Interleukin-13–regulated M2 Macrophages in Combination with Myeloid Suppressor Cells Block Immune Surveillance against Metastasis

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Abstract

CD1-deficient mice reject established, disseminated 4T1 metastatic mammary cancer and survive indefinitely if their primary mammary tumors are surgically removed. This highly effective immune surveillance is due to three interacting mechanisms: (*a*) the generation of inducible nitric oxide synthase (iNOS)–producing M1 macrophages that are tumoricidal for 4T1 tumor cells; (*b*) a rapid decrease in myeloid-derived Gr1$^+$CD11b$^+$ suppressor cells that are elevated and down-regulate the CD3ζ chain when primary tumor is present and that suppress T cells by producing arginase; and (*c*) production of activated lymphocytes. Macrophages from wild-type BALB/c mice are polarized by interleukin-13 (IL-13) towards a tumor-promoting M2 phenotype, thereby inhibiting the generation of tumoricidal M1 macrophages. In contrast, CD1$^{-/-}$ mice, which are deficient for IL-13 because they lack IL-13–producting NKT cells, generate M1 macrophages that are cytotoxic for 4T1 via the production of nitric oxide. Although tumoricidal macrophages are a necessary component of immune surveillance in CD1$^{-/-}$ mice, they alone are not sufficient for tumor resistance because IL-4Rα$^{-/-}$ mice have M1 macrophages and retain high levels of myeloid suppressor cells after surgery; in addition, they are susceptible to 4T1 metastatic disease. These results show that effective immune surveillance against established metastatic disease is negatively regulated by IL-13 and requires the induction of tumoricidal M1 macrophages and lymphocytes combined with a reduction in tumor-induced myeloid suppressor cells. (Cancer Res 2005; 65(24): 11743-51)

Introduction

Recent studies have resurrected the hypothesis that immunosurveillance occurs *in vivo* and protects individuals against spontaneously arising malignant cells (1–4). Various effector mechanisms have been proposed as mediating immunosurveillance, including CD4$^+$ and CD8$^+$ T lymphocytes, natural killer cells, antibodies, and NKT cells (4–6). In addition to their role in protecting against tumor, NKT cells have also been implicated in facilitating tumor progression (7) by their production of the cytokine interleukin-13 (IL-13; refs. 8, 9). Most of the data supporting an inhibitory role for NKT cells derive from experiments using CD1-deficient (CD1$^{-/-}$) mice. CD1$^{-/-}$ mice lack the nonclassic MHC class I CD1d molecule, which is required for the thymus selection of NKT cells (10). Hence, CD1$^{-/-}$ mice lack NKT cells (11). Because NKT cells are a major producer of IL-13, CD1$^{-/-}$ mice are also IL-13 deficient (12).

We (13) and others (8, 9, 14) have reported that CD1$^{-/-}$ mice have enhanced immune surveillance against tumors and have proposed that deletion of the *CD1d* gene removes an inhibitor that blocks antitumor immunity. In our studies, we have used the spontaneously metastatic BALB/c-derived 4T1 mammary carcinoma (15–17). This tumor closely models human breast cancer in many of its characteristics, including its pattern of metastatic spread (18). Also similar to many human cancers (19–23), 4T1 induces a profound immune suppression, which can be partially reversed if the primary tumor is removed (24). Our finding that CD1$^{-/-}$ mice, whose primary tumors are surgically removed survive indefinitely despite the presence of metastatic disease, has led us to hypothesize that immune surveillance is blocked in wild-type mice by two factors: (*a*) an inhibitor that is regulated by the *CD1d* gene and (*b*) immune suppression induced by primary tumor. Terabe et al., using the 15-12RM fibrosarcoma, have also concluded that wild-type mice contain an inhibitor of immune surveillance and have identified the inhibitor as the cytokine IL-13. They argue that IL-13 blocks immune surveillance by activating Gr1$^+$CD11b$^+$ myeloid-derived suppressor cells (MSC) that secrete the immunosuppressive cytokine transforming growth factor β (TGFβ; refs. 8, 9). Although the immune suppression present in mice with 4T1 primary tumors is also mediated by Gr1$^+$CD11b$^+$ MSC, unlike the 15-12RM tumor system, 4T1-induced MSC are not induced by IL-13 (present report) and do not produce TGFβ (25), indicating that resistance to the 15-12RM and 4T1 tumors is mediated by different mechanisms.

Previous studies with the 4T1 tumor in signal transducer and activator of transcription 6–deficient (STAT6$^{-/-}$) mice showed that in addition to MSC, macrophages also regulate tumor growth (25). Macrophages polarized towards an M2 phenotype, produce arginase, and support tumor growth. In contrast, M1 macrophages, which produce inducible nitric oxide synthase (iNOS), are tumoricidal and mediate tumor regression (26).

It is important to clarify the mechanisms that promote immune surveillance and facilitate tumor regression because a better understanding of these mechanisms may lead to strategies that enhance tumor-specific immunity. Therefore, we have studied the pathways leading to effective immune surveillance against the 4T1 mammary carcinoma in CD1$^{-/-}$, mice whose primary tumors have been surgically removed but retain disseminated, metastatic disease. We find that effective immune surveillance requires a combination of three conditions: (*a*) the generation of iNOS-producing tumoricidal M1 macrophages that are produced because CD1$^{-/-}$ mice are deficient for IL-13, which polarizes macrophages to an M2 phenotype; (*b*) a rapid decrease in the quantity of myeloid-derived Gr1$^+$CD11b$^+$ suppressor cells that are

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elevated when primary tumor is present and that suppress $CD4^+$ and $CD8^+$ T-cell activation via the production of arginase and reactive oxygen species; and (*c*) the activation of functional lymphocytes.

Materials and Methods

Mice. $CD1^{-/-}$ (11), $3A9^{+/-}$ TCR-transgenic Vβ8.2 T-cell receptor (TCR)–specific for hen eggwhite lysozyme (HEL) restricted to $I\text{-}A^k$ (27), DO11.10 TCR-transgenic Vβ8-TCR restricted to chicken ovalbumin (OVA) peptide 323-339 restricted by $I\text{-}A^d$ (28), $STAT6^{-/-}$, and BALB/c mice were obtained as described (25). IL-4 receptor α-deficient ($IL\text{-}4R\alpha^{-/-}$) and RAG2-deficient ($RAG^{-/-}$) mice were from The Jackson Laboratory (Bar Harbor, ME) and Taconic Farms (Germantown, MD), respectively. All strains are on a BALB/c background. Female mice of 8 to 16 weeks were used for all studies. Mice were maintained and/or bred in the University of Maryland Baltimore County (UMBC) animal facility according to the NIH. All animal procedures are approved by the UMBC Institutional Animal Care and Use Committee.

Reagents and antibodies. Sodium thioglycolate and lipopolysaccharide (LPS) were from Difco (Detroit, MI); recombinant mouse IFNγ was from Pierce-Endogen (Rockford, IL); dichlorodihydrofluorescein diacetate (DCFDA) and dihydroethidium (DHE) were from Molecular Probes (Eugene, OR). Hemagglutinin (HA) peptide 518-526 and OVA peptide 323-339 were synthesized at the University of Maryland, Baltimore.

