Mouse CD11b^+Gr-1^+ Myeloid Cells Can Promote Th17 Cell Differentiation and Experimental Autoimmune Encephalomyelitis

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Myeloid-derived suppressor cells (MDSCs) have become the focus of intense study for the past few years in the context of cancer (11, 12). MDSCs were originally described as a heterogeneous population of immature cells derived from myeloid progenitors with immune-suppressive functions in tumor-bearing hosts. The immune-suppressive activity of MDSCs is highly pleiotropic and has been shown to involve a variety of mechanisms (13). In mice, MDSCs are broadly characterized as CD11b+Gr-1+ cells, although there are functionally distinct subsets within this population (14, 15). Human MDSCs have been identified as Lin−HLA-DRlow−/−CD33+ (16) or CD11b+CD14+CD33+ (17). It has been reported that MDSCs can convert naive CD4+ T cells ex vivo into Foxp3−expressing regulatory T cells (Tregs) (18, 19). The functional importance of MDSCs in the attenuation of immune responses during cancer progression has been documented (13, 20). In addition to cancer, expansion of MDSCs occurs during mycobacteria-induced infection (21) and in a mouse model of MS (22), implicating their potential regulatory role under inflammatory conditions. However, their specific contribution to the pathological processes associated with inflammatory autoimmune abnormalities remains to be elucidated.

Although MDSCs and Th17 cells represent two major inflammatory cells often seen under conditions associated with inflam-
mation, functional connection between these two cell populations has not been examined. Experimental autoimmune encephalomyelitis (EAE) represents a well-characterized mouse model of human MS, which is induced by immunization of mice with encephalitogenic myelin Ags; that is, myelin oligodendrocyte glycoprotein (MOG) in the presence of adjuvants (23). The critical roles of Th17 cells and IL-17 in the pathogenesis of this chronic inflammatory disease of the CNS have been well documented (24–26). In this report, we show that progression of EAE in mice correlates with concomitant expansion of Th17 cells and MDSCs. We discover that MDSCs can promote Th17 differentiation and IL-17A production highly efficiently under Th17-polarizing conditions in an IL-1β-dependent fashion. We provide the first evidence, to our knowledge, that depletion of MDSCs with gemcitabine markedly reduces the levels of Th17 cell population in vivo and ameliorates the severity of EAE. Furthermore, adoptive transfer of MDSCs to the gemcitabine-treated mice facilitates EAE disease progression, which requires MDSC-derived IL-1β production. Our data establish an important role of MDSCs in modulating the development of a Th17 response in mice during chronic inflammation and suggest that this cell population may serve as a unique target for Th17 cell/IL-17–mediated immunopathology.

Materials and Methods

Mice

C57BL/6 mice were obtained from the National Cancer Institute (Bethesda, MD). IL-1R1 knockout mice (il1r1−/−) and B6129F2 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All experiments and procedures involving mice were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Abs and reagents

Fluorochrome-conjugated mAbs, including FITC–CD4 (GK1.5), PE–CD11b (M1/70), PE-Cy5–Gr-1 (RB6-8C5), PE–Foxp3 (MF-14), PE–IFN-γ (XMG1.2), and PerCP/Cy5.5–IL-17A (TC11–18H10.1), as well as CD16/CD32 (2.4G2), isotype control rat IgG2b (RTK4530), and IgG1 (RTK2071) were purchased from BioLegend (San Diego, CA). Mouse IL-17A and IL-2 ELISA kits were purchased from BioLegend. PE-conjugated anti–ROTYR (AFKJS-9) Abs and mouse IFN-γ and IL-1β ELISA kits were from eBioScience (San Diego, CA). Alexo 594-labeled donkey anti-rat secondary IgG was purchased from Invitrogen (Carlsbad, CA). Rat anti-mouse purified anti–CD11b and biotin-conjugated anti–Gr-1 mAbs for immunofluorescence staining were purchased from BD Biosciences (San Diego, CA).

EAE induction and assessment

Mice were immunized s.c. at the dorsal flanks with 200 μg MOG35–55 peptide (ProSpec, East Brunswick, NJ) emulsified 1:1 in CFA supplemented with 4 mg/ml heat-killed Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI) on day 0. The mice received 200 ng pertussis toxin (Sigma-Aldrich, St. Louis, MO) i.p. on days 0 and 2. After the first immunization, the severity of EAE was monitored and graded in a blinded fashion on a scale of 0–5: 0, no disease; 0.5, decreased tail tone; 1, complete limp tail paralysis (flaccid tail); 2, limp tail and paralysis of one hind limb; 3, paralysis of both hind limbs; 4, complete hind-limb paralysis; 5, morbidity state. Disease incidence was determined by ELISA according to the manufacturer’s instructions. For intracellular IFN-γ staining, splenocytes were cultured at 37°C, 5% CO2 in the presence of PMA (10 nM; Sigma-Aldrich) plus ionomycin (1 μM; EMD Biosciences) for 5 h. Brefeldin A (BioLegend) was added for the last 3 h of culture. After surface staining with FITC-conjugated anti-CD4 mAbs for 30 min at 4°C, cells were fixed, permeabilized, and stained with PerCP/Cy5.5-conjugated anti–IL-17A or PE-conjugated anti–IFN-γ or PE-conjugated anti–ROTYR mAbs for 30 min at 4°C. Foxp3 staining was performed according to the manufacturer’s instructions. For intracellular IL-1β staining, splenocytes were cultured at 37°C, 5% CO2 in the presence of brefeldin A for 4 h. After surface staining with FITC-conjugated CD11b and PE/Cy5-conjugated Gr-1 mAbs for 30 min at 4°C, cells were fixed, permeabilized, and stained with allopochycyanin-conjugated IL-1β mAbs for 30 min at 4°C. Data were then acquired using BD FACS caliber and analyzed using FlowJo software (Tree Star, Ashland, OR).

