Gr-1$^+$CD115$^+$ Immature Myeloid Suppressor Cells Mediate the Development of Tumor-Induced T Regulatory Cells and T-Cell Anergy in Tumor-Bearing Host

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Abstract

The accumulation of myeloid suppressor cells (MSCs) is associated with immune suppression in tumor-bearing mice and in cancer patients. The suppressive activity of MSC correlates with the expression of the myeloid markers Gr-1, CD115 (macrophage colony-stimulating factor receptor), and F4/80. Gr-1$^+$CD115$^+$ MSCs, in addition to being able to suppress T-cell proliferation *in vitro*, can induce the development of Foxp3$^+$ T regulatory cells (Treg) *in vivo*, which are anergic and suppressive. Furthermore, the secretion of interleukin (IL)-10 and transforming growth factor-β by Gr-1$^+$CD115$^+$ MSCs was induced and enhanced, respectively, on IFN-γ stimulation. The development of Treg requires antigen-associated activation of tumor-specific T cells, depends on the presence of IFN-γ and IL-10, and is independent of the nitric oxide–mediated suppressive mechanism by MSC. Our data provide evidence that Gr-1$^+$CD115$^+$ MSC can mediate the development of Treg in tumor-bearing mice and show a novel immune suppressive mechanism by which MSCs can suppress antitumor responses. (Cancer Res 2006; 66(2): 1123-31)

Introduction

The induction of antigen-specific unresponsiveness is one of the mechanisms by which tumor cells evade the immune system. Abundant evidence exists to indicate that tumor-specific T cells undergo inhibitory regulation and become anergic in tumor-bearing hosts (1–6). However, the mechanisms underlying the immune suppression mediated by tumor are as yet ill defined.

As early as the 1980s, a correlation between accumulation of immature myeloid cells and immune suppression was recognized in both tumor-bearing mice and cancer patients (7–9). Because of the suppressive activities and heterogeneity of these myeloid cells, the term myeloid suppressor cell (MSC) was proposed to denote this population (10). MSCs are capable of inhibiting the T-cell proliferative responses induced by alloantigens (11), CD3 ligation (12), or various mitogens (13) and can also inhibit interleukin (IL)-2 utilization by natural killer (NK) cells as well as NK cell activity (14). T-cell inactivation by MSCs *in vitro* can be mediated through several mechanisms: IFN-γ-dependent nitric oxide (NO) production (15), Th2-mediated IL-4/IL-13-dependent arginase 1 synthesis (16), loss of CD3ξ signaling in T cells (17), and suppression of the T-cell response through reactive oxygen species (16, 18–20). Nevertheless, the underlying mechanisms of MSC-mediated immunoregulation in tumor-bearing hosts and the *in vivo* effect of MSC on tumor-specific T cells have not been well defined.

Recently, an additional cell population with immunosuppressive activities has been implicated in the induction of T-cell tolerance (21, 22). T regulatory cells (Treg) play an important role in the control of immune reactivity against self-antigens and non-self-antigens (23, 24). Several Treg subsets have been identified that have the ability to inhibit autoimmune and chronic inflammatory responses and to maintain immune tolerance in tumor-bearing hosts. These subsets include IL-10-secreting Treg type 1 cells, transforming growth factor-β (TGF-β)–secreting Th3 cells, and "natural" CD4$^+$CD25$^+$ Tregs (22, 25, 26). Although no specific surface marker can be associated with Treg cells, the forkhead/winged helix transcription factor (*Foxp3*) has been identified as a key regulatory gene for the development and function of Treg and may implicate a regulatory program for development of Treg (27), and ectopic expression of *Foxp3* in CD4$^+$CD25$^-$ T cells is able to confer suppressive activity (28). Unlike the cell surface markers used to identify Treg (e.g., CD25, CD45RB, CTLA4, and GITR), *Foxp3* is not up-regulated on T-cell activation and thus discriminates Treg cells from activated effector cells.

Very little information is available regarding how tumor-specific Treg cells develop in tumor-bearing hosts. Moreover, the exact Treg subsets that mediate T-cell tolerance and the microenvironment required for tumor-specific T cells to develop into Treg remain to be determined. Many of Tregs studied were generated *in vitro* from naive CD4$^+$CD25$^-$ T cells by T-cell receptor (TCR) engagement in the presence of recombinant cytokines (IL-10, TGF-β, or both) or by stimulation with modulated dendritic cells (IL-10-treated, *RelB* knockout-derived, or plasmacytoid dendritic cells; refs. 29–33). Little is known about the natural signals and antigen-presenting cells (APC) responsible for inducing and maintaining Treg cells in the tumor-bearing host.

Although accumulating evidence suggests that Tregs and MSCs are associated with tumor-mediated suppression, it has not been established whether a possible interaction of MSCs and Treg development exists during tumor progression. We hypothesize that tumors induce the accumulation of MSCs that not only can inhibit clonal expansion of activated effector T cells but also induce tumor-specific Treg to further establish and maintain T-cell tolerance in the tumor-bearing host.

In this report, we show that Gr-1$^+$CD115$^+$ MSCs are important immune regulators that mediate the inactivation of tumor-specific T cells and induce the development of Treg and thus reveal a novel *in vivo* suppressive mechanism mediated by MSC.

Note: B. Huang and P-Y. Pan contributed equally to this work.

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Materials and Methods

Experimental animals. Ten-week-old female congenic Thy1.1$^+$ BALB/c were a gift from Dr. Richard Dutton (Trudeau Institute, Saranac Lake, NY), and C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). Influenza hemagglutinin (HA)–specific I-E^d-restricted CD4 and CD8 TCR transgenic mice (in BALB/c background; Thy1.2) were gifts from Dr. Linda Sherman (Scripps Research Institute, La Jolla, CA) and Dr. Constantin A. Bona (Mount Sinai School of Medicine, New York, NY), respectively. Stat1-deficient BALB/c mice and IL-10 receptor (IL-10R)–deficient mice were established as described before (34, 35). Mice deficient in inducible NO synthase (iNOS; in C57BL/6 background) or IL-4 receptor α chain (IL-4Rα; in BALB/c background) and CD4 ovalbumin-specific TCR transgenic (OT II) C57BL/6 were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were done in accordance with the animal guidelines of the Mount Sinai School of Medicine.

Tumor models. The MCA26 tumor cell line is a BALB/c-derived, chemically induced colon carcinoma line with low immunogenicity (36). To establish a model in which tumor antigen-specific T-cell responses can be tracked *in vivo*, the MCA26 colon tumor cell line was stably transformed with the gene encoding influenza HA (a generous gift from Dr. Adolfo Garcia-Sastre, Mount Sinai School of Medicine). The tumorigenicity of HA-transfected MCA26 (HA-MCA26), clone 44, was confirmed by implantation into syngeneic BALB/c mice. Similar *in vivo* tumor growth rates were observed for control neo plasmid-transfected parental MCA26 and clone 44 cells. The ovalbumin-expressing tumor line used is an ovalbumin-transfected clone derived from the murine B16 (H-2^b) melanoma (37). To generate the tumor model of metastatic colon cancer, MCA26 or HA-MCA26 tumor cells (9×10^4) were inoculated in the liver by intrahepatic implantation of cells as described previously (15). Similar methodology was used for the B16 tumor model.

