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Reduction of Myeloid-Derived Suppressor Cells and Induction of M1 Macrophages Facilitate the Rejection of Established Metastatic Disease[1]

Pratima Sinha, Virginia K. Clements, and Suzanne Ostrand-Rosenberg[2]

More than 60% of STAT6$^{-/-}$ mice immunologically reject spontaneous metastatic mammary carcinoma and survive indefinitely if their primary tumors are removed, whereas 95% of STAT6-competent BALB/c mice succumb to metastatic disease. BALB/c and STAT6-deficient mice with primary tumors have elevated levels of Gr1$^+$CD11b$^+$ myeloid suppressor cells (MSCs), which inhibit T cell activation. After removal of primary tumor, MSC levels revert to baseline in STAT6-deficient mice, but remain elevated in BALB/c mice. The decrease is IFN-γ dependent, as is the reduction in metastatic disease. Neither BALB/c nor STAT6-deficient MSCs produce inducible NO synthase; however, both produce arginase and reactive oxygen species. STAT6-deficient mice produce M1 macrophages, which contain high levels of NO and are tumoricidal, whereas BALB/c mice produce M2 macrophages, which make arginase and are not tumoricidal. Immunity in STAT6-deficient mice requires the activation of NO-producing M1 macrophages that are tumoricidal, the reduction in MSC levels to baseline after surgical removal of primary tumor, and the activation of tumor-specific T cells. These mechanisms occur in STAT6$^{-/-}$ mice because STAT6 deficiency prevents signaling through the type 2 IL-4Rα, thereby blocking the production of arginase and promoting the synthesis of NO. ***The Journal of Immunology,*** **2005, 174: 636–645.**

Recent laboratory and clinical studies suggest that immunosurveillance and immunotherapy may be effective mechanisms for preventing tumor onset and/or limiting the growth and progression of established tumors (1, 2). However, effective anti-tumor immunity is frequently impeded by complicating factors such as 1) host tolerance to tumor Ags (3), 2) down-regulation of MHC molecules on tumor cells, rendering them resistant to cell-mediated immunity (4), 3) tumor cell expression of ligands that mediate T cell destruction or dysfunction (5), 4) production of CD4$^+$CD25$^+$ T regulatory cells that block activation of effector T cells (6), and 5) tumor-induced immune suppression via the production of myeloid-derived suppressor cells (MSCs)[3] (7). The balance between these factors is critical for determining whether the host's immune system rejects tumor or if it is inhibited and is ineffective in controlling tumor growth.

We and others have recently identified a gene whose deletion favors the development of anti-tumor immunity, thereby tipping the balance away from inhibitory processes and toward a productive tumor-specific immune response. Mice that are deficient for the STAT6 gene are resistant to the metastatic 4T1 mammary carcinoma (8, 9), to recurrence of the 15-12RM fibrosarcoma (10), and to growth of the P815 mastocytoma (11). Tumor resistance in STAT6-deficient mice is T cell mediated and CD8 dependent. Because STAT6 is essential for activation of Type 2 CD4$^+$ T cells, it was hypothesized that STAT6-deficient mice have a polarized Type 1 CD4$^+$ T cell response and hence are better able to generate tumor-specific CD8$^+$ T cells than are STAT6$^{+/+}$ mice. Surprisingly, additional studies with the mammary carcinoma and fibrosarcoma tumors did not confirm a role for Type 1 vs Type 2 CD4$^+$ T cell responses, but instead indicated that deletion of the STAT6 gene removed an inhibitor that interfered with the generation of tumor-specific CD8$^+$ T cells. In the fibrosarcoma system, inhibition is IL-13 dependent and involves NKT cells that activate Gr1$^+$CD11b$^+$ MSCs, which in turn produce immunosuppressive TGFβ (12). In contrast, inhibition in the mammary carcinoma system does not involve IL-13, suggesting that STAT6 deficiency can mediate tumor regression via more than one mechanism (9).

Because a better understanding of the pathways by which STAT6 deficiency leads to tumor regression may suggest novel immunotherapy strategies, we are studying the mechanisms that underlie immunity to the BALB/c-derived 4T1 mammary carcinoma. This tumor shares many characteristics with human breast cancer, particularly its ability to spontaneously metastasize to the lungs, brain, liver, blood, lymph nodes, and bone marrow (13, 14). Although >95% of wild-type BALB/c (STAT6$^{+/+}$) mice die from 4T1 metastatic disease even if the primary mammary gland tumor is surgically removed, >60% of STAT6-deficient BALB/c mice survive indefinitely with the same treatment (9). In the present study we find that immunity in postsurgery STAT6-deficient mice is associated with a rapid decrease in a novel MSC population and with the activation of Type 1 tumoricidal macrophages that produce NO. Combined with our earlier studies, these results indicate that immunity to the 4T1 tumor in wild-type mice is blocked by two inhibitors: 1) a novel MSC population that interferes with

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[3] Abbreviations used in this paper: MSC, myeloid-derived suppressor cell; HA, influenza hemagglutinin; HEL, hen eggwhite lysozyme; DCFDA, dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; LCCM, L-cell conditioned medium; BMDM, bone marrow-derived macrophage; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; nor-NOHA, N^W-hydroxyl-nor-L-arginine; L-NMMA, N^G-monomethyl-L-arginine; LDH, lactate dehydrogenase; ATRA, all-trans-retinoic acid; TD, tumor diameter; iNOS, inducible NO synthase.

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$CD4^+$ T cell activation, and 2) macrophages that preferentially produce arginase instead of NO. These studies also demonstrate that suppressor cell characteristics vary between different tumors and hosts, suggesting that multiple subpopulations of MSC exist.

Materials and Methods

Mice

Mice were maintained and/or bred in the University of Maryland Baltimore County (UMBC) animal facility according to the National Institutes of Health guidelines for the humane treatment of laboratory animals. All animal procedures have been approved by the UMBC Institutional Animal Care and Use Committee. BALB/c, STAT6-deficient BALB/c ($STAT6^{-/-}$) and double-deficient $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ BALB/c mice were obtained and/or generated as described (8, 9). BALB/c DO11.10 TCR-transgenic mice expressing an $\alpha\beta$-TCR restricted to chicken OVA peptide 323–339 restricted by $I\text{-}A^d$ (15) were obtained from The Jackson Laboratory. BALB/c clone 4 TCR-transgenic mice expressing an $\alpha\beta$-TCR specific for amino acids 518–526 of influenza hemagglutinin (HA) restricted to $H\text{-}2K^d$ (16) and 3A9 TCR-transgenic mice expressing an $\alpha\beta$-TCR specific for hen eggwhite lysozyme (HEL) peptide 46–61 restricted to $I\text{-}A^k$ (17) were kindly provided by Drs. E. Fuchs (Johns Hopkins, Baltimore, MD) and B. Wade (Dartmouth, Hannover, NH), respectively.

Reagents and Abs

Dichlorodihydrofluorescein diacetate (DCFDA) and dihydroethidium (DHE) were from Molecular Probes; recombinant mouse IFN-γ and LPS were from Pierce-Endogen and Difco, respectively. $OVA_{323-339}$ peptide was synthesized in the Biopolymer Core Facility at the University of Maryland, Baltimore. Diff-Quik stain set was from Dade Behring.