Vβ8.1,2-PE, CD1d1.1-PE, Gr1-PE, rat IgG2a-PE isotype, and rat IgG2a-FITC isotype were from BD PharMingen (San Jose, CA). CD3ζ-FITC was from Abcam (Cambridge, MA). CD11b-FITC and KJ1-26, an anti-clonotypic monoclonal antibody (mAb) that recognizes the DO11.10 TCR (29), were from Caltag (Burlingame, CA). mAb to arginase 1 and rat anti-mouse Gr-1 antibody for magnetic-affinity cell sorting (MACS; clone RB6-8C5) were from BD PharMingen.

Cell lines, tumor challenges, surgery, and metastasis assay. The J774 macrophage cell line (American Type Culture Collection, Manassas, VA) was maintained in DMEM (Biofluids, Rockville, MD; ref. 25). Mice were inoculated in the abdominal mammary gland with 7,000 4T1 cells, and primary tumor growth and lung metastases were measured (17, 18, 24). Tumor size was measured on the day of surgery, tumor diameter was calculated as the square root of length × width, and primary tumors were surgically removed (30). For experiments comparing non-surgery versus post-surgery groups, mice were inoculated with 4T1 on day 0, and tumor diameters were measured on the day of surgery. Mice were then divided into two groups so that the average tumor diameters for the groups were not significantly different. Primary tumors were removed from one group ("post-surgery") and left in place for the other group ("non-surgery").

T-cell and macrophage depletions. Mice were depleted for $CD4^+$ (mAb GK1.5) or $CD8^+$ (mAb 2.43) T cells or with irrelevant antibodies as described (31). Liposomes loaded with clondronate or control liposomes without clondronate were used to deplete macrophages (32). Briefly, mice were injected i.p. on days 1 and 4 after surgery with 0.2 mL of clodronate or control PBS liposomes and thereafter once a week with 0.1 mL of clodronate or control PBS liposomes. Treatment continued until all of the experimental mice were moribund.

Flow cytometry. Live cells were labeled for cell surface molecules by direct immunofluorescence (18). Samples were analyzed on an Epics XL flow cytometer and analyzed using Expo32 ADC software (Beckman Coulter, Miami, FL).

Myeloid suppressor cells and reactive oxygen species. Splenic MSC were positively purified by magnetic bead sorting using LS columns and rat anti-mouse Gr1 antibody with anti-rat IgG microbeads (ref. 25; Miltenyi Biotec, Auburn, CA). Purifed MSC were assayed by flow cytometry and were >90% $Gr1^+CD11b^+$. Reactive oxygen species (ROS) production was measured by DCFDA and DHE (25).

Macrophage assays. Peritoneal macrophages were generated by injecting 1 mL of sterile 3% Brewer thioglycolate medium (Difco) in distilled water i.p. Five days later, mice were euthanized by CO_2 asphyxiation, their abdomens were wiped with 70% alcohol, 10 mL of sterile PBS were injected into the peritoneal cavity, and the resulting peritoneal fluid was withdrawn aseptically. Contaminating RBC were lysed with Gey's solution, and the peritoneal exudate cells were washed twice and plated at 1.5×10^6/mL in 0.5 mL DMEM containing 10% FCS in 24-well plates. Nonadherent cells were removed after a 3-hour incubation at 37°C in 5% CO_2. The resulting macrophages were activated with IFNγ and LPS at final concentrations of 2 and 100 ng/mL, respectively, for 16 hours in DMEM, 5% FCS. In some experiments macrophages were stimulated with IL-4 or IL-13 at 50 ng/mL for 16 hours in DMEM containing 5% FCS before their activation with IFNγ and LPS.

Western blots. Macrophages were washed with excess PBS and resuspended in 200 μL of lysis buffer [one tablet of proteinase inhibitor mix (Roche, Indianapolis, IN), 2 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L HEPES, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 5% Triton X-100 in 10 mL H_2O]. Lysates were microfuged (3,000 × *g* for 10 minutes at 4°C), the clarified supernatants were electrophoresced in 12% SDS-PAGE gels, and the proteins were blotted onto Hybond-polyvinylidene difluoride membranes (Amersham, Piscataway, NJ) and immunoblotted with mAbs to arginase 1 (33). Proteins were detected using Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Nitric oxide and cytotoxicity assays. Nitric oxide (NO) was measured using Griess reagent (34) as described (25). Data are the mean ± SD of triplicate wells. Macrophage cytotoxicity was determined by the procedure of Decker and Lohmann-Matthes (35) as described (25). Values are the average of triplicates ± SD. Background values for media were subtracted from each point. Activated and nonactivated macrophages without 4T1 gave no lactate dehydrogenase release.

$$\%\ \text{specific lysis} = 100\% \times [(A490_{\text{Experimental}} - A490_{\text{Spontaneous}})/(A490_{\text{Maximum}})]$$

T-cell proliferation assay. T-cell proliferation and transwell experiments were done as described (25). All points were run in triplicates. Data are expressed as:

$$\%\ \text{suppression} = 100\%\ [1 - (\text{cpm of spleen} + \text{peptide} + \text{MSC}/\text{cpm of spleen} + \text{peptide})].$$

CD3ζ expression. Cells were mixed with peptide and with or without irradiated MSC (5,000 rad) in 24-well plates [5×10^5 T cells, 10^6 MSC, in 500 μL HL1 culture medium (Bio Whittaker, Walkersville, MD) per well]. After 3 days of culture, cells were harvested, labeled for cell surface markers [KJ1-26-tricolor mAb for D011.10 with CD4-PE; or Vβ8-PE mAb for clone 4 with CD8-tricolor (all at a 1:50 dilution)], fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, and stained with a 1:20 dilution of CD3ζ-FITC mAb. Labeled cells were analyzed for expression of CD3ζ by gating on double-positive ($CD4^+KJ1\text{-}26^+$ or $CD8^+V\beta8^+$) cells.

Statistical analysis. Student's *t* test for unequal variance was done using Microsoft Excel 2000.

Results

CD1-deficient mice survive indefinitely after surgical removal of primary 4T1 mammary carcinoma. The 4T1 mammary carcinoma is a BALB/c-derived tumor that spontaneously metastasizes following inoculation into the mammary gland. Similar to human breast cancer, metastatic disease progresses while the primary tumor is present and after the primary tumor is surgically removed. We have previously used 4T1 to study tumor immunity in a setting comparable with that of breast cancer patients whose primary tumors have been removed but have residual, disseminated metastatic disease (18, 30, 36). To confirm our earlier findings that $CD1^{-/-}$ mice are resistant to 4T1 metastatic disease, $CD1^{-/-}$ and control syngeneic CD1-competent BALB/c mice were injected

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s.c. in their abdominal mammary gland with 7,000 4T1 cells, primary tumors were either left in place (non-surgery group) or surgically removed 2 to 3 weeks later (post-surgery group), and mice were followed for survival (tumor diameter at surgery: BALB/c, 4.93 ± 0.98; $CD1^{-/-}$, 4.9 ± 1.2 mm). As shown in Fig. 1*A*, 100% of post-surgery $CD1^{-/-}$ mice survived >180 days, whereas 89% of the BALB/c mice died with a mean survival time (MST) of 53.4 days. To determine if the difference in survival time between $CD1^{-/-}$ and BALB/c mice was due to differences in metastatic disease, the lungs of non-surgery and post-surgery $CD1^{-/-}$ and BALB/c mice were removed 30 to 39 days after 4T1 challenge (9-11 days after surgery for the surgery groups) and tested for metastatic tumor cells. Non-surgery and post-surgery $CD1^{-/-}$ and BALB/c mice have very similar levels of metastatic cells (Fig. 1*B*). Therefore, despite the presence of high levels of metastatic tumor, $CD1^{-/-}$ mice whose primary tumors are removed survive, whereas BALB/c mice die. To determine if $CD1^{-/-}$ mice survive because they eliminate metastatic cells, lung metastases were quantified in long-term (4-10 months) $CD1^{-/-}$ survivors. These mice had no detectable 4T1 cells, and splenic MSC levels were in the reference range (<8%), indicating that post-surgery $CD1^{-/-}$ mice are resistant because they reject 4T1 tumor cells.