Peptide and antibodies. CD4 HA peptide (^{110}SFERFEIFPKE^{120}), CD8 HA peptide (^{533}IYSTVASSL^{541}), and CD4 ovalbumin peptide (^{323}ISQAVHAAHAEINEAGR^{339}) were purchased from Washington Biotechnology, Inc. (Baltimore, MD). Neutralizing anti-mouse IL-10, IL-13, and IFN-γ antibodies were purchased from R&D Systems (Minneapolis, MN). Anti-Thy1.2-FITC, anti-Gr-1-APC/FITC, anti-CD115-phycoerythrin, anti-F4/80-FITC, anti-CD11b-APC/FITC, anti-CD25-APC, and isotype-matched monoclonal antibodies were purchased from eBioscience (San Diego, CA).

Carboxyfluorescein diacetate succinimidyl ester labeling. Splenocytes from transgenic BALB/c mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR). Briefly, the cells were suspended in serum-free RPMI 1640 and incubated with CFSE (5 μmol/L) at 37°C for 10 minutes followed by quenching with an equal volume of cold FCS and washing thrice with complete medium and twice with cold PBS.

Isolation of fraction 2 MSC. Mice with tumor sizes greater than 10×10 mm^2 were sacrificed and their spleen, tibias, and femurs were harvested. After lysis of RBC, bone marrow cells and splenocytes were fractionated by centrifugation on a Percoll (Amersham Biosciences, Uppsala, Sweden) density gradient as described (15). Cells were collected from the gradient interfaces. Cell bands between 40% and 50% were labeled as fraction 1, between 50% and 60% as fraction 2, and between 60% and 70% as fraction 3.

Cell sorting. In all of the sorting experiments, very stringent gating conditions were used (FACSVantage with FACSDiVa). The purity of the sorted cells was checked by flow cytometry and sorted cell populations that were >97% to 98% pure MSC or T cells were chosen for the following experiments.

MSC suppression assay. The suppressive activity of MSC was assessed in a peptide-mediated proliferation assay of TCR transgenic T cells as described previously (38). Briefly, splenocytes (1×10^5) from TCR transgenic mice were cultured in the presence of serial dilutions of irradiated MSCs in 96-well microplates. [^{3}H]thymidine was added during the last 8 hours of 72-hour culture.

Cytokine detection by ELISA and NO measurement. Cytokine ELISAs were done on culture supernatants using the mouse IL-2, IL-4, IL-10, IL-13, IFN-γ, and TGF-β ELISA kits (R&D Systems) according to the manufacturer's instructions. In addition, NO was measured by Greiss reagent (Sigma-Aldrich, St. Louis, MO).

Mice irradiation. Mice were irradiated with high-dose radiation (850 rad) to eradicate endogenous MSC and T cells, which was confirmed by flow cytometric analysis of Gr-1$^+$CD115$^+$ cells and T cells in the bone marrow and spleen of irradiated mice, which showed that <0.5% of T cells and MSC was present in the recipient mice.

Adoptive transfer experiments. Thy1.2 congenic CD4 or CD8 HA-specific TCR transgenic T cells were enriched by T-cell enrichment columns according to the manufacturer's instructions (R&D Systems) for adoptive transfer through tail vein injection (5×10^6 per mouse). As for MSC, sorted Gr-1$^+$CD115$^+$ bone marrow fraction 2 cells (2.5×10^6 per mouse) or single Gr-1$^+$ fraction 2 cells (5×10^6 per mouse) from large tumor-bearing mice were used.

HA-MCA26 cells (or neo-transfected parental MCA26 cells as a control; 9×10^4) were inoculated into Thy1.1$^+$ BALB/c mice. Six days later, the mice with tumor size of $\sim 5 \times 5$ mm^2 were irradiated. The following day, the sorted MSC and T cells were coadoptively transferred through tail vein. Mice were sacrificed at day 7 after the adoptive transfer and Thy1.2$^+$ T cells were recovered from spleen and lymph nodes of recipient mice by cell sorting.

Proliferation assay. The sorted Thy1.2$^+$ or column-enriched T cells (1×10^4) with irradiated (2,500 rad) naive splenic cells (4×10^3) as APC were cocultured with or without HA peptide (5 μg/mL) in 96-well microplates. [^{3}H]thymidine was added during the last 8 hours of 72-hour culture.

Reverse transcription-PCR and quantitative real-time PCR. Target cells were homogenized in TRIzol reagent (Invitrogen) and total RNA was extracted according to the manufacturer's instructions. A reverse transcription-PCR (RT-PCR) procedure was used to determine relative quantities of mRNA (One-step RT-PCR kit; Qiagen). Twenty-eight PCR cycles were used for all of the analyses. The intensity of each amplified DNA bands was further analyzed by IQ Mac version 1.2 software and relatively quantitated using *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as the internal control. The primers for all genes tested, including internal control *GAPDH*, were synthesized by Gene Link: *GAPDH* 5′-GTGGAGATTGTTGCCATCAACG-3′ (sense) and 5′-CAGTGGATGCAGGGATGATGTTCTG-3′ (antisense), *TGF-β1* 5′-GTGGTATACTGAGACACCTTGG-3′ (sense) and 5′-CCTTAGTTTGGACAGGATCTGG-3′ (antisense), *IL-10* 5′-CTCTTACTGACTGGCATGAGG-3′ (sense) and 5′-CCTTGTAGACACCTTGGTCTTGGAG-3′ (antisense), *Foxp3* 5′-CAGCTGCCTACAGTGCCCCTAG-3′ (sense) and 5′-CATTTGCCAGCAGTGGGTAG-3′ (antisense), *arginase 1* 5′-CAGAGTATGACGTGAGAGACCAC-3′ (sense) and 5′-CAGCTTGTCTACTTCAGTCATGGAG-3′ (antisense), and *iNOS* 5′-GAGATTGGAGTTCGAGACTTCTGTG-3′ (sense) and 5′-TGGCTAGTGCTTCAGACTTC-3′ (antisense). For quantitative real-time PCR, cDNA (2 μL) reverse transcribed from total RNA was amplified by real-time quantitative PCR with 1× SYBR Green Universal PCR Mastermix (Bio-Rad, Richmond, CA). Each sample was analyzed in duplicate with the IQ-Cycler (Bio-Rad) and the normalized signal level was calculated based on the ratio to the respective *GAPDH* housekeeping signal.