Fluorescently labeled anti-mouse Abs Gr1-PE, CD3-FITC, CD4-PE, CD8-FITC, B220-PE, CD11c-PE, $I\text{-}A^d/I\text{-}E^d$, D^d-FITC, CD86-PE, CD80-FITC, CD40-PE, CD44-FITC, CD14-FITC, CD23-FITC, CD31-FITC, CD34-FITC, CD16/CD32-FITC, rat IgG2a-PE isotype control, and rat IgG2a-FITC isotype control were from BD Pharmingen. CD11b-FITC, F4/80-FITC, and KJ1-26, an anti-clonotypic mAb that recognizes the DO11.10 TCR (18), were from Caltag; PDL2-PE was from eBioscience; CD33-FITC was from Biocarta; DEC205-FITC was from Cedarlane; and rat anti-mouse Gr-1 Ab for MACS sorting (clone RB6-8C5) was from BD Pharmingen.

Cell lines

The J774 macrophage and L929 fibroblast cell lines were obtained from the American Type Culture Collection and maintained in DMEM (Biofluids) supplemented with 10% FBS (HyClone), 1% penicillin, 1% streptomycin (Biofluids), and 1% Glutamax (Invitrogen Life Technologies). The 4T1 mammary carcinoma was maintained as described (8).

L-cell conditioned medium (LCCM)

L929 cells were grown in 75-cm^2 T flasks in bone marrow-derived macrophage (BMDM) medium (DMEM, 10% FBS, 1% penicillin, 1% streptomycin, and 1% Glutamax) at 37°C in 5% CO_2. One confluent T flask was split into five 75-cm^2 T flasks and the cells were cultured for 48 h or until confluent. Culture supernatants were collected, filtered through 0.22-μm filters, and diluted 5-fold with BMDM medium. Resulting LCCM was stored frozen until used.

BMDMs

BMDMs were prepared as described (19). Briefly, femurs were removed from euthanized mice and flushed with DMEM. The resulting cells were pelleted at 290 × *g* and incubated in BMDM medium at 37°C in 5% CO_2. Twenty-four hours later the adherent cells, containing mostly fibroblasts and stromal cells, were discarded, and the nonadherent cells were replated in 10-cm dishes in 10 ml of LCCM. Four days later, another 5 ml of LCCM was added to each dish. Cultures were maintained for 10–20 days. Resulting cells were assayed by flow cytometry and were >90% $CD11b^+$ or $F4/80^+$.

Splenic MSCs

Splenocytes were depleted of RBCs (20) and washed twice with degassed, cold MACS buffer (0.5% BSA in PBS with 2 mM EDTA). Washed cells were resuspended at 1–2 × 10^9 cells in 2 ml of MACS buffer, incubated with 100 μl of rat anti-mouse Gr1 Ab for 30 min at 4°C, and then washed twice with MACS buffer. Gr1-labeled splenocytes (in 400 μl of MACS buffer in a 50-ml tube) were then incubated at 4°C with 100 μl of goat anti-rat IgG microbeads (Miltenyi Biotec) for 15 min. The tube was then filled with 45 ml of MACS buffer and centrifuged for 10 min at 290 × *g*. Pelleted cells were resuspended in 5 ml of MACS buffer and sequentially applied to two MACS-LS columns for positive selection according to the manufacturer's instructions (Miltenyi Biotec). The resulting cells were assayed by flow cytometry and were >90% $Gr1^+CD11b^+$.

Tumor inoculation, surgery, metastasis (clonogenic) assay, and carrageenan treatment

Female BALB/c mice were inoculated in the abdominal mammary gland with 7 × 10^3 4T1 tumor cells in 50 μl of serum-free IMDM (Biofluids) as described (13). Surgical resection of primary tumors, measurement of primary tumor diameters, and quantification of metastatic disease using the clonogenic assay were performed as previously described (13). Day of tumor inoculation is day 0. Carrageenan-treated (Sigma-Aldrich) mice were inoculated with 1 mg/mouse i.p. on days −6 and −4 and every 14 days thereafter and were followed for survival for 73 days. Treated mice were assayed for depletion of phagocytic cells by measuring reduced susceptibility to LPS-induced toxic shock as described (21). Rapidly progressing primary tumors are defined as those that are >4 mm in diameter by day 25–30.

Cytokine assays

4T1 cells at 5 × 10^5 cells/well/4 ml growth medium (IMDM, 10% Fetal Clone I, 1% penicillin, 1% streptomycin, and 1% Glutamax) were cultured in six-well plates. Supernatants were collected after 48 h and assayed in triplicate by the Cytokine Core Facility at the University of Maryland, Baltimore (IL-6, IL-10, GM-CSF, and activated TGFβ) or by using an ELISA kit from R&D Systems according to the manufacturer's guidelines vascular endothelial growth factor (VEGF).

Flow cytometry

Cells were labeled for direct immunofluorescence as described (13). Abs were diluted in HEPES buffer (0.01 M, pH 7.35) with 2% FCS (HyClone). Samples were analyzed on an Epics XL flow cytometer (Beckman Coulter) and analyzed using Expo32 ADC software (Beckman Coulter).

Reactive oxygen species (ROS)

ROS production was measured by DCFDA and DHE as described (22). Briefly, 10^6 MSCs were incubated at 37°C in serum-free DMEM containing 2 μM DCFDA for 20 min to measure ROS or with 2 μM DHE for 60 min to measure superoxide. To block ROS production, MSCs were incubated at 37°C with the arginase inhibitor N^W-hydroxyl-nor-L-arginine (nor-NOHA; 500 mM) for 10 min, followed by a 20-min incubation with DCFDA (2 μM). Treated cells were washed twice with excess cold PBS and analyzed by flow cytometry.

NO and arginase assays

BMDMs or MACS-sorted MSCs were activated by culturing 2 × 10^5 cells/200-μl well in DMEM containing 5% FBS and IFN-γ and LPS at final concentrations of 2 and 100 ng/ml, respectively, in 96-well flat-bottom plates for 18 h. The supernatants of individual wells were then removed for the NO assay, and the remaining attached cells were used for the arginase assay.

NO assay

NO was measured using Griess reagents (23). Briefly, 100 μl of culture supernatant was incubated for 10 min at room temperature with 50 μl of Griess reagent A (1% sulfanilamide in 2.5% H_3PO_4) plus 50 μl of Griess reagent B (0.1% naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4) per well in 96-well flat-bottom plates. Absorbance at 540 nm was measured using a Biotek 311 microplate reader. Data are the mean ± SD of triplicate wells.

Arginase assay

Arginase was quantified by measuring the production of urea as described (24, 25) with the following modifications. BMDMs were washed twice with 0.5% BSA in PBS, resuspended in 50 μl of lysis buffer (10 ml of 0.1% Triton X-100 in water with one tablet of protease inhibitor mixture; Roche), and incubated at 37°C for 30 min. Lysates were transferred to 1.5-ml microfuge tubes and arginase was activated by adding 50 μl of 25 mM Tris-HCl and 10 μl of 2 mM $MnCl_2$ per tube and heating the mixture at 56°C for 10 min. One hundred microliters of 500 mM L-arginine (pH 9.7) was added per tube, and the tubes were incubated at 37°C for 30 min

to hydrolyze the L-arginine. L-Arginine hydrolysis was stopped by adding 800 μl of acid solution (H_2SO_4 (96%):H_3PO_4 (85%):H_2O (1:3:7)) per tube. To measure the degradation of L-arginine to urea, 40 μl of α-isonitrosopropiophenone (dissolved in 100% ethanol) was added to each tube and the resulting precipitate was dissolved by heating the tubes at 100°C for 15 min. A calibration curve was run in parallel with the experimental samples and consisted of 50 μl of serial dilutions of urea dissolved in lysis buffer. Two hundred microliters from each tube was transferred to wells of a 96-well flat-bottom plate, and the urea concentration was measured at 540 nm using a Biotek microplate reader. Nor-NOHA, L-norvalin, N^G-monomethy-L-arginine (L-NMMA; Calbiochem) were used as described (26). The inactive enantiomer D-NMMA served as a negative control for L-NMMA. Data are the mean ± SD from triplicate wells.