A trivial explanation for the resistance of $CD1^{-/-}$ mice to 4T1 is that 4T1 tumor cells contain CD1 protein that functions as an alloantigen. To eliminate this possibility, 4T1 tumor cells were tested for CD1 expression. As shown in Fig. 1*C*, 4T1 cells do not contain CD1. Therefore, survival of $CD1^{-/-}$ mice is not due to an immune response against the knockout gene product.

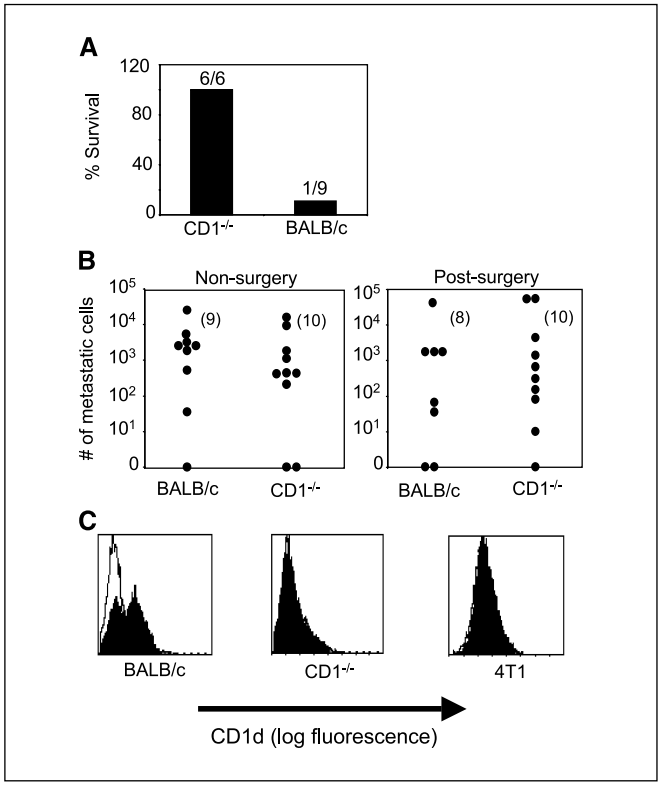

Figure 1. $CD1^{-/-}$ mice are resistant to disseminated metastatic disease. $CD1^{-/-}$ and BALB/c mice were inoculated in the mammary gland on day 0 with 7,000 4T1 tumor cells, and primary tumors were left in place (non-surgery group) or removed 2 to 3 weeks later (post-surgery group). *A,* post-surgery mice were followed for survival. *Numbers,* number of mice surviving >180 days per total mice. From one of two independent experiments. *B,* lungs were harvested, and the number of metastatic cells was quantified by the clonogenic assay. ●, individual mouse. From two independent experiments. *C,* BALB/c or $CD1^{-/-}$ splenocytes or 4T1 cells were stained with FITC-conjugated CD1d mAb (*filled histograms*) or isotype-matched control mAb (*open histograms*) and analyzed by flow cytometry. From one of two independent experiments.

Myeloid suppressor cell levels return to normal in CD1-deficient mice after removal of primary tumor. MSC accumulate in some tumor-bearing patients and animals and are potent inhibitors of cell-mediated, tumor-specific immunity (19–23). These cells are immature cells that are in the process of differentiating into mature granulocytes, dendritic cells, or macrophages and are identified by their expression of Gr1 and CD11b. We have previously found that 4T1 tumor progression is associated with the accumulation of MSC (25). To determine if the resistance of $CD1^{-/-}$ mice is related to MSC activity, MSC levels were measured in tumor-bearing $CD1^{-/-}$ and CD1-competent mice. $CD1^{-/-}$ and wild-type BALB/c mice were inoculated with 4T1 tumor cells, and splenocytes were harvested 30 to 39 days later and analyzed for $CD11b^+$ $Gr1^+$ cells (tumor diameter at surgery: BALB/c, 6.05 ± 0.75 mm; $CD1^{-/-}$, 6.38 ± 0.8 mm). Tumor-free BALB/c and $CD1^{-/-}$ mice have <8% splenic MSC, whereas tumor-bearing (non-surgery) mice have elevated levels of MSC (Fig. 2*A*; BALB/c, 23 ± 11%; $CD1^{-/-}$, 26 ± 5%). Therefore, non-surgery $CD1^{-/-}$ and CD1-competent mice both have elevated levels of MSC relative to tumor-free mice.

To determine if removal of primary tumor differentially affects MSC levels, BALB/c and $CD1^{-/-}$ mice were inoculated with 4T1, primary tumors were removed 21 to 28 days later, and splenocytes were analyzed 9 to 11 days later (days 30-39 after initial tumor inoculation; tumor diameter at surgery: BALB/c, 6.12 ± 0.81 mm; $CD1^{-/-}$, 5.99 ± 0.90 mm). After surgery, MSC levels in 90% of post-surgery $CD1^{-/-}$ mice are within the reference range (<8%), whereas only 21% of post-surgery BALB/c mice have <8% MSC (Fig. 2*A*). Therefore, although MSC levels are elevated in both BALB/c and $CD1^{-/-}$ mice when primary tumor is present, there is a drop to the normal level in most post-surgery $CD1^{-/-}$ mice.

The accumulation of MSC is most likely driven by tumor-secreted factors (37). Therefore, the decrease of MSC in post-surgery $CD1^{-/-}$ mice may be due to less metastatic disease in the $CD1^{-/-}$ versus wild-type mice. To test this hypothesis, we plotted the number of metastatic cells versus the percentage of MSC for individual post-surgery mice (Fig. 2*B*; similar results were obtained for mice with >35% MSC; data not shown). Both BALB/c and $CD1^{-/-}$ mice have extensive metastatic disease, and there is no correlation between percentage of MSC and the number of metastatic cells. Therefore, the decrease in MSC in post-surgery $CD1^{-/-}$ mice is independent of metastatic disease.

Lymphocytes may also play a role in driving MSC levels. To determine if lymphocytes are involved, splenic MSC levels were determined for BALB/c $RAG^{-/-}$ mice inoculated with 4T1 according to the schedule in Fig. 2*A* (tumor diameter: non-surgery, 5.07 ± 1.2 mm; post-surgery, 5.57 ± 0.95 mm). Likewise, tumor diameter of non-surgery $RAG^{-/-}$, BALB/c, and $CD1^{-/-}$ mice were similar when MSC levels were measured ($RAG^{-/-}$, 9.3 ± 1.5 mm; BALB/c, 9.5 ± 0.73; $CD1^{-/-}$, 10.24 ± 1.10). The baseline level of $Gr1^+CD11b^+$ splenocytes in tumor-free $RAG^{-/-}$ mice is <8%, whereas non-surgery $RAG^{-/-}$ mice have significantly ($P < 0.01$) more MSC than BALB/c or $CD1^{-/-}$ mice (Fig. 2; $RAG^{-/-}$ MSC, 51.8 ± 6%). After surgery, MSC in $RAG^{-/-}$ mice remain significantly higher than in BALB/c or $CD1^{-/-}$ mice ($P < 0.01$).