Results

Increase in Gr-1$^+$CD115$^+$F4/80$^+$ MSCs in tumor-bearing mice and induction of Treg cells *in vitro*. Historically, Gr-1 and CD11b (Mac-1) markers have been used to identify MSCs. However, other cell lineages can also express these markers. Identification of more specific cell markers will facilitate the study of the functional activities of MSCs. Because of the myeloid origin of MSCs, we chose the myeloid cell markers CD115 [macrophage colony-stimulating factor (M-CSF) receptor] and F4/80, in addition to Gr-1, to further identify this myeloid suppressor population. Percoll fraction 2 cells derived from bone marrow and spleen of naive or tumor-bearing mice were labeled with fluorochrome-conjugated antibodies. The Gr-1-gated flow cytometric profile (Fig. 1*A, left*)

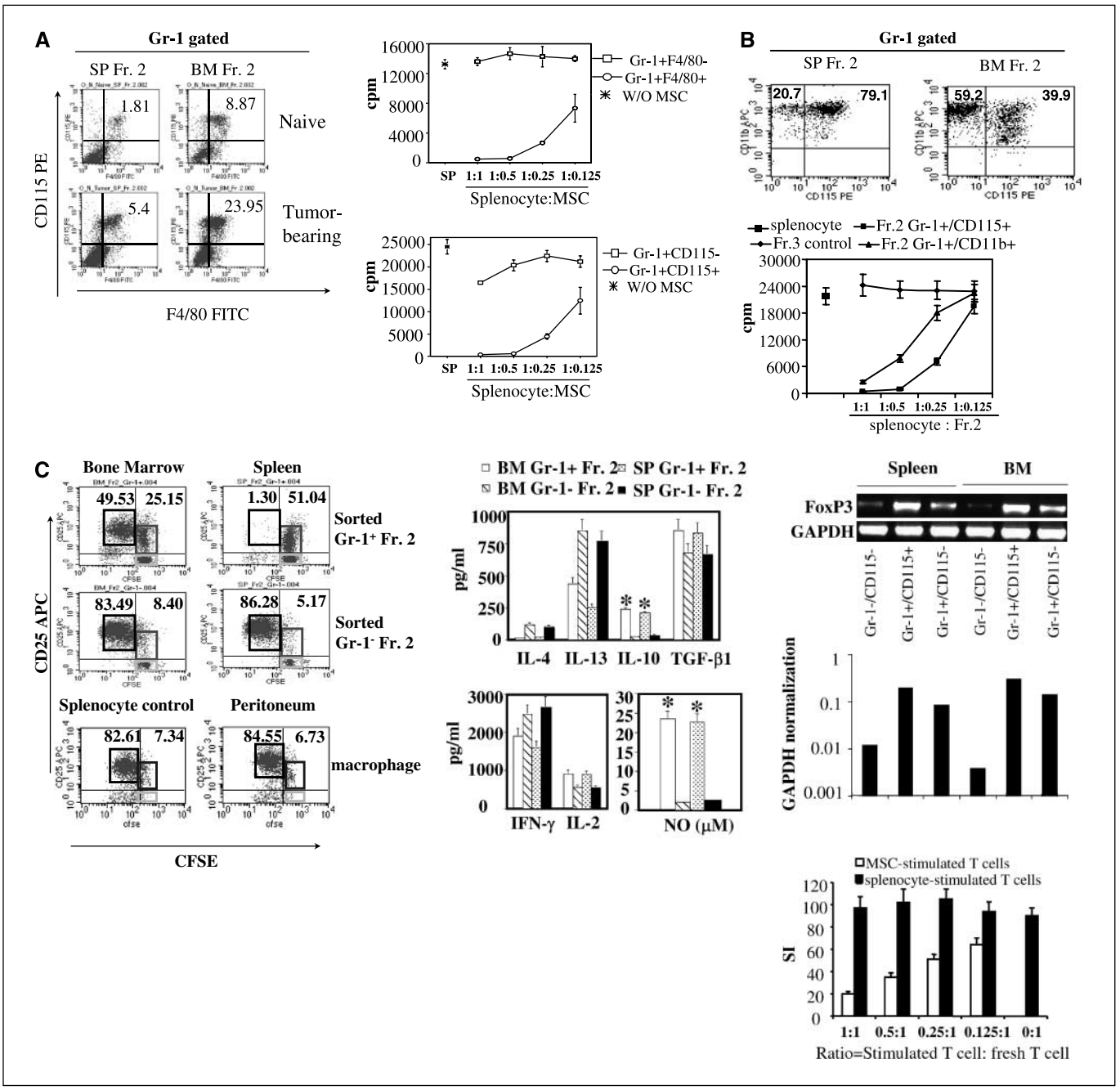

Figure 1. Accumulation of Gr-1$^+$CD115$^+$F4/80$^+$ MSCs in tumor-bearing mice and induction of Treg cells *in vitro. A,* increase of Gr-1$^+$CD115$^+$F4/80$^+$ cell population in bone marrow and spleen fraction 2 from tumor-bearing animals. Gr-1 gated dot plots are presented (*left*) and suppressive activity of Percoll fraction 2 cells correlates with Gr-1 and CD115 markers. The suppressive activities were assessed by HA peptide-mediated HA CD4 TCR splenocyte proliferation responses (*right*). *B,* comparison of Gr-1$^+$CD115$^+$ MSC with Gr-1$^+$CD11b$^+$ MSC. *Top,* Gr-1 gated dot plots; *bottom,* suppression of MSC on CD4$^+$ HA-specific TCR splenocytes. *C,* sorted Gr-1$^+$ cells inhibited the proliferation of CD4$^+$ T cells. CFSE-labeled CD4$^+$ HA-specific TCR splenocytes were cocultured for 72 hours with irradiated Gr-1$^-$ or Gr-1$^+$ fraction 2 cells or peritoneal macrophages in the presence of HA peptide. Viable cells were isolated for staining. Representative two-variable (CFSE versus CD25 APC) dot plots gated on CD4$^+$ cells (*left*). *D,* cytokine and NO secretion. The cytokine profile and NO levels in above culture supernatants were measured by ELISA and Greiss reagent, respectively (*right*). *E* and *F,* induction of Foxp3$^+$ Treg cells by Gr-1$^+$CD115$^+$ MSC. CD4$^+$ HA-specific TCR splenocytes were cocultured with HA-peptide and sorted Gr-1$^+$CD115$^+$ cells for 5 days. The viable cells were harvested and some were used for total RNA isolation and the expression of *Foxp3* was assessed by RT-PCR (*top left*) and real-time PCR (described in Materials and Methods; *bottom left*). Thy1.2 T cells were sorted from the remaining viable cells and cocultured with CD4$^+$ HA-specific TCR splenocytes at various ratios in the presence of HA peptide. The suppressive activity is shown in (*F*).

showed a significantly increased percentage of Gr-1$^+$CD115$^+$F4/80$^+$ cells in tumor-relative bone marrow (23.95%) and spleen (5.4%) fraction 2 compared with naive bone marrow (8.87%) and spleen (1.81%) fraction 2, and the absolute number of cells was even higher in the former due to an increased total cell number. To determine whether the increased Gr-1$^+$CD115$^+$F4/80$^+$ cells have suppressive function, we sorted tumor bone marrow Percoll fraction 2 cells into Gr-1$^+$F4/80$^+$ versus Gr-1$^+$F4/80$^-$ or Gr-1$^+$CD115$^+$ versus Gr-1$^+$CD115$^-$ populations for analysis of their suppressive activities in HA peptide-mediated proliferation assays.

