Myeloid-Derived Suppressor Cells Attenuate T_{H1} Development through IL-6 Production to Promote Tumor Progression

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Abstract

Collaborative action between tumor cells and host-derived suppressor cells leads to peripheral tolerance of T cells to tumor antigens. Here, we showed that in tumor-bearing mice, generation of tumor antigen-specific effector T-helper cells (T_{H1}) was significantly attenuated, and impaired T_{H1} differentiation was restored by the temporal blockade of interleukin (IL)-6 activity at the T-cell priming phase. Furthermore, we found that Gr-1^+ myeloid-derived suppressor cells (MDSC) served as a source of IL-6 in tumor-bearing mice. Adoptive transfer of effector CD4^+ T cells revealed that MDSC-sensitized effector CD4^+ T cells were less potent in mounting antitumor immune responses, although effector T cells generated together with Gr-1^+ cells from tumor-free mice eradicated established tumors. CD8^+ T cells, IFN-γ, and MHC-class II expression in host mice were indispensable for the antitumor activity initiated by effector CD4^+ T cells. Despite comparable suppressive activity of IL-6^{+/−} and IL-6^{−/−} MDSC on primary T-cell activation, transfer of IL-6^{+/−} MDSC, but not IL-6^{−/−} MDSC, dampened the efficient induction of effector T_{H1} cells and counteracted CD4^+ T cell-mediated antitumor immunity including cognate help for CD8^+ T cells in vivo. These findings suggest that, apart from the inhibitory effects on primary T-cell activation, MDSC promote tumor progression by attenuating functional differentiation of tumor-specific CD4^+ T cells into effector T_{H1} cells through IL-6 production to promote tumor progression. This novel mode of MDSC-induced tolerance of effector CD4^+ T cells should be considered as the basis for the rational design of effective T cell-mediated antitumor therapies. Cancer Immunol Res; 1(1); 64–76. ©2013 AACR.

Introduction

T cells represent a key effector arm of the immune system required for cancer control. Significant progress has been made in the identification of antigens expressed in tumor cells that are recognized by T cells (1). These findings have led us to develop tumor-derived antigen-specific vaccine strategies including further exploration of tumor-associated antigens (2) and dendritic cell vaccines for patients with cancer (3, 4). Currently, a potential role of CD4^+ T cells for cancer immunotherapy is being increasingly appreciated (5–9). Accumulating evidences have shown that tumor-specific CD4^+ T cells eradicate tumors via orchestrating host-derived immune components, especially CD8^+ T cells (7, 10–12) and natural killer cells (13). On the basis of these studies, the efficient induction of tumor-specific, multifunctional effector CD4^+ T cells that provoke antitumor function of other immune cells through their CD40 ligand, interleukin (IL)-2, and IFN-γ (5, 6, 11, 14) is now recognized as an important aim of antitumor vaccination strategies.

The most important problematic issue is that tumor microenvironment allows tumor cells to escape from the attack by T cells in vivo. During tumor progression, tumor-specific T cells result in a dysfunctional or “exhausted” state with impaired effector functions (7, 15–17). The discordant antitumor T-cell responses in tumor-bearing recipients have been explained by diverse mechanisms, such as the induction of tumor-induced suppressor cells including regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC; refs. 16, 18–20), alternations in T-cell signaling (21, 22), and induction of T-cell apoptosis (23). In particular, IFN-γ production by tumor-specific T cells is shown to be inhibited in tumor-bearing hosts (7, 15, 17). This is consistent with the epigenetic silencing of ifng gene in tumor-infiltrating CD4^+ effector T cells (24). Moreover, a positive correlation between the survival of patients with cancer and the mRNA expression of T-helper cell (T_{H1})-associated markers such as IFN-γ and T-box expressed in T cells (T-bet) in tumor tissues has been observed (25). Therefore, a T_{H1}-skewed antitumor response is beneficial, and the strategy to overcome immunosuppression including attenuated T_{H1} development in tumor-bearing hosts will be required for improving the efficacy of therapy for an existing tumor burden as well as prophylaxis (5, 7–9).

Chronic inflammation and increased levels of inflammatory cytokines including IL-6 have been reported in patients with...
various type of tumors (26), and an elevated level of IL-6 fostering progressive expansion of tumor cells (27) has been associated with poor clinical outcome (28–31). Here, we focused on the association between the functional impairment of CD4+ T cells and immune-suppressive effects of the tumor-induced inflammation-related factors, IL-6 and MDSC. When T cells are primed, MDSC attenuate the proliferation and cytokine production of T cells or induce apoptosis (16, 18, 19). Although a most of the attention on the inhibitory action of MDSC has been focused on to their effects on the primary T-cell activation, the consequences of MDSC-sensitized CD4+ T cells after their priming remains to be investigated. Our results in this study suggest that MDSC-derived IL-6 cause the intrinsic dysfunction of tumor-specific effector CD4+ T cells, which is characterized by their decreased ability to produce IFN-γ and failure to attack the tumor at the subsequent effector phase. Therefore, we propose that inappropriate responsiveness of effector CD4+ T cells to tumor antigens induced by MDSC imposes a significant barrier to therapeutic tumor vaccine strategies.

