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\(^+\) \( T \) cells from tumor-free mice eradicated established tumors. CD8\(^+\) \( T \) cells, IFN-\( \gamma \), 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.
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, 19, 20). 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-\(\gamma\) 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 of Medicine, Tokyo, Japan). C57BL/6 mice were purchased from Balewa (BioBred Laboratories, Holliston, MA). Cell culture media and reagents were purchased from Millipore (Billerica, MA). Unless noted otherwise, all antibodies were purchased from BD Biosciences (San Diego, CA). The reagents: Alexa Fluor 488, 546, and 647, Streptavidin Alexa Fluor 555, and PE-Cy7 were purchased from Invitrogen (Carlsbad, CA). Immunologic reagents were purchased from Sanquin Diagnostics (Amsterdam, The Netherlands). OVA and OT-1 cells were purchased from Becton Dickinson (San Jose, CA). The in vivo luciferase substrate D-luciferin was purchased from PerkinElmer (Boston, MA). afternoon withdrawal of 8/10 were purchased from rabbit anti-IFN-\(\gamma\) (R&D Systems), nitric oxide synthase (NOS) inhibitor (L-NMMA; Sigma), Cox-2 inhibitor (NS-398; Sigma), anti-IL-2 antibody (eBioscience), anti-IL-6 antibody (BioXCell), anti-Ly6G (clone: IA8; eBioscience), anti-CD8 or anti-CD4 antibody injection was injected intravenously together with dendritic cell.

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

Naïve CD4+ T cells were isolated from OT-II TCR-transgenic mice using a naïve 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 \(\times\) 10^5) and MDSC (3–6 \(\times\) 10^5) were injected intravenously into naïve mice or mice bearing 5 mm \(\times\) 5 mm tumors. Six hours after T-cell and MDSC transfer, mice were immunized by intravenous transfer of 3 \(\times\) 10^6 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-V\(\beta\)5 or anti-Ly6G (1A8), anti-CD11b (Caltag) or anti-IL-6, anti-CD4 or PerCP–anti-IL-10 antibody (eBioscience; ref. 34).

The H-2Kb/SIINFEKL-OVA tetramer-phycoerythrin (PE) was purchased from MBL. For intracellular staining, following isolation of effector CD4+ T cells from the in vivo cultures or from mice using anti-CD34 beads, CD4+ T cells were stimulated with cognate peptide-pulsed dendritic cell or phorbol 12-myristate 13-acetate (PMA)/ionomycin in vitro and were stained with Alexa Flour 488–anti-IFN-\(\gamma\), 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’ instruction. Immunofluorescence images were analyzed using a FACScaliber (Becton Dickinson). Data were analyzed using FlowJo software (Treestar).

**OT-II T cells and MDSC coculture experiments**

Naïve OT-II cells (1.0 \(\times\) 10^6/well) were stimulated with dendritic cell (5.0 \(\times\) 10^5/well) and 5 \(\mu\)m/L OVA peptide. MDSC (3 \(\times\) 10^5/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-CD8 antibody (BioXCell), anti-TGF-\(\beta\) antibody (R&D Systems), nitric oxide synthase (NOS) inhibitor (L-NMMA; Sigma), Cox-2 inhibitor (NS-398; Sigma), anti-IL-10 antibody (eBioscience), anti-IL-6 antibody (BioXCell), control rat Ig (Pierce), recombinant IL-2...
(15 ng/mL), 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 × 10^5/well) were stimulated with OVA peptide-pulsed and mitomycin C–treated dendritic cell (5 × 10^5) 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 an RNeasy kit (Qiagen), digested with DNase I and reverse-transcribed into cDNA using a TaqMan probes for NOS2 (Mm01257348), indoleamine 2,3-dioxygenase (IDO; Mm00494449), Arginase 1 (Mm00475988), IL-10 (Mm00483241), 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 (C_T) values for each reaction were determined and averaged using TaqMan SDS analysis software. Changes in gene expression were calculated by the comparative C_T method (fold changes = 2^{ΔΔC_T}).

**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. T11 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 T11 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 T11 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 T11 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^+ T11 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...
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 M04 melanoma-implanted mice (Supplementary Fig. S2A).