Isolation of mononuclear cells from spinal cords

Mice were perfused with 25 ml ice-cold PBS containing 2 mM EDTA, and two intact spinal cords per group were pooled. The spinal cords were cut into several small pieces and homogenized with a Dounce glass homogenizer in 3 ml 1× HBSS. Cell suspensions were collected after filtration through 80-μm mesh and subjected to Percoll gradient (70/30%) centrifugation (500 g) at 18°C for 30 min. Mononuclear cells were collected from the 30/70% interface and washed, resuspended in FACS buffer or T cell medium, and used for subsequent experiments.

Isolation of MDSCs and subsets

Spleen-derived MDSCs were purified from EAE mice using CD11b magnetic beads (Miltenyi Biotech, Auburn, CA). Approximately 98% of CD11b+ cells are Gr-1+, and the purity of CD11b+ Gr-1+ cell fractions was typically greater than 95%. In some experiments, CD11b+Gr-1− cells were sorted from splenocytes, and CD11b+Gr-1− and CD11b−Gr-1− cells sorted were from bone marrow using the BD Biosciences FACSaria II cell sorter.

Immunosuppression assays by MDSCs and cytokine detection

Control splenocytes (2 × 106 cells/well) were cocultured with purified MDSCs at different ratios (as indicated in the figures) in the presence of 1 μg/ml of plate-bound anti-CD3 mAb (145-2C11; BioXcell, West Lebanon, NH) and 0.5 μg/ml soluble anti-CD28 mAb (37.51; BioLegend) in a 96-well flat-bottom plate for 72 h. Cells were pulsed with 0.5 μCi/well [3H]thymidine for the last 16 h of incubation. Proliferation was measured by [3H]thymidine incorporation in triplicate wells using an LS 6500 multiscatter scintillation counter (Beckman Coulter). Supernatant was collected 48 h after culture, and levels of cytokines IL-2 and IFN-γ were determined by ELISA according to the manufacturer’s instructions.

In vitro Th17 cell differentiation

CD4+CD25−CD62Lhi naive T cells were FACS sorted (>98% pure), and 5 × 105 cells/well were activated with plate-bound 5 μg/ml anti-CD3 plus 1 μg/ml soluble anti-CD28 Abs in the presence of 2.5 ng/ml hTGF-β (Peprotech, Rocky Hill, NJ), 20 ng/ml IL-6 (Peprotech), 5 μg/ml anti–IFN-γ (XMG1.2, BioXcell), and anti–IL-4 (11B11; BioLegend) mAbs in 24-well plates. MDSCs were added to the culture on day 0 at a ratio of 1:1 (MDSC/T cell). Cells were cultured in triplicate in RPMI 1640 media supplemented with 10% FCS, 50 μM 2-mercaptoethanol, and 2 mM L-glutamine/1% penicillin/streptomycin. After three days of activation, supernatants were collected for IL-17A or IL-1β cytokine assays by ELISA according to the manufacturer’s instructions. Cells were washed and
MDSCs profoundly expand during the progression of EAE and display T cell suppressive activity. (A) EAE was induced in C57BL/6 mice (n = 5) by immunization with MOG35-55/CFA followed by pertussis toxin injection. The presence of IL-17A–producing CD4+ T cells was examined by flow cytometry 23 d after EAE induction. Percentage of CD11b+Gr-1+ MDSCs in the splenocyte (SP) and peripheral blood (PB) was also analyzed. Representative histograms from at least three independent experiments with similar results are shown. (B) Kinetic changes of CD11b+Gr-1+ MDSCs during EAE. (C) MDSCs exhibit immunosuppressive activity against T cell proliferation. MDSCs were isolated from EAE mice using magnetic beads and cocultured with splenocytes stimulated with anti-CD3/CD28 mAbs at indicated ratios for 72 h. Cell proliferation was assessed using [3H]thymidine (TdR) incorporation assays. **p < 0.005 (MDSCs versus no MDSCs). (D) and (E) Inhibition of IFN-γ and IL-2 production by MDSCs. After MDSCs coculture with splenocytes stimulated with anti-CD3/CD28 mAbs, IFN-γ (D) and IL-2 (E) levels in the culture supernatant were examined using ELISA. The results represent three independent experiments. **p < 0.005 (MDSCs versus no MDSCs).