Materials and Methods

Mice

IL-6−/− deficient mice were generated by M. Kopf (Institute of Molecular Health Sciences, Zurich, Switzerland) and provided by Y. Iwakura (University of Tokyo, Tokyo, Japan). Ovalbumin (OVA)-specific OT-II T-cell receptor (TCR)–transgenic mice were provided by S. Koyasu (Keio University School of Medicine, Tokyo, Japan). C57BL/6 mice were purchased from Nihon Clea. CD45.1+ mice were provided by S. Koyasu (Keio University School of Medicine, Tokyo, Japan). C57BL/6 mice were purchased from Japan SLC, Inc. B6.SJL-PtprcaPep3b/BoyJ mice and genetically mice were provided by S. Koyasu (Keio University School of Medicine, Tokyo, Japan). C57BL/6 mice were purchased from Nihon Clea. CD45.1+ mice were provided by S. Koyasu (Keio University School of Medicine, Tokyo, Japan). C57BL/6 mice were purchased from Japan SLC, Inc. B6.SJL-PtprcaPep3b/BoyJ mice and genetically mice were provided by S. Koyasu (Keio University School of Medicine, Tokyo, Japan). C57BL/6 mice were purchased from Japan SLC, Inc.

Tumor inoculation and antibody treatment in vivo

MCA205 cells were transfected with an OVA expression vector, and stable clones expressing OVA protein (MCA205-OVA) were established as described previously (3). Mice were inoculated subcutaneously in the right flank with 8 × 10³ MCA205-OVA or 3 × 10⁵ OVA-expressing melanoma cell line, MO4 (32). Tumor sizes are expressed as a tumor index: square root (length × width) as described previously (3). In the pulmonary metastatic model, mice were injected intravenously with 1 × 10⁶ firefly luciferase-transfected MO4 cells. For measurement of tumor progression in the lung, 2 mg r-luciferin was injected intravenously into the mice and luminescence images were analyzed using an in vivo imaging system (NightOwl II; Berthold Technologies). Two hundred and 50 µg of anti-IL-6, antibody MP5-20F3 (BioXCell) or control rat immunoglobulin G (IgG) antibody (Millipore) was injected 1 day before and after immunization with dendritic cell. Daily intraperitoneal injection of 200 µg anti-IFN-γ antibody (ebioscience) was conducted for 3 days after T-cell transfer. For depletion, mice were injected with 200 µg anti-Gr-1 antibody (RB6-8C5; BioXCell), anti-Ly6G (clone: 1A8; eBioscience), anti-CD8 or anti-CD4 antibody 4 to 5 days before T-cell transfer, immunization, or tumor inoculation. Recombinant soluble IL-6R α-chain [soluble form of IL-6 receptor (sIL-6R); 500 ng; PeproTech] was injected intravenously together with dendritic cell.

Preparation of CD4+ T cells and MDSC, adoptive transfer, and immunization

Naive CD4+ T cells were isolated from OT-II TCR-transgenic mice using a naive CD4+ T-cell isolation kit (Miltenyi Biotec). Gr-1+ control cells or MDSC were isolated using an MDSC isolation kit or anti-Ly6G microbeads (Miltenyi Biotec) from pooled spleens of tumor-free or tumor-bearing mice, respectively. The resultant Gr-1+CD11b+ MDSC population was more than 90%. Isolated OT-II cells (5 × 10⁵) and MDSC (3–6 × 10⁶) were injected intravenously into naïve mice or mice bearing 5 mm × 5 mm tumors. Six hours after T-cell and MDSC transfer, mice were immunized by intravenous transfer of 3 × 10⁶ bone marrow-derived dendritic cell (33) pulsed with the OT-II TCR-specific peptide (ISQAVHAAHAEINEAGR).

Flow-cytometric analysis

Antibodies used in flow-cytometric analysis were as follows: anti-Vβ5 or anti-Ly6G (1A8), anti-CD11b (Calltag) or anti-IL-6; anti-CD4 or PerCP–streptavidin, biotinylated anti-Vβ5 (BD Biosciences), anti-IL-4R, anti-M-CSFR, anti-MHC-II, anti-CD45.1, anti-Ly6C (HK1.4; Biolegend), or anti-Gr-1 antibodies reactive to both Ly6G and Ly6C (RB6-8C5; eBioscience). The H-2Kb/SIINFEKL-OVA tetramer-phycocerythrin (PE) was purchased from MBL. For intracellular staining, following isolation of effector CD4+ T cells from the in vitro cultures or from mice using anti-CD4 beads, CD4+ T cells were stimulated with cognate peptide-pulsed dendritic cell or phosphorl 12-myristate 13-acetate (PMA)/ionomycin in vitro and were stained with Alexa Fluor 488–anti-IFN-γ, PE–anti-IL-2 or -anti-IL-17A antibody (ebioscience; ref. 34). For the analysis of IL-6 production in MDSC, splenocytes harvested from tumor-free or –bearing mice were cultured with Brefeldin A (Sigma) in vitro, and 12 hours later, cells were analyzed for intracellular IL-6. The cell-permeant reagent, Deep Red (Molecular Probes) was used for reactive oxygen species (ROS) detection according to manufacturers’ instructions. Immunofluorescence images were analyzed using a FACSCaliber (Becton Dickinson). Data were analyzed using FlowJo software (Treestar).

