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

Bo Huang,1 Ping-Ying Pan,1 Qingsheng Li,1 Alice I. Sato,1 David E. Levy,3 Jonathan Bromberg,1 Celia M. Divino,2 and Shu-Hsia Chen1

Departments of 1Gene and Cell Medicine and 2General Surgery, Mount Sinai School of Medicine; 3Departments 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⁺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 CD3ε, 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 Treg studies 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.
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<sup>d</sup>-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 methodology was used for the B16 tumor model.

Peptide and antibodies. CD4 HA peptide (151STFEFIEFKE<sup>159</sup>) and CD8 HA peptide (323ISQAVHAA-<sup>331</sup>LYSTVASSL<sup>341</sup>) were purchased from Sigma-Aldrich, St. Louis, MO. Anti-CD11b/TCR-3 antibody was a gift from Dr. Richard Dutton (Trudeau Institute, Saranac Lake, NY), and CD4 ovalbumin peptide (CD4<sup>104</sup>-<sup>112</sup>) and CD8 TCR transgenic HA-specific 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<sup>6</sup> per mouse). As for MSC, sorted Gr-1<sup>+</sup>CD115<sup>+</sup> bone marrow fraction 2 cells (2.5×10<sup>6</sup> per mouse) or single Gr-1<sup>+</sup> fraction 2 cells (5 × 10<sup>6</sup> per mouse) from large tumor-bearing mice were used.

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<sup>6</sup> per mouse). As for MSC, sorted Gr-1<sup>+</sup>CD115<sup>+</sup> bone marrow fraction 2 cells (2.5×10<sup>6</sup> per mouse) or single Gr-1<sup>+</sup> fraction 2 cells (5 × 10<sup>6</sup> per mouse) from large tumor-bearing mice were used.

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).

Cytokine or NO measurement was done by ELISA or NO measurement of the supernatant in vitro. 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).

Results

Increase in Gr-1<sup>+</sup> CD115<sup>+</sup>/F4/80<sup>+</sup> 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. 1A, left)
showed a significantly increased percentage of Gr-1⁺CD115⁺F4/80⁺ cells in tumor-bearing animals compared to naive controls. 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⁻/CD11b⁻ 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. 1A, 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 (~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 (IL-2R 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. 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). 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. 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 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. 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 coadotransfected 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-

suggest that IL-10 and IFN-
treated with anti-IL-13 antibodies. Taken together, the results
expression were still detected in the tumor tissues from mice
were detectable, however, at a lower level in the tumors from

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 coadoptionally 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. 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, along with IFN-γ, 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, 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. 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). 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-β-1, 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-bearng 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.
iNOS is required for MSC-mediated immune suppression but not for Treg induction. Previous studies showed that IFN-γ-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. 5B). However, a significant level of Foxp3 expression was still detectable in the coculture with iNOS-deficient MSC (Fig. 5A). 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 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. 5C 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. 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+ 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 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-β1 and

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 spleens. 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.

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.

Unpublished results.
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 dependence may be due to differences in the tumor models studied.

References


Gr-1^+CD115^+ 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, et al.


Updated version  Access the most recent version of this article at:  
http://cancerres.aacrjournals.org/content/66/2/1123

Cited articles  This article cites 49 articles, 27 of which you can access for free at:  
http://cancerres.aacrjournals.org/content/66/2/1123.full#ref-list-1

Citing articles  This article has been cited by 100 HighWire-hosted articles. Access the articles at:  
http://cancerres.aacrjournals.org/content/66/2/1123.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.