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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 been a focus of recent study on tumor-mediated immune suppression. However, its role in Th17 cell differentiation and the pathogenesis of autoimmune diseases (e.g., multiple sclerosis) has not been determined. We show in this study that development of experimental autoimmune encephalomyelitis (EAE) in mice is associated with a profound expansion of CD11b+Gr-1+ MDSCs, which display efficient T cell inhibitory functions in vitro. Unexpectedly, these MDSCs enhance the differentiation of naive CD4+ T cell precursors into Th17 cells in a highly efficient manner under Th17-polarizing conditions, as indicated by significantly increased number of Th17 cells, elevation of IL-17A production, and upregulation of the orphan nuclear receptor RORA and RORC. Mechanistic studies show that IL-1β represents a major mediator of MDSC-facilitated Th17 differentiation, which depends on the IL-1 receptor on CD4+ T cells but not MDSCs. Selective depletion of MDSCs using gemcitabine results in a marked reduction in the severity of EAE (e.g., decreased clinical scores and myelin injury), which correlates with reduced Th17 cells and inflammatory cytokines (IL-17A and IL-1β) in the lymphoid tissues and spinal cord. Adoptive transfer of MDSCs after gemcitabine treatment restores EAE disease progression. Together, we demonstrate for the first time, to our knowledge, that excessive and prolonged presence of MDSCs can drive a Th17 response and consequently contributes to the pathogenesis of EAE. These new findings provide unique insights into the pleiotropic functions of MDSCs and may help explain the failure of immunosuppressive MDSCs to control Th17/IL-17–dependent autoimmune disorders.

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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 mouse mAbs, including FITC–CD4 (9.1, 2H4; BD Biosciences), PE–CD11b (M1/70), PerCP–Cy5.5–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-ROSyt (AFKJS-9) Abs and mouse IFN-γ and IL-1β ELISA kits were from eBioScience (San Diego, CA). Alex4/9 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 an initial perfusion with ice-cold PBS, mice were perfused transcardially with 4 mg/ml heat-killed Mycobacterium H37Rv (ProSpec, East Brunswick, NJ) emulsified 1:1 in CFA supplemented with 10% FCS, 50 μg/ml gentamicin, and 2 ml/mouse of 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 in the last 16 h of incubation. Proliferation was measured by [3H]thymidine incorporation in triplicate wells using an LS 6500 multipurpose 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.

Histology and immunofluorescence

After an initial perfusion with ice-cold PBS, mice were perfused transcardially with 4% paraformaldehyde, and spinal cords with vertebrae were removed. Paraffin-embedded sagittal sections of cervicothoracic spinal cord were stained with HE and examined for cellular infiltration or Luxol fast blue/periodic acid–Schiff for determining demyelination. For immunofluorescence, after deparaffinizing in xylene (2x3), blue/periodic acid–Schiff for determining demyelination. For immunofluorescence staining were purchased from BD Biosciences. Alexa 594-labeled donkey anti-rat secondary IgG at room temperature and IL-17A (1:50) overnight at 4˚C, followed by incubation with Alexa 594-labeled donkey anti-rat secondary IgG at room temperature for 1 h. A negative staining control was performed by incubation with isotype control Abs. The coverslips were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and images were taken using an Olympus BX41 fluorescent microscope (Tokyo, Japan).

Flow cytometry analysis

For surface staining, splenocytes, lymph node cells, or mononuclear cells from spinal cord were prepared as single-cell suspensions. Cells resuspended in FACS buffer (PBS containing 0.1% BSA and 0.04% EDTA-Na2) were blocked with anti-mouse CD16/CD32 mAbs (2.4G2; BD Biosciences) and then incubated on ice for 30 min with fluorochrome-conjugated Abs or the appropriate isotype controls. For intracellular cytokine staining, cells were stimulated with MOG35-55 (1 μg/ml) overnight at 37˚C, 5% CO2, and treated with 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-ROSyt 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 allop yococyanin-conjugated IL-1β mAbs for 30 min at 4˚C. Data were then acquired using BD FACSCalibur 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 Biotec, Auburn, CA). Approximately 98% of CD11b+ cells are Gr-1+, and the purity of CD11b+ Gr-1+ cells was typically greater than 95%. In some experiments, CD11b+Gr-1+ were sorted from splenocytes, and CD11b+Gr-1hi and CD11b+Gr-1lo cells were sorted 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 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 multipurpose scintillation counter (Beckman Coulter). Supernatant was collected 48 h after culture, and levels of cytokines IL-2 and INF-γ were determined by ELISA according to the manufacturer’s instructions.

In vitro Th17 cell differentiation

CD4+CD25−CD62L+ naive T cells were FACS sorted (>98% pure), and 5 × 104 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 μg/ml gentamicin, and 2 ml/mouse of IL-2 and IL-17A cytokine assays by ELISA according to the manufacturer’s instructions. Cells were washed and equilibrated in citrate buffer (10 mM, pH 6) for 3 min then boiled for Ag retrieval for 10 min in a microwave oven, cooled to room temperature, and then rinsed with PBS–TWEEN 20. After serum blocking for 60 min, the sections were stained with primary mAbs for Gr-1 (1:10), CD4 (1:100), and IL-1β (1:50) and IL-17A (1:50) overnight at 4˚C, followed by incubation with Alexa 594-labeled donkey anti-rat secondary IgG at room temperature for 1 h. A negative staining control was performed by incubation with isotype control Abs. The coverslips were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and images were taken using an Olympus BX41 fluorescent microscope (Tokyo, Japan).
stimulated with PMA plus ionomycin in the presence of Golgi-stop for 4 h, followed by intracellular staining for IL-17A and IFN-γ-producing CD4+ cells. Intracellular staining for Foxp3 was performed with a Foxp3 staining kit (eBioscience). Cell proliferation was assessed using CFSE dilution assays. In some experiments, 10 ng/ml IL-1β (BioLegend), 30 μg/ml IL-1β mAbs (BioLegend), 200 ng/ml IL-1 receptor antagonist (IL-1ra; ProSpec), or 10 μg/ml anti-β7 mAbs (BioLegend) were added.