OT-II T cells and MDSC coculture experiments

Naive OT-II cells (1.0 × 10⁶/well) were stimulated with dendritic cell (5.0 × 10⁶/well) and 5 μmol/L OVA peptide. MDSC (3 × 10⁶/well) purified from tumor-bearing mice or Gr-1+ cells from tumor-free mice as control cells were added to T-cell cultures. In some experiments, anti-PO-1 antibody (BioXCell), anti-TGF-β antibody (R&D Systems), nitric oxide synthase (NOS) inhibitor (L-NAME; Sigma), Cox-2 inhibitor (NS-398; Sigma), anti-HL-10 antibody (ebioscience), anti-IL-6 antibody (BioXCell), control rat Ig (Pierce), recombinant IL-2

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IL-12 (5 ng/mL), and IL-6 (30 ng/mL) with or without IFN-γ (20 ng/mL) were added to the cultures on day 0. Recombinant murine cytokines were purchased from Wako Pure Chemical Industries. After 6 days of coculture, T cells were used in further experiments. To assess the primary T-cell expansion, naïve OT-II cells (2.5 × 10⁴/well) were stimulated with OVA peptide-pulsed and mitomycin C-treated dendritic cell (5 × 10⁴) or plate-coated anti-CD3 antibody (2 μg/mL) and anti-CD28 antibody (5 μg/mL). The indicated numbers of MDSC were added to each well. Proliferation was determined by [³H] thymidine incorporation as described previously (34).

ELISA and real-time reverse transcription PCR

For the measurement of IFN-γ secretion, supernatants of T-cell culture were collected 36 hours after stimulation and assayed by ELISA (eBioscience). Serum was collected from tumor-free or -bearing mice, and IL-6 or sIL-6R was determined by ELISA (R&D Systems). Total RNA was extracted from isolated MDSC using a RNeasy kit (Qiagen), digested with DNase I and reverse-transcribed into cDNA using a cDNA Synthesis Kit (Roche Diagnostics GmbH). TaqMan probes for NOS2 (Mm01257348), indoleamine 2,3-dioxygenase (IDO; Mm00494449), Arginase 1 (Mm00475988), IL-10 (Mm00474338), IL-6 (Mm00446190), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems. All samples analyzed in triplicate using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) and were normalized to GAPDH expression levels. Threshold cycle (Ct) values for each reaction were determined and averaged using TaqMan SDS analysis software. Changes in gene expression were calculated by the comparative Ct method (fold changes = 2⁻¹ΔΔCt).

In vitro generation of human MDSC and coculture with CD4⁺ T cells

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers by density gradient centrifugation using Ficoll Hypaque (Sigma). Tumor-associated MDSC were generated by the coculture of PBMC with ES-2 tumor cells for 6 days (35). Resultant MDSC (CD33⁺HLA-DR⁻CD11b⁺CD15⁻ phenotype; data not shown) were then isolated using anti-CD33 microbeads (Miltenyi Biotec). CD33⁺MDSC or control CD33⁺ cells that were freshly isolated from PBMC without coculture with tumor cells were cultured with autologous naïve CD4⁺ T cells at 1:1 ratio. T₃₁L differentiation was induced by stimulation with anti-human CD3 and CD28 antibodies (BD Biosciences) in the presence of recombinant IL-12 and sIL-6R, or IL-6 (R&D Systems). Intracellular cytokine staining using fluorescein isothiocyanate (FITC)–anti-IFN-γ, PE–anti-IL-2, and PerCP-Cy5.5–anti-CD4 (eBiosciences) were conducted in effector CD4⁺ T cells restimulated with PMA/ionomycin 7 days after the first stimulation.

Statistical analysis

All statistical analyses were conducted using the Prism 4.0 software (GraphPad). Multiple group comparisons were conducted by one-way ANOVA followed by Tukey post hoc tests; Kruskal–Wallis test was the nonparametric alternative to ANOVA. Values of P < 0.05 were considered significant.