T cell proliferation assay

Splenocytes from DO11.10, clone 4, or 3A9 mice were depleted of RBCs and cocultured in 96-well plates at 37°C in proliferation medium (HL1 medium (BioWhittaker), 1% penicillin, 1% streptomycin, 1% Glutamax, and 5×10^{-5} M 2-ME) at 10^5 cells/well with 2500 Rad irradiated MACS-sorted MSCs and 14 μM OVA peptide, 28 μM HA peptide, or 5 μg of HEL protein in a total volume of 200 μl/well in 5% CO_2. Cells were pulsed with 1 μCi [^{3}H]thymidine/well (ICN Biochemicals) on day 3, and 18 h later the cells were harvested onto glass fiber filter mats using a Packard Micromate 196 cell harvester. Filter mats were sealed in plastic bags with 4 ml of Betaplate scintillation fluid (PerkinElmer) and [^{3}H]thymidine incorporation was measured using a Wallac 1450 Microbeta liquid scintillation counter (PerkinElmer). Data are expressed as cpm (mean ± SD) of triplicate cultures. Transwell chambers (0.02-μm pores) were from Nunc.

percent suppression = 100% [1 − (cpm of spleen + peptide + MSC/cpm of spleen + peptide)].

Cytotoxicity assay

Cytotoxicity was measured as described (27). IFN-γ- and LPS-activated BMDMs (10^5/well) were washed twice with cytotoxicity medium (IMDM, 3% FBS) and cocultured with 4T1 target cells (10^4/well) in 200 μl/well for 24 h at 37°C in 5% CO_2. Lactate dehydrogenase (LDH) activity in the culture supernatants was determined using a Cytotoxicity Detection kit (Roche) per the manufacturer's guidelines. The absorbance at 490 nm was measured using a Biotek 311 microplate reader. Spontaneous LDH release was obtained from nonactivated BMDMs incubated with 4T1 target cells. Experimental LDH release was obtained from activated BMDMs incubated with 4T1 target cells. Maximum release was obtained by adding 100 μl of 2% Triton X-100 (in cytotoxicity medium) to wells of 4T1 target cells alone. Values are the average of triplicates ± SD. Background values for media were subtracted from each point. Activated and nonactivated BMDMs without 4T1 were routinely run and gave no LDH release.

Percent specific lysis = 100%

$$\times [(A_{490}\,\text{Experimental} - A_{490}\,\text{Spontaneous})/(A_{490}\,\text{Maximum})]$$

All-trans-retinoic acid (ATRA) treatment

Pellets with or without ATRA (Innovative Research of America) were implanted in the neck on the day of removal of primary tumor as described (28). Lung metastases were quantified on day 38 after 4T1 inoculation.

Statistical analysis

Student's one-tailed (see Fig. 2, *A* and *B*) or two-tailed (see all other figures) *t* test for unequal variance was performed using Microsoft Excel 2000.

Results

MSC levels return to normal in STAT6-deficient mice, but not in BALB/c mice, after surgical removal of primary tumor

Immature myeloid cells or MSCs frequently accumulate in mice and patients as tumor burden increases, resulting in immune suppression. To determine whether deletion of the STAT6 gene affects MSC levels in 4T1 tumor-bearing mice, wild-type BALB/c and STAT6$^{-/-}$ mice were inoculated in the mammary gland with 4T1 tumor cells, and splenocytes were harvested 30–39 days later, stained, and analyzed by flow cytometry for Gr1$^+$CD11b$^+$ MSCs (nonsurgery group). Because immune suppression has been shown to decrease if primary tumor is removed (29), we also assessed MSC levels in mice whose primary tumors were surgically resected (postsurgery group). For the postsurgery group, primary tumors were resected on day 21–28 after initial tumor inoculation, and splenic MSC levels were assessed 9–11 days later. The nonsurgery BALB/c and STAT6$^{-/-}$ groups were matched for primary tumor diameter (TD) (6.05 ± 0.75 mm and 5.4 ± 0.97 mm, respectively) as were the BALB/c and STAT6$^{-/-}$ surgery groups at the time of surgery (TD: 6.11 ± 0.81 mm and 5.56 ± 0.95 mm, respectively) to minimize differences due to primary tumor burden. Tumor-free BALB/c (Fig. 1*A*) and STAT6$^{-/-}$ (Fig. 1*B*) mice have <8% splenic MSCs, whereas BALB/c and STAT6$^{-/-}$ nonsurgery mice have similarly high levels of splenic MSCs. Although both postsurgery groups showed decreases in splenic MSCs, the decrease in STAT6$^{-/-}$ mice is significantly greater than in BALB/c mice in that 67% of STAT6$^{-/-}$ mice vs 21% of BALB/c mice have <8% splenic MSCs 11 days after surgery ($p < 0.036$). The average MSC levels between the two postsurgery groups are also statistically significantly different (BALB/c: 12% ± 2.5; STAT6$^{-/-}$: 7% ± 1.9; $p < 0.01$). Therefore, although BALB/c and STAT6-deficient mice have comparably high levels of splenic MSCs while primary tumor is present, postsurgery STAT6$^{-/-}$ mice have fewer MSCs.

STAT6-deficient mice have less metastatic disease than do BALB/c mice

Tumor cells are known to secrete cytokines that stimulate the accumulation of MSCs (30). Therefore, the different levels of MSCs in postsurgery BALB/c vs STAT6-deficient mice could be due to differences in cytokine production by 4T1 tumor cells as they grow in the two mouse strains. To test this hypothesis, supernatants from in vitro-cultured 4T1 cells were compared with supernatants from ex vivo-cultured 4T1 cells harvested from primary tumor or from lungs of nonsurgery mice. As measured by ELISA, 24-h supernatants from in vitro-cultured 4T1 cells contained IL-6 (265 ± 16 pg/ml), GM-CSF (177 ± 9 pg/ml), VEGF (88 ± 14 pg/ml), and activated TGFβ (2410 ± 27 pg/ml). Although supernatants from ex vivo-cultured primary and metastatic 4T1 cells contained variable levels of IL-6, GM-CSF, VEGF, and activated TGFβ, there

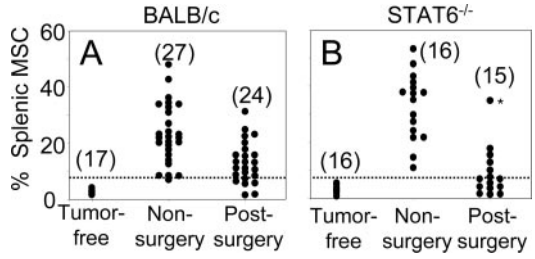

FIGURE 1. Gr1$^+$CD11b$^+$ splenic MSC levels are elevated in nonsurgery BALB/c and STAT6$^{-/-}$ mice, but return to normal in STAT6$^{-/-}$ mice after removal of primary tumor. BALB/c (*A*) or STAT6-deficient (*B*) mice were inoculated on day 0 with 4T1 tumor cells in the mammary gland, and primary tumors either were left in place (nonsurgery) or were surgically resected on approximately day 28 (postsurgery). Tumor-free groups were not inoculated with 4T1. Percent splenic MSCs is the percent of splenocytes that were CD11b$^+$Gr1$^+$ on approximately day 38. Postsurgery groups have statistically significantly fewer MSCs than do their corresponding nonsurgery groups ($p < 0.05$). Postsurgery STAT6-deficient mice have statistically fewer MSCs than do postsurgery BALB/c mice ($p < 0.036$). (Outlier value denoted with an asterisk was omitted for the *t* test). Each symbol represents an individual mouse. Numbers indicate the number of mice in each group. Data are pooled from four independent experiments. Dotted lines indicate the level of MSCs in tumor-free mice.

were no significant differences between cytokine levels from BALB/c and STAT6$^{-/-}$ mice (data not shown). Therefore, 4T1 tumor cells secrete several cytokines known to induce MSCs; however, the difference in MSC levels in postsurgery BALB/c and STAT6$^{-/-}$ mice is not due to differential secretion of these cytokines.