To determine if $CD4^+$ and/or $CD8^+$ T cells are involved in resistance, post-surgery $CD1^{-/-}$ mice were *in vivo* depleted for

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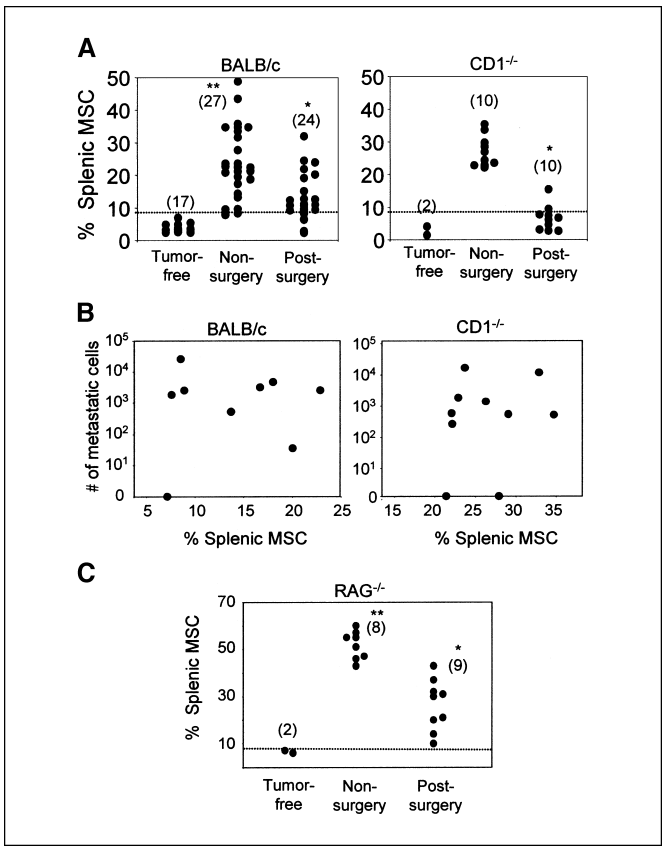

Figure 2. Splenic MSC levels return to baseline after removal of primary tumor from CD1$^{-/-}$ mice. BALB/c or CD1$^{-/-}$ (*A* and *B*) or RAG$^{-/-}$ (*C*) mice were inoculated on day 0 with 4T1 tumor cells, and primary tumors were left in place (non-surgery groups) or removed on day ~28 (post-surgery groups). Tumor-free groups were not given 4T1. On day ~38, lungs and spleens were harvested, splenocytes were labeled with Gr1-PE and CD11b-FITC to determine percent MSC (*A* and *C*), and the number of metastatic cells in the lungs were quantified by the clonogenic assay (*B*). ●, individual mouse. *Numbers in brackets,* number of mice per group. *Dotted lines,* maximum amount of MSC in tumor-free mice (<8%). From two independent experiments. *, statistically significantly different from each other. **, $P < 0.05$, statistically significantly different from each other.

CD4$^+$ or CD8$^+$ T cells or treated with irrelevant antibodies. Both CD4$^+$ and CD8$^+$ T cells are required for tumor resistance because 100% of CD4-depleted (three of three) and 80% of CD8-depleted (four of five) post-surgery CD1$^{-/-}$ mice but none of the irrelevant antibody treated mice (three of three) die. Therefore, lymphoid cells are essential for tumor rejection and may reduce MSC in post-surgery BALB/c and CD1$^{-/-}$ and mice.

Myeloid suppressor cells inhibit T cells by an arginase-dependent mechanism. CD1$^{-/-}$ mice may have greater tumor immunity because their MSC are less suppressive than MSC of BALB/c mice. To test this possibility, splenocytes from non-surgery BALB/c and CD1$^{-/-}$ mice were MACS purified for Gr1 (>91% and 93% Gr1$^+$CD11b$^+$ for BALB/c and CD1$^{-/-}$, respectively). The resulting MSC were then cocultured with antigen-specific CD4$^+$ or CD8$^+$ syngeneic T cells or CD4$^+$ allogeneic T cells plus the appropriate peptide (*H-2^d* D011.10 with OVA-peptide, *H-2^d* clone 4 with HA-peptide, or *H-2^k* 3A9 with HEL, respectively), and T-cell activation was measured by [^{3}H]thymidine uptake (Fig. 3*A*). On a per cell basis, purified BALB/c and CD1$^{-/-}$ MSC equally suppress syngeneic CD4$^+$ or CD8$^+$ or allogeneic CD4$^+$ T cells.

MSC are thought to suppress via arginase and/or iNOS (38). To ascertain the role of these molecules, DO11.10 transgenic T cells were cocultured with CD1$^{-/-}$ MSC in the presence of OVA peptide and the arginase inhibitor N^w-hydroxyl-nor-L-arginine (nor-NOHA) or the iNOS inhibitor L-NMMA, and T-cell proliferation was measured by [^{3}H]thymidine uptake. The arginase inhibitors, but not the iNOS inhibitor, reverses the suppression (Fig. 3*B*). Therefore, CD1$^{-/-}$ MSC inhibit T-cell activation via arginase production.

To determine if suppression requires direct contact between MSC and T cells, CD1$^{-/-}$ MSC were suspended in transwell chambers containing OVA peptide–pulsed DO11.10 T cells (Fig. 3*C*). Proliferation of DO11.10 cells was not inhibited when the MSC were separated from the T cells by a semipermeable membrane. Therefore, suppression requires direct contact between the MSC and the affected T cells.

Myeloid suppressor cells down-regulate T-cell receptor–associated zeta chain in CD4$^+$ but not CD8$^+$ T cells. Rodriguez et al. (39) and Zabaleta et al. (40) have shown that T-cell dysfunction caused by macrophages or bacteria is associated with the down-regulation of the TCR-associated CD3ζ chain. To determine if MSC induce suppression by this mechanism, OVA

