributed to the absence of AM.

In view of recent studies demonstrating the nonhematopoietic origin of TRM (5), functions of these cells in health and disease require further investigation. Our current work suggests the contribution of these cells represented by AM to the premetastatic niche in the lungs (Fig. 7). In summary, in the presence of primary breast tumors, AM acquire T cell suppressive functions, facilitating metastasis to their resident organ. Selective depletion of AM leads to a reduction of metastases in the lungs and synergizes with CsA inhibition in preventing lung metastasis. Therefore, this study points to AM as a novel target for anticancer therapy.

Acknowledgments

We thank Dr. Laurence Wood, Texas Tech University Health Science Center, for providing quantitative PCR primers and the B16 cell line.

Disclosures

The authors have no financial conflicts of interest.

References

2. Qian, B. Z., J. Li, H. Zhang, T. Kitamura, J. Li, J. Li, E. A. Kaiser, L. A. Snyder, and J. W. Pollard. 2011. CCL2 recruits inflammatory monocytes to the lungs by CCL2 and differentiate into macrophages during inflammation or in the tumor-bearing host (2, 44). However, the functional and phenotypic characteristics of these macrophages are different from those of AM (2, 10). Importantly, we found that clodronate liposomes administered intranasally efficiently depleted AM but did not affect monocyte-derived macrophages. Therefore, the consequences of clodronate liposome treatment on lung immunity and metastasis should be attributed to the absence of AM.

In view of recent studies demonstrating the nonhematopoietic origin of TRM (5), functions of these cells in health and disease require further investigation. Our current work suggests the contribution of these cells represented by AM to the premetastatic niche in the lungs (Fig. 7). In summary, in the presence of primary breast tumors, AM acquire T cell suppressive functions, facilitating metastasis to their resident organ. Selective depletion of AM leads to a reduction of metastases in the lungs and synergizes with CsA inhibition in preventing lung metastasis. Therefore, this study points to AM as a novel target for anticancer therapy.