The strong suppressive effect of sorted Gr-1$^+$F4/80$^+$ and Gr-1$^+$CD115$^+$ cells, but not Gr-1$^+$F4/80$^-$ or Gr-1$^+$CD115$^-$ cells, was observed (Fig. 1*A, right*). Based on the facts that (*a*) the majority of Gr-1$^+$CD115$^+$ cells also expressed F4/80 and (*b*) CD115 is an earlier marker of myeloid progenitor cell than F4/80 (39), we used Gr-1 and CD115 to purify MSC from Percoll fraction 2 in this study. To address whether Gr-1 and CD115 are better markers for MSC than classic Gr-1 and CD11b, we further compared the percentage and suppressive function between the conventional MSC markers Gr-1/CD11b and Gr-1/CD115 in fraction 2 cells. All of the Gr-1$^+$CD115$^+$ cells expressed CD11b makers. A stronger suppressive activity ($\sim$2-fold increase) was observed in sorted Gr-1$^+$CD115$^+$ cells when compared with sorted Gr-1$^+$CD11b$^+$ cells (Fig. 1*B*). Taken together, the results indicate Gr-1 and CD115 may be better markers to further enrich MSCs.

To determine the outcome of antigen-mediated T-cell activation in the presence of MSC, sorted Gr-1$^+$ or Gr-1$^-$ tumor bone marrow or non-MSC peritoneal macrophages were cocultured for 3 days with CFSE-labeled CD4$^+$ HA-specific TCR splenocytes in the presence of HA antigens. Cell division and CD25 (IL-2Rα) expression of HA-specific T cells were analyzed by flow cytometry. The result showed that Gr-1$^+$ cells significantly inhibited the proliferation of CD4$^+$ T cells but not Gr-1$^-$ cells (49% versus 83% from bone marrow and 1.3% versus 86% from spleen; Fig. 1*C*) or non-MSC macrophages or splenocyte. More interestingly, a population of nonproliferating CD4$^+$cells that expressed a lower level of CD25 was observed in the coculture with Gr-1$^+$ fraction 2 (25% from bone marrow and 51% from spleen), whereas a very low percentage of CD4$^+$CD25$^+$ nondividing T cells was seen in the coculture with control cells (8.4% from bone marrow and 5.17% from spleen). These data reveal that a population of nondividing CD4$^+$CD25$^+$T is induced by Gr-1$^+$ fraction 2 MSCs. Consistent with these results, the evaluation of cytokine profiles and NO production in the supernatant showed significantly higher levels of IL-10 and NO and substantially higher levels of TGF-β and IL-2 in the coculture with Gr-1$^+$ fraction 2 MSCs (Fig. 1*D*). In contrast, higher levels of IFN-γ, IL-4, and IL-13 were detected in the supernatant of the coculture with Gr-1$^-$ fraction 2 cells.

The phenotype (CD25$^+$ and nonproliferating) of the T cells in the coculture with Gr-1$^+$ fraction 2 MSC prompted us to examine whether Treg cells can be induced by MSC. Tumor bone marrow and spleen Percoll fraction 2 cells were sorted into Gr-1$^-$CD115$^-$, Gr-1$^+$CD115$^-$, and Gr-1$^+$CD115$^+$ populations. Sorted cells were irradiated and cocultured with CD4$^+$ HA-specific TCR splenocytes for 6 days in the presence of irradiated HA-MCA26. The expression of *Foxp3* was significantly induced by Gr-1$^+$CD115$^+$ MSC, whereas no significant *Foxp3* expression was detected by RT-PCR and real-time PCR in the coculture with Gr-1$^-$CD115$^-$ fraction 2 cells (Fig. 1*E*). A substantially lower level of *Foxp3* was detected following the stimulation of Gr-1$^+$CD115$^-$ fraction 2 cells. A similar pattern of *Foxp3* expression was observed from tumor spleen Percoll fraction 2 (Fig. 1*E*). To confirm the development of Treg cells in T-cell/MSC coculture, Thy1$^+$ T cells were sorted from the cocultures by fluorescence-activated cell sorting (FACS) and the suppressive activity of sorted Thy1$^+$ T cells was assessed in proliferation assays using CD4$^+$ HA-TCR$^+$ splenocytes stimulated with HA peptide. Only the sorted MSC cocultured T cells significantly suppressed the proliferation of CD4$^+$ HA-TCR$^+$ T cells (Fig. 1*F*). Taken together, these data (the expression of *Foxp3* and suppressive activity) provide strong evidence that Gr-1$^+$CD115$^+$ MSC can induce the development of Treg cells *in vitro*.

***In vivo* development of Treg cells induced by MSCs.** We further examined whether antigen-specific immune suppression in tumor-bearing mice was mediated through MSCs. The sorted Gr-1$^+$CD115$^+$ fraction 2 MSCs and Gr-1$^+$CD115$^-$ or Gr-1$^-$CD115$^-$ fraction 2 cells in conjunction with congenic Thy1.2$^+$CD4$^+$ HA-TCR$^+$ T cells were adoptively transferred into Thy1.1$^+$ mice bearing HA-MCA26 tumors (5×5 mm^2). Before adoptive transfer, mice were irradiated to eradicate endogenous MSCs and T cells. Seven days later, Thy1.2$^+$ T cells were sorted for the analysis of *Foxp3* gene expression and proliferation assay. As shown in Fig. 2*A*, a significantly higher level of *Foxp3* expression was detected in the Gr-1$^+$CD115$^+$ MSC group. In parallel with Foxp3 induction, T cells from Gr-1$^+$CD115$^+$ group responded poorly to HA peptide stimulation, whereas T cells from Gr-1$^-$CD115$^-$ group proliferated vigorously. T cells from Gr-1$^+$CD115$^-$ group proliferated on stimulation by HA peptide but at a significantly lower level when compared with Gr-1$^-$CD115$^-$ group. More strikingly, the residual tumor weights were much lower in the control splenocyte group or Gr-1$^-$CD115$^-$ group (tumor mass 0-25 mg) when compared with the Gr-1$^+$CD115$^+$ group (tumor mass 250-300 mg; Fig. 2*B*). To clarify whether tumor progression is ascribed to the effect of MSC-induced Treg, we did *in vivo* depletion of CD4$^+$CD25$^+$ Treg by peritoneal injection of anti-CD25 antibody (PC-61, 100 μg/mouse). The depletion efficiency was confirmed by flow cytometry (>97%). The experimental group in which CD25$^+$ T cells were depleted showed a significant reduction in tumor growth (Fig. 2*C, upper panel*). The adoptively transferred tumor-specific CD4$^+$ and/or CD8$^+$ T cells from the CD25 depletion groups (Fig. 2*C, bottom*), but not from the group without CD25 depletion, remained functional, indicating that MSC-induced CD25$^+$ Treg are involved in the suppression of antitumor responses. Taken together, the data suggest that adoptively transferred Gr-1$^+$CD115$^+$ MSCs can render tumor (HA)-specific T cells unresponsive to *in vitro* peptide stimulation, induce the development of CD25$^+$ T cells that express Foxp3, and suppress antitumoral T-cell responses.

