

Cross-Talk between Myeloid-Derived Suppressor Cells and Macrophages Subverts Tumor Immunity toward a Type 2 Response¹

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Although the immune system has the potential to protect against malignancies, many individuals with cancer are immunosuppressed. Myeloid-derived suppressor cells (MDSC) are elevated in many patients and animals with tumors, and contribute to immune suppression by blocking CD4⁺ and CD8⁺ T cell activation. Using the spontaneously metastatic 4T1 mouse mammary carcinoma, we now demonstrate that cross-talk between MDSC and macrophages further subverts tumor immunity by increasing MDSC production of IL-10, and by decreasing macrophage production of IL-12. Cross-talk between MDSC and macrophages requires cell-cell contact, and the IL-12 decrease is dependent on MDSC production of IL-10. Treatment with the chemotherapeutic drug gemcitabine, which reduces MDSC, promotes rejection of established metastatic disease in IL-4R α ^{-/-} mice that produce M1 macrophages by allowing T cell activation, by maintaining macrophage production of IL-12, and by preventing increased production of IL-10. Therefore, MDSC impair tumor immunity by suppressing T cell activation and by interacting with macrophages to increase IL-10 and decrease IL-12 production, thereby promoting a tumor-promoting type 2 response, a process that can be partially reversed by gemcitabine. *The Journal of Immunology*, 2007, 179: 977–983.

Tumor-induced immune suppression is widespread in patients and experimental animals with malignant tumors and is likely to be a significant impediment to immunotherapy and immunosurveillance of cancer. Multiple mechanisms are thought to facilitate tumor-induced immune suppression, with myeloid-derived suppressor cells (MDSC)³ (1), previously called immature myeloid cells (2) or myeloid suppressor cells (3–5), being a major contributor. MDSC are found in many cancer patients, including those with head and neck, breast, nonsmall cell lung, and renal cancers (6–10), and are induced by a variety of factors, including vascular endothelial growth factor (11), GM-CSF (12, 13), and proinflammatory cytokines such as IL-1 β (14, 15). They interfere with tumor immunity and promote tumor growth by inhibiting tumor cell cytotoxicity mediated by NK cells (16), and by blocking the activation of tumor-reactive CD4⁺ and CD8⁺ T cells (2, 3, 5, 17). MDSC are also thought to facilitate immune suppression and tumor progression by inducing the accumulation of immunosuppressive T regulatory cells (18).

In addition to T and NK cells, macrophages are also important effector cells in tumor immunity, and depending on their phenotype can either promote or prevent tumor progression. Two nomenclatures have been used to describe macrophage phenotypes. So-called classically activated macrophages are activated by IFN- γ and LPS and have an IL-12^{high}, IL-10^{low} phenotype, whereas alternatively activated macrophages are activated by IL-4 and IL-13 and have an IL-12^{low}, IL-10^{high} phenotype (19). Because their cytokine profile for IL-12 and IL-10 is similar to that of CD4⁺ Th1 and Th2 cells, respectively (20), classically activated and alternatively activated macrophages have also been referred to as M1 and M2 macrophages, respectively (20–22). M1 macrophages are tumoricidal and promote tumor rejection, whereas M2 macrophages facilitate tumor progression (21–23). Many progressively growing solid tumors contain tumor-associated macrophages, which promote tumor progression through a variety of mechanisms (24, 25), and which have the phenotype of M2 macrophages (26). Because MDSC inhibit T cells in a non-MHC-restricted fashion and without regard for T cell specificity (5), we hypothesized that they may also suppress tumor immunity by altering macrophage phenotype and subsequent activity. Using the spontaneously metastatic mouse 4T1 mammary carcinoma, we now report that MDSC and macrophages of tumor-bearing individuals engage in cross-talk that results in a reduction in IL-12 production by macrophages and an increase in IL-10 production by MDSC, thereby skewing innate and adaptive immunity toward a type 2 response that favors tumor progression.

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³ Abbreviations used in this paper: MDSC, myeloid-derived suppressor cell; DC, dendritic cell; HA, influenza hemagglutinin; nor-NOHA, N^w-hydroxyl-nor-L-arginine; PDCA, plasmacytoid DC; PEC, peritoneal exudate cell.

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Materials and Methods

Mice

BALB/c, IL-4R α -deficient (IL-4R α ^{-/-}), and IL-10-deficient (IL-10^{-/-}) (both on a BALB/c background) mice were purchased from The Jackson Laboratory. TS1 mice on a BALB/c background and transgenic for a TCR reactive to influenza hemagglutinin (HA) peptide 110–119 (SFERFEIFPK;