Results

Therapeutic effect of CD4⁺ T cells on tumor progression and T₃₁L differentiation are improved by IL-6 blockade

First, we investigated the effect of tumor-bearing hosts on the generation of tumor-specific effector CD4⁺ T cells. For this purpose, we established an MCA205 fibrosarcoma cell line expressing OVA protein (MCA205-OVA), in which OVA served as a model tumor-associated antigen. In this system, antigen presentation to CD4⁺ T cells relies on endogenous or transferred antigen-presenting cells (APC), because the tumor cells do not express MHC-II (data not shown). Despite the lack of direct antigen presentation, adoptive transfer of OVA-specific OT-II TCR-transgenic CD4⁺ T cells induced a significant reduction in tumor growth in response to immunization with dendritic cells pulsed with an OT-II TCR-specific OVA peptide (Fig. 1A). The importance of CD4⁺ T cells was further confirmed by the increase in tumor size after depletion of CD4⁺ T cells in vivo with anti-CD4 antibody treatments. (Supplementary Fig. S1A).

We were particularly interested in IL-6 because this pleiotropic cytokine was produced abundantly in patients with advanced malignancies (26). To investigate the involvement of IL-6 in therapeutic efficacy, we treated tumor-bearing mice with an anti-IL-6–neutralizing antibody combined with the transfer of tumor-specific CD4⁺ T cells and immunization with peptide-pulsed dendritic cells. In addition to active immunization, the temporal blockade of IL-6 activity at the T-cell priming phase significantly enhanced the efficacy of CD4⁺ T-cell transfer therapy (Fig. 1A).

IFN-γ-producing T₃₁L cells are one of the potent antitumor components (5, 9, 12, 14). Therefore, we analyzed the development of OVA-specific IFN-γ–producing donor cells following in vivo priming in tumor-bearing mice. As shown in Fig. 1B, 7 days after T-cell transfer, the number of donor OT-II cells was comparably increased by dendritic cell immunization in both tumor-bearing and tumor-free mice. Anti-IL-6 antibody treatment slightly decreased the expansion of OT-II T cells and did not affect the number of Treg in tumor-bearing mice (Fig. 1B and C). However, the frequency of IFN-γ–producing donor OT-II cells in tumor-bearing mice was significantly lower than those in tumor-free mice. The frequency of IL-2–producing T cells was not altered regardless of the presence of dendritic cells or anti-IL-6 antibody, whereas the generation of IFN-γ–producing T cells was significantly improved by IL-6 blockade in tumor-bearing mice (Fig. 1D and E and Supplementary Fig. S1B). The development of tumor-specific T₃₁L cells strongly correlated with the extent of tumor regression. Thus, IL-6–mediated signaling in tumor-bearing mice seemed to be detrimental to the generation of functional effector CD4⁺ T₃₁L cells and subsequent tumor regression.

IL-6 is produced by Gr-1⁺ MDSCs in tumor-bearing mice

The systemic increase of IL-6 concentrations in patients with cancer may have a negative impact on tumor regression.
(28, 29). Indeed, several types of tumors produce IL-6 (26, 28, 36). Consistent with these studies, a marked increase in serum IL-6 levels was observed in MCA205-bearing mice, whereas tumor-free mice exhibited much lower levels of IL-6 (Fig. 2A). Although IL-6 expression was intact in tumor cells and the degree of tumor growth was comparable in IL-6+/+ versus IL-6−/− mice (Fig. 2B), IL-6 levels increased slightly in tumor-bearing IL-6−/− mice as compared with tumor-free IL-6+/+ mice. These results raised the possibility that a source of IL-6 in tumor-bearing mice could be the host-derived cells in addition to tumor cells.

Given that in tumor-bearing animals or in patients with cancer, elevated accumulation of MDSC coincides with tumor progression as well as increased levels of IL-6 (30, 31), we assessed the contribution of Gr−1 MDSC to the high levels of IL-6 in tumor-bearing mice. Thus, tumor-bearing mice were treated with anti-Gr-1 antibody or anti-Ly6G antibody to deplete MDSC and then the IL-6 concentration was determined. On day 5 when cell depletion–provoked inflammation had calmed down, IL-6 level was reduced in anti-Gr-1 antibody- or anti-Ly6G antibody–treated tumor-bearing mice as compared with the control tumor-bearing mice (Fig. 2A). In contrast, the suppressive effect of anti-Gr-1 antibody treatment on IL-6 concentration was not observed in tumor-bearing IL-6−/− mice. Reduced IL-6 concentration by MDSC depletion was reproducible in MO4 melanoma-implanted mice (Supplementary Fig. S2A).