Involvement of IFN-γ and IL-10 in MSC-mediated immune suppression and Treg development *in vivo*. Although MSC has the ability to induce Treg generation *in vivo*, the actual requirements for *in vivo* development of Treg cells remain to be determined. Because the high concentrations of IL-10, IL-13, and IFN-γ were detected in the supernatant of HA-specific CD4$^+$ T cells cocultured with MSCs and HA peptide (Fig. 1*D*), we asked whether these cytokines were necessary for the T-cell anergy and Treg development induced by MSCs *in vivo*. Using the same model, the MSC and T-cell coadoptively transferred Thy1.1 tumor mice were simultaneously given i.p. injections of control antibody (rat Ig), anti-IL-10, anti-IL-13, or anti-IFN-γ neutralizing antibodies. After 9 days, the adoptively transferred T cells were recovered by sorting for Thy1.2$^+$ cells, and their proliferative responses to HA peptide were evaluated and the level of *Foxp3* gene expression was determined. Neither control antibody nor anti-IL-13 could reverse the hypoproliferative response of sorted Thy1.2$^+$ T cells (P = 0.1093, ANOVA; Fig. 3*A*). In contrast, treatment with anti-IL-10 or anti-IFN-γ antibodies significantly enhanced the proliferative response ($P < 0.01$, ANOVA), which was accompanied by a significantly reduced level of *Foxp3* (Fig. 3*A*). In line with the above observation, the weight of dissected tumor tissue from the anti-IL-10 and anti-IFN-γ groups was significantly lower than that in mice from the control Ig-treated group

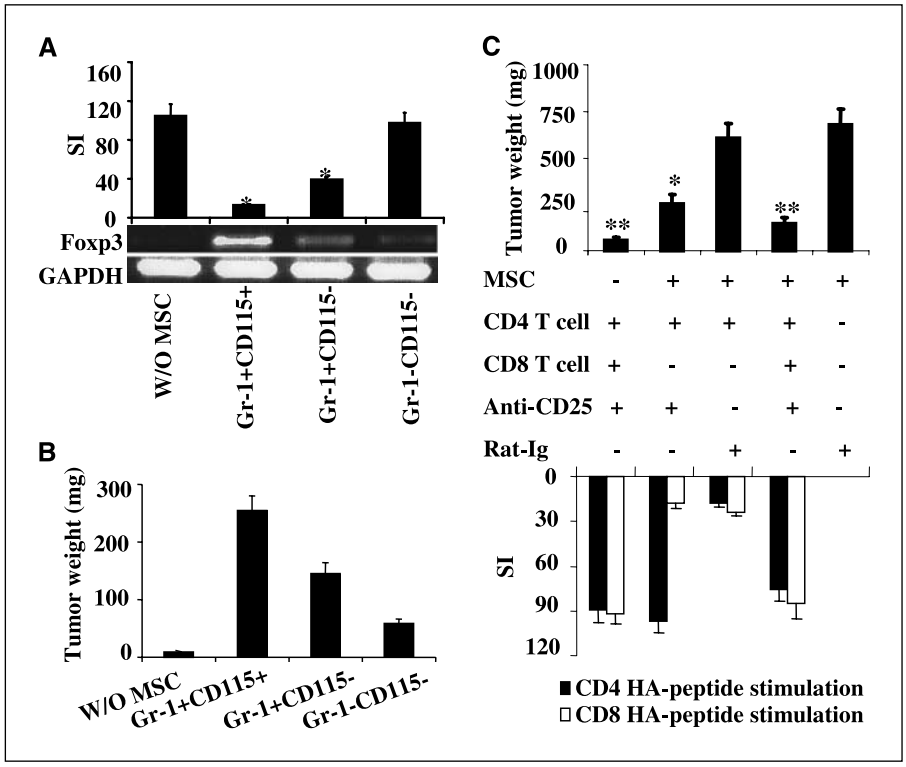

Figure 2. Development of Treg cells mediated by MSC *in vivo*. *A* and *B,* MSC induces Treg development. Sorted Percoll fraction 2 Gr-1$^-$CD115$^-$ cells, Gr-1$^+$CD115$^-$ cells, and Gr-1$^+$CD115$^+$ MSCs (2.5×10^6 per mouse) and Thy1.2$^+$ CD4 HA-specific TCR T cells (5×10^6) were coadoptively transferred into irradiated Thy1.1$^+$ HA-MCA26 tumor-bearing mice. One group only received T-cell adoptive transfer but did not receive MSC as a negative control for Treg development. After 7 days, the HA-specific T cells were recovered from spleen by sorting for Thy1.2$^+$. The sorted cells showed *Foxp3* expression by RT-PCR and proliferative activity (*A*). *, $P < 0.01$ (ANOVA). Stimulation index (*SI*) was calculated by dividing the proliferation count in the presence of HA peptide by that in the absence of HA peptide. Representative of two reproducible experiments. *Columns,* mean of three individual animals; *bars,* SD. The residual tumor weight from each group was measured (*B*). Depletion of CD4$^+$CD25$^+$ Treg enhances the tumor regression and proliferation response. The sorted Gr-1$^+$ MSC (5×10^6) and CD4$^+$ HA-TCR$^+$ T cells were coadoptively transferred into HA-MCA 26 tumor-bearing mice using the same strategy outlined above. Two days later, CD4$^+$CD25$^+$ cells were deleted by the i.p. injection of anti-CD25 antibody (PC-61; 100 μg/mouse). On the second day of depletion, CD8$^+$ HA-TCR$^+$ T cells were adoptively transferred. Nine days later, five mice per group were sacrificed and the tumor weight was measured (*C, top*). **, $P < 0.01$; *, $P < 0.05$ compared with the group without CD25 depletion and only T-cell and MSC transfer (ANOVA). Meanwhile, CD4 and CD8 T cells were recovered from spleen and stimulated with CD4 or CD8 HA-peptide for proliferative responses (*C, bottom*).

(Fig. 3*B*; $P < 0.01$). Although the trend in the anti-IL-13 treatment group was toward some suppression of tumor growth, the observed decrease in tumor size did not reach statistical significance ($P > 0.05$). The tumors were completely eradicated by the adoptively transferred HA-specific T cells in the mice that did not receive adoptive transfer of MSC ($P < 0.001$). To determine the effect of anti-cytokine treatment on the tumor microenvironment, the expression levels of the TGF-β, iNOS, and arginase 1 genes in the tumor tissue from animals in the various treatment groups were analyzed by RT-PCR. Anti-IL-10 treatment resulted in a 12-fold decrease in TGF-β gene expression and, to a lesser degree, *iNOS* (3-fold decrease) and *arginase 1* (4-fold decrease) gene expression when compared with treatment with the control antibody, rat Ig (Fig. 3*C*). IFN-γ is required for *iNOS* expression in the tumor, as anti-IFN-γ treatment completely inhibited the expression of iNOS. TGF-β and arginase 1 mRNAs were detectable, however, at a lower level in the tumors from mice treated with anti-IFN-γ antibody when compared with rat Ig treatment. Substantial levels of TGF-β, iNOS, and arginase 1 gene expression were still detected in the tumor tissues from mice treated with anti-IL-13 antibodies. Taken together, the results suggest that IL-10 and IFN-γ are required for the suppression of antitumor responses and the development of Treg cells mediated by MSC in recipient tumor-bearing mice.