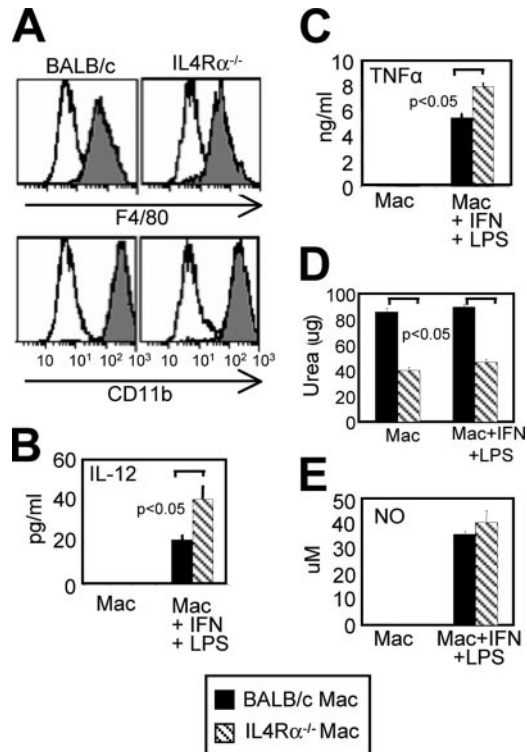


FIGURE 1. IL-4R $\alpha^{-/-}$ mice constitutively make M1 macrophages, whereas BALB/c mice make M2 macrophages. *A*, Peritoneal macrophages from BALB/c and IL-4R $\alpha^{-/-}$ mice stained with mAbs to F4/80 or CD11b (gray peaks) or isotype control mAb (white peaks). Data are from individual mice and are representative of six mice tested. Supernatants of activated or not activated BALB/c and IL-4R $\alpha^{-/-}$ macrophages (Mac) assayed: *B*, by ELISA for IL-12; *C*, by ELISA for TNF- α ; *D*, for arginase activity by arginase-catalyzed formation of urea; or *E*, for NO using the Greiss reagent. Data for *B*–*E* are the average \pm SD of triplicates from individual mice, and are representative of four independent experiments.

HA_{110–119} restricted to I-E^d (27) were provided by E. Fuchs (Johns Hopkins University, Baltimore, MD). Mice were bred and maintained according to National Institutes of Health guidelines in the University of Maryland animal facility. All animal procedures have been approved by the university's Institutional Animal Care and Use Committee.

Reagents and Abs

Heparin sodium salt (grade IA) from porcine intestinal mucosa was purchased from Sigma-Aldrich. Anti-mouse mAb to *c-kit* PE, CD3 FITC, CD4 PE, CD8 PE, CD11c FITC, Gr1 PE, CD11b FITC, isotype control rat IgG FITC, and rat IgG PE were from BD Pharmingen; mAb F4/80 FITC was from Caltag Laboratories. Rabbit anti-asialo GM1 was from Wako Pure Chemical. PE anti-mouse Fox P3 staining set and B220 mAb were from eBioscience. FITC anti-mouse plasmacytoid DC (PDCA)-1 mAb was from Miltenyi Biotec. ELISA duo set mAbs for IL-12, IL-10, and TNF- α were from R&D Systems. Influenza peptide HA_{110–119} was synthesized in the Biopolymer Core Facility at the University of Maryland. Diff-Quik stain set was from Dade Behring. Gemcitabine (Eli Lilly) was obtained from the University of Pennsylvania pharmacy.

Gemcitabine treatment

Mice were inoculated i.p. with 1.5 mg of gemcitabine in 50 μ l of saline twice per week for the first week and once per week thereafter. Treatment was started 1 day after 4T1 inoculation (primary tumor growth groups) or 1 day after surgery (postsurgery groups).

The 4T1 inoculation, tumor measurements, and surgery

Female mice were inoculated in the abdominal mammary gland with 7000 4T1 tumor cells, tumor diameters were calculated, and primary tumors were surgically removed, as described (28).

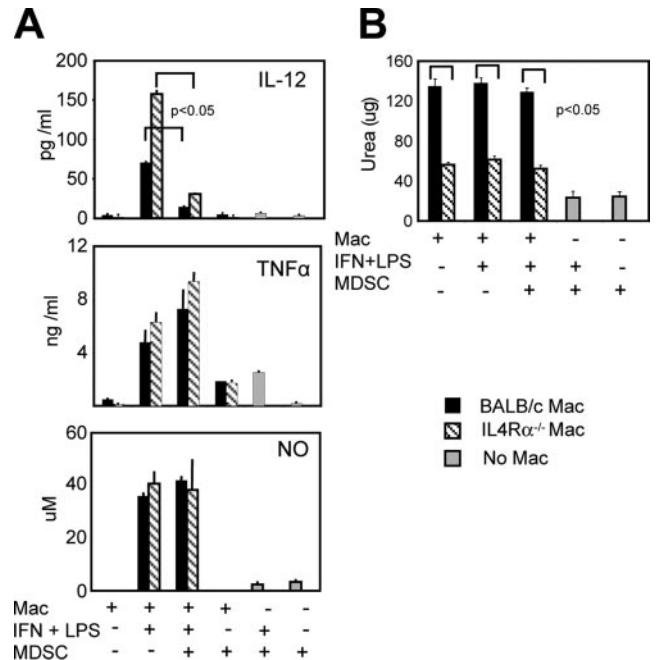


FIGURE 2. MDSC decrease macrophage production of IL-12. Peritoneal macrophages from BALB/c and IL-4R $\alpha^{-/-}$ mice were not activated or activated with LPS and IFN- γ , and cocultured with purified splenic MDSC from 4T1 tumor-bearing BALB/c mice. Culture supernatants were assayed for IL-12, TNF- α , or NO by ELISA (*A*) or for arginase activity by arginase-mediated catalysis of urea (*B*). Data are the average \pm SD of triplicates from two mice in each group and are representative of three independent experiments.

Flow cytometry and blood MDSC histology

Tumor-free and 4T1 tumor-bearing mice were bled from the tail into 500 μ l of a 0.008% heparin solution, and the RBC was removed by lysis, as described for splenocytes (5). Cells were stained with mAbs to MDSC markers Gr1 and CD11b and analyzed by flow cytometry, as described (5). Blood MDSC were stained using a Diff-Quik kit, as described (5).

T cell activation

T cell activation assays were performed, as described (5), except splenocytes were obtained from BALB/c TS1 mice and HA_{110–119} peptide was used at 10 μ M. MDSC used in the suppression assays were 85–90% Gr1⁺CD11b⁺ and were obtained from BALB/c mice with 8- to 10-mm-diameter 4T1 primary tumors.

