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Cutting Edge: Mast Cells Critically Augment Myeloid-Derived Suppressor Cell Activity

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Myeloid-derived suppressor cells (MDSCs) are primarily recognized for their immunosuppressive properties in malignant disease. However, their interaction with other innate immune cells and their regulation of immune responses, such as in parasitic infection, necessitate further characterization. We used our previously published mouse model of MDSC accumulation to examine the immunoregulatory role of MDSCs in B16 melanoma metastasis and Nippostrongylus brasiliensis infection. In this study, we demonstrate that the activity of MDSCs is dependent on the immune stimuli and subset induced. Monocytic MDSCs predictably suppressed antitumor immune responses but granulocytic MDSCs surprisingly enhanced the clearance of N. brasiliensis infection. Intriguingly, both results were dependent on MDSC interaction with mast cells (MCs), as demonstrated by adoptive-transfer studies in MC-deficient (KitW$^{-}$/W$^{-}$) mice. These findings were further supported by ex vivo cocultures of MCs and MDSCs, indicating a synergistic increase in cytokine production. Thus, MCs can enhance both immunosuppressive and immunosupportive functions of MDSCs. The Journal of Immunology, 2012, 189: 511–515.

Acute inflammatory reactions stimulate the development and recruitment of myeloid lineage cells, including neutrophils and macrophages. Unresolved inflammation increases myelopoiesis, shifting the distribution of myeloid subpopulations. This results in the premature bone marrow release of a heterogeneous population of mononuclear (CD11b$^{+}$Ly6Chigh) and polymorphonuclear (CD11b$^{+}$Ly6-G$^{+}$) cells, known as myeloid-derived suppressor cells (MDSCs). These cells are widely studied in the context of neoplasia. MDSCs exert their prooncstatic effects through the release of small soluble oxidizers, the depletion of essential amino acids from the local extracellular environment, and the impairment of T cell Ag recognition, all ultimately leading to T cell suppression (1). This MDSC-mediated immune suppression and impairment of cancer immunotherapy has been extensively investigated (2). However, by limiting immune activation, MDSCs can serve a beneficial role in transplantation, autoimmunity, and sepsis (3). This dual role of MDSCs depends upon NO production, the cytokine milieu, and the interaction among MDSCs, T cells, and NK cells (3–6).

Another interaction that may be physiologically significant is MDSC/mast cell (MC) collaboration. Although MCs have been well documented to mediate allergic inflammation, their demonstrated involvement in neoplastic disease is more recent. MCs were shown to accumulate in the tumor microenvironment and correlate with poor prognosis (7). This is largely due to the secretion of various proinflammatory cytokines produced by the MCs (8). Additionally, MCs contribute to MDSC recruitment to the tumor site via the production of 5-lipoxygenase and IL-17 (9, 10). Given that MC-deficient mice have weakened tumor growth and that MCs recruit MDSCs to the tumor microenvironment, it is necessary to further examine the contribution of this interaction to tumor metastasis. Furthermore, although several studies indicate the expansion, recruitment, and activity of MDSCs in parasitic infections, they do not clarify the extent to which MDSCs regulate this immune response (11).

Therefore, we analyzed the effect of MDSCs in anti-helminth immunity and B16 metastasis, as well as the interaction with MCs during the immune response. We used our previously published mouse model of MDSC accumulation in a tumor-free environment (12). In these mice, the overexpression of a distichetrin and metalloproteinase 10 (ADAM10) promotes the expansion of MDSCs that are analogous to tumor-induced MDSCs. Adoptive-transfer (AT)

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Abbreviations used in this article: ADAM10, a distichetrin and metalloproteinase 10; AIT, adoptive immunotherapy; AT, adoptive transfer; A10Tg, a distichetrin and metalloproteinase 10 Tg; BMMC, bone marrow-derived mast cell; CYP, cyclophosphamide; GEM, gemcitabine; L3, stage 3 larvae; MC, mast cell; MDSC, myeloid-derived suppressor cell; VCU, Virginia Commonwealth University; WT, wild-type.
studies using either C57BL/6 (wild-type [WT]) or MC-deficient Kit−/− mice were performed in parallel. Using two model systems, we demonstrate that the activity of MDSCs is variable, because granulocytic MDSCs enhanced antiparasite immunity, whereas monocytic MDSCs promoted B16 metastasis. Intriguingly, both activities depend upon the presence of MCs. This intercellular communication leads to augmentation of Th2-polarizing cytokines that are beneficial in the case of parasitic infection but maladaptive in neoplasia. Thus, our findings indicate that MDSCs have an immunomodulatory role that is largely dependent on the type of stimuli, the MDSC population involved, and the presence of MCs.