Because tumor load can affect MSC levels, we have used the clonogenic assay to quantify lung metastatic disease in the nonsurgery and postsurgery BALB/c and STAT6-deficient mice of Fig. 1. Although STAT6$^{-/-}$ mice have significantly ($p < 0.01$) reduced metastatic disease relative to BALB/c mice, there is no direct correlation between number of metastatic cells and percent MSCs (Fig. 2).

MSCs from STAT6$^{-/-}$ and BALB/c mice are phenotypically and functionally equivalent and their suppressive activity is due to arginase production

Qualitative differences between MSCs of BALB/c and STAT6$^{-/-}$ mice may also contribute to the increased anti-tumor immunity of STAT6-deficient animals. To test this hypothesis, BALB/c and STAT6$^{-/-}$ mice were inoculated in the mammary gland with 4T1 tumor cells; 21–28 days later splenocytes were removed and MSCs were purified by MACS sorting and phenotyped by Ab staining. To minimize differences due to primary tumor load, BALB/c and STAT6$^{-/-}$ donors were matched for primary 4T1 TD at the time of sacrifice (8.96 ± 0.35 mm and 9.01 ± 0.33 mm, respectively). MACS-sorted MSCs from both BALB/c and STAT6$^{-/-}$ mice were ≥95% pure (Gr1$^+$CD11b$^+$) (Fig. 3*A*) and had the morphology of immature myeloid cells (Fig. 3*B*). Although the MSCs from both strains are phenotypically similar, MSCs from STAT6-deficient mice express more CD16/CD32 and CD80, whereas MSCs from BALB/c mice express more CD11c, DEC205, and CD8 (Fig. 3*C*). Therefore, there are subtle differences between the MSCs of tumor-bearing BALB/c and STAT6-deficient mice.

To determine whether the MSCs of BALB/c and STAT6-deficient mice have similar immunosuppressive activity, equal numbers of MACS-sorted Gr1$^+$CD11b$^+$ cells from tumor-free, nonsurgery, or postsurgery mice were cocultured with OVA peptide-pulsed splenocytes from DO11.10 transgenic mice, and T cell proliferation was measured by [^{3}H]thymidine incorporation. Gr1$^+$CD11b$^+$ cells from all three groups and from both strains are highly suppressive, whereas control J774 cells do not suppress (Fig. 4*A*). Purified MSCs similarly inhibit HA-specific CD8$^+$ T cells and HEL-specific allogeneic CD4$^+$ T cells (Fig. 4*B*). Culture supernatants were also assayed for IL-2 activity. In the presence of MSCs, IL-2 levels were reduced ~50%; however, IL-2 levels rebounded completely when the arginase inhibitor nor-NOHA was added (data not shown). Therefore, the suppressive capacity of Gr1$^+$CD11b$^+$ cells on a per cell basis is similar in BALB/c and STAT6-deficient mice, is independent of whether tumor is present, involves a reduction in IL-2 production, and is not MHC-, CD4-, or CD8-restricted.

To determine whether differences in anti-tumor immunity between BALB/c and STAT6$^{-/-}$ mice could be due to differential expression of ROS, as suggested by Kusmartsev et al. (31), MACS-purified Gr1$^+$CD11b$^+$ MSCs from tumor-free and nonsurgery mice were treated with DCFDA or DHE and were analyzed by flow cytometry. DCFDA is oxidized by hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), peroxynitrile ($ONOO^-$), or superoxide to yield a fluorescent compound, and thus measures ROS.

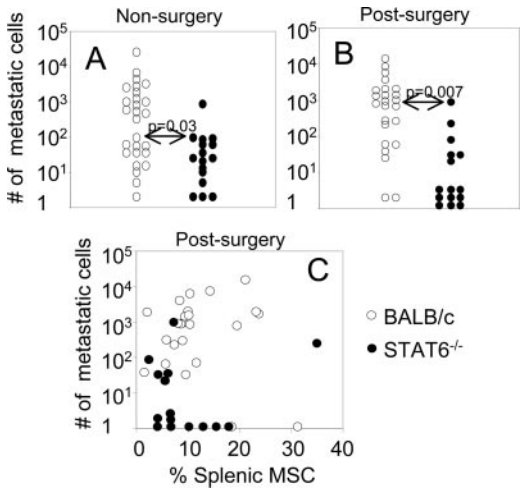

FIGURE 2. STAT6-deficient mice have less metastatic disease than do BALB/c mice. Mice were inoculated with 4T1 tumor as indicated in Fig. 1. Lungs were harvested from the nonsurgery (*A*) or postsurgery (*B* and *C*) groups, and the number of metastatic cells was quantified using the clonogenic assay. Each symbol represents the number of metastatic tumor cells in the lungs of an individual mouse. *C*, MSC levels do not directly correlate with number of metastatic cells. Data are pooled from four independent experiments.

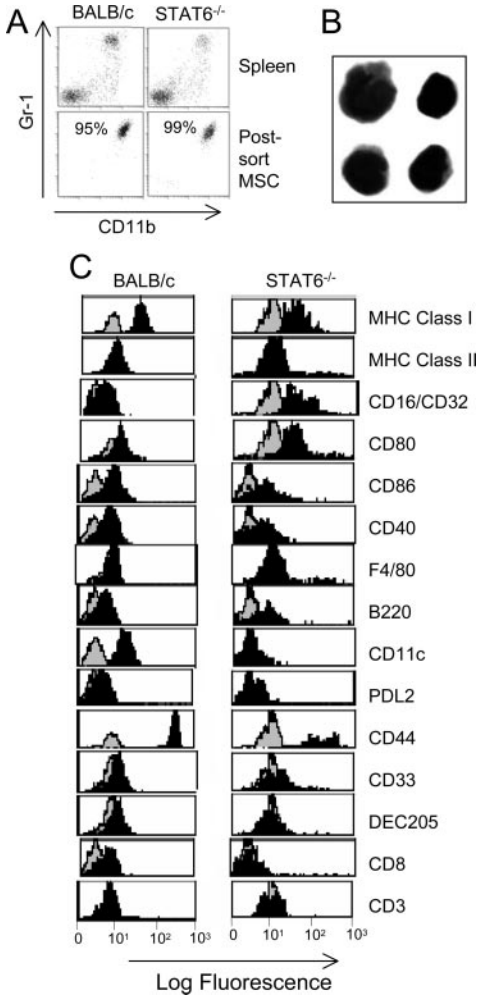

FIGURE 3. MSCs from STAT6-deficient and BALB/c mice have different phenotypes. *A*, Spleen cells from BALB/c and STAT6$^{-/-}$ nonsurgery mice stained with Gr1 and CD11b mAbs before and after MACS sorting. *B*, Wright-Giemsa-stained, MACS-purified Gr1$^+$CD11b$^+$ cells (magnification ×630). *C*, MACS-sorted MSCs stained with the indicated mAbs. CD3 represents the staining by CD4, CD14, CD23, CD31, and CD34 mAbs. Gray and black peaks are the isotype control and Ag-specific staining, respectively. Data are from one of two independent experiments.