Peritoneal macrophages, NO, arginase, IL-12, IL-10, and TNF assays

Mice were inoculated i.p. with 1 ml of 3% thioglycolate, and 4 days later peritoneal exudate cells (PEC) were harvested and RBC were lysed. PEC were >90% macrophages, as measured by F4/80 staining. Contaminating cells were dendritic cells (DC; CD11c⁺; \leq 8.3%), mast cells (*c-kit*⁺; \leq 1.6%), plasmacytoid DC (PDCA⁺; \leq 0.2%), and/or MDSC (CD11b⁺Gr1⁺; \leq 7%). Macrophages were plated at 7.5×10^5 cells/well/500 μ l DMEM with 10% FBS in 24-well plates, and incubated at 37°C in 5% CO₂ for 3 h. Nonadherent cells were then removed, and the attached cells were washed once with macrophage medium (DMEM with 5% FBS), then activated with 2 ng/ml IFN- γ and 100 ng/ml LPS for 16 h, as described (29). Culture supernatants were analyzed for NO using the Greiss reagent, and for TNF- α , IL-12, and IL-10 using ELISA kits (R&D Systems) in the University of Maryland cytokine core facility, or in our laboratory, as recommended by the manufacturer. Cells were lysed and arginase was quantified by measuring production of urea, as described (5).

Peritoneal macrophages and MDSC coculture experiments

MDSC were isolated from 4T1 tumor-bearing mice by magnetic bead sorting of Gr1⁺ cells using Miltenyi Biotec magnetic beads, as described (5). Purified MDSC were >90% Gr1⁺CD11b⁺. The 10% contaminating cells

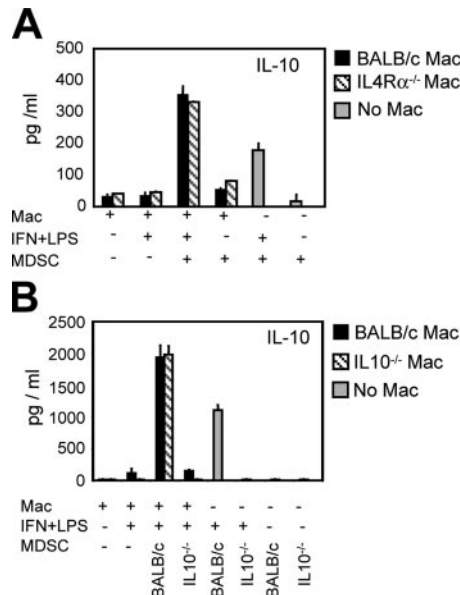
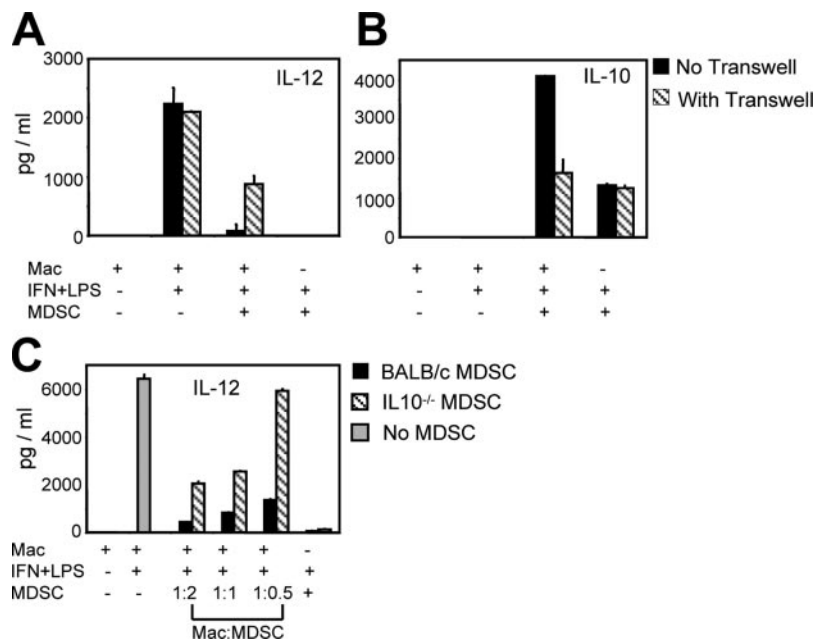


FIGURE 3. MDSC constitutively make IL-10 and are induced by macrophages to make more IL-10. *A*, Supernatants of the cultures from Fig. 2 were assayed by ELISA for IL-10. *B*, Peritoneal macrophages from BALB/c or IL-10 $^{-/-}$ mice were not activated or activated with LPS and IFN- γ , and cocultured with purified splenic MDSC from 4T1 tumor-bearing BALB/c or IL-10 $^{-/-}$ mice. Culture supernatants were assayed for IL-10 by ELISA. Data are the average \pm SD of triplicates from two mice in each group and are representative of two to three independent experiments.

included CD3 $^{+}$ CD4 $^{+}$ ($\leq 3.6\%$), CD3 $^{+}$ CD8 $^{+}$ ($\leq 2.1\%$), DC (CD11c $^{+}$; $\leq 2.5\%$), mast cells (*c-kit* $^{+}$; $\leq 4.4\%$), plasmacytoid DC (PDCA $^{+}$; $\leq 2.2\%$), and/or macrophages (F4/80 or CD11b single positive; $\leq 4.8\%$). MDSC were irradiated (2500 rad) and added to wells (1.5×10^6 MDSC/well/500 μ l macrophage medium) containing peritoneal macrophages. Culture supernatants were collected 16 h later and assayed for NO using the Greiss reagent, and for TNF- α , IL-12, and IL-10 by ELISA. Cells were washed once with excess PBS and lysed, and arginase was quantified by measuring production of urea, as described (5). The arginase inhibitor *N* $^{\omega}$ -hydroxyl-nor-L-arginine (nor-NOHA) or inducible NO synthase inhibitor *N* G -monomethyl-L-arginine was added to some wells. For some experiments, MDSC were placed in a 0.4- μ m Transwell chamber (Costar) in the 24-well plates.