Materials and Methods

Mice

Mice were kept at Virginia Commonwealth University (VCU) in accordance with the humane treatment of laboratory animals set forth by the National Institute of Health and the American Association for the Accreditation of Laboratory Animal Care. C57BL/6 ADAM10 Tgs (A10Tgs) were generated with the VCU Transgenic Mouse Core, as previously described (12), and littermates were used as controls. Pmel-1 and Kit−/− mice were purchased from The Jackson Laboratory. All mouse protocols were approved by the VCU Institutional Animal Care and Use Committee.

Cell lines

The LLC cell line was obtained from the American Type Culture Collection. B16 melanoma and GM-CSF–B16 cells were provided by H.D.B. and maintained as previously described (13). Mouse bone marrow-derived MCs (BMMCs) were generated from mice through isolation and subsequent culture of bone marrow cells in complete RPMI 1640.

Isolation of MDSCs and AT

Spleens were harvested from A10Tg or LLC-bearing WT mice, depending upon the experimental protocol. They were then dispersed into single-cell suspensions and filtered through 70-μm nylon mesh strainers (Invitrogen). Erythrocytes were lysed using an ammonium chloride potassium lysing buffer (Quality Biological). Gr-1+, Ly6G+, or Ly6C+ cells were purified from A10Tg spleens using the EasySep PE-Selection kit (Stem Cell Technologies), according to the manufacturer’s protocol. For AT studies, 5–10 × 10⁶ naive A10Tg MDSCs were injected into the tail vein of each experimental group. For T cell depletion, mice were injected i.p. with 200 μg anti-CD4 (GK1.5) and anti-CD8 (2.43) Abs on days

Arginase and NO detection

MDSC cytoplasmic extracts were prepared from isolated A10Tg- and LLC-bearing hosts and tested for urea production (DARG-200; BioAssay Systems). NO was measured using Greiss Reagent (G7921; Molecular Probes).

T cell suppression assays

For polyclonal T cell activation, CD90.2+ T cells were sorted from spleen and activated with immobilized anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/ml) in 96-well plates. Sorted MDSCs (Ly6G+, Gr-1+, and Ly6G−) from A10Tg or LLC tumor-bearing mice were then added increasing T cell/MDSC ratios. For Ag-specific T cell suppression assays, soluble gp100 (1 μg/ml) was added to defined ratios of pml-1 transgenic splenocytes and A10Tg MDSCs. After 54 h of culture, 1 μCi [3H]thymidine was added to each well for an additional 18 h, and thymidine incorporation was measured. BMMC coculture with A10Tg MDSCs

BMMCs were derived from femurs of WT naive mice cultured in complete RPMI 1640 containing 20% WEHI-3–conditioned medium over a 4-wk period. They were resuspended at 0.5 × 10⁶ cells/ml, loaded with 0.5 μg/ml mouse IgE, and cultured with MDSCs at a 1:1 ratio in 10 ng/ml IL-3 and 10 ng/ml GM-CSF overnight. MCs were activated by DNP-human serum albumin (20–100 ng/ml), and supernatants were collected 6 or 18 h after cross-linking for ELISA.

Adaptive immunotherapy of B16 melanoma

T cells were prepared for adoptive immunotherapy (AIT), as previously described (13). Recipient mice were injected i.v. with 0.25 × 10⁶ B16 cells. One day prior to AIT, mice were treated i.p. with 2 mg cyclophosphamide (CYP) and 1.2 mg gemcitabine (GEM) and were treated every 5 d with GEM only. After 14 d, lung nodules were counted, as previously described (13, 14); if too many to count, they were assigned a count of 250.

N. brasiliensis infection, GEM treatment, and AT

N. brasiliensis larvae were generously provided by Joe Urban (Agriculture Research Station, Beltsville, MD) and were maintained as previously described (15). Each experimental group of mice was infected with ~650 stage 3 larvae (1.3) N. brasiliensis. Fecal egg burdens were enumerated on days 5–13, and serum was collected by tail vein nick. For mice injected with GEM, 1.2 mg was injected i.p. on day 0 and repeated every 5 d throughout the experiment. For AT studies, WT mice were injected with 5–10 × 10⁶ naive A10Tg MDSCs via tail vein injection starting at day 0 of infection and repeated every 3 d thereafter. Additional groups of animals were sacrificed on day 7 postinfection, and the proximal halves of the small intestines were harvested. Adult worms were purified and enumerated.