In the MCA205-OVA model, MDSC exhibited the classical phenotype, CD11b+Gr-1+IL-4Ra−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, naïve 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 Tfl development was not restored by the addition of IL-12 (Fig. 3B).

Figure 1. Temporal IL-6 blockade enhances tumor-specific effector CD4+ T cell-mediated protective antitumor immunity. A, CD45.1+ naïve OT-II T cells were transferred into MCA205-OVA−bearing CD45.2+ mice, which were then immunized by the transfer of dendritic cell (DC) pulsed with OVA peptide specific for OT-II TCR. Tumor-bearing mice were treated with control antibody (Ab) or anti-IL-6 antibody twice (at day −1 and 1) during T-cell priming. Data for tumor growth shown here represent mean ± SEM with n = 5 to 7 mice per group. B−D, 1 week after immunization as described in A, donor OT-II cells were harvested from host spleens and lymph nodes. The number of donor OT-II cells (B) and CD4+ Foxp3+ Treg cells (C), and the frequencies of IFN-γ− (D) and IL-2+ cells (E) cells in donor OT-II cells restimulated with OVA-peptide pulsed dendritic cell in vitro, were determined. The values represent mean ± SEM with n = 5 per group; *, P < 0.05; **, P < 0.01. The data shown are representative of 3 or more independent experiments with similar results.
We next evaluated the effect of IL-6 on the ability of MDSC to suppress T<sub>H1</sub> differentiation. Addition of anti-IL-6 antibody ameliorated the MDSC-mediated suppression of T<sub>H1</sub> differentiation. In contrast, exogenous IL-6 drastically inhibited the development of IFN-γ-producing effector CD<sub>4</sub><sup>+</sup> 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<sup>+</sup> cells- and MDSC-sensitized effector CD<sub>4</sub><sup>+</sup> T cells (data not shown).

Similar phenomena were observed when human polyclonal CD<sub>4</sub><sup>+</sup> T cells were stimulated with anti-CD3/CD28 antibodies in coculture with tumor-associated CD33<sup>+</sup> MDSC (Fig. 3C).

This result excluded the possibility that MDSC-mediated attenuation of T<sub>H1</sub> 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 CD<sub>4</sub><sup>+</sup> T cells toward T<sub>H1</sub> effectors through IL-6 production in both mice and humans.

**MDSC-sensitized, tumor-specific effector CD<sub>4</sub><sup>+</sup> T cells are ineffective for tumor regression**

To determine the antitumor effect of MDSC-sensitized effector CD<sub>4</sub><sup>+</sup> T cell, we transferred into tumor-bearing mice OT-II effector T cells generated with control Gr-1<sup>+</sup> 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 CD<sub>4</sub><sup>+</sup> 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 CD<sub>4</sub><sup>+</sup> T cells into MHC-II<sup>-</sup> 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 CD<sub>4</sub><sup>+</sup> 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/ionomycin 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/ionomycin. Representative data of 3 independent experiments with similar results are shown (mean ± SEM).

**Figure 4.** CD4⁺ T-cell–mediated antitumor activity is increased by MDSC depletion. 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/ionomycin 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/ionomycin. 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 Th1 differentiation of CD4\(^+\) T cells.

**Antitumor effect of Th1 effector CD4\(^+\) T cells is attenuated by MDSC-derived IL-6 in vivo**

Defective Th1 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 Th1 development (Fig. 6). To determine whether the defect in Th1 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 Th1 cells. Transfer of 6 \(\times\) 10\(^6\) MDSC, inhibited the primary expansion of naive OT-II cells in response to vaccination with dendritic cell (Supplementary Fig. S4A). In

![Figure 4](https://example.com/figure4.png)