FIGURE 4. MDSC-induced decrease in macrophage production of IL-12 and macrophage-induced increase in MDSC production of IL-10 require contact between MDSC and macrophages. Peritoneal macrophages from BALB/c mice were not activated or activated with LPS and IFN- γ , and cocultured with MDSC from 4T1 tumor-bearing mice or with MDSC from 4T1 tumor-bearing mice contained in a Transwell chamber. IL-12 (*A*) or IL-10 (*B*) was measured by ELISA. *C*, MDSC-induced decrease in macrophage production of IL-12 is IL-10 dependent. BALB/c peritoneal macrophages were not activated or activated with LPS and IFN- γ , and cocultured with decreasing quantities of MDSC from 4T1 tumor-bearing BALB/c or IL-10 $^{-/-}$ mice. IL-12 was measured by ELISA. Data are the average \pm SD of triplicates from two mice in each group and are representative of two independent experiments.



Statistics

Student's *t* test or the log rank test was used to determine statistical significance between experimental groups.

Results

MDSC decrease macrophage production of IL-12

If MDSC promote tumor progression by altering macrophage phenotype, then coculture of MDSC with M1 macrophages could skew macrophages toward an M2 phenotype. Polarization of macrophages toward the M2 or alternatively activated phenotype is regulated by IL-4 and IL-13, which bind with high affinity to plasma membrane receptors containing the IL-4R α chain, and signal through STAT6 (19). Herbert et al. (30) have demonstrated that in vitro activation of PEC from IL-4R $\alpha^{-/-}$ mice yields M1 (or classically activated) macrophages, because the PEC cannot respond to IL-4 and IL-13, whereas wild-type BALB/c mice produce M2 (or alternatively activated) macrophages. We have made use of these mouse strains to generate M1 and M2 macrophages for testing with MDSC. BALB/c and IL-4R $\alpha^{-/-}$ mice were inoculated i.p. with thioglycolate, and PEC were harvested 4 days later. Harvested cells were incubated in vitro for 16 h with LPS and IFN- γ (and their supernatants were assayed by ELISA for cytokines characteristic of M1 vs M2 macrophages). More than 90% of the in vitro PEC were macrophages as quantified by staining for the macrophage markers F4/80 and CD11b (Fig. 1A). Consistent with earlier reports, macrophages from IL-4R $\alpha^{-/-}$ mice are M1-like because they are IL-12 high (Fig. 1B), TNF- α^{high} (Fig. 1C), and arginase low (Fig. 1D), whereas macrophages from BALB/c mice are M2-like (IL-12 low , TNF- α^{low} , and arginase high). IL-10 was below the level of detection (< 31.2 pg/ml) in both macrophage populations, and NO levels did not differ (Fig. 1E).

To determine whether MDSC inhibit tumor immunity by altering macrophage phenotype, irradiated MDSC were cocultured with IFN- γ - and LPS-activated macrophages from IL-4R $\alpha^{-/-}$ or BALB/c mice, and culture supernatants or cell lysates were tested for IL-12, IL-10, TNF- α , NO, and arginase, molecules characteristic of the M1 and M2 phenotypes (22). To generate MDSC, BALB/c mice were inoculated in the abdominal mammary gland with 4T1 mammary carcinoma cells, and their spleens were

removed 3 wk later when their primary tumors were 9.47 ± 1.02 mm in diameter. MDSC were purified by magnetic bead sorting of the splenocytes for Gr1⁺ cells. The sorted population was >90% Gr1⁺CD11b⁺ as assessed by flow cytometry. IL-4R $\alpha^{-/-}$ macrophages were IL-12^{high}, and BALB/c macrophages were IL-12^{low} as previously seen, whereas MDSC made insignificant amounts of IL-12, even in the presence of IFN- γ and LPS (Fig. 2A). Addition of irradiated BALB/c MDSC decreased IL-12 production by both BALB/c and IL-4R $\alpha^{-/-}$ macrophages by 84 and 80%, respectively. In contrast, coculture of BALB/c MDSC with either BALB/c or IL-4R $\alpha^{-/-}$ macrophages did not alter macrophage production of TNF- α , NO (Fig. 2A), or arginase (Fig. 2B). MDSC derived from IL-4R $\alpha^{-/-}$ mice similarly reduced IL-12 production by IL-4R $\alpha^{-/-}$ and BALB/c macrophages (data not shown). The decrease in IL-12 is not due to irradiation, which is known to reduce IL-12 production by myeloid cells (31, 32), because only the MDSC and not the macrophages were irradiated in these experiments. Therefore, tumor-induced Gr1⁺CD11b⁺ MDSC reduced IL-12 production by both M1 and M2 macrophages, thereby skewing M1 macrophages toward a M2 phenotype and accentuating the phenotype of M2 macrophages.