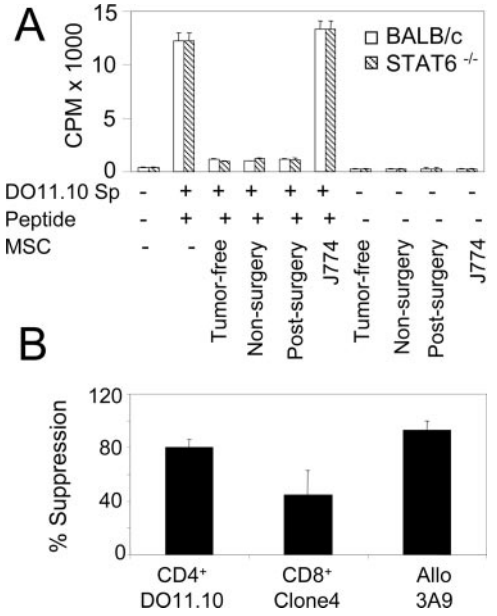

FIGURE 4. MSCs suppress Ag-specific $CD4^+$ and $CD8^+$ T cell proliferation and are not MHC restricted. *A*, DO11.10 transgenic splenocytes were stimulated with $OVA_{323–339}$ peptide in the presence or absence of MACS-sorted $Gr1^+CD11b^+$ MSCs from tumor-free, nonsurgery, or postsurgery STAT6-deficient or BALB/c mice, or with control J774 cells. *B*, D011.10, clone 4, or 3A9 transgenic splenocytes were stimulated with $OVA_{323–339}$ or $HA_{518–526}$ peptide or HEL protein, respectively, in the presence of nonsurgery BALB/c MSCs. MSCs from two to three mice were pooled for each group. Data are from one of two independent experiments.

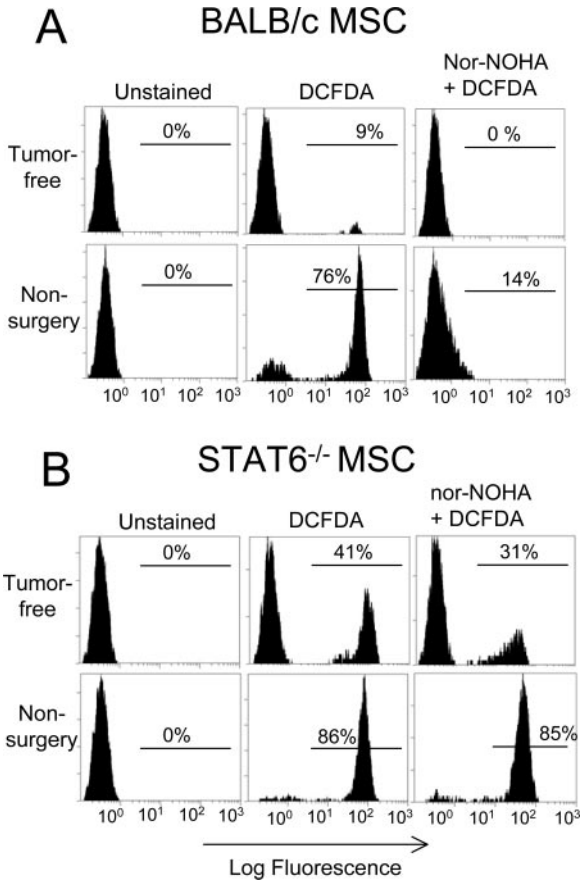

FIGURE 5. MSCs from tumor-bearing BALB/c and STAT6-deficient mice produce ROS; however, ROS production is arginase dependent in BALB/c mice and arginase independent in STAT6-deficient mice. MACS-purified MSCs from tumor-free or nonsurgery BALB/c (*A*) or STAT6-deficient (*B*) mice were incubated with DCFDA in the presence or absence of the arginase inhibitor nor-NOHA. Data are from one of two independent experiments.

Likewise, DHE is oxidized by superoxide to a fluorescent species. MSCs from nonsurgery BALB/c (Fig. 5*A*) and STAT6-deficient (Fig. 5*B*) mice contain more ROS than do MSCs from tumor-free mice. Neither MSC population stains with DHE (data not shown), indicating that the MSCs do not make superoxide. To determine whether arginase is required for ROS production, MSCs from nonsurgery mice were treated with the arginase inhibitor nor-NOHA before staining with DCFDA. Nor-NOHA blocks the production of ROS from MSCs of BALB/c nonsurgery mice, but has no effect on ROS expression by MSCs from nonsurgery $STAT6^{-/-}$ mice. In addition, $STAT6^{-/-}$ MSCs have a high baseline level of ROS in tumor-free mice, so there is only a 2-fold increase in ROS in MSCs from tumor-free vs nonsurgery $STAT6^{-/-}$ MSCs, whereas there is an 8-fold increase in BALB/c MSCs. Therefore, MSCs from tumor-free BALB/c and STAT6-deficient mice contain different baseline levels of ROS, whereas ROS levels are comparable in nonsurgery mice; however, the ROS produced by BALB/c mice are arginase dependent, whereas the ROS produced by STAT6-deficient MSCs are arginase independent.

Bronte et al. (26, 32) have shown that MSC activity is dependent on arginase and/or inducible NO synthase (iNOS). To determine whether MSCs from nonsurgery mice differ because of selective expression of arginase and/or iNOS, OVA peptide-pulsed splenocytes from DO11.10 transgenic mice were cocultured with MSCs in the presence or absence of inhibitors of arginase or iNOS. BALB/c and $STAT6^{-/-}$ MSCs inhibit DO11.10 proliferation, and this inhibition is reversed by the arginase inhibitors norvalin and nor-NOHA, but not by the iNOS inhibitor L-NMMA (Fig. 6*A*). Similar inhibition was seen for clone 4 $CD8^+$ T cells and for allogeneic 3A9 $CD4^+$ T cells (data not shown). To determine whether suppression requires cell contact, peptide-pulsed DO11.10 cells were cocultured with MSCs contained in transwell chambers. Proliferation of DO11.10 cells separated from MSCs by a semipermeable membrane was not inhibited, indicating that MSCs must directly contact the target cells they are suppressing (Fig. 6*B*). Therefore, in agreement with earlier studies with other tumors (33), 4T1-induced MSCs suppress via a contact-dependent mechanism involving arginase

Reduction of MSC levels and decrease in metastatic disease in $STAT6^{-/-}$ mice is IFN-γ dependent

Previous studies established that resistance to the 4T1 tumor in STAT6-deficient mice requires IFN-γ because $STAT6^{-/-}$IFN-$\gamma^{-/-}$ mice were just as susceptible to metastatic disease as were BALB/c mice (9). If tumor resistance in postsurgery $STAT6^{-/-}$ mice is dependent on the rapid decrease in MSCs and if IFN-γ is involved in that decrease, then postsurgery $STAT6^{-/-}$IFN-$\gamma^{-/-}$ mice should have relatively high levels of MSCs. To test this hypothesis, $STAT6^{-/-}$IFN-$\gamma^{-/-}$ mice were inoculated with 4T1 on day 1, and tumors were either left in place (nonsurgery group) or they were removed 21–28 days later (postsurgery group). Nine to 11 days after the surgery date, all mice were sacrificed, their splenocytes were stained for Gr1 and CD11b, and their lungs were assayed by the clonogenic assay for metastatic 4T1 cells. The nonsurgery and postsurgery groups were matched for primary TD on the day of surgery (nonsurgery: 6 ± 2.02 mm; postsurgery: 6.22 ± 1.74 mm). There is a modest decrease in MSCs in postsurgery $STAT6^{-/-}$IFN-$\gamma^{-/-}$ mice (Fig. 7*A*); however, the decrease is