In addition, a comparable approach with mice deficient in the signaling of Stat1 (Stat1$^{-/-}$), IL-4/IL-13 (IL-4Rα$^{-/-}$), or IL-10 (IL-10R$^{-/-}$) was used to confirm the role of IFN-γ, IL-13, and IL-10 in the suppression of antitumor responses mediated by MSCs. MCA26 and B16 tumor models were used in knockout mice with BALB/c and C57BL/6 backgrounds, respectively. The MSCs from wild-type or knockout tumor mice were coadoptively transferred with T cells (HA-TCR in BALB/c and ovalbumin-TCR in C57BL/6) into irradiated tumor (HA-MCA26 or ovalbumin-B16)–bearing mice. Seven days later, the adoptively transferred T cells were recovered by FACS (Thy1.2; BALB/c) or by T-cell enrichment column (C57BL/6). The proliferative response of recovered T cells to peptide stimulation was assessed. Consistent with the data from experiments using neutralizing antibodies, T cells recovered from mice that received MSCs deficient in Stat1 (IFN-γ signaling) or IL-10R exhibited normal proliferative responses to peptide stimulation when compared with those recovered from the mice that did not receive MSCs (Fig. 3*D*). T cells recovered from mice receiving wild-type or IL-4/IL-13 signaling-deficient MSCs were hypoproliferative in response to peptide stimulation. Moreover, the tumor mass of the mice that received IL-4Rα$^{-/-}$ or wild-type MSCs was larger than that in mice that were injected with Stat1$^{-/-}$ or IL-10R$^{-/-}$ MSCs (data not shown).

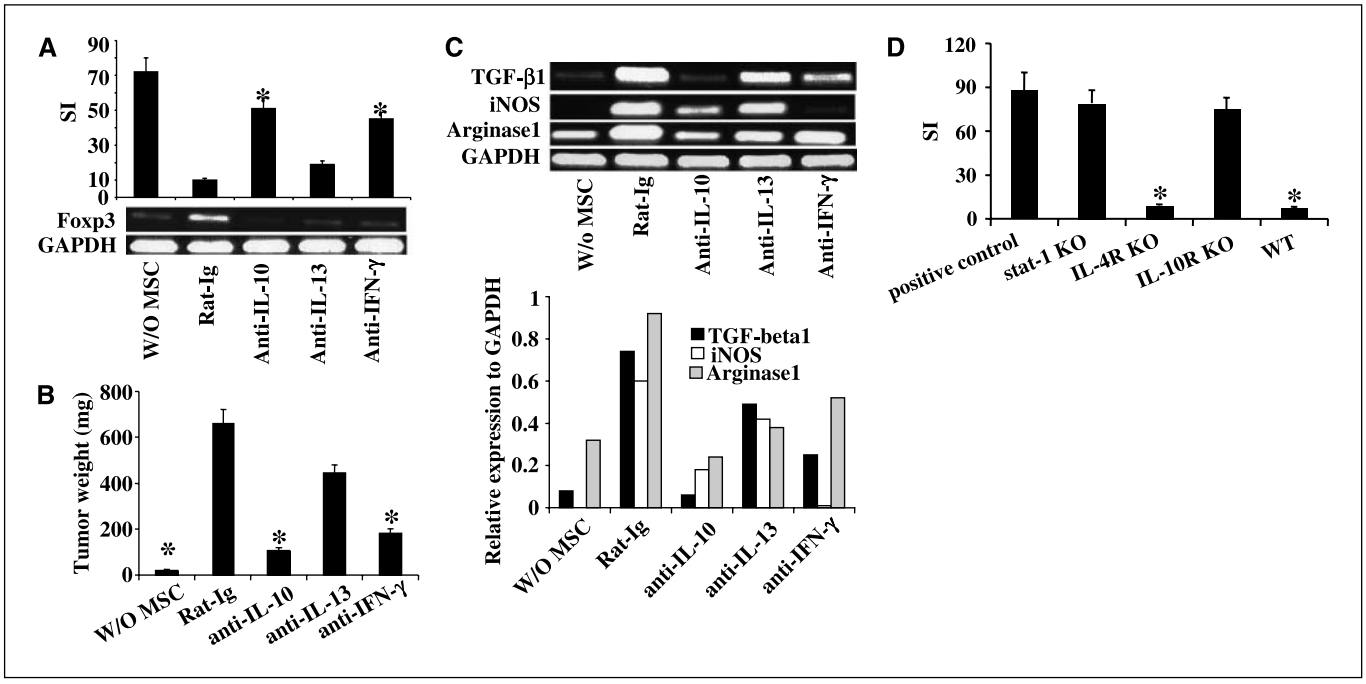

Figure 3. Requirement of IL-10 and IFN-γ for MSC-mediated T-cell tolerance *in vivo*. Similar T-cell and MSC adoptive transfers were done, except with the administration of different cytokine neutralizing antibodies i.p. every 3 days (150 μg/mouse/dose). One group of mice received only T cells and control splenocytes without MSC as positive control. *A,* proliferation and *Foxp3* expression levels of sorted T cells. The proliferative responses of adoptive sorted T cells from anti-IFN-γ and anti-IL-10 groups against HA peptide are significantly higher than those from the control Ig group. *, $P < 0.01$ (ANOVA). *B,* tumor weight. The residual tumor weight from each group was measured. The results were combined from three reproducible experiments. The tumor weight of animals in the anti-IFN-γ and anti-IL-10 groups is significantly lower than that of those in the control IG group. *, $P < 0.01$ (ANOVA). *C,* TGF-β1, iNOS, and arginase 1 gene expression in tumor tissues. The intensity of amplified DNA bands was analyzed by IQ Mac version 1.2 software and relative expression levels were compared with the internal control *GAPDH*. *D,* deficiency of IFN-γ or IL-10 signaling impaired the development of Treg cells mediated by MSC. MSC from tumor-bearing knockout (*KO*) mice was injected into irradiated HA-MCA26 or ovalbumin-B16 tumor-bearing mice along with HA or ovalbumin TCR transgenic T cell transfer. After 7 days, the adoptively transferred T cells were isolated from the spleen and assayed for proliferative response against HA or ovalbumin peptide. *Columns,* mean (expressed as stimulation index); *bars,* SD.