Macrophages increase MDSC production of IL-10

Because IL-10 is a hallmark of type 2 responses, supernatants of the cultures of Fig. 2A were also assayed for IL-10 (Fig. 3A). When activated with LPS and IFN- γ , irradiated BALB/c MDSC made significant amounts of IL-10, and addition of BALB/c or IL-4R $\alpha^{-/-}$ macrophages further increased the production of IL-10. The increased production of IL-10 in the presence of both MDSC and macrophages was synergistic because it exceeded the sum of the IL-10 levels by the separate MDSC and macrophage populations. Similar results were obtained using nonirradiated MDSC (data not shown). Therefore, there is cross-talk between MDSC and M1 or M2 macrophages that results in increased production of the type 2 cytokine IL-10.

Although the data of Fig. 3A demonstrate synergy between MDSC and macrophages, they did not indicate whether the MDSC and/or the macrophages were making the additional IL-10. To address this question, peritoneal macrophages and 4T1-induced MDSC were cocultured as per Fig. 2A, except the MDSC and peritoneal macrophages were obtained from BALB/c wild-type or IL-10 knockout mice (Fig. 3B). Activated BALB/c MDSC cocultured with BALB/c or IL-10^{-/-} macrophages produced synergistic levels of IL-10. In contrast, IL-10^{-/-} MDSC cocultured with either BALB/c or IL-10^{-/-} macrophages did not produce significant amounts of IL-10. Therefore, macrophages induce MDSC to produce elevated levels of the type 2 cytokine IL-10.

Cross-talk between MDSC and macrophages is cell contact dependent

Figs. 2 and 3 demonstrate that there is cross-talk between MDSC and macrophages with MDSC down-regulating IL-12 production by macrophages, and macrophages increasing IL-10 production by MDSC. To determine whether the cross-talk requires physical contact between the MDSC and macrophages, BALB/c macrophages and MDSC were cocultured in the presence (with Transwell) or absence (no Transwell) of a semipermeable membrane that separated the two cell populations, and supernatants were assayed for IL-10 and IL-12 (Fig. 4). Macrophage production of IL-12 was reduced >95% in the absence of the Transwell and only reduced by 58% in the presence of the Transwell (Fig. 4A). Macrophage-induced MDSC production of IL-10 was 60% less in the presence of a Transwell and was not significantly different from MDSC production of IL-10 in the absence of macrophages (Fig. 4B).

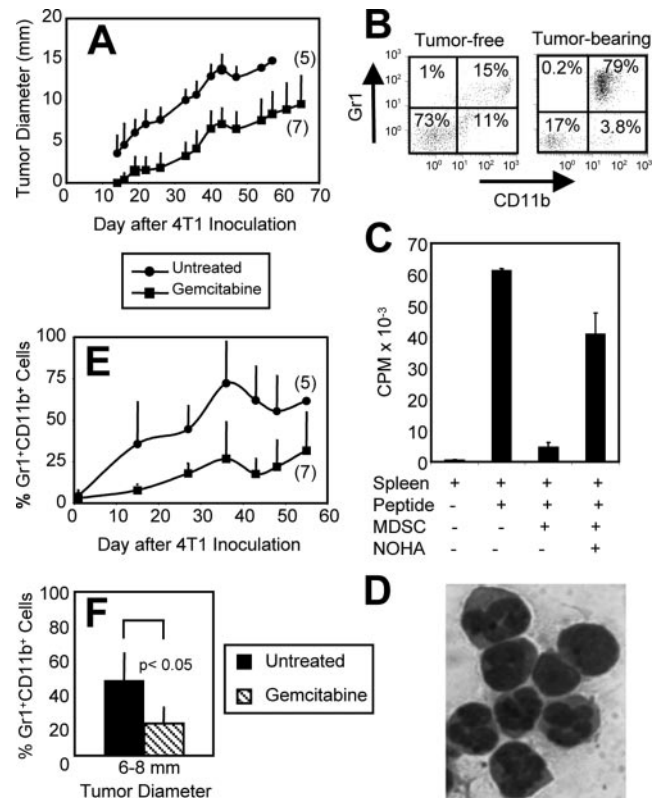


FIGURE 5. Gemcitabine treatment delays growth of 4T1 primary tumors and reduces the quantity of MDSC. *A*, BALB/c mice inoculated with 4T1 tumor cells were either treated or not treated with gemcitabine, and primary tumor growth was measured by calipers. *B*, Blood of tumor-bearing mice contains high levels of Gr1⁺CD11b⁺ MDSC. White blood cells from tumor-free BALB/c mice or BALB/c mice with 29-day-old 4T1 tumors were stained for Gr1 and CD11b. Percentage of cells in each quadrant by the production of arginase. CD4⁺ TS1 transgenic T cells were cocultured with HA peptide \pm irradiated MDSC from BALB/c tumor-bearing mice, and T cell proliferation was measured by [³H]thymidine uptake. nor-NOHA, an inhibitor of arginase, was added to some wells. *D*, MDSC in the blood of tumor-bearing BALB/c mice are a heterogeneous population of immature myeloid cells. Gr1⁺CD11b⁺ MDSC from 4T1 tumor-bearing mice stained with Diff-Quik stain. *E*, Gemcitabine treatment reduces the quantity of MDSC in tumor-bearing mice. BALB/c mice were inoculated with 4T1 tumor cells on day 0, started on gemcitabine treatment on day 1, and bled 1 day after each gemcitabine treatment to determine the percentage of Gr1⁺CD11b⁺ MDSC in the blood. *F*, Values from *E* plotted as tumor diameter vs percentage of MDSC. Numbers in parentheses indicate the number of mice/group. Data are representative of two to three independent experiments.

Therefore, optimal MDSC-induced reduction of macrophage-produced IL-12 and macrophage-induced up-regulation of MDSC-produced IL-10 require cell-to-cell contact between the MDSC and macrophages.