**Results**

Expansion of Th17 cells and MDSCs during EAE progression

Th17 cells play a prominent role in the pathogenesis of autoimmune diseases (e.g., EAE). As expected, a substantial increase of the percentage of IL-17A–producing CD4+ T cells was detected after EAE induction, as indicated by intracellular cytokine staining and flow cytometry analysis of splenocytes in EAE mice (Fig. 1A, top). Notably, the increase in Th17 cells was concomitant with marked expansion of CD11b+Gr-1+ CD4+Th17 cells in the spleen (Fig. 1A, middle) and peripheral blood (Fig. 1A, bottom). The increase of the CD11b+Gr-1+ cell population in peripheral blood during the disease progression in EAE mice was time-dependent (Fig. 1B).

The phenotype of CD11b+Gr-1+ cells expanded during the EAE disease progression is highly similar to previously defined tumor-expanded MDSCs. These cells can also be subdivided into two subsets, that is, CD11b+Ly6ChighLy6G− and CD11b+Ly6ChighLy6G+. These two myeloid-derived suppressor cells (MDSCs) and granulocytic myeloid-derived suppressor cells (G-MDSCs), respectively, and both of which expanded during the development of EAE (Supplemental Fig. 1A, 1B). Phenotypic similarity is also supported by analyses of light scatter characteristics and morphology. In contrast to inflammatory monocytes that often express F4/80 and CD80 (27), these myeloid cells do not express these two markers, and also do not express CD11c and MHC class II, which resembles the immature or undifferentiated myeloid cells (Supplemental Fig. 1C). However, they share some phenotypic characteristics with inflammatory monocytes; for example, expression of CD11b, CCR2, and CD62L (Supplemental Fig. 1D).

This CD11b+Gr-1+ cell population is also functionally similar to CD11b+Gr-1+ MDSCs often seen in cancer-bearing hosts, as evidenced by their highly efficient activity in suppressing the
proliferation and cytokine (IFN-γ and IL-2) production of T cells after TCR engagement or Ag (MOG35-55)-specific stimulation (Fig. 1C–E, Supplemental Fig. 1E–G). These results are also consistent with a recent report showing that CD11b+ Ly6C+ cells increased during the inflammatory phase of EAE and induced potent inhibition of T cell activation (22). Therefore, CD11b+ Gr-1+ cells (henceforth called MDSCs) that are enriched and accumulated in peripheral lymphoid tissues of the EAE mice may play a regulatory role in development of a Th17 response.

MDSCs promote Th17 cell differentiation under Th17-polarizing conditions

To determine the potential effect of MDSCs on Th17 cell differentiation, we cultured MACS bead-isolated MDSCs with sorted naive CD4+CD25−CD62L+ T cells ex vivo for 3 d in Th17-polarizing media, which contained IL-6, TGF-β, anti–IFN-γ, and IL-4 mAbs in the presence of plate-bound anti-CD3 mAbs and soluble anti-CD28 mAbs. Strikingly, the presence of MDSCs profoundly increased the percentage of CD4+IL-17A+ Th17 cells but not CD4+IFN-γ+ Th1 cells or CD4+Foxp3+ Tregs (Fig. 2A). The increased percentage of Th17 cells correlated with a significant increase in the expansion of Th17 cells, indicated by CFSE dilution assays and changes of the absolute number of Th17 cells in the culture (Fig. 2B). MDSC-enhanced Th17 polarization was also demonstrated by the elevated protein levels of cytokine IL-17A in the supernatant (Fig. 2C), which correlated with increased gene transcription of il17a and rora during Th17 differentiation, indicated by real-time PCR analysis (Fig. 2D). Flow cytometry analysis revealed that the presence of MDSCs also increased the protein levels of RORγt, a key transcription factor that determines the Th17 differentiation (28) (Fig. 2E).

We next assessed the efficiency of MDSCs to promote Th17 polarization by culturing naive CD4+ T cells with MDSCs at different ratios. MDSCs efficiently enhanced the differentiation of Th17 cells even at a ratio of 1:4 (Fig. 2F). It was found that MDSCs displayed reduced effectiveness in promoting Th17 cell differentiation when added at the later time points (data not shown), suggesting that the effect of MDSCs mainly occurs in the
early phase of Th17 cell differentiation. We showed that MDSCs isolated using MACS beads or by cell sorting showed similar activity in driving Th17 cell polarization (Supplemental Fig. 2A). Notably, tumor-expanded MDSCs also promoted Th17 differentiation in vitro efficiently (Supplemental Fig. 2B). In addition to facilitating Th17 polarization, MDSCs could also enhance the proliferation and IL-17A production of the differentiated Th17 cells (Supplemental Fig. 2C–E).

Considering that MDSCs in mice are broadly grouped into two subpopulations, CD11b+Gr-1low and CD11b+Gr-1high, which resemble monocytic MDSCs and granulocytic MDSCs, respectively (29, 30), we compared the capacity of these two cell subsets in promoting Th17 cell differentiation. CD11b+Gr-1low cells appeared to be much more effective than CD11b+Gr-1high cells in enhancing Th17 polarization (Fig. 2G).