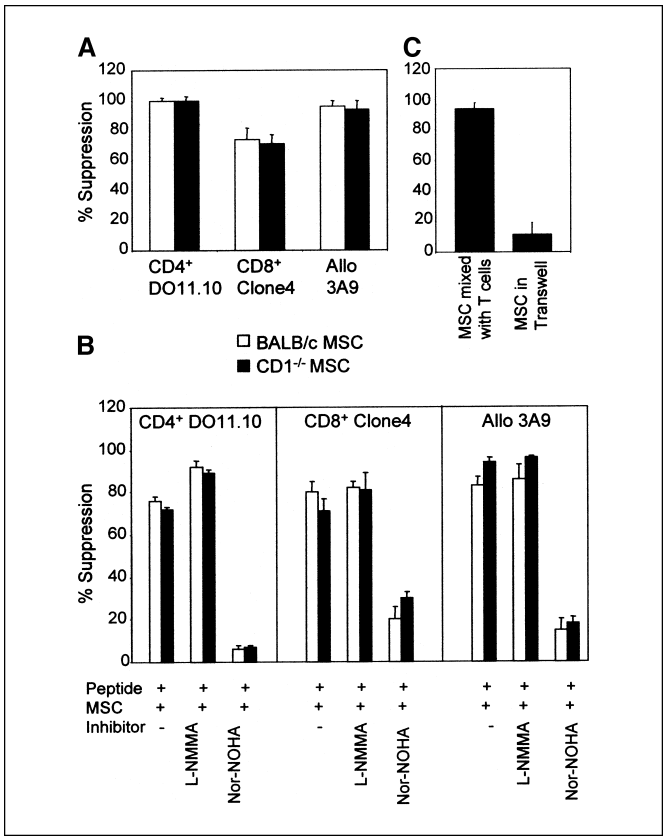

Figure 3. MSC inhibit T-cell activation by an arginase-dependent mechanism. *A,* syngeneic DO11.10 or clone 4 or allogeneic 3A9 transgenic splenocytes were stimulated with OVA$_{323-339}$, HA$_{518-526}$, or HEL protein, respectively, in the presence of non-surgery BALB/c (*open columns*) or CD1$^{-/-}$ (*filled columns*) MSC. *B,* the experiment of (*A*) was repeated with or without the inhibitors for iNOS (L-NMMA) or arginase (nor-NOHA). MSC from two to three mice were pooled for each group. Control J774 cells gave no suppression (data not shown). *C,* DO11.10 T cells were stimulated with OVA$_{323-339}$ peptide and cocultured in the same well with CD1$^{-/-}$ MSC, or the MSC were contained in a transwell chamber suspended in the well containing the T cells. From one of two independent experiments.

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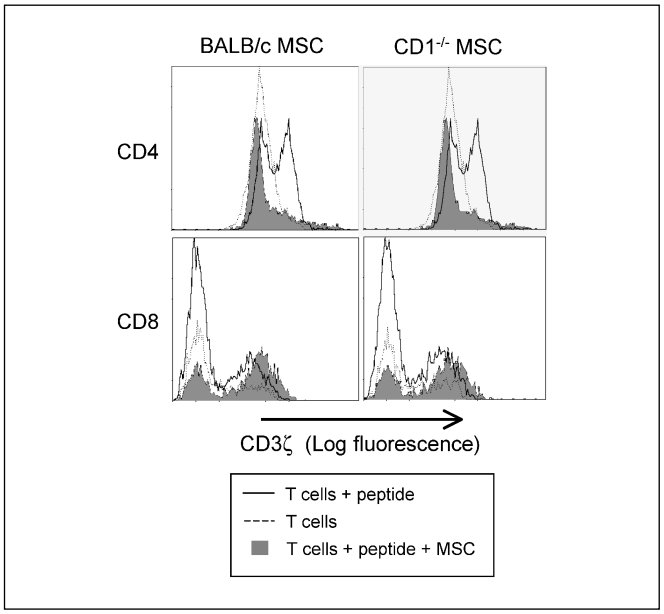

Figure 4. MSC down-regulate CD3ζ chain in CD4$^+$ but not CD8$^+$ T cells. CD4$^+$ DO11.10 or CD8$^+$ clone 4 transgenic splenocytes were stimulated with $OVA_{323-339}$ or $HA_{518-526}$ peptide, respectively, in the presence of non-surgery BALB/c or CD1$^{-/-}$ MSC. Cultures were harvested, and cells were stained with CD3ζ-FITC, KJ1-26-tricolor, and CD4-PE (CD4$^+$ T cells) or CD3ζ-FITC, Vβ8-PE, and CD8-tricolor (CD8$^+$ T cells). CD3ζ expression was determined by gating on double-positive cells (CD4$^+$KJ1-26$^+$ or CD8$^+$Vβ8$^+$, respectively). *Dotted lines,* unstimulated T cells (no peptide); *solid lines,* stimulated T cells (with peptide); *filled histograms,* stimulated T cells with MSC. From one of two independent experiments.

peptide–pulsed CD4$^+$ DO11.10 T cells were cocultured with MSC from BALB/c or CD1$^{-/-}$ mice. Following 3 days of incubation, the cultures were harvested, and the cells were triple labeled for CD3ζ, CD4, and the D011.10 clonotype (KJ1-26). The cells were analyzed by flow cytometry by gating on the DO11.10$^+$ CD4$^+$ double-positive population and assessing CD3ζ expression. Fifty-three percent of D011.10 transgenic T cells cocultured with OVA peptide have elevated CD3ζ chain (Fig. 4, *top*). If BALB/c or CD1$^{-/-}$ MSC are added to the cultures, then only 17% and 15% of the T cells, respectively, have elevated CD3ζ expression. Therefore, BALB/c and CD1$^{-/-}$ MSC reduce CD3ζ chain expression, which probably inhibits T-cell activation by inhibiting signal transduction.

To determine if MSC also suppress the activation of CD8$^+$ T cells via the down-regulation of CD3ζ, CD8$^+$ clone 4 T cells were cultured with HA peptide. The resulting cells were gated on the CD8$^+$ Vβ8$^+$ double-positive population and analyzed for CD3ζ expression (Fig. 4, *bottom*). More than half of the activated CD8$^+$ T cells had elevated CD3ζ. In contrast to CD4$^+$ T cells, CD3ζ did not decrease following coculture with either BALB/c or CD1$^{-/-}$ MSC. Therefore, BALB/c and CD1$^{-/-}$ MSC suppress CD4$^+$ T cells by down-regulating CD3ζ chain but suppress CD8$^+$ T cells via a different mechanism.

BALB/c and CD1$^{-/-}$ myeloid suppressor cells produce reactive oxygen species. Kusmartsev et al. (41) have shown that ROS are a characteristic of MSC, and we (25) have previously noted that ROS production characterizes different MSC populations. To determine if ROS are differentially expressed in BALB/c versus CD1$^{-/-}$ MSC, splenic MSC were MACS purified from tumor-free and non-surgery mice and analyzed for ROS. Staining with DHE, which measures superoxide, was negative (data not shown). Staining with DCFDA, which measures hydrogen peroxide, hydroxyl radical, peroxynitrile, and superoxide, shows that Gr1$^+$CD11b$^+$ splenic cells from non-surgery BALB/c (Fig. 5*A*) and CD1$^{-/-}$ (Fig. 5*B*) mice contain more ROS than MSC from the corresponding tumor-free mice. To assess if arginase is involved in ROS production, the arginase inhibitor nor-NOHA was added to the purified Gr1$^+$CD11b$^+$ cells before their staining with DCFDA. Although nor-NOHA has no effect on ROS expression in CD1$^{-/-}$ MSC, it inhibits ROS expression in BALB/c MSC. Therefore, MSC from both BALB/c and CD1$^{-/-}$ mice contain ROS; however, ROS expression in the CD1$^{-/-}$ MSC is arginase independent, whereas in BALB/c MSC, it is arginase dependent.