In the MCA205-OVA model, MDSC exhibited the classical phenotype, CD11b+ Gr−1 IL−4Rα+ ‘M-CSFR+ MHC-II+’ (Supplementary Fig. S2B and S2C). To directly confirm IL-6 production by Gr−1 MDSC that were divided into 2 subpopulations Ly6Ghi or Ly6Glow− cells (19), intracellular IL-6 in these populations were evaluated by flow-cytometric analysis. A relatively high frequency of IL-6–producing cells was detected in Ly6Ghi MDSC that accumulated in tumor-bearing mice, whereas few IL-6+ Gr−1− cells from tumor-free mice were detected (Fig. 2C). This was confirmed by the results that the levels of IL-6 production from isolated Ly6G+ cells were higher than those from Gr−1+ cells (containing CD11b+Ly6Ghi MDSC and CD11b+Ly6Glow− MDSC) or CD11b+ cells (containing CD11b+Gr−1− cells, Ly6Ghi MDSC and Ly6Glow− MDSC; Supplementary Fig. S2D). Collectively, these findings suggest that MDSC serve as a substantial source of IL-6 in tumor-bearing mice.

**Attenuated IFN-γ production in effector CD4+ T cells is programmed by murine and human MDSC in vitro**

To study directly the effects of MDSC and IL-6 on lineage commitment of CD4+ T cells after primary activation, naive OT-II cells were stimulated in vitro with OVA peptide-pulsed dendritic cells for 6 days in the presence of control Gr−1− cells isolated from tumor-free mice or Gr−1+ MDSC from tumor-bearing mice. Cytokine production was assessed in resultant effector OT-II T cells restimulated in MDSC-free condition. As shown in Fig. 3A, MDSC-sensitized effector OT-II cells produced a lower level of IFN-γ than did control T cells. Because neither exogenous IL-2 nor IFN-γ rescued the MDSC-mediated decrease in the differentiation of IFN-γ–producing effectors, this phenotype was not due to reduced IFN-γ or IL-2 production at their priming phase. Furthermore, the suppressive effect of MDSC on Tα1 development was not restored by the addition of IL-12 (Fig. 3B).
We next evaluated the effect of IL-6 on the ability of MDSC to suppress T_{H1} differentiation. Addition of anti-IL-6 antibody ameliorated the MDSC-mediated suppression of T_{H1} differentiation. In contrast, exogenous IL-6 drastically inhibited the development of IFN-γ–producing effector CD4^+ T cells in the absence of MDSC (Fig. 3B and C). No significant differences in IL-17, IL-2, TNF-α, IL-21, or IL-4 production was observed between control Gr-1^+ cells- and MDSC-sensitized effector CD4^+ T cells (data not shown).

Similar phenomena were observed when human polyclonal CD4^+ T cells were stimulated with anti-CD3/CD28 antibodies in coculture with tumor-associated CD33^+ MDSC (Fig. 3C). This result excluded the possibility that MDSC-mediated attenuation of T_{H1} differentiation via IL-6 might be a mouse-specific event and that it might be induced through APC. These data suggest that MDSC inhibited the polarization of CD4^+ T cells toward T_{H1} effectors through IL-6 production in both mice and humans.

**MDSC-sensitized, tumor-specific effector CD4^+ T cells are ineffective for tumor regression**

To determine the antitumor effect of MDSC-sensitized effector CD4^+ T cell, we transferred into tumor-bearing mice OT-II effector T cells generated with control Gr-1^+ cell-coculture, which resulted in the regression of the established tumor (Fig. 4A). In contrast, the therapeutic effect of MDSC-sensitized effector OT-II cells on tumor burden was much less potent. The inhibitory effect of MDSC on primary T-cell activation is due to robust production of immune-suppressive factors such as NO, ROS, inhibitory cytokines, and IDO (18, 19). However, the lack of antitumor efficacy of MDSC-sensitized effectors was not rescued by the blockade of these factors when the effectors were generated in vitro (Fig. 4A). Importantly, MDSC-cocultured effector CD4^+ T cells that were generated under IL-6–neutralized condition, provoked antitumor activity to a similar extent as did control effector T cells (Fig. 4B).

Next, to evaluate the involvement of antigen presentation by host APC in tumor regression, we transferred in vitro generated effector CD4^+ T cells into MHC-II–deficient tumor-bearing mice. As shown in Fig. 4C, tumor growth was substantially enhanced in MHC-II–deficient mice compared with wild-type mice, indicating that in vivo restimulation of effector CD4^+ T cells through MHC-II on host APC was necessary to induce immune responses against tumor cells.

Similar to MHC-II deficiency, when IFN-γ activity was neutralized by antibody treatment in vivo, neither control- nor...
MDSC-sensitized effector CD4⁺ T cells exerted their antitumor activities (Fig. 4D). These results confirmed the importance of IFN-γ in tumor regression mediated by tumor-specific effector CD4⁺ T cells, and that the MDSC-sensitized effector T cells lacking IFN-γ cannot eliminate tumors. Furthermore, depletion of host CD8⁺ T cells also abrogated the ability of effector CD4⁺ T cells to repress the tumor burden (Fig. 4E), suggesting that host-derived CD8⁺ T cells were required for effector CD4⁺ T cell–induced tumor elimination.