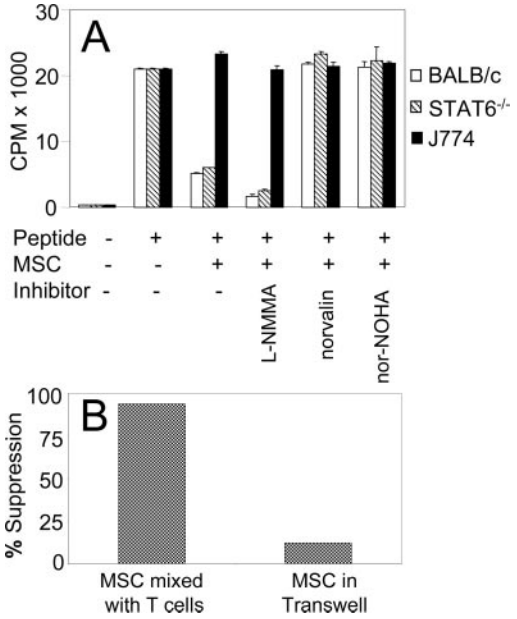

FIGURE 6. MSC-induced suppression of Ag-specific T cells is arginase dependent and cell contact dependent. *A*, DO11.10 transgenic splenocytes were cocultured with $OVA_{323–339}$ peptide in the presence or absence of MSCs from nonsurgery mice or with control J774 cells in the presence or absence of the iNOS inhibitor L-NMMA, or with the arginase inhibitors nor-NOHA or norvalin. *B*, MSCs were contained in a transwell chamber or directly mixed with D011.10 T cells plus peptide. Data are from one of three to five independent experiments.

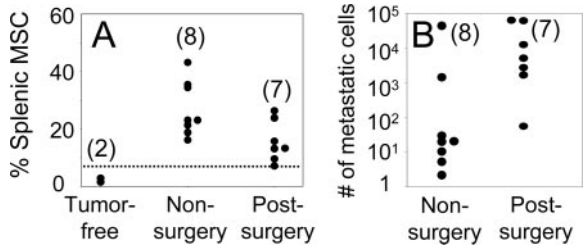

FIGURE 7. Regression of MSCs and metastatic disease in STAT6-deficient mice is IFN-γ dependent. $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ mice were inoculated with 4T1 tumor as described in Fig. 1. *A*, Percent splenic MSCs is the percent of splenocytes that stained $Gr1^{+}CD11b^{+}$ on approximately day 38 after tumor inoculation. Dotted line indicates the level of MSCs in tumor-free mice. MSCs in the postsurgery $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ group are significantly higher than in the postsurgery $STAT6^{-/-}$ group ($p \leq 0.01$), but they are not significantly different from the postsurgery BALB/c group (see Fig. 1*A* for BALB/c data). *B*, Lungs from the mice in *A* were harvested on approximately day 38 and were assayed by the clonogenic assay for the number of metastatic tumor cells. Each symbol represents an individual mouse. Numbers in parentheses indicate the number of mice in each group. Data are pooled from two independent experiments.

comparable to that seen in tumor-susceptible BALB/c mice (14% and 21% have <8% MSCs, respectively; see Fig. 1*A* for BALB/c data) and does not approach the larger decrease seen in $STAT6^{-/-}$ mice, in which 67% of mice have <8% MSCs (see Fig. 1*B* for $STAT6^{-/-}$ data). The average MSC levels between the $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ and $STAT6^{-/-}$ postsurgery groups are also statistically significantly different ($STAT6^{-/-}IFN\text{-}\gamma^{-/-}$: 14.8 ± 5.6; $STAT6^{-/-}$: 7 ± 1.9; $p < 0.01$). In contrast, the average MSC levels between postsurgery $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ and BALB/c mice are not significantly different ($STAT6^{-/-}IFN\text{-}\gamma^{-/-}$: 14.8 ± 5.6; BALB/c: 12 ± 2.5). In agreement with the high MSC count, the number of metastatic cells in the $STAT6^{-/-}IFN\text{-}\gamma^{-/-}$ mice is also significantly ($p = 0.05$) elevated (Fig. 7*B*) compared with $STAT6^{-/-}$ postsurgery mice. However, there is no direct correlation between number of metastatic cells and percent MSCs (data not shown). Therefore, resistance to metastatic disease in $STAT6^{-/-}$ mice requires IFN-γ, and IFN-γ is essential for the rapid decrease in MSCs after surgery.

$STAT6^{-/-}$ macrophages produce high levels of iNOS, are cytotoxic for 4T1 tumor cells, and do not produce arginase

In addition to cytotoxic $CD8^{+}$ T cells, macrophages can have tumoricidal activity. Type 1 macrophages (M1 macrophages), which make iNOS and do not make arginase, are particularly tumoricidal, whereas M2 macrophages, which make arginase and do not make iNOS, are not tumoricidal (34–36). Previous studies indicated that phagocytic cells are an important component of immune surveillance against the 4T1 tumor (21), suggesting that macrophages may be involved in the enhanced immunity of STAT6-deficient mice. To determine whether macrophages are involved in the heightened immunity in STAT6-deficient mice, BMDMs were prepared from tumor-free, nonsurgery, and postsurgery BALB/c and $STAT6^{-/-}$ mice and were tested in vitro for arginase and iNOS production and for cytotoxicity against 4T1 tumor cells. The nonsurgery and postsurgery groups were matched for primary TD at the time of surgery (BALB/c nonsurgery, 7.75 ± 0.8 mm vs postsurgery, 8.08 ± 0.8 mm; $STAT6^{-/-}$ nonsurgery, 7.46 ± 0.59 mm vs postsurgery, 7.6 ± 0.41 mm). Both activated and nonactivated BALB/c macrophages from nonsurgery and postsurgery mice synthesize arginase, as measured by urea production, whereas $STAT6^{-/-}$ macrophages, regardless of activation state, do not produce arginase (Fig. 8*A*). Nonactivated macrophages from both strains do not produce iNOS, as measured by NO production. iNOS production by BALB/c macrophages decreases with tumor progression, whereas STAT6-deficient macrophages maintain high iNOS production regardless of the presence of primary tumor or

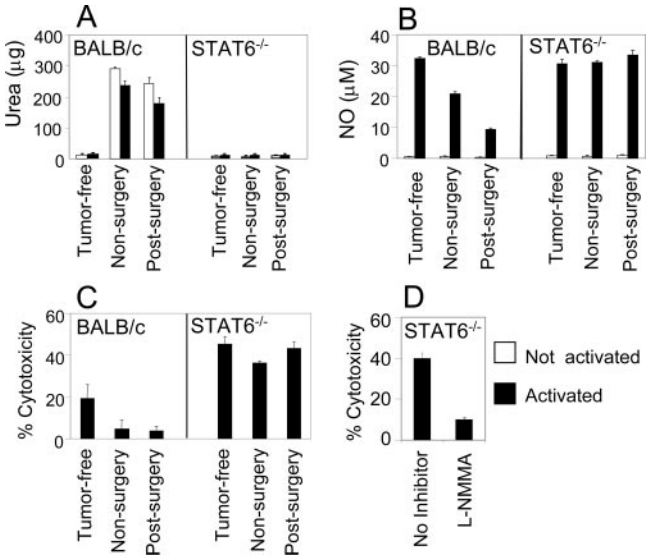

FIGURE 8. STAT6-deficient mice make M1 BMDMs, which produce NO and are tumoricidal, whereas BALB/c mice make M2 BMDMs, which produce arginase and are not tumoricidal. BMDMs from tumor-free, nonsurgery, or postsurgery mice were not activated or activated with IFN-γ and LPS and were assayed for urea production (*A*) as a measure of arginase content or for NO production (*B*) as a measure of iNOS. *C* and *D*, BMDMs were cocultured with 4T1 target cells and percent cytotoxicity was determined. Data are from one of two independent experiments.

metastatic disease (Fig. 8*B*). Activated STAT6$^{-/-}$ macrophages are also more tumoricidal for 4T1 cells compared with activated BALB/c macrophages (Fig. 8*C*), and the cytotoxic activity is iNOS dependent (Fig. 8*D*). Collectively, these data indicate that BALB/c mice make an M2 macrophage response that is ineffective in destroying 4T1 tumor, whereas STAT6-deficient mice make an M1 macrophage response that kills 4T1 tumor cells.