CD1$^{-/-}$ mice have tumoricidal M1 macrophages. iNOS-producing M1 macrophages are associated with heightened anti-tumor immunity and inhibition of tumor progression (26, 42, 43). IL-4 and IL-13 polarize macrophages towards an M2 phenotype (26, 43). Because CD1$^{-/-}$ mice lack NKT cells, which are major sources of IL-13 (11, 12, 44, 45), they may preferentially generate M1 macrophages, which may contribute to tumor resistance. To test this hypothesis, peritoneal macrophages from BALB/c and CD1$^{-/-}$ mice were activated *in vitro* with LPS and IFNγ and assayed for iNOS production. LPS- and IFNγ-activated macrophages from STAT6$^{-/-}$ and IL-4Rα$^{-/-}$ mice were used as controls. The IL-4Rα is a common chain that is shared between the receptors for IL-4 and IL-13 and hence is required for transmitting signals for both of these cytokines (46, 47). STAT6 is a transcription factor that transmits signals through the IL-4Rα (48–50). Therefore, STAT6$^{-/-}$ and IL-4Rα$^{-/-}$ macrophages should make

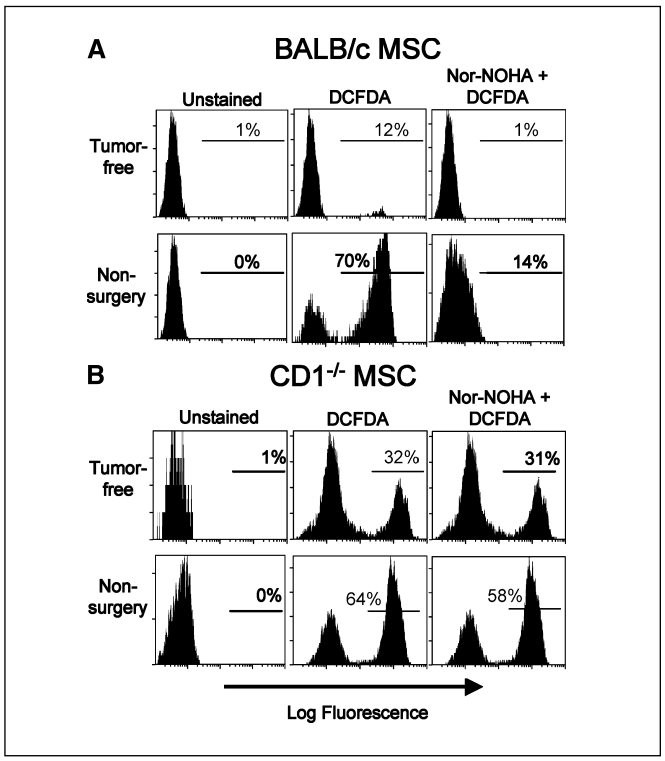

Figure 5. ROS production by BALB/c but not CD1$^{-/-}$ MSC is arginase dependent. Purified BALB/c (*A*) and CD1$^{-/-}$ (*B*) MSC from tumor-free and non-surgery mice were unstained, or incubated with DCFDA in the presence or absence of the arginase inhibitor nor-NOHA. From one of two independent experiments.

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iNOS regardless of the presence or absence of IL-4 and/or IL-13 (51). Macrophages from all four strains that are activated *in vitro* in the absence of IL-4 or IL-13 produce iNOS (Fig. 6). However, if the macrophages are treated with IL-4 or IL-13 before activation with LPS and IFNγ, then BALB/c and CD1$^{-/-}$ macrophages make less iNOS, whereas iNOS production by STAT6$^{-/-}$ and IL-4Rα$^{-/-}$ is unaffected. Because BALB/c mice produce IL-4 and/or IL-13 *in vivo*, their macrophages will not make significant levels of iNOS; hence, BALB/c mice will not have M1 macrophages. In contrast, CD1$^{-/-}$ mice will have iNOS-producing M1 macrophages *in vivo* because they have diminished levels of IL-4 and IL-13 because they lack NKT cells.

The production of arginase has been associated with M2 type macrophages, which are thought to promote tumor progression (26, 42, 43). To determine if arginase production by macrophages is associated with tumor progression, BALB/c, CD1$^{-/-}$, and IL-4Rα$^{-/-}$ peritoneal macrophages were tested for arginase by Western blot (Fig. 6*B*). Macrophages were either not activated, activated with LPS plus IFNγ, pretreated with IL-4 before LPS and IFNγ activation, pretreated with IL-13 before LPS and IFNγ activation, unactivated and treated with IL-4, or not activated and treated with IL-13. BALB/c and CD1$^{-/-}$ macrophages, regardless of treatment, contain arginase, whereas IL-4Rα$^{-/-}$ macrophages contain very little, if any arginase.

Macrophage tumoricidal activity is attributed to iNOS production (26); thus, CD1$^{-/-}$ macrophages may be tumoricidal, although they also contain arginase. To test this hypothesis, BALB/c, CD1$^{-/-}$, and IL-4Rα$^{-/-}$ peritoneal macrophages were harvested, activated *in vitro* with LPS and IFNγ, and tested for cytotoxic activity against 4T1 cells. CD1$^{-/-}$ and positive control IL-4Rα$^{-/-}$ macrophages are significantly more cytotoxic than BALB/c macrophages (Fig. 6*C*; $P < 0.05$). The cytotoxicity is due to iNOS, because addition of the iNOS inhibitor L-NMMA eliminates the cytotoxic effect, whereas the inactive inhibitor D-NMMA has no effect. Therefore, although CD1$^{-/-}$ macrophages contain both iNOS and arginase, they have strong tumoricidal activity, indicating that they are polarized towards the M1 phenotype. To confirm the role of macrophages in tumor resistance, macrophages were depleted from post-surgery CD1$^{-/-}$ mice by treatment with liposomes loaded with clodronate. Macrophage-depleted (three of three) mice were dead by 42 days after injection of primary tumor, whereas mice treated with PBS loaded liposomes survived (MST > 83 days). Therefore, macrophages are essential for the survival of post-surgery CD1$^{-/-}$ mice.

Interleukin-4 receptor α–deficient mice are tumor susceptible and maintain elevated levels of myeloid suppressor cells after surgery. If the presence of M1 macrophages is sufficient for tumor resistance, then IL-4Rα$^{-/-}$ mice, which have tumoricidal M1 macrophages, may survive after removal of primary tumor. To test this possibility, BALB/c and IL-4Rα$^{-/-}$ mice were inoculated with 4T1, primary tumors were surgically removed 2 to 3 weeks later, and the mice were followed for survival. IL-4Rα$^{-/-}$ mice are just as susceptible as BALB/c mice (five of six IL-4Rα$^{-/-}$ versus seven of eight BALB/c mice die), indicating that despite the presence of M1 macrophages, IL-4Rα$^{-/-}$ mice do not have heightened tumor immunity. Because MSC decrease to baseline in post-surgery CD1$^{-/-}$ (see Fig. 2) and STAT6$^{-/-}$ (25) mice, we assessed MSC levels in tumor-bearing, non-surgery and post-surgery IL-4Rα$^{-/-}$ mice (tumor diameter for non-surgery mice: BALB/c, 6.1 ± 1.7 mm; IL-4Rα$^{-/-}$, 7.1 ± 1.1 mm; tumor diameter at surgery for the post-surgery groups: BALB/c, 6.5 ± 1 mm; IL-4Rα$^{-/-}$, 7.5 ± 0.43 mm). Non-surgery IL-4Rα$^{-/-}$ mice have elevated MSC (Fig. 7*A*), and MSC remain elevated after surgery similar to BALB/c ($P > 0.05$), with only 14% of IL-4Rα$^{-/-}$ mice having normal levels (<8% MSC). Likewise, post-surgery IL-4Rα$^{-/-}$ mice contain high levels of metastatic cells (Fig. 7*B*). Therefore, although IL-4Rα$^{-/-}$ mice generate tumoricidal M1 macrophages, they are not tumor resistant and have elevated levels of MSC even after removal of primary tumor.

