Gr-1<sup>+</sup>CD115<sup>+</sup> Immature Myeloid Suppressor Cells Mediate the Development of Tumor-Induced T Regulatory Cells and T-Cell Anergy in Tumor-Bearing Host

Bo Huang, Ping-Ying Pan, Qingsheng Li, Alice I. Sato, David E. Levy, Jonathan Bromberg, Celia M. Divino, and Shu-Hsia Chen

Departments of Gene and Cell Medicine and General Surgery, Mount Sinai School of Medicine; Departments of Pathology and Microbiology, New York University School of Medicine, New York, New York

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<sup>+</sup>CD115<sup>+</sup> MSCs, in addition to being able to suppress T-cell proliferation in vitro, can induce the development of Foxp3<sup>+</sup> 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<sup>+</sup>CD115<sup>+</sup> 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<sup>+</sup>CD115<sup>+</sup> MSCs 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 tumors 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 immunosuppression 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 reaction 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<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD115<sup>+</sup> 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.
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-Ek-restricted CD4 and CD8 TCR transgenic mice (in BALB/c background; Thy1.2) were gifts from Dr. Linda Sherman (Scirpps Research Institute, La Jolla, CA) and Dr. Constantin A. Bona (Mount Sinai School of Medicine, New York, NY), respectively. Stat-1-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 (IL-4R)–a 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-2b) melanoma (37). To generate the tumor model of metastatic colon cancer, MCA26 or HA-MCA26 tumor cells (9 × 10^6) were inoculated in the liver by intraperitoneal implantation of cells as described previously (15). Similar methodology was used for the B16 tumor model.

Peptide and antibodies. CD4 HA peptide (191SFEREFEFPEK229), CD8 HA peptide (533IYSTVASSL541), and CD4 ovalbumin peptide (323ISQAVHAA-FQ179) were purchased from Washington Biotechnology, Inc. (Baltimore, MD). Neutralizing anti-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-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 mm² were sacrificed and their spleen, thymus, 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 × 10^6) from TCR transgenic mice were cultured in the presence of serial dilutions of irradiated MSCs in 96-well microplates. [3H]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 were 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 × 10⁶ per mouse). As for MSC, sorted Gr-1+CD115+ bone marrow fraction 2 cells (2.5 × 10⁶ per mouse) or single Gr-1+ fraction 2 cells (5 × 10⁶ per mouse) from large tumor-bearing mice were used.

HA-MCA26 cells (or neo-transfected parental MCA26 cells as a control; 9 × 10⁶) were inoculated into Thy1.1+ BALB/c mice. Six days later, the mice with tumor size of −5 × 5 mm² were irradiated. The following day, the sorted MSC and T cells were coadministered 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 × 10⁶) with irradiated (2,500 rad) naive splenic cells (4 × 10⁶) as APC were cocultured with or without HA peptide (5 μg/mL) in 96-well microplates. [3H]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 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. L4, left)
showed a significantly increased percentage of Gr-1+/CD115+/F4/80+ cells 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-/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 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).
The strong suppressive effect of sorted Gr-1+CD4+CD25+ cells and Gr-1+CD115+ cells, but not Gr-1+CD4+CD25− or Gr-1+CD115− cells, was observed (Fig. 1A, right). Based on the facts that (a) the majority of Gr-1+CD115+ cells also expressed CD4+ 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 (~2-fold increase) was observed in sorted Gr-1+CD115+ cells when compared with sorted Gr-1+CD11b+ cells (Fig. 1B). 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 expression 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. 1C) 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). This data reveal that a population of nondividing CD4+CD25− T cells 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. 1D). 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. 1E). 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. 1E). 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. 1F). 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 congeneric Thy1.2+CD4+ HA-TCR+ T cells were adoptively transferred into Thy1.1+ mice bearing HA-MCA26 tumors (5 × 5 mm2). 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. 2A, 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. 2B). 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. 2C, upper panel). The adoptively transferred tumor-specific CD4+ and/or CD8+ T cells from the CD25 depletion groups (Fig. 2C, 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 vivo 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. 1D), 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 coadministered 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. 3A). 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. 3A). 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.
expression in the tumor, as anti-IFN-γ treat-ment completely inhibited the expression of IFN-γ 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 coadaptively transferred with T cells (HA-TCR in BALB/c and 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. 3D). 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).
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 in the supernatants of the coculture of MSCs and CD4 HA TCR transgenic splenocytes (Fig. 1C). 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, 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 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. 4A). 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. 4B). 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. 4A). 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 IFN-γ secreted from activated T cells, Gr-1+CD115+ MSCs can secrete IL-10, TGF-β, and NO.
**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. 1B). 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. 1A). 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. 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 I 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-β1 and...
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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References


Myeloid Suppressor Cells Mediated Treg Induction


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