**IL-6 is not necessary for the maintenance and suppressive activity of MDSC on primary T-cell activation**

To determine the role of IL-6 in the suppressive activity of MDSC, we evaluated the accumulation of MDSC in IL-6–deficient tumor-bearing mice. Similar to IL-6⁺/+ mice, tumor-bearing IL-6⁻/- mice accumulated MDSC even if mice were treated with anti-IL-6 antibody (Fig. 5A), suggesting that IL-6 might not be essential to maintain MDSC.

We next examined the expression of effector molecules known to correlate with the suppressive role of MDSC. As expected, a substantial amount of IL-6 was produced by tumor cells (MCA205-OVA) and IL-6–sufficient MDSC, but not by IL-6–deficient MDSC (Supplementary Fig. S3A). As shown in Fig. 5B, the expression of IDO, NOS2, Arginase 1, and IL-10 mRNA did not vary, regardless of IL-6 status in MDSC. Although the levels of ROS in MDSC were higher than that in control CD33⁺ cells or in vitro–generated, tumor-associated CD33⁺ MDSC. During coculture, recombinant IL-12 and anti-IL-6 antibody were added. Plots show the IL-2– and IFN-γ–producing effector CD4⁺ T cells when restimulated with PMA/ionomycin.

Representative data of 3 independent experiments with similar results are shown (mean ± SEM).

**Figure 3.** MDSC-mediated suppression of TH1 differentiation in vitro is rescued by IL-6 blockade. A and B, effector T cells were generated by stimulating from naïve OT-II cells by stimulating in vitro for 6 days with OVA peptide-pulsed dendritic cells in the presence of MDSC from tumor-bearing mice or control Gr-1⁺ cells from tumor-free mice. Cytokines and antibodies were added to the culture as indicated. Data represent the plots for IFN-γ and IL-17A expression in effector OT-II cells restimulated with PMA/iomycin under MDSC-free condition. C, human polyclonal CD4⁺ T cells were stimulated for 7 days with plate-bound anti-CD3 and CD28 antibodies in the presence of control CD33⁺ cells or in vitro–generated, tumor-associated CD33⁺ MDSC. During coculture, recombinant IL-12 and anti-IL-6 antibody were added. Plots show the IL-2- and IFN-γ-producing effector CD4⁺ T cells when restimulated with PMA/iomycin.

Representative data of 3 independent experiments with similar results are shown (mean ± SEM).
together, these data show that mere ablation of IL-6 production in MDSC does not contribute to the suppressive activity for primary T-cell activation but it affects the subsequent T_{H1} differentiation of CD4\(^+\) T cells.

**Antitumor effect of T_{H1} effector CD4\(^+\) T cells is attenuated by MDSC-derived IL-6 in vivo**

Defective T_{H1} development in tumor-bearing mice was restored by MDSC-depletion via anti-Gr-1 antibody infusion, confirming that MDSCs were at least in part responsible for the defective T_{H1} development (Fig. 6). To determine whether the defect in T_{H1} development was caused by MDSC-derived IL-6 or by other tumor-induced factors, we infused tumor-free mice with MDSC and assessed the differentiation of tumor-specific CD4\(^+\) T cells into T_{H1} cells. Transfer of 6 \times 10^6, but not 3 \times 10^6 MDSC, inhibited the primary expansion of naive OT-II cells in response to vaccination with dendritic cell (Supplementary Fig. S4A).
contrast, transfer of $6 \times 10^6$ Gr-1 cells from tumor-free mice had no effect on primary T-cell activation. When $3 \times 10^6$ MDSC were transferred, T-cell priming as indicated by CD44 upregulation was not abrogated, and the frequency of Treg was unchanged (Supplementary Fig. S4C and S4D). This condition allowed us to elucidate whether an infusion of MDSC into tumor-free mice would be sufficient for the attenuation of TH1 differentiation and antitumor immunity without inhibiting primary T-cell activation in vivo (Fig. 7A).

In cells lacking IL-6R expression on the cell-surface, IL-6 signals can be transmitted by the gp130 transmembrane receptor engaged in a complex with IL-6 and sIL-6R, known as trans-signaling (37). Consistent with previous reports (29, 38), a significant increase in sIL-6R concentration was detected in the serum of MCA205-OVA–bearing mice (Supplementary Fig. S4B), suggesting that the sIL-6R in tumor-bearing mice could complex with IL-6 and contribute to the attenuation of Th1 development. Furthermore, the combination of MDSC transfer and administration of recombinant sIL-6R could facilitate IL-6 trans-signaling, which in turn, enhanced donor OT-II T cells suppression of TH1 differentiation (Supplementary Fig. S4E and Fig. 7B). This suppression was reversed by the treatment of anti-IL-6 antibody. As in the case of broad inhibition of IL-6 activity, concomitant transfer of IL-6–/– MDSC and sIL-6R also failed to attenuate the production of TH1 cells (Fig. 7B). Proportions of donor OT-II cells were comparable under all conditions tested (Fig. 7C and Supplementary Fig. S4F), suggesting that MDSC-derived IL-6 is necessary for polarizing CD4+ T cells away from TH1 responses.