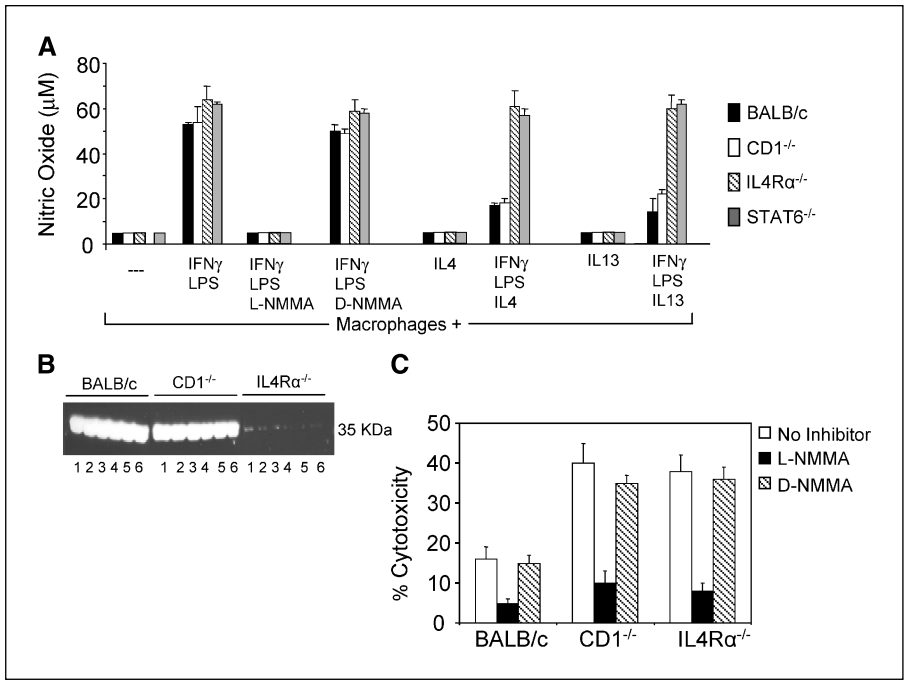

Figure 6. CD1$^{-/-}$ mice have tumoricidal iNOS-producing M1 macrophages. *A,* peritoneal macrophages from BALB/c, CD1$^{-/-}$, IL-4Rα$^{-/-}$, or STAT6$^{-/-}$ mice were not activated, activated with LPS and IFNγ, and/or treated with IL-4 or IL-13 in the presence or absence of the iNOS inhibitor L-NMMA or its inactive enantiomere D-NMMA. NO was measured using the Griess reagent. *B,* peritoneal macrophages were prepared as in (*A*), and cell lysates were Western blotted with mAb to arginase. *Lane 1,* not activated; *lane 2,* activated with LPS and IFNγ; *lane 3,* pretreated with IL-4 before activation with LPS and IFNγ; *lane 4,* pretreated with IL-13 before activation with LPS and IFNγ; *lane 5,* pretreated with IL-4, not activated; *lane 6,* pretreated with IL-13, not activated. *C,* LPS- and IFNγ-activated BALB/c, CD1$^{-/-}$, or IL-4Rα$^{-/-}$ peritoneal macrophages were cocultured with 4T1 cells in the presence or absence of the iNOS inhibitor L-NMMA, and percent cytotoxicity was measured by LDH release. Activated CD1$^{-/-}$ or IL-4Rα$^{-/-}$ macrophages are significantly more cytotoxic than BALB/c macrophages ($P < 0.05$).

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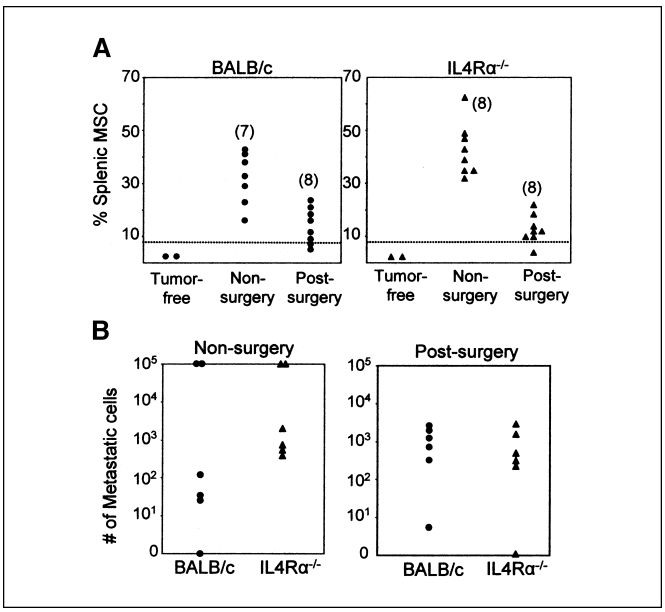

Figure 7. IL-4Rα$^{-/-}$ mice are not resistant to 4T1 metastatic disease and retain elevated levels of MSC after surgery. IL-4Rα$^{-/-}$ and BALB/c mice were inoculated in the mammary gland on day 0 with 7,000 4T1 tumor cells, and primary tumors were left in place (non-surgery groups) or removed on day ~28 (post-surgery groups). Tumor-free mice were not given 4T1. *A,* lungs and spleens were harvested on day ~38, and splenocytes were labeled with Gr1-PE and CD11b-FITC to determine percent MSC. *B,* the number of metastatic cells in the lungs was quantified by the clonogenic assay. *A* and *B,* from two independent experiments (n = 6 mice per group). *Dotted lines,* maximum amount of MSC in tumor-free mice (<8%). ● and ▲, individual mouse.

Discussion

Despite extensive metastatic mammary carcinoma at the time of surgery, CD1$^{-/-}$ mice survive indefinitely if 4T1 primary tumor is removed. Resistance is associated with three phenomena: (*a*) the production of iNOS-producing M1 macrophages, (*b*) a rapid decrease to baseline in the levels of MSC, and (*c*) the presence of functional lymphocytes. Resistance to metastatic disease requires the coordinate interaction of the three conditions; neither mechanism alone is sufficient to mediate tumor rejection.

iNOS-producing M1 macrophages with tumoricidal activity have been described in numerous tumor systems (26, 42, 43). They are cytotoxic because iNOS converts arginine and oxygen to NO, which is toxic. The tumoricidal macrophages of CD1$^{-/-}$ mice are unusual in that they produce iNOS and arginase. Typically, M1 macrophages produce less arginase because it degrades arginine and therefore limits the amount of substrate available for conversion to NO (52, 53). Despite the coexpression of arginase, CD1$^{-/-}$ peritoneal macrophages produce sufficient NO to mediate tumor cell destruction. El-Gayar et al. (53) have shown that IL-13 prevents iNOS production, thereby polarizing macrophages towards a M2 phenotype. Because CD1$^{-/-}$ mice lack NKT cells, which are a major producer of IL-13, it is likely that CD1$^{-/-}$ mice have M1 macrophages because they are deficient for IL-13. This hypothesis is supported by two findings: (*a*) Addition of IL-13 to cultures of CD1$^{-/-}$ macrophages produces a M2 phenotype; and (*b*) Macrophages from IL-4Rα$^{-/-}$ mice, which lack the receptor for IL-13, are M1-type iNOS producers and tumoricidal. Although both IL-13 and IL-4 signal through the IL-4Rα and STAT6, it is unlikely that IL-4 is the inhibitor of M1 macrophage generation *in vivo* because IL-4 is produced by activated Th2 cells in addition to NKT cells (54, 55), and CD1$^{-/-}$ mice are only deficient for NKT cells. Therefore, CD1$^{-/-}$ mice, which are deficient for IL-13 (12), constitutively generate iNOS-producing M1 macrophages that are cytotoxic for 4T1 tumor, whereas BALB/c mice produce M2 macrophages under the induction of IL-13.