Because IL-10 is a type 2 cytokine, we tested whether MDSC down-regulation of macrophage-synthesized IL-12 was IL-10 dependent. A fixed number of IFN- γ and LPS-activated macrophages was cocultured with decreasing numbers of wild-type BALB/c or IL-10^{-/-} 4T1-induced MDSC (Fig. 4C). Macrophage production of IL-12 was inversely proportional to the number of wild-type BALB/c MDSC, and MDSC from IL-10^{-/-} mice were much less effective than MDSC from BALB/c mice in reducing IL-12 levels. Therefore, MDSC-mediated reduction of macrophage production of IL-12 is dependent on MDSC production of IL-10.

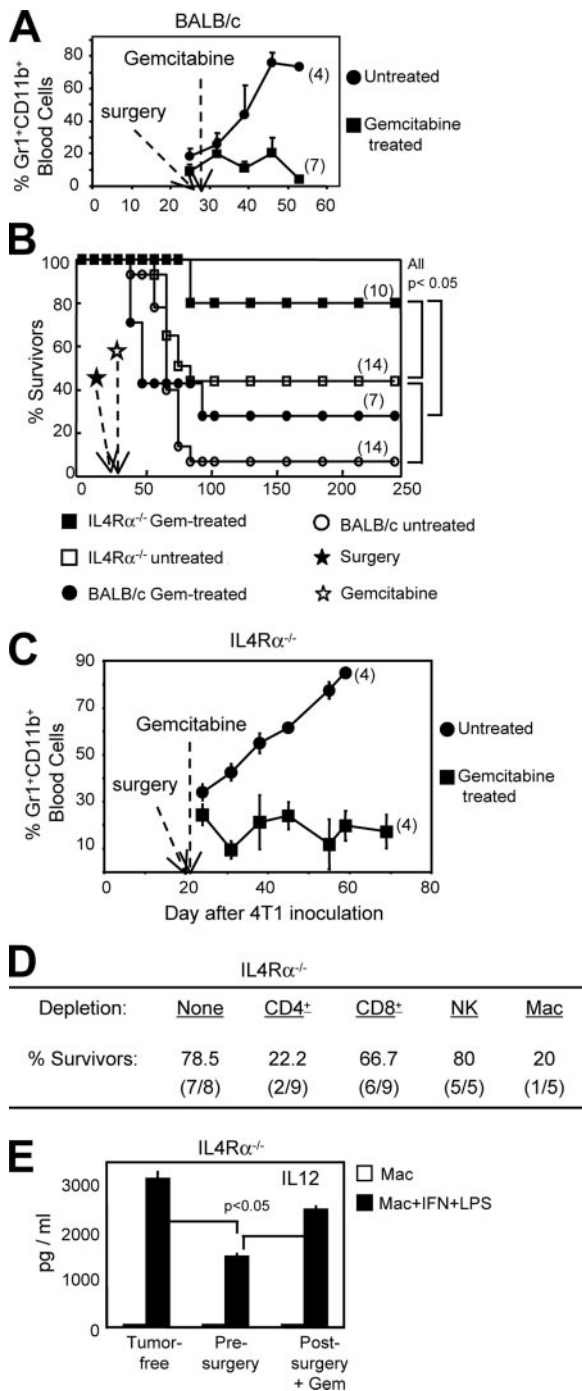


FIGURE 6. Reduction of MDSC in IL-4R $\alpha^{-/-}$ mice with M1 macrophages enables rejection of metastatic disease. *A*, BALB/c mice were inoculated on day 1 with 4T1 tumor cells, their primary tumors were removed on day 27 (tumor diameter: 6.1 \pm 1.7 mm), and gemcitabine treatment was started 1 day later. Mice were bled 1 day after each gemcitabine treatment, and their white blood cells were stained for Gr1 and CD11b. Data are representative of two independent experiments. *B*, IL-4R $\alpha^{-/-}$ and BALB/c mice were inoculated with 4T1 on day 0, and their primary tumors were removed on day 23 (tumor diameters: 5.9 \pm 1.2 mm and 5.3 \pm 1.7 mm, respectively). Half of each group was treated with gemcitabine, and all mice were observed for survival. Data are pooled from two independent experiments. *C*, IL-4R $\alpha^{-/-}$ mice were inoculated with 4T1 tumor cells on day 0, primary tumors were removed on day 20 (tumor diameter: 4.8 \pm 1.04 mm), gemcitabine treatment was started 1 day later, and mice were bled 1 day after each gemcitabine treatment to determine the percentage of MDSC. Data are representative of two independent experiments. Numbers in parentheses

MDSC suppress CD4⁺ and CD8⁺ T cell activation by their production of arginase and/or NO (5, 10, 33–38). To determine whether their effects on macrophages also involve arginase and/or NO, BALB/c and IL-4R $\alpha^{-/-}$ peritoneal macrophages were activated in vitro with LPS and IFN- γ and cocultured with or without BALB/c-derived MDSC in the presence of inhibitors of arginase (nor-NOHA) and/or NO (*N*^G-monomethyl-L-arginine). Neither inhibitor affected IL-12 or IL-10 production by BALB/c or IL-4R $\alpha^{-/-}$ macrophages (data not shown). Therefore, MDSC effects on macrophages and on T cell activation are mediated by different mechanisms.