In cells lacking IL-6R expression on the cell-surface, IL-6 signals can be transmitted by the gp130 transmembrane receptor engaged in a complex with IL-6 and sIL-6R, known as trans-signaling (37). Consistent with previous reports (29, 38), a significant increase in sIL-6R concentration was detected in the serum of MCA205-OVA–bearing mice (Supplementary Fig. S4B), suggesting that the sIL-6R in tumor-bearing mice could complex with IL-6 and contribute to the attenuation of Th1 development. Furthermore, the combination of MDSC transfer and administration of recombinant sIL-6R could facilitate IL-6 trans-signaling, which in turn, enhanced donor OT-II T cells suppression of Th1 differentiation (Supplementary Fig. S4E and Fig. 7B). This suppression was reversed by the treatment of anti-IL-6 antibody. As in the case of broad inhibition of IL-6 activity, concomitant transfer of IL-6–/– MDSC and sIL-6R also failed to attenuate the production of Th1 cells (Fig. 7B). Proportions of donor OT-II cells were comparable under all conditions tested (Fig. 7C and Supplementary Fig. S4F), suggesting that MDSC-derived IL-6 is necessary for polarizing CD4+ T cells away from Th1 responses.

Given that CD8+ T cells are essential for CD4+ T cell–mediated tumor regression, we analyzed the ability of MDSC-sensitized effector CD4+ T cells to promote tumor antigen-specific CD8+ T-cell response when vaccinated mice were challenged with tumor cells (Fig. 7A). The transferred MDSC transiently affected the generation of CD4+ effector T cells, but not CD8+ T cells, in the dendritic cell–vaccinated
tumor-free mice; the dendritic cell used in this vaccination protocol were pulsed only with an OT-II TCR-specific peptide, and the transferred MDSC had disappeared by the time of challenge with MCA205-OVA (data not shown). Six days after tumor challenge, mice that were infused with CD4\(^+\) T cells primed with peptide-pulsed dendritic cells exhibited more drastic induction of OVA-specific CD8\(^+\) T cells than did mice infused with naive OT-II cells alone (Fig. 7D). Notably, much less OVA-specific CD8\(^+\) T cells were induced in mice infused with MDSC-sensitized effector T cells. In contrast, helper activity for OVA-specific CD8\(^+\) T cells was not impaired in IL-6-deficient MDSC-transferred mice.

We finally addressed whether antitumor activity of effector CD4\(^+\) T cells was repressed by MDSC-derived IL-6 in vivo. Consistent with successful Th1 differentiation and their helper activity for CD8\(^+\) T cells, recipient mice vaccinated with OT-II TCR-specific peptide-pulsed dendritic cell led to dramatic eradication of challenged tumors (Fig. 7E). This was also case for the mice transferred with IL-6\(^-/-\) MDSC, whereas no effect on tumor progression was observed in the mice transferred with IL-6\(^-/-\) MDSC. A similar trend was observed when mice were challenged with the aggressive MO5 melanoma cells in a pulmonary metastatic model (Fig. 7F) or in a subcutaneous transplant model (Supplementary Fig. S4G). Collectively, these data suggest that the IL-6 produced by MDSC does not affect the primary activation of tumor-specific CD4\(^+\) T cells, but rather it inhibits their differentiation into functional effector CD4\(^+\) T cells, and the subsequent induction of tumor-specific CD8\(^+\) T cells and antitumor immunity.

**Discussion**

It has been suggested that immune tolerance against tumor cells in patients with cancer accounts for the inadequate clinical responses observed with cancer treatments, despite a measurable frequency of tumor-reactive T cells (7, 16). This phenomenon is thought to be caused, in part, by the suppressive effects of MDSC on primary T-cell activation (18–20). Here, we propose a novel mode of immune suppression by tumor-associated MDSC on secondary CD4\(^+\) T-cell activation that initiates antitumor immunity. The major findings of our present study are as follows (Supplementary Fig. S5): (i) systemic increases of IL-6 and sIL-6R in tumor-bearing mice serve as a causative mechanism underlying the defective Th1 differentiation observed in tumor-bearing animals. (ii) MDSCs accumulated in tumor-bearing mice produce IL-6. (iii) IL-6 enables MDSC attenuation of Th1 development but not suppression of primary T-cell activation, rather than an immunosuppressive molecule on primary T-cell activation. (iv) Effector CD4\(^+\) T cells sensitized by MDSC-derived IL-6 are defective in eliminating tumors because of their decreased ability to produce IFN-\(\gamma\) and their dampened helper activity for cognate tumor-specific CD8\(^+\) T cells.