Figure 4. MDSC-sensitized effector T cells fail to provoke antitumor immunity that depends on CD8\(^+\) T cells, IFN-\(\gamma\), and MHC-II expression. A and B, effector OT-II cells were generated *in vitro* by coculture of control Gr-1\(^{+}\) cells or MDSC with the indicated inhibitors or blocking antibody (Ab). Effector OT-II cells (1 \(\times\) 10\(^5\)) were transferred into MCA205-OVA–bearing mice. Subsequent tumor growth was monitored. C–E, generation and transfer of effector OT-II cells were conducted as in A, and then tumor growth was monitored. MHC-II–sufficient or–deficient mice were used as recipients for T-cell transfer (C). Anti-IFN-\(\gamma\) antibody was injected on days 0, 1, 3, and 5 after T-cell transfer (D). In vivo anti-CD8 antibody treatment was carried out 4 days before T-cell transfer (E). One representative of 2 or 3 experiments with similar results is presented. Error bars represent SEM with 5 to 9 mice per group. *, \(P < 0.05\); **, \(P < 0.01\); KO, knockout.
contrast, transfer of 6 × 10^6 Gr-1 cells from tumor-free mice had no effect on primary T-cell activation. When 3 × 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.

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 MICA205-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 M04 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-γ 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.
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-1β-mediated signal is considered to function as a substitute for tumor-derived IL-6 in MDSC accumulation in tumor-bearing mice (40). To make a definitive conclusion, further investigation asking whether IL-6-deficient or -knocked down tumor cells drive the accumulation of MDSC even in IL-6-deficient mice will be needed.

There is mounting evidence that tumor-specific T<sub>H</sub>1 CD4<sup>+</sup> T cells enhance antitumor activity mediated by CD8<sup>+</sup> T cells (10, 12, 14). We show here that MDSC-sensitized effector CD4<sup>+</sup> 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<sup>+</sup> T cells failed to provide help for endogenous tumor-reactive CD8<sup>+</sup> T cells that were required for the tumor elimination. On the basis of this result, impaired IFN-γ production in MDSC-sensitized effector CD4<sup>+</sup> T cells was the most likely cause for their inadequate helper activity for cognate CD8<sup>+</sup> T-cell response and the subsequent antitumor activity. This is consistent with the finding by Nakanishi and colleagues that IFN-γ produced by CD4<sup>+</sup> T cells contributes to recruitment of CD8<sup>+</sup> 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 T<sub>H</sub>1 differentiation of tumor-specific CD4<sup>+</sup> 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 T<sub>H</sub>1 immune response (43). However, this is unlikely because MDSC-mediated inhibition of T<sub>H</sub>1 differentiation was still observed when exogenous IL-12 was added, or even under APC-free stimulatory conditions. The second possibility is that attenuated T<sub>H</sub>1 differentiation result from other lineages. It is also well known that IL-6 together with TGF-β converts Treg into IL-17-producing CD4<sup>+</sup> T cells, namely T<sub>H</sub>17 (44). However, in our system, the development of T<sub>H</sub>17 or Treg was not altered by the presence of MDSC in vivo and in vitro. Other cytokines, such as IL-4, IL-21, and lineage-specific transcriptional factor, Bel-6, ROR-γ, or GATA3 expression in effector CD4<sup>+</sup> T cells were not drastically changed in the presence or absence of MDSC (data not shown). The third possibility is that MDSC-derived IL-6 has inherent inhibitory effect on T<sub>H</sub>1 development or IFN-γ production, rather than its secondary effect due to redirected differentiation into other lineages.

In this regard, IL-6 was found to inhibit IFN-γ receptor-STAT1 signaling-dependent T<sub>H</sub>1 differentiation directly through the upregulation of suppressor of cytokine signaling 1 (45). MDSC-induced loss of STAT1 activation in response to IFN-γ or IFN-γ has been reported in 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 T<sub>H</sub>1 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<sup>+</sup> T cells (H. Tsukamoto; unpublished data). The detailed molecular mechanism(s) by which MDSC-derived IL-6 constrains the T<sub>H</sub>1 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<sup>+</sup> T cells. Moreover, recent clinical trial using a humanized anti-IL-6 antibody, CNTO 328 provided promising effects in some patients with cancer with high levels of IL-6 (28, 48). Therefore, combined application of temporal IL-6 blockade by this antibody or transient chemotherapies with gemcitabine or 5-fluorouracil to deplete MDSC (49) may significantly enhance T cell–based cancer immunotherapy.

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

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Development of methodology: H. Tsukamoto, R. Nishikata

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Tsukamoto

Analysis and interpretation of data (e.g., statistical analysis, biosstatistics, computational analysis): H. Tsukamoto, S. Senju

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Tsukamoto, R. Nishikata

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References


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