Depletion of macrophages reduces survival

To determine whether macrophages are critical for increased survival of STAT6-deficient mice, STAT6$^{-/-}$ mice were either untreated or depleted of phagocytic cells by carrageenan treatment, inoculated with 4T1 tumor, had primary tumors removed, and were followed for survival. As shown in Table I, only 45% of carrageenan-treated mice survived, whereas 75% of nontreated mice survived. The macrophage-depleted mice that died also died more rapidly (37 ± 14 vs 47 ± 21 days for the carrageenan-treated vs nontreated groups, respectively); however, these values were not statistically signficantly different. Before surgery, the two groups were also followed for primary tumor growth. Sixty-three percent and 29% of carrageenan-treated and untreated mice, respectively, had rapidly progressing primary tumors. Therefore, macrophages are essential for survival and for enhanced immunity to primary and metastatic disease in STAT6-deficient mice.

To determine whether reduction in MSCs is sufficient for tumor rejection, postsurgery BALB/c mice were implanted with pellets containing ATRA, which has been shown to reduce MSC levels (28). Although MSC levels were reduced to baseline in 50% of the postsurgery BALB/c mice implanted with the ATRA pellets, metastatic tumor levels in these mice remained high (data not shown). Therefore, reduction in MSC levels in the absence of cytotoxic macrophages is not sufficient for metastasis rejection.

Discussion

Global deletion of the STAT6 gene provides potent immunity/immunosurveillance to a variety of tumors (8, 10, 11). The enhanced immunity is particularly impressive against metastatic disease in that 60–80% of mice whose primary mammary tumors are removed reject their established metastases and survive indefinitely. In contrast, <5% of STAT6-competent BALB/c mice survive (9). Previous studies established that CD8$^+$ T cells are critical for immunity (8). The current study identifies two additional cell populations that are also involved: 1) immunosuppressive MSCs, which rapidly and irreversibly decrease to background levels after surgical removal of primary tumor, and 2) tumoricidal M1-type macrophages that produce NO and do not produce arginase.

Fig. 9 shows a model of how these effectors and inhibitors may interact to mediate tumor regression vs tumor progression. In both STAT6-competent and STAT6-deficient mice, 4T1 cells of the primary tumor secrete cytokines (IL-6, TGFβ, GM-CSF, and VEGF) that stimulate the accumulation of MSCs. MSCs are thought to inhibit T cell function by secreting arginase, which depletes L-arginine, causing a loss of CD3ζ chain expression (37–39). Therefore, the high levels of arginase-producing MSCs block activation of tumor-reactive CD4$^+$ and CD8$^+$ T cells, which would normally be activated to tumor Ags by cross-presentation by professional APCs. In the absence of functional CD4$^+$ T cells, potent tumor-specific CD8$^+$ T cells are not generated and tumor growth progresses. However, after removal of 4T1 primary tumor from STAT6-deficient mice, MSC levels decrease to baseline, allowing tumor-specific CD4$^+$and CD8$^+$ T cells to be activated. In contrast, MSC levels in postsurgery STAT6-competent mice do not return to baseline, so T cells remain suppressed. In addition, macrophages of STAT6-deficient mice are tumoricidal because they make NO, whereas BALB/c macrophages are not cytotoxic and make arginase, which supports tumor growth (35). Therefore, complete rejection of metastatic disease and survival of STAT6-deficient mice requires the reduction in MSC levels, coupled with the presence of NO-secreting macrophages and tumor-specific T cells. This model is consistent with the results presented in this report, as well as with earlier studies demonstrating a requirement for CD8$^+$ T cells (8) and phagocytic cells (21).

Previous studies in patients have demonstrated that MSCs decrease after surgical removal of tumor, presumably due to the reduction of tumor cells secreting factors that mediate MSC accumulation (reviewed in Refs. 7 and 30). The rapid decrease of MSCs in postsurgery STAT6-deficient mice identified in the current study demonstrates that MSC levels after surgery are not exclusively regulated by tumor burden. In agreement with others, our working hypothesis is that MSC regression is regulated by a ligand that binds to a receptor on the MSCs and causes them to accumulate or regress. Because STAT6 deficiency favors rapid regression to baseline levels, the relevant ligand must signal through STAT6. Although others have proposed that the IL-4Rα regulates MSC levels (10, 12, 26), this receptor is not relevant in the 4T1 system because IL-4Rα knockout mice have the same levels of MSCs as do wild-type mice (P. Sinha and S. Ostrand-Rosenberg, unpublished observations). Therefore, an alternative receptor on 4T1-induced MSCs that signals through STAT6 must mediate the regression to baseline in postsurgery mice.

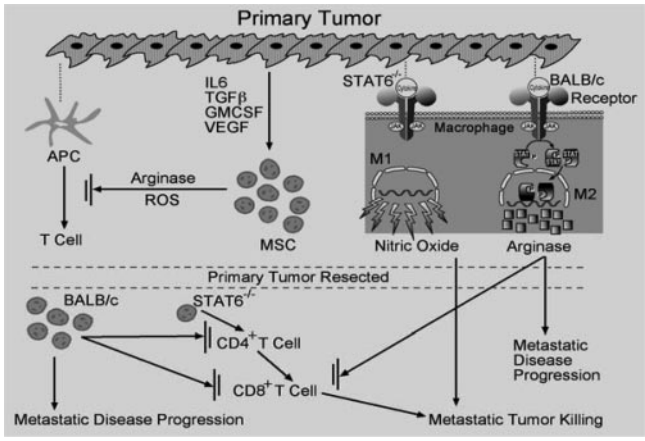

FIGURE 9. Proposed model for tumor resistance of STAT6-deficient mice. Resistance to metastatic disease in postsurgery STAT6-deficient mice requires the regression of MSCs and the presence of M1 macrophages and activated T cells. See details for a *Discussion* of the roles of NO, arginase, MSCs, macrophages, and T cells.

Table I. *Macrophage-depleted STAT6-deficient mice have decreased survival*[a]

Treatment	Percent Survival	Percentage of Mice with Rapid Primary Tumor Growth
None	75% (9/12)	29% (4/14)
Carrageenan	45% (5/11)	63% (10/16)

[a] STAT6-deficient mice were inoculated in the abdominal mammary gland with 7000 4T1 cells on day 0, primary tumors were removed 3–4 wk later, and mice were followed for 73 days. Macrophages were depleted by inoculation of 1 mg/mouse carrageenan on days −6, −4, and every 14 days thereafter for the duration of the experiment. Rapid primary tumor growth is defined as tumors that are >4 mm in diameter by days 25–30.