Production of IL-10 and TGF-β by MSC on stimulation with IFN-γ. IL-10 and TGF-β have been shown to induce the development of Treg cells (26, 40–44). We detected significant levels of IL-10 and TGF-β, along with IFN-γ, in the supernatants of the coculture of MSCs and CD4 HA TCR transgenic splenocytes (Fig. 1*C*). Hence, we further hypothesized that MSC can secrete IL-10 and TGF-β in response to the stimulation of IFN-γ secreted by activated T cells. To test this hypothesis, Gr-1$^+$CD115$^+$ MSCs were sorted, by FACS, from Percoll fraction 2 derived from mice with large tumor burdens and cultured in the presence or absence of IFN-γ. After stimulation for 24 hours, the expression of IL-10, TGF-β, arginase 1, and *iNOS* genes and the secretion of IL-10 and TGF-β were assessed. TGF-β was expressed by sorted MSCs even in the absence of stimulation by IFN-γ (Fig. 4*A*). The expression of IL-10 was not detectable without stimulation but was induced in the presence of IFN-γ. Consistent with previous findings using bulk Percoll fraction 2 cells, the expression of *iNOS* by sorted Gr-1$^+$CD115$^+$ MSCs was significantly induced on stimulation with IFN-γ. No arginase 1 mRNA was detected in the absence or presence of IFN-γ. In agreement with the RT-PCR results, significant levels of IL-10 and TGF-β were secreted by sorted MSC on stimulation with IFN-γ (Fig. 4*B*). Interestingly, the secretion of TGF-β by sorted MSCs was further enhanced in the presence of IFN-γ. The fact that there was no significant difference in TGF-β gene expression on stimulation by IFN-γ when measured by RT-PCR is probably due to saturated amplification of primers (Fig. 4*A*). No IL-2, IL-4, or IL-13 was detected in the culture supernatants in the absence or presence of IFN-γ. The data suggest that, on stimulation by IFNγ secreted from activated T cells, Gr-1$^+$CD115$^+$ MSCs can secrete IL-10, TGF-β, and NO.

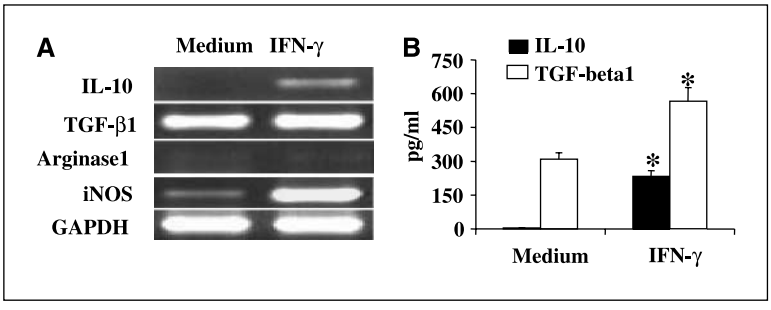

Figure 4. Production of IL-10 and TGF-β by MSC on stimulation by IFN-γ. Gr-1$^+$CD115$^+$ MSCs were sorted from Percoll fraction 2 derived from mice with large tumor burdens and cultured in the absence or presence of IFN-γ (100 ng/mL). After stimulation for 24 hours, cells were harvested for the analysis of gene expression by RT-PCR and culture supernatants were collected for the determination of cytokine concentrations by ELISA. *A,* gene expression of IL-10, TGF-β, arginase 1, and iNOS by sorted MSCs. *B,* ELISA of secreted IL-10 and TGF-β from sorted Gr-1$^+$CD115$^+$ MSC with or without IFN-γ stimulation. *, $P < 0.05$ compared with unstimulated group (Student's *t* test).

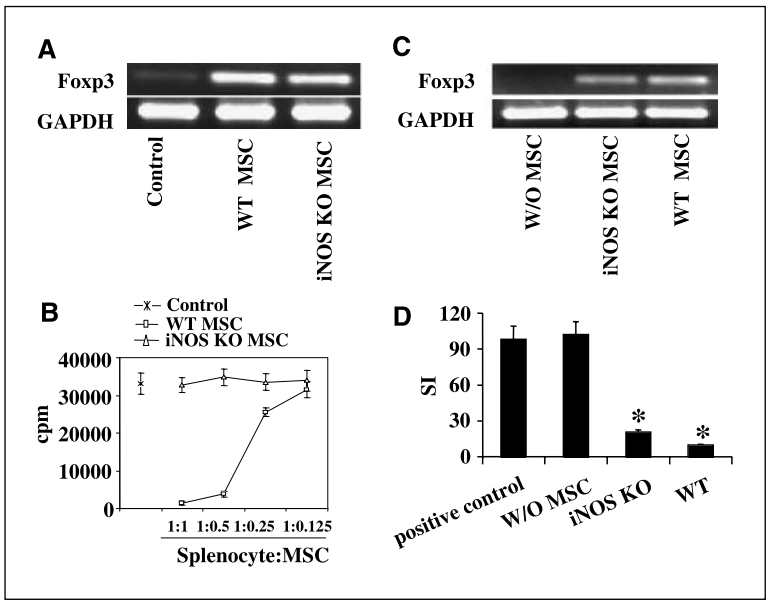

Figure 5. Treg induction by MSC independent on NO pathway. *A* and *B,* iNOS was required for *in vitro* suppression of T-cell proliferation but not Treg induction mediated by MSC *in vitro*. Suppressive activity and Foxp3 gene induction by MSC derived from wild-type or iNOS-deficient tumor-bearing C57BL/6 mice were analyzed. *A,* expression of *Foxp3* and *GAPDH* was analyzed by RT-PCR. *B,* MSC-mediated suppressive activity was assessed by coculture of CD4 ovalbumin transgenic splenocytes and MSC at various ratios. *Points,* mean of triplicate cultures; *bars,* SD. *C* and *D,* iNOS was not required for the development of Treg cells *in vivo*. Bone marrow Percoll fraction 2 MSC from tumor-bearing iNOS knockout or wild-type C57BL/6 mice was injected i.v. into irradiated ovalbumin-B16 tumor-bearing mice that also received tumor (ovalbumin)–specific transgenic T cells. After 7 days, the adoptively transferred T cells were isolated from spleen. One-step RT-PCR kits were used to analyze *Foxp3* expression (*C*). Proliferative response of tumor-specific T cells recovered from recipient tumor-bearing mice was assessed (*D*). *, $P < 0.01$ (ANOVA). *Columns,* mean (expressed as stimulation index); *bars,* SD.

iNOS is required for MSC-mediated immune suppression but not for Treg induction. Previous studies showed that IFNγ-dependent NO production was required for the suppression of *in vitro* T-cell proliferation mediated by MSC. In this next experiment, we asked whether NO production by MSCs is necessary for the development of Treg cells. CD4 ovalbumin TCR transgenic splenocytes were cocultured with Percoll fraction 2 Gr-1$^+$ MSCs derived from wild-type or iNOS-deficient tumor-bearing mice in the presence of irradiated ovalbumin-B16 melanoma cells. Percoll fraction 3 cells derived from wild-type tumor-bearing mice were used as negative control. Six days later, cells were harvested and the expression of *Foxp3* was analyzed by RT-PCR. In addition, the ability of iNOS-deficient MSC to suppress T-cell proliferation was assessed. Consistent with previous findings, iNOS-deficient MSC completely lacked suppressive activities (Fig. 5*B*). However, a significant level of *Foxp3* expression was still detectable in the coculture with iNOS-deficient MSC (Fig. 5*A*). To further verify whether the expression of iNOS by MSC is required for the development of Treg cells *in vivo*, MSCs were isolated from iNOS-deficient tumor-bearing mice and injected via the tail vein into irradiated ovalbumin-B16 tumor-bearing mice that also received CD4 ovalbumin TCR transgenic T cells. At day 7 after adoptive transfer, ovalbumin TCR transgenic T cells in the spleen were recovered. The proliferative response and *Foxp3* expression of recovered T cells were assessed. A similar level of *Foxp3* expression by T cells recovered from mice that received iNOS-deficient MSCs was detected when compared with those from mice that received wild-type MSCs and the T cells still exhibited a hypoproliferative response to peptide stimulation (Fig. 5*C* and *D*). The data suggest that the production of NO by MSCs is not required for the induction of *Foxp3* expression and that both wild-type and iNOS-deficient MSCs can induce the hypoproliferation of T cells isolated from tumor-bearing mice.