**MDSC-enhanced Th17 differentiation is mediated by IL-1β and requires IL-1 receptor on T cells**

We next investigated the potential factors responsible for MDSC-enhanced Th17 differentiation. IL-6 and TGF-β have been re-
ported as the minimal requirements for murine Th17 cell polarization (31, 32). We initially cocultured naive CD4+ T cells with MDSCs in the presence of TGF-β plus IL-6, IL-6 alone, or TGF-β alone. Lack of exogenous TGF-β disrupted the MDSC-facilitated Th17 differentiation, as indicated by markedly decreased percentage of Th17 cells and IL-17A production (Fig. 3A, 3B, left). However, a very low level of Th17 cells and IL-17A could still be detected in the MDSC–T cell culture in the presence of MDSCs (Fig. 3A, 3B, left). Use of TGF-β blocking Abs interfered with the effect of MDSCs when only IL-6 was present in the culture (Fig. 3B, right). Given the previously documented TGF-β expression by MDSCs (33, 34), MDSCs may be able to compensate partially for the absence of exogenous TGF-β. In sharp contrast, absence of exogenous IL-6 completely abolished Th17 polarization in the presence of MDSCs (Fig. 3A, 3B, left), indicating an indispensable role of this cytokine in the Th17 cell lineage commitment.

In light of recent reports of the importance of IL-1 signaling in the early Th17 differentiation (9, 35, 36), we examined the role of IL-1β in the MDSC-enhanced Th17 differentiation. Blockade of IL-1β using neutralizing mAbs markedly reduced the percentage of Th17 cells (Fig. 3C), which was mirrored by a significant decrease in the IL-17A levels in the supernatants (Fig. 3D). The presence of IL-1R antagonist, the naturally occurring inhibitor of IL-1β, also decreased MDSC-induced IL-17A production (Fig. 3E). Indeed, IL-1β levels were significantly higher in the Th17-polarizing culture system containing MDSCs than in the culture without MDSCs (Fig. 3F), indicating that MDSC represents the major source of IL-1β. The expression of IL-1β in MDSCs was also confirmed by intracellular staining assays (Fig. 3F, inset).

We next asked whether IL-1 signaling in T cells or MDSCs was required for the enhanced Th17 differentiation. MDSCs were sorted from wild-type (WT) or il1r1−/− mice and cocultured with naive T cells derived from WT or il1r1−/− mice, respectively. Il1r1 deficiency in MDSCs had little effect on Th17 polarization. However, lack of il1r1 in T cells abolished the stimulating effect of MDSCs on Th17 cell differentiation, indicated by the reduced percentage of Th17 cells and production of IL-17A (Fig. 3G, left and right).

It was recently reported that CD11b+ myeloid cells derived from normal mice and EAE mice exhibited suppressive activity in vitro (37). We therefore compared the efficiency of these CD11b+Gr-1+ cells to influence Th17 polarization. MDSCs from EAE mice appeared to be more effective than those from normal mice in stimulating Th17 cell differentiation (Fig. 3H, left). Indeed, EAE mice-derived MDSCs expressed higher levels of IL-1β compared with those from normal mice (Fig. 3H, right), suggesting that MDSCs may be stimulated by inflammatory signals in EAE mice. Although inducible NO synthase 2 and arginase 1 are known to mediate the suppressive activity of MDSCs, we showed that inhibition of arginase 1, not NO synthase 2, diminished the Th17-polarizing activity of MDSCs (Supplemental Fig. 3). Notably, blocking of arginase 1 activity did not appear to alter the expression of IL-1β expression in MDSCs (Supplemental Fig. 3).

Selective depletion of MDSCs reduced Th17 cells in vivo and EAE severity

It has been reported that gemcitabine can specifically reduce the number of MDSCs in tumor-bearing mice with no significant