Macrophages are a heterogenous population of cells whose phenotype, characteristics, and functions are determined by the cytokine milieu in which they reside. M1 macrophages produce NO and reduce tumor growth, whereas M2 macrophages produce arginase and facilitate tumor progression (34–36). In activated macrophages, iNOS converts arginine and oxygen to citrulline and NO. If arginine is not available, NO is not produced. In M2 macrophages, where arginase production is high, the arginine pool is depleted, resulting in low levels of iNOS and minimal NO production (32, 40). Because the production of arginase requires IL-4 and IL-13 signaling through the JAK3/STAT6 pathway (19, 41), STAT6-deficient mice should not produce arginase and hence should not make M2 macrophages. Our finding that macrophages from STAT6-deficient mice are strongly polarized toward an M1 phenotype is consistent with the role of STAT6 in regulating arginase production and suggests that, in the absence of arginase, activated macrophages default toward a tumoricidal M1 phenotype. Because signaling by IL-4, presumably through the IL-4Rα and the JAK-STAT pathway, is thought to be essential for the production of arginase (35, 42), the preferential generation of M1 macrophages by STAT6-deficient mice is entirely consistent with the deletion of the STAT6 gene.

In addition to its ability to mediate cytotoxicity, NO is also important in $CD8^+$ T cell differentiation. Decreased NO levels due to depletion of arginine by arginase have been associated with CD3ζ chain deficiencies and defects in $CD8^+$ T lymphocytes (39, 43). Because NO preferentially induces type 1 T cell differentiation (44, 45), $CD8^+$ T cells of STAT6-deficient mice may be more efficacious because $STAT6^{-/-}$ macrophages are polarized toward NO production. This scenario is consistent with earlier observations that STAT6-deficient mice have 4T1-specific cytotoxic $CD8^+$ T cells, whereas STAT6-competent mice do not (8). Given the multiple inhibitory effects of arginase, strategies that specifically block or degrade arginase in macrophages may be novel and useful cancer immunotherapy approaches.

NO production by activated macrophages has also been shown to stimulate differentiation of MSCs to normal myeloid-derived cells (46, 47). We find a direct correlation between elevated levels of NO-producing macrophages and decreased MSC levels in STAT6-deficient mice, suggesting that NO-producing macrophages may also enhance immunity by reducing MSC levels.

Arginase production by MSCs is thought to be triggered by IL-4 and/or IL-13 binding to the cell's IL-4Rα, followed by signaling through the JAK3/STAT6 pathway (19, 26, 32). Our finding that MSCs from STAT6-deficient and STAT6-competent mice make comparable levels of arginase suggests that there is an additional mechanism for induction of arginase by MSCs that is independent of the IL-4Rα JAK3/STAT6 pathway. This hypothesis is supported by our observation that MSCs from IL-4R-deficient mice also suppress via the production of arginase (P.S. and S.O.-R., unpublished observations).

Myeloid-derived cells with suppressor activity have been reported in many tumor systems in both experimental animals and patients (7, 30, 48). Although these cells have the common feature of suppressing T cells, there are significant differences in their phenotype and function. Bronte et al. (26) describe MSCs that express MHC class II, B220, F4/80, CD86, CD16/32, and DEC205, whereas Gabrilovich et al. (33) report the expression of MHC class I and the absence of MHC class II and costimulatory molecules. Not only do the MSCs described in this report differ between BALB/c and STAT6-deficient mice, but they also differ from these previously described MSCs with respect to their cell surface markers. The 4T1-induced STAT6-deficient and BALB/c MSCs also differ in the mechanism by which they generate ROS. Although ROS production by 4T1-induced MSCs from BALB/c mice is arginase dependent in agreement with Kusmartsev et al. (31), ROS production by $STAT6^{-/-}$ MSCs is arginase independent, suggesting that there are multiple pathways for stimulating ROS biosynthesis in MSCs.

MSCs are also heterogeneous with respect to function. Some MSCs inhibit MHC class I-restricted $CD8^+$ T cells and have no effect on $CD4^+$ T cells (33, 49, 50), whereas others inhibit $CD4^+$ T cells (51). The MSCs reported here inhibit both $CD4^+$ and $CD8^+$ T cells. Kusmartsev et al. (31) report that $Gr1^+CD11b^+$ splenic cells from tumor-free mice are not suppressive, whereas the splenic $Gr1^+CD11b^+$ cells from tumor-free mice in this report are as suppressive on a per cell basis as are $Gr1^+CD11b^+$ cells from tumor-bearing mice.

The role of IFN-γ in suppressor cell development/activity is also variable. In the MSCs described by Mazzoni et al. (51), IFN-γ enhances MSC suppressive activity by inducing production of NO from MSCs. Kusmartsev and Gabrilovich (30) find that IFN-γ increases the production of reactive oxygen intermediates by MSCs, which in turn decreases the net amount of IFN-γ by down-regulating IFN-γ production by $CD4^+$ and $CD8^+$ T cells. Because IFN-γ is critical for effective anti-tumor immunity (52), these authors conclude that this net decrease in IFN-γ is one of the mechanisms by which MSCs mediate immune suppression. In the 4T1 system described in this report, IFN-γ is essential for the postsurgery decrease in MSCs in $STAT6^{-/-}$ mice, supporting the concept that MSC function is IFN-γ dependent.

Different mechanisms of suppression have also been postulated. Suppression is most frequently attributed to overexpression of arginase and the resulting loss of NO production (26, 31, 51), although it has also been ascribed to TGFβ production (12). The MSCs of this report do not produce TGFβ (data not shown) and their suppressive activity is blocked by inhibitors of arginase. 4T1-induced MSCs also differ from other described suppressor cell populations in that they express B220 in addition to Gr-1 and CD11b. This phenotype is characteristic of plasmacytoid dendritic cells, which are also immunosuppressive (53). These variations in $Gr1^+CD11b^+$ MSCs suggest that there are multiple subpopulations of MSCs. Not only do the various subpopulations differ in their phenotype and characteristics, but they also suppress different target cells. Although it is unclear why there is such variation, it is likely that the variation is due to differential cytokine production by the various tumors and the interaction of these cytokines with the host.

Jensen et al. (54) have suggested that enhanced immunity in $STAT6^{-/-}$ mice is due to $CD8^+$ T cell reactivity against tumor-expressed STAT6 protein, and they report rejection of primary 4T1 tumors by STAT6-deficient mice. In contrast, we find that, although 4T1 primary tumor growth is slightly delayed in STAT6-deficient mice, all mice die (8), indicating that immunity in the presence of primary tumor is only modestly effective, presumably due to the presence of high levels of MSCs. We also find heightened immunity in STAT6-deficient mice against STAT6-negative tumors because $STAT6^{-/-}NeuT^{+/-}$ mice, which spontaneously develop $STAT6^{-/-}$ mammary tumors, have significantly extended survival times relative to $STAT6^{+/+}NeuT^{+/-}$ mice (55). We also do not find evidence for $CD8^+$ T cell reactivity against STAT6 protein because CTLs from 4T1 immune $STAT6^{-/-}$ mice are not cytotoxic for $H\text{-}2^d$ $STAT6^+$ tumors other than 4T1 (V.K.C. and S.O.-R., unpublished observations). Therefore, heightened immunity in STAT6-deficient mice is not due to tumor-expressed STAT6 protein.

Anti-tumor immunity is a careful balance between the presence of anti-tumor effector mechanisms such as $CD4^+$ and $CD8^+$ T cells, NK cells, and tumoricidal macrophages and counterproductive inhibitory phenomena such as MSCs and $CD4^+CD25^+$ T regulatory cells. Ultimately, the ability to exploit the immune system to control tumor growth depends on tipping the balance toward the effector mechanisms and away from the inhibitory mechanisms, and deletion of the STAT6 gene achieves this polarization.

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