Considering a mechanistic rationale for specific targeting of IL-6 signaling and for subsequent improvement of CD4\(^+\) T cell–mediated anticancer immune therapy, it is intriguing to identify the source of IL-6 and sIL-6R in tumor-bearing animal. The systemic increase of IL-6 levels in the plasma of patients with cancer (28, 29) markedly correlated with proportional accumulation of MDSC (30, 31). These reports support our findings and previous reports (39, 40) that MDSC produce IL-6 in tumor-bearing mice. Tumor cells also produce IL-6, which promotes their own survival by an autocrine mechanism (36). It is worth noting that IL-6 production by MDSC is largely responsible for a systemic increase of IL-6 concentration in addition to the local production by tumor cells in our model. However, it remains possible that other cell populations may also contribute to the systemic increase of IL-6 in tumor-bearing mice because detectable levels of IL-6 were produced by them ex vivo.

![Figure 6. Gr-1\(^+\) MDSC suppress Th1 differentiation in vivo. Naive OT-II cells were primed in vivo as in Fig. 1. Mice were treated with control or anti-Gr-1 antibody (Ab) 4 days before immunization. Seven days after immunization, donor OT-II cells were restimulated with PMA/ionomycin. Representative plots (left) and the frequencies of IFN-\(\gamma\)\(^+\) cells in donor OT-II cells (right) are shown. *\(P < 0.05\); **\(P < 0.01\). DC, dendritic cell.](image-url)
The importance of sIL-6R in immune suppression was also underscored by the fact that the MDSC-induced decrease in the development of IFN-γ-producing effector CD4⁺ T cells was drastically exacerbated by simultaneous infusion of sIL-6R even in tumor-free mice. Various studies have shown that sIL-6R was also produced by the tumor itself (29, 38). In addition to tumor cells, we found that host-derived cells in tumor-bearing mice also produced sIL-6R (H. Tsukamoto; unpublished data). These data suggest that in tumor-bearing mice, sIL-6R-producing tumor cells and host-derived cells could synergize to impair Th1 differentiation through increasing spatial/temporal availability of MDSC-derived IL-6 in vivo.

Although IL-6 has been reported to facilitate the development or accumulation of MDSC (35, 40, 41), we showed that IL-6 deficiency and blockade of IL-6 activity in vivo had no detectable effect on MDSC in tumor-bearing mice. Indicating that tumor-derived IL-6 was not essential for maintenance of
MDSC. Our interpretation is supported by a previous study showing that host-derived factor(s) induced by IL-1R-mediated signal is considered to function as a substitute for tumor-derived IL-6. MDSC accumulation in tumor-bearing mice failed to provide help for endogenous tumor-reactive CD8 T cells (10, 12, 14). We show here that MDSC-sensitized effector CD4 T cells exhibited intact responses (normal expansion and IL-2 production) except for the IFN-γ production when secondary stimulation was given. However, MDSC-sensitized CD4 T cells failed to provide help for endogenous tumor-reactive CD8 T cells that were required for the tumor elimination. On the basis of this result, impaired IFN-γ production in MDSC-sensitized effector CD4 T cells was the most likely cause for their inadequate helper activity for cognate CD8 T-cell response and the subsequent antitumor activity. This is consistent with the finding by Nakanishi and colleagues that IFN-γ produced by CD4 T cells contributes to recruitment of CD8 T cells into draining lymph nodes (42), which may be beneficial for anti-tumor immune responses.

Several mechanisms underlying the detrimental consequences of MDSC-derived IL-6 on TH1 differentiation of tumor-specific CD4 T cells could be reasonably speculated. First, it is possible that IL-6 could abolish the function of APCs through downregulation of their IL-12 production, and subsequently, the Th1 immune response (43). However, this is unlikely because MDSC-mediated inhibition of Th1 differentiation was still observed when exogenous IL-6 did not downregulate T-bet expression in effector CD4 T cells (H. Tsukamoto; unpublished data). The detailed molecular mechanism(s) by which MDSC-derived IL-6 constrains the Th1 response is now under investigation.

Viewed from the perspective of the antitumor immunity, the levels of MDSC-derived IL-6 and sIL-6R in patients with cancer could be useful markers to determine the therapeutic efficacy of tumor-specific CD4 T cells. Moreover, recent clinical trials using a murine adenocarcinoma model (46), further supporting a link between IFN-γ signaling and MDSC-derived IL-6. The IFN-γ–STAT1 signaling regulates its downstream transcriptional factor, T-bet, which is a critical step in Th1 differentiation (47). T-bet expression in effector T cells was significantly repressed when cocultured with MDSC (data not shown). However, contrary to this scenario, neutralization of IL-6 produced by MDSC did not rescue T-bet expression, and stimulation with exogenous IL-6 did not downregulate T-bet expression in effector CD4 T cells (H. Tsukamoto; unpublished data). The detailed molecular mechanism(s) by which MDSC-derived IL-6 constrains the Th1 response is now under investigation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: H. Tsukamoto, Y. Nishimura
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Tsukamoto
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Myeloid-Derived Suppressor Cells Attenuate $T_H1$ Development through IL-6 Production to Promote Tumor Progression

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