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Depletion of MDSCs by gemcitabine (GEM) reduces the frequency of Th17 cells in vivo and ameliorates EAE. (A) Efficient depletion of MDSCs by gemcitabine. C57BL/6 mice (n = 5) were immunized with MOG35–55/CFA on day 0. Gemcitabine (100 mg/kg) was administered i.p. once on day 11. The percentages of CD11b+Gr-1+ cells in peripheral blood (PB) and spleen (SP) on day 13 (i.e., 2 d after gemcitabine treatment) were examined by flow cytometry. (B) Blocking of EAE progression by gemcitabine administration. Gemcitabine was administered after EAE induction on days 4, 8, 12, and 16. EAE development in mice (n = 10) was followed, and clinical scores were recorded (**p < 0.001, GEM versus PBS). Data are representative of three independent experiments. (C) Reduced IL-17A levels in gemcitabine-treated mice. Sixteen days after MOG immunization, IL-17A levels in serum (pooled from three mice per group) were measured by ELISA. (D) and (E) Decreased percentage of Th17 cells in gemcitabine-treated mice. CD4+IL-17A+ T cells in the spleen and draining lymph nodes were examined 23 d after MOG immunization by intracellular staining. The numbers indicate the percentages of Th17 cells in wild-type (WT) or il1r1−/− mice and cocultured with naive T cells derived from WT or il1r1−/− mice, respectively. Il1r1 deficiency in MDSCs had little effect on Th17 polarization. However, lack of il1r1 in T cells abolished the stimulating effect of MDSCs on Th17 cell differentiation, indicated by the reduced percentage of Th17 cells and production of IL-17A (Fig. 3G, left and right).
decrease in T cells, NK cells, macrophages, or B cells (38–40). To obtain direct evidence that MDSCs exacerbate EAE disease, we administered gemcitabine to mice after EAE induction. Treatment with gemcitabine at the dose of 100 mg/kg effectively reduced the MDSC population in peripheral blood and spleen (Fig. 4A) but not other immune cell subsets in lymphoid tissues and peripheral blood (Supplemental Fig. 4A, 4B). C57BL/6 mice developed severe EAE after immunization with MOG35-55 and CFA. However, gemcitabine treatment after EAE induction efficiently blocked EAE development in mice compared with PBS treatment (Fig. 4B). Gemcitabine administration at a lower dose (50 mg/kg) was equally effective, as indicated by complete inhibition of EAE incidence. Treatment with gemcitabine at 10 mg/kg was still able to delay the onset and decreased the severity of the disease (Supplemental Fig. 4C, 4D). The efficiency of gemcitabine in inhibiting the EAE disease correlated with the levels of MDSCs (Supplemental Fig. 4E).

MDSC depletion by gemcitabine resulted in significantly reduced concentrations of IL-17A in the serum (Fig. 4C) and a substantial decrease in the percentage of MOG35-55-specific Th17 cells in the lymph nodes and spleen (Fig. 4D, 4E). Quantitative RT-PCR showed that gene transcription levels of rora, rorc, and il17a in the spleen (Fig. 4F, left) and lymph nodes (Fig. 4F, right) were significantly reduced after gemcitabine treatment. In addition, the mRNA levels of il1b in the spleen were significantly lower in gemcitabine-treated EAE mice compared with PBS-treated counterparts (Fig. 4G).

Adoptive transfer assays were conducted to demonstrate further the role of MDSCs in the EAE progression in vivo. MOG35-55/CFA immunized mice were treated with gemcitabine after disease onset, followed by adoptive transfer of EAE-expanded MDSCs. We showed that gemcitabine treatment after disease onset also inhibited the EAE progression efficiently. However, the transfer of MDSCs restored the disease progression (Fig. 5A). The frequency of MOG35-55-specific Th17 cells also increased significantly in the spleen (Fig. 5B) and lymph nodes (data not shown) in mice receiving MDSCs compared with those that were not transferred with MDSCs. To block IL-1β production from MDSCs, we pretreated MDSCs with the caspase-1 inhibitor prior to cell transfer. These MDSCs failed to accelerate the progression of EAE, which correlated with little change of the frequency of Th17 cells in recipient mice (Fig. 5B). Additionally, transfer of MDSCs or MDSC-pretreated with caspase-1 inhibitor did not appear to influence Th1 and Th2 cells in gemcitabine-treated EAE mice (Fig. 5C).

**FIGURE 5.** Restoring the EAE disease progression by adoptively transferred MDSCs after gemcitabine (GEM) treatment is dependent on MDSC-derived IL-1β. (A) Gemcitabine (50 mg/kg) was administered to EAE mice twice at 4-d intervals after disease onset (arrows). Four days after gemcitabine treatment, mice (n = 5) received MDSCs or MDSCs pretreated with the caspase-1 inhibitor twice at a 1-wk interval (arrowheads). Gemcitabine-treated mice that received PBS served as controls. The progression of EAE disease was followed. *p < 0.05 (MDSCs versus PBS or inhibitor-treated MDSCs). (B) The frequency of Th17 cells in the spleen was examined 28 d after MOG immunization by intracellular cytokine staining assays. *p < 0.05. (C) The presence of Th1 and Th2 cells in the spleen was examined using intracellular staining for IFN-γ or IL-4 in CD4+ T cells, respectively. Bar graphs indicate the changes of Th17, Th1, or Th2 cells before and after MDSC transfer. The results shown represent two independent experiments.
MDSC depletion with gemcitabine reduces inflammatory cellular infiltration in spinal cords

We examined the effect of gemcitabine treatment on the infiltration of immune cells into the spinal cords, which is generally correlated with disease progression in EAE mice. Flow cytometry analysis of the cellular infiltrates from spinal cords showed that gemcitabine treatment resulted in a marked reduction of infiltrating CD11b+Gr-1+ MDSCs (Fig. 6A, top), which was associated with a significant decrease in the percentage of IL-17A-producing CD4+ cells (Fig. 6A, bottom). The decreased Th17 response in gemcitabine-treated EAE mice was also indicated by the reduced gene expression of il17a in the spinal cords, indicated by quantitative RT-PCR (Fig. 6B).