Statistical analysis

The p values were calculated using unpaired two-tailed Student t tests or unpaired Mann–Whitney analysis. Error bars represent the SEM between samples.

Results and Discussion

MDSCs from A10Tg mice are phenotypically and functionally analogous to tumor-induced MDSCs

Although MDSC accumulation is a byproduct of ADAM10 overexpression in early hematopoietic progenitors, ADAM10 expression is not altered in these cells (12). To ascertain whether A10Tg-derived MDSCs are functional, ex vivo T cell suppression assays were performed. A10Tg MDSCs inhibited T cell proliferation of both A10Tg and WT purified T cells under polyclonal (Supplemental Fig. 1A, 1B) and Ag-specific (Supplemental Fig. 1C) conditions in a manner parallel to tumor-derived MDSCs (Supplemental Fig. 1D). Tumor-derived MDSCs can be divided into granulocytic (CD11b+Ly6G±Ly6Chigh) and more immunosuppressive monocyteic (CD11b+Ly6G−Ly6Chigh) subsets (16). Accordingly, monocytic MDSCs possessed more suppressive potential. Furthermore, similar to T cells from tumor-bearing hosts, A10Tg T cells exhibited reduced levels of L-selectin (CD-62L), a receptor required for homing of naive T cells to the lymph nodes (Supplemental Fig. 1E, 1F) (17). MDSCs impair the T cell response through the activity of arginase 1 and inducible NO synthase. Accordingly, A10Tg MDSCs had levels of these enzymes that were comparable to tumor-derived MDSCs (Supplemental Fig. 1G, 1H) (2). These ex vivo observations indicate that MDSCs present in tumor-free A10Tg animals behave similarly to tumor-derived MDSCs.

Monocytic MDSCs promote B16 metastasis

We used the B16 melanoma model to examine in vivo activities of MDSCs from A10Tg mice. B16 was injected i.v.; although not a true metastasis, this allows comparison of tumor colonization into the lung with and without MDSCs and is commonly used to assess AIT (13). When challenged with B16, A10Tg animals were more susceptible to metastasis than were WT controls (Fig. 1A). The A10Tg animals had tumor nodules that were too numerous to count and were consequently assigned a count of 250. Parallel to the ex vivo
assay, the suppressive activity of MDSCs was attributed to the monocytic population. When WT mice were challenged with B16 and AT of either monocytic or granulocytic MDSCs, melanoma metastasized more aggressively in mice given the monocytic subset, and granulocytic MDSCs had no effect (Supplemental Fig. 2A). AIT exerted a minimal effect on A10Tg mice, suggesting that the presence of MDSCs diminished the activity of adoptively transferred T cells. Although AIT treatment did not completely abolish metastasis in WT lungs, it induced a significant decrease in metastatic lesions. Given that lymphodepleting chemotherapy is known to enhance AIT (18), the protocol was modified to incorporate CYP and GEM. CYP lymphodepletes recipient mice, permitting homeostatic proliferation of transferred T cells; GEM preferentially decreases MDSC levels in tumor-bearing mice (19, 20). CYP and GEM alone and in combination in WT mice did not significantly alter metastasis, indicating that they have minimal effects on tumor cells (Supplemental Fig. 2C). However, GEM selectively diminished the MDSC population in A10Tg mice without affecting lymphocyte levels (Supplemental Fig. 2B). A10Tg mice treated with GEM alone exhibited an antitumor response comparable to WT counterparts, demonstrating that T cell function is restored upon MDSC depletion (Fig. 1B, 1C). Additionally, the combination of GEM, CYP, and AIT (tritherapy) resulted in complete regression of metastasis in both WT and A10Tgs (Fig. 1B, 1C). FIGURE 1. MDSC depletion restores the antitumor response and prevents metastatic progression of B16 melanoma in A10Tg mice. (A) Number of lung metastases in WT and A10Tg animals challenged with B16 melanoma with (filled bars) or without (open bars) AIT (A), as described in Materials and Methods. Number of lung metastases of WT (B) or A10Tg (C) mice challenged with B16 and treated as in (A), with the addition of CYP (C) and GEM (G). (D) Representative lungs of WT and A10Tg mice with AIT, with and without CYP and GEM. Data represent five mice/group. *p < 0.05 versus respective untreated controls. ND, None detected.