Discussion

A significantly increased number of MSC, also known as immature myeloid cells, has been observed in tumor-bearing mice and in patients with squamous cell carcinoma, non–small cell lung carcinoma, breast cancer, and head and neck cancer (8, 45). MSCs consist of a heterogeneous population of cells with myeloid lineage markers Gr-1 and Mac-1 (CD11b). However, these markers also exist on non-MSC cells, such as granulocytes and monocytes. In this study, we identified a more specific population within Percoll fraction 2 MSCs that expresses the myeloid markers Gr-1, CD115 (M-CSF receptor), and F4/80, which has much stronger suppressive activity compared with the classic Gr-1$^+$CD11b$^+$ MSC (Fig. 1*B*). Although Gr-1$^+$ immature myeloid cells from the spleens of tumor-bearing mice have been shown to suppress the proliferation of CD4$^+$ and CD8$^+$ T cells (18, 46), the sorted Gr-1 single positive cells of Percoll fraction 2 did not suppress HA-mediated proliferation of CD4 (Fig. 1*A*). Because the only marker used for sorting immature myeloid cells in previously published studies was Gr-1, it is possible that the observed suppressive activity was mediated by the Gr-1$^+$CD115$^+$ MSCs within the sorted Gr-1$^+$ population.

The *in vivo* mechanisms underlying MSC-mediated tumor-specific T-cell immune suppression are not completely clear. Furthermore, the development of Treg in tumor-bearing animals is not well studied. In this report, we provide the first evidence that MSCs can induce the development of Treg *in vitro* and in tumor-bearing mice, which requires IFN-γ and IL-10 but not IL-13. In addition, we have observed that IL-10 and IFN-γ, but not IL-13, up-regulate MHC class II and ligands for several costimulatory molecules (CD86, ICOSL, and PD-L1/B7-H1) on MSC.[4] These costimulatory molecule ligands on MSC may provide signals important for the development of Treg cells. It has been shown that the Th2 cytokine IL-13/IL-4R pathway can up-regulate arginase 1 expression by MSC, leading to arginase 1–mediated T-cell tolerance (16, 18). However, IL-13 signaling may not affect the MSC-mediated development of Treg cells. MSCs have been shown to block immunosurveillance of CTLs, resulting in tumor recurrence (47). This immunosuppressive activity was mediated through IL-13 secreted by NK T cells, which then acts on MSC to induce TGF-β1 secretion. In our system, however, IFN-γ, rather than IL-13, is required for the enhanced production of TGF-β and

[4] Unpublished results.

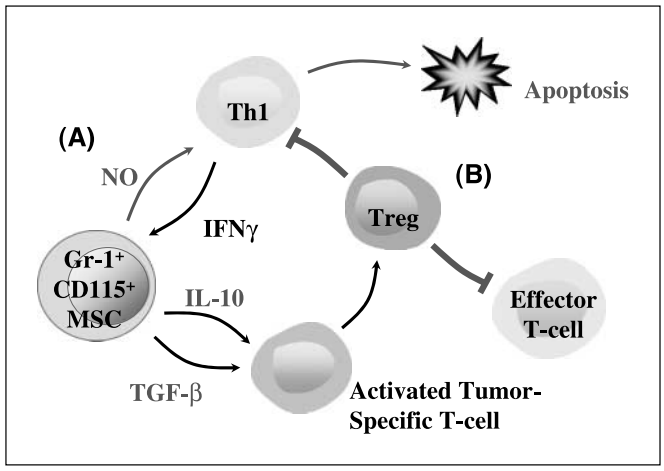

Figure 6. A schematic model depicting a possible mechanism by which MSC can suppress antitumor responses. MSC can suppress tumor-specific T-cell responses directly and indirectly through (*A*) the production of NO at early stages at the encounter of activated T cells and (*B*) the induction of Treg development at later stages to control immune suppression in tumor-bearing host.

induction of IL-10 secretion by MSC and the subsequent development of Treg cells. The difference in IFN-γ versus IL-13 dependence may be due to differences in the tumor models studied (no spontaneous regression is observed in our tumor model) and in the interactions between MSC and NK T cells or T effector cells. MSC may mediate the development of Treg cell through a combination of various pathways dependent on TGF-β, IL-10, and cell-cell contact. Although activation of T cells is required for the development of Treg cells induced by MSC in our system, whether these Treg cells are derived from activated effector Th1 and Th2 cells is currently under study.

MSC induced by tumor may promote tumor growth and metastasis through multiple mechanisms. MSC may suppress T-cell responses through an IFN-γ-dependent iNOS pathway for the inhibition of Th1 cells (15), a Th2 cytokine-dependent arginase 1 pathway (17), and iNOS and arginase 1–dependent free radical–mediated cell death (16, 18–20). Recently, Gr-1$^+$Mac-1$^+$ immature myeloid cells have been shown to promote tumor angiogenesis by directly incorporating into the tumor endothelium and thus promote tumor development and growth (48). In this report, we show a novel mechanism—the induction of Treg by which tumor-induced Gr-1$^+$CD115$^+$ MSC can suppress the antitumor response.

Based on the results reported here, we propose a novel mechanism by which tumor-induced MSC can suppress tumor-specific T-cell responses (Fig. 6). MSCs not only can inhibit the activation and clonal expansion of tumor-specific T cells directly through the secretion of IL-10, TGF-β, and NO but also mediate the development of Treg cells, which can induce and maintain T-cell tolerance in tumor-bearing hosts. Furthermore, Treg induction and NO-dependent suppressive activity mediated by MSC seem to be independent pathways because iNOS-deficient MSC lost *in vitro* suppressive activity but could still induce the development of Tregs both *in vitro* and in tumor-bearing mice (Fig. 5). Our findings identify MSC as a potential target for intervention in multiple tumor evasion mechanisms simultaneously. Immune modulatory therapy is less effective in treating large tumors partly due to immune suppression associated with MSC (38, 49). Therapeutic approaches directed toward the manipulation of the MSC population and their function may improve immune enhancing therapy for advanced malignancy.

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