Histologic analysis showed a massive infiltration of mononuclear cells in the spinal cords of PBS-treated EAE mice. In contrast, mononuclear cell infiltrates and inflammatory foci formation were significantly reduced in the white matter of spinal cords from gemcitabine-treated EAE mice (Fig. 6C), which is consistent with the reduced clinical scores. MOG35–55 immunization-induced myelin injury was indicated by reduced Luxol fast blue staining and development of widespread vacuolation in the white matter (Fig. 6D). Surprisingly, gemcitabine administration effectively improved myelination of the spinal cord, as almost no demyelination was observed in the white matter of gemcitabine-treated EAE mice (Fig. 6D). The increase in the density and quality of the myelin in the spinal cords after gemcitabine treatment was also associated with significantly reduced levels of inflammatory cell infiltrates (Fig. 6D).

Immunofluorescence staining was used to examine further the infiltration of inflammatory cells in the spinal cords from EAE mice treated with or without gemcitabine. The spinal cord tissues from gemcitabine-treated mice showed substantially decreased infiltration of CD4+, Gr-1+, and IL-17A+ cells compared with PBS-treated EAE mice (Fig. 6E). Gemcitabine treatment similarly caused a pronounced reduction in IL-1β+–producing cells in the spinal cords of EAE mice (Fig. 6E).

**Discussion**

Most of the current knowledge regarding the role of MDSCs in immune responses has come from studies in the context of cancer. MDSCs in cancer patients and tumor-bearing mice can subvert immune surveillance by dampening T cell immunity (11, 12). Although expansion of MDSCs has been observed in a mouse model of MS (22), the potential role of the MDSCs in the pathological conditions (e.g., EAE) has not been determined. Our results in this study unveil a complicated inflammatory network in autoimmune disorders, such as EAE. We discover that mouse MDSCs can drive the differentiation of Th17 cells under Th17-polarizing conditions (e.g., in the presence of cytokines IL-6 and TGF-β). Instead of curbing T cell activation or facilitating the resolution of inflammation, the excessive expansion and the prolonged accumulation of MDSCs may have a detrimental effect by enhancing development of Th17 cells, which can further drive tissue inflammation and aggravate tissue damage in autoimmune diseases.

We provide here the first evidence, to our knowledge, that MDSCs stimulate Th17 cell polarization and IL-17A production in a highly efficient manner. This finding is striking, considering the well-established immunosuppressive activity of MDSCs. It should be noted that we specifically examine the effect of MDSCs...
on the expansion of CD4⁺IL-17⁺ T cells under Th17-polarizing conditions or during the differentiation of naive T precursor cells, whereas all other reports only examine the immunosuppressive activity of MDSCs on T cells in the context of Ag stimulation or TCR engagement. Together with the previous reports of MDSC-facilitated conversion of naive CD4⁺ T cells into Foxp3⁺-expressing Tregs ex vivo (18, 19), our results indicate that MDSCs can display pleiotropic functions under different physiological and pathological conditions, and the pro-Th17 effect of MDSCs may be uncoupled from its T cell suppressive activity. We postulate that the capability of MDSCs to regulate T cells differentially or drive their lineage commitment depends on the inflammatory environment and cytokines existing in the inflamed sites. Our new findings may provide an explanation why MDSCs mobilized under pathological conditions (e.g., EAE) “fail” to materialize their immunosuppressive potential. Instead, these MDSCs can facilitate an inflammatory Th17 response or IL-17A production and further promote chronic inflammation as well as the pathogenesis of autoimmune diseases. Therefore, caution should be used in applications of MDSCs for treatment of autoimmune diseases. Our findings also indicate that understanding of the action of MDSCs in the host responses and disease progression is far from complete, and future studies should not only investigate the regulatory role of MDSCs in the development as well as activities of T cells of different lineages in different contexts but also explore other potential effects of MDSCs beyond the established immunosuppressive activity.

Our results from independent systems demonstrate that IL-1β plays an important role in mediating MDSC-stimulated Th17 cell generation. We confirm that IL-1R expression in CD4⁺ T cells, but not on MDSCs, is needed for MDSC-promoted Th17 cell development. This observation supports the recent finding of Dong and colleagues (9) that IL-1 signaling in T cells was required for early Th17 cell differentiation. Notably, human myeloid cells (e.g., monocytes) isolated from the site of rheumatoid arthritis were also shown to increase a Th17 response, however in an IL-1β-independent fashion (41). Although our studies support the notion that IL-1β represents a major factor involved in MDSC-facilitated Th17 polarization, other IL-1β-independent mechanisms underlying this phenomenon remain the possibility, given the plasticity and heterogeneity of this cell population. In the current study, we also observe that arginase 1 is needed for the effect of MDSCs in Th17 differentiation. However, inhibition of arginase 1 activity does not appear to affect IL-1β production from MDSCs, suggesting that MDSC-associated IL-1β or arginase 1 regulates Th17 polarization via independent mechanisms. It is possible that excessive NO production in the presence of arginase 1 inhibitor (42) may be able to suppress Th17 differentiation, as recently documented by Niedbala et al. (43). Given the multiple effects of arginase activity and potential different outcomes (42), further studies are necessary to understand the precise role of arginase 1 in MDSC-driven Th17 polarization.