Previous studies showed that macrophages are essential for immune surveillance against the 4T1 tumor (25, 56). Although M1 macrophages are necessary, their presence is not sufficient for tumor rejection. For example, IL-4Rα$^{-/-}$ mice, which have tumoricidal M1 macrophages, die from metastatic disease, and CD1$^{-/-}$ mice are only resistant if their primary tumor is removed, although tumoricidal M1 macrophages are present before surgery. In pre-surgery and post-surgery IL-4Rα$^{-/-}$ mice and in pre-surgery CD1$^{-/-}$ mice, MSC levels are elevated, suggesting that M1 macrophages are ineffective in the presence of large quantities of MSC. In contrast, post-surgery CD1$^{-/-}$ mice with M1 macrophages have baseline levels of MSC and reject metastatic disease. Depletion of M1 macrophages from post-surgery CD1$^{-/-}$ mice makes them susceptible to tumor. Therefore, effective immunity against metastasis requires M1 macrophages coupled with baseline levels of MSC, a condition that only exists in post-surgery CD1$^{-/-}$ mice.

Resistance to 4T1 metastatic disease in CD1$^{-/-}$ mice is reminiscent of resistance to 4T1 in STAT6$^{-/-}$ mice (13, 25, 31). In both strains, tumoricidal M1 macrophages are produced, MSC levels decrease drastically after surgery, and lymphocytes are required. It is likely that IL-13 plays the same role in both strains because IL-13 signaling through the IL4Rα is via the STAT6/Janus-activated kinase 3 pathway (57). Although IL-13 plays an important role in blocking the production of M1 macrophages, it is not involved in accumulation of MSC or maintaining elevated MSC levels, because non-surgery CD1$^{-/-}$ or IL-4Rα$^{-/-}$ and post-surgery IL-4Rα$^{-/-}$ mice have high levels of MSC. Therefore, in addition to their effect on the IL-13/IL4Rα pathway, CD1 and STAT6 deficiencies also affect another pathway that regulates MSC cell retention.

MSC are present in many patients and experimental animals with cancer and are uniformly immunosuppressive (19–23). Although MSC from different individuals share the ability to suppress, they seem to be a heterogeneous population that suppress via a variety of mechanisms. Down-regulation of the CD3-associated ζ chain and the resulting dysfunction of T cells is a common phenomenon in many patients and experimental mice (reviewed by ref. 58). Rodriguez et al. have shown that such a down-regulation is mediated by macrophages (39, 59). Our findings support this mechanism for the suppression of CD4$^+$ T cells by MSC. However, CD8$^+$ T cells are not down-regulated for CD3ζ chain in our experiments, suggesting that there are additional mechanisms by which MSC inhibit T-cell activation. Other studies also support the concept that MSC are a functionally heterogeneous population of cells. For example, some MSC inhibit the activation of CD4$^+$ T cells and not CD8$^+$ T cells (60), whereas others inhibit CD8$^+$ T cells and have no effects on CD4$^+$ T cells (20, 22, 61), and others inhibit both CD4$^+$ and CD8$^+$ T cells (ref. 25; current report). The heterogeneity of MSC is further supported by the varied phenotypes that have been reported for these cells. Although many mouse MSC are characterized by their expression of Gr1 and CD11b, other mouse MSC express CD31 and do not express Gr1 and/or CD11b

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(19). Some MSC express MHC class II, B220, F4/80, CD86, CD16/32, and DEC205 (38), whereas others express MHC class I and do not express MHC class II or costimulatory molecules (20), and others express MHC I and costimulatory molecules but not MHC II (25). Differences also exist in ROS production between the different MSC populations studied. Kusmartsev et al. (41) have shown that ROS production by MSC is arginase dependent. ROS production by the BALB/c MSC described in this report are arginase dependent, whereas ROS production by $CD1^{-/-}$ MSC are arginase independent. These phenotypic differences probably characterize subpopulations of MSC, and it is possible that the different subpopulations have different target cells (e.g., $CD4^+$ versus $CD8^+$ T cells), thereby explaining the functional heterogeneity observed in the different tumor systems.

Others have also shown that $CD1^{-/-}$ mice have enhanced tumor immune surveillance (8, 9, 14, 62), supporting the concept that a deficiency in variant or invariant NKT cells facilitates tumor immunity. Preliminary data with $J\alpha18^{-/-}$ mice, which are deficient for invariant NKT cells, indicates that both variant and invariant NKT cells inhibit tumor immunity.[1] Terabe et al. also observe that blocking of IL-13 or interfering with signal transduction through the IL4Rα causes rejection of the CT26 colon carcinoma or block recurrence of the 15-12RM fibrosarcoma. In the 15-12RM fibrosarcoma, IL-13 inhibits tumor immunity by inducing MSC that produce high levels of the immunosuppressive cytokine TGFβ (9). However, this mechanism is not responsible for resistance to the 4T1 tumor because (*a*) 4T1-induced MSC do not produce TGFβ (25); (*b*) IL-4R$\alpha^{-/-}$ mice, which are unable to respond to IL-13 because they lack the requisite receptor, are susceptible to 4T1 metastatic disease; and (*c*) wild-type BALB/c mice treated with an inhibitor of IL-13 remain susceptible (13). Therefore, although we concur that IL-13 is a potent inhibitor of tumor immunity, we find that suppressing IL-13 is not sufficient for generating effective immune surveillance. We also do not agree that IL-13 inhibits immune surveillance by inducing MSC that produce high levels of TGFβ, because 4T1-derived MSC do not contain TGFβ (25). Our data indicate that IL-13 counteracts immune surveillance by polarizing macrophages away from a tumoricidal M1 phenotype. Although these differences in interpretation of IL-13 function may be due to differences in the tumor systems used, they may also indicate that IL-13 is a pleiomorphic cytokine that blocks immune surveillance via multiple mechanisms.

These studies show that immune surveillance can eliminate metastatic disease in a post-surgery setting. Although effective immunity is a complex process that requires the activation of multiple effector cells (macrophages and lymphocytes) coupled with the down-regulation of suppressive/inhibitory cells (MSC), a better understanding of these mechanisms may reveal strategies for facilitating tumor immunity and extending survival.

[1] P. Sinha and S. Ostrand-Rosenberg, unpublished results.

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