Reduction of MDSC in BALB/c mice with spontaneous metastatic disease delays growth of primary tumor and modestly increases the number of survivors

Because MDSC are potent suppressive agents of T cells and polarize both innate and adaptive immunity toward a tumor-promoting type 2 phenotype, their elimination may facilitate tumor rejection by eliminating T cell suppression and reducing IL-10 levels. To test this hypothesis, we have used the drug gemcitabine. In addition to its established anti-DNA replication effects, gemcitabine has recently been shown to reduce MDSC levels in vivo (16). Female BALB/c mice were inoculated on day 0 in the abdominal mammary gland with 4T1 tumor cells and either treated or not treated with gemcitabine. Primary 4T1 tumors progressed more slowly in gemcitabine-treated BALB/c mice vs untreated mice (Fig. 5A). To confirm that the gemcitabine treatment reduced MDSC, treated and untreated mice were periodically bled and the white cells were tested by flow cytometry for Gr1⁺CD11b⁺ MDSC. In previous studies, we have used splenic MDSC of tumor-bearing mice (5, 29). MDSC in the blood are similar to MDSC in the spleens in that both populations are strongly Gr1⁺CD11b⁺ (Fig. 5B), block peptide-specific activation of transgenic T cells via the production of arginase (Fig. 5C), and are a heterogeneous mixture of cells that have the morphology of immature myeloid cells (Fig. 5D). Gemcitabine treatment significantly reduced MDSC accumulation in the blood of BALB/c mice with 4T1 tumors (Fig. 5E), and the reduction was independent of tumor burden because gemcitabine-treated mice had fewer MDSC as compared with untreated mice with the same size primary tumors (Fig. 5F).

Because gemcitabine is a pleiotropic drug that can also affect other immune cells, the levels of other potentially relevant cells were also examined. These experiments were performed using lymph nodes because it is likely that tumor-reactive T cells are activated in the draining lymph nodes so alterations in cell numbers would be most relevant in this study. In addition, the very high numbers of MDSC in the blood and spleen make it statistically difficult to assess changes in these other cell populations that are present in relatively small quantities. Gemcitabine treatment restored the levels of CD4⁺ T cells in the lymph nodes of tumor-bearing BALB/c mice to that seen in tumor-free BALB/c mice

indicate the number of mice/group in A–C. *D*, IL-4R $\alpha^{-/-}$ mice were inoculated with 4T1 tumor cells; primary tumors were removed on day 21 (tumor diameter: 5.1 \pm 0.5 mm); and depletions for T cells, NK cells, and macrophages were started on the day of surgery. Numbers in parentheses indicate the number of survivors in each group. Data are pooled from two independent experiments. *E*, Peritoneal macrophages were isolated from tumor-free, presurgery 4T1 tumor-bearing (tumor diameter: 5.4 \pm 0.7 mm), or postsurgery gemcitabine-treated IL-4R $\alpha^{-/-}$ mice, and activated or not activated with IFN- γ and LPS. IL-12 was measured by ELISA. Data are representative of four mice in each group.

(56 ± 3.6 , 47.3 ± 1.7 , and $26.35 \pm 5.2\%$ CD3⁺CD4⁺ T cells in gemcitabine-treated tumor-bearing, tumor-free, and untreated tumor-bearing mice, respectively; values significantly different at $p \# 0.03$). Gemcitabine treatment also appeared to restore the levels of CD8⁺ T cells (23.7 ± 3 , 22.8 ± 1.4 , and $14.5 \pm 5.5\%$ CD3⁺CD8⁺ T cells in gemcitabine-treated tumor-bearing, tumor-free, and untreated tumor-bearing mice, respectively); however, the values were not statistically significantly different. Levels of plasmacytoid DC, T regulatory cells, and mast cells were low in tumor-free mice (2.05 ± 1.2 , 4.5 ± 2.1 , and $0.85 \pm 0.4\%$, respectively), and showed only slight differences that were not statistically significantly different in tumor-bearing mice or gemcitabine-treated tumor-bearing mice. Therefore, gemcitabine treatment reduces MDSC, restores the homeostatic levels of CD4⁺ T cells in lymph nodes, and slows tumor progression; however, gemcitabine-treated BALB/c mice do not reject primary tumors.

The 4T1 primary tumors spontaneously metastasize to the lungs, liver, brain, lymph nodes, and bone marrow starting at approximately day 14 after 4T1 inoculation in the abdominal mammary gland. Removal of primary tumor does not alter the progression of established metastatic disease (28). To determine whether reduction of MDSC is sufficient to increase survival of BALB/c mice with metastatic disease whose primary tumors have been surgically removed, mice were inoculated with 4T1 tumor cells and the primary tumors were excised 3 wk later when metastatic disease was established (28). Gemcitabine treatment was started 1 day after surgery, and the mice were followed for survival and monitored for MDSC levels. BALB/c mice treated with gemcitabine had fewer MDSC (Fig. 6A), and a higher percentage of treated BALB/c mice survived (Fig. 6B) compared with untreated mice (28 vs 7% for treated vs untreated mice). Therefore, gemcitabine treatment reduced MDSC and modestly increased survival; however, most mice died, indicating that the antiproliferative effects of gemcitabine and a reduction in MDSC did not confer resistance to metastatic disease in BALB/c mice.