Gemcitabine has been used clinically as an approved therapeutic agent and was recently shown to improve anti-tumor immune responses by selectively targeting MDSCs in several preclinical models (38–40). Our results demonstrate that MDSC depletion after gemcitabine administration effectively ameliorates the clinical symptoms in EAE mice, which is attributed to significantly reduced levels of IL-17A-producing Th17 cells in peripheral tissues and inflamed disease sites (e.g., spinal cords). Furthermore, adoptive transfer of EAE-expanded MDSCs to gemcitabine-treated mice can restore the EAE disease progression, whereas pretreatment of MDSCs with caspase-1 inhibitor for blocking of IL-1β production disrupts this effect. This result strongly supports the critical role of MDSC-derived IL-1β in enhanced Th17 response and EAE progression in vivo. Given the potency of gemcitabine in blockade of MDSC-driven Th17 response, the potential benefits of using this agent for treatment of inflammatory autoimmune diseases (e.g., EAE) warrant further investigation. However, more studies are necessary to define the sensitivity of MDSCs and other cell subsets to the gemcitabine effect.

Recently, Ioannou et al. (44) showed that CD11b⁺Ly6G⁺ cells (G-MDSCs or a subset of neutrophils) decreased the severity of EAE via immune suppression induced by PD-L1–PD-1 interactions. Adoptive cell transfer of G-MDSCs in this study was performed starting 4 d after MOG immunization, whereas transfer of total MDSCs in our study occurred after disease onset. It is possible that MDSCs exhibit distinct biological activities depending upon the microenvironment at the different pathological stages of autoimmune diseases. As a result, the disease stage must be taken into consideration when MDSCs are exploited for the disease intervention. Ioannou et al. (44) also showed that only G-MDSCs expanded during the EAE progression, and were able to ameliorate the disease compared with CD11b⁺Ly6G⁻ cells (presumably M-MDSCs). However, other report (22) and our data from the current study demonstrate that CD11b⁺Ly6C⁹⁻ or CD11b⁺Gr-1low cells, mainly composed of M-MDSCs, also expanded in this mouse model of multiple sclerosis. It has been well documented that M-MDSCs exhibited more potent suppressive activity against T cells compared with CD11b⁺Gr-1high cells, represented mostly by G-MDSCs (29, 30). Intriguingly, we have shown that CD11b⁺Gr-1low cells promote Th17 polarization more efficiently than CD11b⁺Gr-1high cells. Therefore, the disparity in these results could be attributed to the plasticity of the phenotype and functions of heterogeneous MDSCs, which may be determined by the environmental or inflammatory milieu.

The current study reveals a previously unknown role of MDSCs during the expansion of Th17 cells and IL-17A production, indicating that the specificity of MDSCs as an immune regulator of T cell differentiation can be dictated by the environment or cytokines present in the inflammatory milieu. Our data demonstrate that MDSCs can contribute to the pathogenesis of autoimmune diseases (e.g., EAE) by promoting Th17 differentiation and advance our understanding of the biological and pathological roles of MDSCs. These unexpected findings highlight the importance of MDSC–T cell interactions in the shaping of inflammatory T cell responses and suggest that therapeutic targeting of MDSCs may lead to potentially effective intervention of Th17/IL-17–dependent autoimmune disorders.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary data

Supplementary Figure S1. Characterization of MDSCs expanded during the EAE progression. C57BL/6 mice were immunized with MOG35-55/CFA. Two weeks post immunization, the phenotype of splenic MDSCs was analyzed using FACS. A. Ly6C and Ly6G expression on CD11b+ cells was examined. B. The morphology of CD11b+Gr-1+ cells was examined using standard Giemsa staining and microscope. C. Expression of CD11c, F4/80, MHC II and CD80 on CD11b+Gr-1+ cells. D. FACS analysis of the expression of CD115, CCR2, CD62L and Ly6C. E-G. MDSCs from EAE mice suppress T cell activation by CD3/CD28 engagement or antigen stimulation. E. CFSE labeled splenocytes from naïve C57BL6 mice were treated with anti-CD3/CD28 antibodies (1 μg/mL) in the absence or presence of MDSCs for 3 days. T cell proliferation was analyzed using CFSE dilution assay (left). Cell viability was determined by FACS after staining with 7-AAD (right). Filled grey: T cells without stimulation; red: T cells with stimulation; black, T cells with stimulation in the presence of MDSCs (1:1). F. IFN-γ production from CD4 cells was analyzed by intracellular staining assays. G. For antigen-specific activation, BMDCs loaded with MOG35-55 peptide (1 μg/mL) were co-cultured with MOG35-55-specific CD4+ T cells derived from the 2D2 transgenic mice (1:100) in the absence or presence of MDSCs. CD4+ T cell proliferation was determined using ^3H thymidine incorporation assay. *, p < 0.05