Next, we examined the effect of MDSCs on the immune response in antiparasite immunity. Although MDSC accumulation following N. brasiliensis infection has been reported (11), the direct immunomodulatory role of MDSCs in anti-N. brasiliensis responses has not been examined. In N. brasiliensis infection, a WT response is characterized by production of Th2-associated cytokines that ultimately mediate adult worm clearance 10–12 d postinoculation (21). Following N. brasiliensis challenge, A10Tg animals had significantly reduced adult worm burden in A10Tgs (Fig. 2B). This indicated that MDSCs may facilitate parasite clearance. To determine whether the increased worm clearance correlated with an increase in Th2 cytokines, we examined a panel of Th2 cytokines. Analysis of peak infection (day 7) serum levels of IL-4, IL-5, and IL-13 demonstrated significantly enhanced levels in N. brasiliensis-infected A10Tg animals compared with infected littermates (Fig. 2C). Additionally, IL-17 and IL-33, which were reported to recruit MDSCs, were also elevated in serum of A10Tg mice (9, 22). These serum cytokines decreased as infection began to decline (data not shown).

To determine whether enhanced N. brasiliensis clearance is dependent on MDSC activity, MDSCs were depleted from A10Tg mice. GEM treatment resulted in elevated egg counts, and GEM selectively diminished the MDSC population in A10Tg mice without affecting lymphocyte levels (Supplemental Fig. 2B). A10Tg mice treated with GEM alone exhibited an antitumor response comparable to WT counterparts, demonstrating that T cell function is restored upon MDSC depletion (Fig. 1B, 1C). Additionally, the combination of GEM, CYP, and AIT (tritherapy) resulted in complete regression of metastasis in both WT and A10Tgs (Fig. 1B, 1C). FIGURE 1. MDSC depletion restores the antitumor response and prevents metastatic progression of B16 melanoma in A10Tg mice. (A) Number of lung metastases in WT and A10Tg animals challenged with B16 melanoma with (filled bars) or without (open bars) AIT (A), as described in Materials and Methods. Number of lung metastases of WT (B) or A10Tg (C) mice challenged with B16 and treated as in (A), with the addition of CYP (C) and GEM (G). (D) Representative lungs of WT and A10Tg mice with AIT, with and without CYP and GEM. Data represent five mice/group. *p < 0.05 versus respective untreated controls. ND, None detected.

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in A10Tg mice similar to WT levels (Fig. 2A). Additionally, *N. brasiliensis* infection resulted in a modest 4–5-fold increase in MDSCs in WT spleens from days 0 to 14. This level of WT MDSCs is sufficient to promote *N. brasiliensis* clearance and prevent a chronic infection. However, GEM-mediated MDSC depletion in WT mice significantly exacerbated the peak level of *N. brasiliensis* infection (data not shown). Taken together, this data indicate that the enhanced immune response in A10Tgs is a consequence of elevated MDSC levels. Next, to eliminate off-target effects of GEM, A10Tg MDSCs were purified and adoptively transferred to WT mice over the course of *N. brasiliensis* infection. Like the MDSCs that were observed accumulating following *N. brasiliensis* infection, the transferred MDSCs also contained both Ly-6G+ and Ly6C+ populations. AT of MDSCs into WT mice significantly increased the level of WT MDSCs is sufficient to promote *N. brasiliensis* expulsion. Surprisingly, AT of MDSCs into MC-deficient mice did not enhance *N. brasiliensis* expulsion, indicating that an MDSC/MC interaction is critical for MDSC-mediated anti-*N. brasiliensis* responses (Fig. 2F).

To assess this interaction ex vivo, MCs were cocultured with MDSCs. This resulted in a synergistic increase in IL-6, IL-13, TNF-α, and MIP-1α (Fig. 3). MCs also contribute to MDSC-mediated immune suppression in B16 melanoma

Given that MDSCs require MCs to enhance anti-*N. brasiliensis* immunity, the B16 metastasis study was re-evaluated in MC-deficient mice. Several groups demonstrated that MCs recruit MDSCs, which accumulate in the tumor microenvironment and correlate with poor prognosis. In addition, MC-deficient mice have decreased rates of tumor growth that increases to WT levels subsequent to MC reconstitution (26). Therefore, we anticipated that B16 melanoma cells would metastasize more slowly in KitWsh/Wsh mice compared with WT controls, but it would be enhanced with MDSC AT, KitWsh/Wsh and WT mice were injected with B16, with and without AT of MDSCs. WT mice were more susceptible to B16 metastasis compared with KitWsh/Wsh mice. Upon MDSC AT, WT mice exhibited similar levels of tumor nodules as did B16 A10Tg, indicating the direct contribution of MDSCs to the immune response. However, much to our surprise, AT of MDSCs into KitWsh/Wsh mice failed to increase B16 metastasis (Fig. 4). Thus, these results demonstrate that MDSCs promote B16 melanoma metastasis in an MC-dependent manner and indicate that MDSC/MC interactions significantly enhance the MDSC-mediated suppression of antitumor immunity.