Gemcitabine-treated IL-4R α ^{-/-} mice are resistant to metastatic disease

M1 macrophages can be critical effectors for tumor rejection (5, 22, 25, 29). Therefore, gemcitabine-treated BALB/c mice may not be fully tumor resistant because their macrophages are skewed to an M2 phenotype, even in the absence of MDSC. However, IL-4R α ^{-/-} mice may be resistant to established metastatic disease because gemcitabine treatment will not only facilitate T cell activation and reduce IL-10 levels, but will also maintain the M1 macrophage phenotype. To test this possibility, IL-4R α ^{-/-} mice were inoculated in the abdominal mammary gland with 4T1 tumor cells, primary tumors were surgically removed on day 23 when they were 5.9 ± 1.2 mm in diameter, and gemcitabine treatment was started on day 24. Mice were periodically bled, and their white blood cells were tested for Gr1⁺CD11b⁺ MDSC. MDSC levels were significantly reduced in the gemcitabine-treated IL-4R α ^{-/-} postsurgery mice (Fig. 6C), and 80% of the gemcitabine-treated IL-4R α ^{-/-} mice survived indefinitely (Fig. 6B). Survival of IL-4R α ^{-/-} mice required both macrophages and T cells because in vivo depletion of either population significantly reduced the percentage of survivors (Fig. 6D). As hypothesized, macrophages of IL-4R α ^{-/-} mice with primary tumors (day 20 tumors, primary tumor diameter 5.4 mm; presurgery) were IL-12^{low} relative to tumor-free IL-4R α ^{-/-} mice, whereas macrophages of gemcitabine-treated postsurgery mice were IL-12^{high} (Fig. 6E). Therefore, in addition to its known ability to block DNA replication and to reduce MDSC levels, gemcitabine treatment restores macrophage production of IL-12 in IL-4R α ^{-/-} mice.

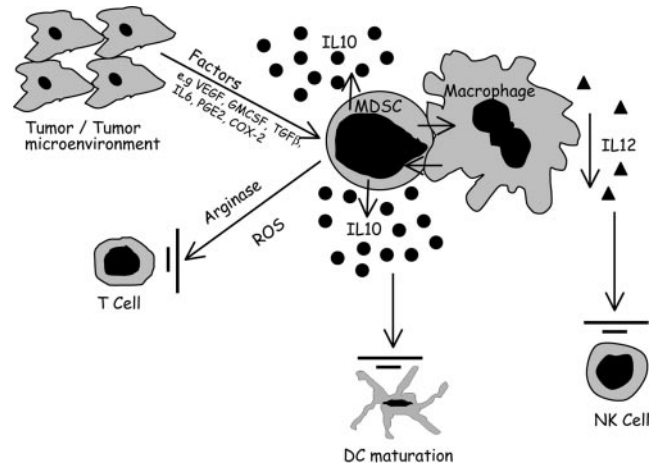


FIGURE 7. Schematic illustration of the mechanisms by which MDSC subvert antitumor immunity. MDSC are induced by growth factors, proinflammatory cytokines, and/or inflammatory agents. They produce arginase that blocks the activation of CD4⁺ and CD8⁺ T cells. Macrophages induce MDSC to produce more IL-10 that skews T cell immunity toward a tumor-promoting type 2 response and decreases DC maturation. IL-10 production by MDSC reduces IL-12 production by macrophages, thereby skewing M1 macrophages toward an M2 phenotype. The decrease in IL-12 production may also limit NK cell activity.

Discussion

The high frequency of MDSC in many individuals and experimental animals with tumors suggests that MDSC are a critical cell population that mediates immune suppression in cancer patients. Their inhibitory role in adaptive immunity via their suppression of T cell activation is well established (2, 3, 5, 17). The results presented in this study demonstrate that in addition to their direct suppression of T cells, MDSC down-regulate IL-12 production by macrophages, and increase their own production of IL-10 in response to signals from macrophages. This cross-talk between MDSC and macrophages polarizes M1 macrophages toward a type 2 phenotype and accentuates the M2 phenotype of M2 macrophages, and may establish an environment that is likely to skew CD4⁺ and CD8⁺ T cell immunity toward a tumor-promoting type 2 response (39) (see Fig. 7 for a schematic illustration of these mechanisms). As a result, MDSC directly, and through their cross-talk with macrophages, suppress both adaptive and innate antitumor immunity, and facilitate tumor growth.

In addition to the direct effects of MDSC on macrophages and T cells, decreased IL-12 and increased IL-10 production may also indirectly affect tumor immunity. For example, because macrophage-produced IL-12 promotes tumoricidal NK activity (39), MDSC may minimize NK activity by down-regulating macrophage production of IL-12. Similarly, because IL-10 interferes with DC maturation (40), MDSC secreting high levels of IL-10 may indirectly block DC function. Therefore, MDSC acting independently and in conjunction with macrophages suppress tumor immunity through multiple direct and indirect mechanisms (see Fig. 7).

The cross-talk between MDSC and macrophages that results in increased IL-10 production and decreased IL-12 production requires cell-to-cell contact. Similarly, MDSC suppression of T cell activation requires cell-to-cell contact (5, 33, 37). Whether ligand-receptor complexes mediate the cell contact is unknown, as is whether MDSC use the same ligand/receptor to interact with T cells and macrophages.

Gemcitabine is a commonly used agent in combination chemotherapy for the treatment of several types of cancers (e.g., mammary, bladder, nonsmall cell lung, and pancreatic cancers), and

was originally identified as an inhibitor of DNA synthesis (41). Although it has been reported that gemcitabine reduces MDSC levels (16), the current study demonstrates that this reduction has the added benefit of lowering IL-10 levels, and restoring macrophage production of IL-12. The therapeutic efficacy of gemcitabine most likely depends on all of these effects. Because gemcitabine treatment restores IL-12 production in both M1 and M2 macrophages, and reduces IL-10 production, it may be a useful drug for preparing cancer patients for active immunotherapies aimed at generating type 1 cell-mediated responses.

MDSC are potent regulatory cells that suppress adaptive and innate antitumor immunity via a variety of direct and indirect mechanisms that target T cells and macrophages. They are a significant impediment to any active immunotherapy, and their elimination or reduction should facilitate immune surveillance and immune-mediated rejection of resident tumor cells.

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