Supplementary Figure S2. Effects of MDSCs from different sources on Th17 differentiation. A. Similar effect of MDSCs isolated using MACS beads or FACS sorting on Th17 cell polarization. Naïve CD4+ T cells were induced for Th17 cell differentiation in the presence of MDSCs prepared using different methods. The frequency of Th17 cells and IL-17 levels were analyzed by flow cytometry and ELISA, respectively. N.S. Not significant. B. MDSCs derived from tumor-bearing mice are highly efficient in promoting Th17 differentiation. Naïve CD4+CD25-CD62L+ T cells (5×10^5 per well in 24-well plates) were co-cultured with or without MDSCs from B16 tumor bearing mice under Th17-polarizing conditions for 3 days. Cells were washed and examined for IL-17A or IFN-γ expression by intracellular staining assays and FACS. Data represent two independent experiments. C-E. MDSCs promote Th17 cell expansion. Naïve CD4+ T cells were induced for Th17 cell differentiation in the presence of MDSCs for 3 days. CD4+ T cells were then sorted, CFSE-labeled, and seeded in 24-well plates pre-coated 1 μg/ml anti-CD3 Abs (5×10^5 cells per well). Cells were cultured in the presence or absence of MDSCs (1:1) for additional 72 hours. Cell proliferation was analyzed using CFSE dilution assays (C). Cells were subjected to intracellular IL-17A staining and FACS analysis, and the number of Th17 cells are calculated (D). The levels of IL-17A in the supernatants were assessed using ELISA assays (E). Data represent two independent experiments.

Supplementary Figure S3. Arginase1, not NOS2, is also involved in MDSC-enhanced Th17 differentiation. MDSCs were co-cultured with naïve CD4+CD25-CD62L+ T cells (5×10^5 per well in 24-well plates) cells at 1:1 ratio under Th17 polarizing conditions in the presence of NOS2 inhibitor, L-N^6-(1-iminoethyl)-lysine (L-NIL, 500 μM), IL-17A expression was examined using intracellular staining and ELISA assays (A). Alternatively, arginase1 inhibitor, nor-NOHA (300 μM) was used in the co-culture (B). Intracellular IL-17A expression was examined using
FACS analysis. Additionally, IL-1β production from MDSCs was also assessed using intracellular staining assays.

**Supplementary Figure S4. Effects of GEM treatment on immune cell subsets and EAE disease progression.** A-B. GEM treatment does not alter the overall levels of T, B, NK cells and DCs. Mice (n=3) were immunized with MOG\textsubscript{35-55}/CFA, followed by administration of pertussis toxin. GEM (100 mg/kg) was injected 11 days after EAE induction. At the indicated time points, cells prepared from the peripheral blood (A), spleen and lymph nodes (B) were analyzed by flow cytometry for potential influence of GEM-mediated MDSC depletion on various immune cell subsets, including CD4 T cells (CD3\textsuperscript{+}CD4\textsuperscript{+}), CD8 T cells (CD3\textsuperscript{+}CD8\textsuperscript{+}), NK cells (CD3\textsuperscript{−}NK1.1\textsuperscript{+}), B cells (B220\textsuperscript{+}), cDCs (CD11c\textsuperscript{+}B220\textsuperscript{−}), and pDCs (CD11c\textsuperscript{+}B220\textsuperscript{+}). Data represent two independent experiments. C. Effect of different doses of GEM on EAE incidence and progression. C57BL/6 mice (n=5) were immunized with MOG\textsubscript{35-55}/CFA, followed by intraperitoneal administration of pertussis toxin on days 0 and 2. Mice received GEM treatment at the doses of 10 mg/kg or 50 mg/kg as indicated on days 4, 8, 12, 16. EAE incidence (C) and mean clinical scores (D) were recorded. E. MDSCs in the peripheral blood were analyzed 2 days after the last GEM treatment using FACS. Data represent two independent experiments.
Supplementary Figure S1

A

B

C

D

E

F

G

Supplementary Figure S1
Supplementary Figure S2

A

- MDSC

+ MDSC

MACS bead Cell sorting

IL-17A

IFN-γ

B

No MDSC

MDSC

from B16 tumor-bearing mice

IL-17A

IFN-γ

C

- MDSC/56%

+ MDSC/94%

CFSE

Th17 cell number (X10^5)

p<0.001

D

IL-17A (ng/ml)

0 5 10 15

E

p<0.001
Supplementary Figure S3

A

- MDSC

+ MDSC

vehicle

L-NIL

10.8%

25.9%

25.7%

IL-17A

IFN-γ

+ MDSC

vehicle

L-NIL

N.S

IL-17A (ng/ml)

0 50 100 150

B

- MDSC

+ MDSC

vehicle

nor-NOHA

13.2%

33%

18%

IL-17A

IFN-γ

nor-NOHA

vehicle

IL-1β
Supplementary Figure S4

A

Percentage (%)

PBS  GEM

one week

two weeks

four weeks

Percentage (%)

PBS  GEM

B

four weeks

spleen

lymph node

Percentage (%)

PBS  GEM

C

Incidence (%)

Days after immunization

PBS  10mg/kg  50mg/kg

D

Clinical score

Days after immunization

PBS  10mg/kg  50mg/kg

E

Percentage of MDSCs in PB

Days after immunization

PBS  GEM (mg/kg)

p = 0.001

p = 0.045