The A10Tg mouse affords a unique tool to characterize the immune-modulatory potential of MDSCs in an environment free of established tumor. Because the A10Tgs suffer no confounding pathology, this was an ideal system in which to elucidate the immunomodulatory roles of MDSCs. We

**FIGURE 3.** MC/MDSC interaction augments cytokine production. BMMCs were cocultured with A10Tg MDSCs, as described in Materials and Methods. Supernatants were collected and analyzed for the production of IL-6 (A), IL-13 (B), MIP-1α (C), and TNF-α (D). The data represent pooled spleens of at least three mice/group. *p < 0.05 versus MCs alone.

**FIGURE 4.** MC/MDSC interaction is required for MDSC-mediated immune suppression. Photographic representation (A) and quantification (B) of B16 lung metastasis in control C57 and KitWsh/Wsh mice, with and without AT of MDSCs. The data are representative of at least five mice/group. *p < 0.05 versus WT alone or KitWsh/Wsh with MDSCs.
used this system to investigate roles for MDSCs in both neoplasia and helminth infection. In B16 metastasis, monocyctic MDSCs strongly suppressed the immune response, whereas granulocytic MDSCs failed to promote metastasis. However, in N. brasiliensis infection, the granulocytic MDSCs enhanced the antiparasitic immune response, and the monocyctic MDSCs had no effect. AT studies demonstrated that this differential immune regulation by MDSCs occurs in an MC-dependent manner. The ex vivo coculture studies indicate that MDSC/MC interactions may modulate immune responses by increasing cytokine production. MDSCs traffic to MCs, affording a synergistic Th2-skewed immune response. Although undesirable in the context of neoplasia, the observed response is ideal in helminth infections. Overall, our data indicate that inhibition of MC function may serve as a novel target to inhibit MDSC activity.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure 1. MDSCs from ADAM10Tg mice are phenotypically and functionally analogous to tumor-derived MDSCs. (A) Proliferation of WT and ADAM10Tg (B) T cells or (C) pmel-1 TCR transgenic splenocytes in the presence of increasing amounts of CD11b+ MDSCs (Ly6G+,Ly6G-, or Gr-1+); WT and ADAM10Tg T cells were stimulated with immobilized anti-CD3 and soluble anti-CD28. (D) Tumor derived MDSCs were purified from LLC bearing mice and used in suppression assays with Pmel1 splenocytes at increasing ratios (T cells or splenocytes: MDSCs, *p<0.05). All Pmel-1 splenocytes were stimulated with soluble gp100. (E) Cell surface expression of L-selectin (CD62L) by CD4+ and CD8+ (F) gated T cells from peripheral lymph nodes (PLNs). Lysates of MDSCs derived from Lewis Lung Carcinoma bearing WT hosts and ADAM10Tg MDSCs were analyzed for the activity of (G) Arginase by urea production and (H) Nitric oxide by Greiss Reagent. The data is representative of at least three independent experiments with splenocytes from three or more mice.
Supplementary Figure 2

A

Average Number of Mets

WT  LY6G  LY6C

B

Total Cell Number (10^6)

Day

0  5  10  15  20

MDSCs

B Cells

T Cells

C

Average Number of Mets

Untreated  GEM Only  CYP Only  CYP + GEM  AIT + CYP + GEM

0  100  200  300

/ g2 / g1 / g6 / g17 / g14 / g11 / g8 / g12 / g8 / g13 / g16 / g7 / g15 / g18 / g1 / g5 / g10 / g9 / g17 / g15 / g8 / g1 / g19 / g1 / g3 / g1
Supplementary Figure 2. Gemcitabine selectively depletes MDSCs, which allows for effective AIT with tumor specific T cells. (A) Quantification of B16 lung metastasis in WT C57 with AT of either granulocytic (CD11b^+Ly6G^+) or monocytic (CD11b^+Ly6C^+) MDSCS. (B) Cytometric analysis of peripheral blood leukocyte levels in ADAM10Tg mice following i.p. injections with gemcitabine (upward arrow) every five days for three weeks, *p<0.05. (C) Number of B16 lung metastases in mice treated with AIT comprised of pmel-1 transgenic T cells and chemotherapeutics as described in Methods. More than five mice were used per group in three independent experiments. *p<0.05 in comparison to respective untreated controls and #p<0.05 in comparison to respective AIT+ CYP treatment.