Real-time PCR analysis

Total RNAs were prepared using TRIzol reagent (Invitrogen) from spleen, lymph nodes, spinal cord, or cultured Th17 cells. RNA was treated with RNase-free DNase I (Invitrogen) to eliminate the contamination of genomic DNA and quantified using an Ultra-Spec spectrophotometer (Amersham Bioscience, Piscataway, NJ). cDNA was synthesized with Reverse Aid MMLV Reverse Transcriptase (Fermentas, Glen Burnie, MA) in 20 μl reaction volumes containing 1 μg of total RNA and 50 ng oligo-deoxythymidine primers (Invitrogen). For quantitative PCR analysis, transcription profiles of Il17a, rora, rorc, and Il1b were assessed on an ABI prism 7900HT Sequence Detection System using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primers and FAM-labeled probe sets were obtained as predeveloped assay reagents from Applied Biosystems: il17a, Mm00439618_m1; rora, Mm00443103_m1; rorc, Mm01261022_m1; il1b, Mm01336189_m1. The PCR was started with 2 min at 50˚C and an initial 15 min denaturation at 94˚C, followed by a total of 40 cycles of 15 s denaturation at 94˚C, and 1 min of annealing and elongation at 60˚C. All measurements were performed in triplicate wells and repeated three times. Gene expression was quantified relative to the expression of the housekeeping gene gpdh and normalized to that measured in control cells by standard 2−ΔΔCT calculation.

Adoptive cell transfer

MDSCs derived from EAE mice were treated with or without caspase-1 inhibitor (100 μM; Cayman Chemical) prior to adoptive transfer. C57BL/6 mice (n = 5) were immunized with MOG35–55/CFA, followed by gemcitabine (50 mg/kg) administration after the disease onset twice at 4-d intervals. Four days after gemcitabine treatment, mice were transferred i.v. with MDSCs (8 × 106) twice at a 1-wk interval. The progression of EAE disease was followed, and clinical scores were recorded.

Statistical analysis

Statistical analysis was performed with Prism Software (GraphPad, San Diego, CA). Data are shown as the mean ± SEM. Unpaired two-tailed Student t test was used for two-group comparisons. Where there were more than two groups, differences between groups were tested with ANOVA test. The p values < 0.05 were considered statistically significant.

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+ myeloid 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+Ly6ClowLy6G+, which correspond to monocyctic myeloid-derived suppressor cells (M-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 CD115, 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. MDSCs promote Th17 cell differentiation under Th17-polarizing conditions.

**FIGURE 2.** MDSCs are highly efficient in promoting Th17 differentiation in the presence of IL-6 and TGF-β. (A) Naive CD4+CD25−CD62L+ T cells (5 × 10⁵ per well in 24-well plates) were cocultured with or without MDSCs in Th17-polarizing medium for 3 d. Cells were washed and examined for IL-17A or IFN-γ expression by intracellular staining using flow cytometry after stimulation with PMA plus ionomycin in the presence of brefeldin A. CD4+Foxp3+ cells were also examined by FACS analysis. (B) The percentage of CD4+IL-17A+ T cells (left) and total number of CD4+IL-17A+ T cells (right) after 3-d culture are shown. Cell proliferation in the presence or absence of MDSCs during Th17 polarization in the culture was analyzed using CFSE dilution assays (middle). (C) Three days after coculture, supernatant was harvested and assessed for IL-17A levels by ELISA. (D) Relative mRNA levels of il17a or rora were measured using real-time RT-PCR. (E) RORγt expression during Th17 differentiation was determined using intracellular staining and flow cytometry analysis (top, gray filled, isotype control; dashed line, without MDSCs; solid line, with MDSCs). The frequency changes of CD4+RORC+ T cells in the presence or absence of MDSCs during Th17 cell polarization in the culture was analyzed using CFSE dilution assays (middle). (F) Three days after coculture, supernatant was harvested and assessed for IL-17A levels by ELISA. (G) Relative mRNA levels of il17a or rora were measured using real-time RT-PCR. (H) RORγt expression during Th17 differentiation was determined using intracellular staining and flow cytometry analysis (top, gray filled, isotype control; dashed line, without MDSCs; solid line, with MDSCs). The frequency changes of CD4+RORC+ T cells in the presence or absence of MDSCs during Th17 cell polarization in the culture was analyzed using CFSE dilution assays (middle).
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-1^low and CD11b^Gr-1^high, 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-1^low cells appeared to be much more effective than CD11b^Gr-1^high 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-

![Diagram](http://www.jimmunol.org/)
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 Abs 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 antagonists, 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 il1rl−/− mice and cocultured with naive T cells derived from WT or il1rl−/− mice, respectively. Il1rl deficiency in MDSCs had little effect on Th17 polarization. However, lack of il1rl 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

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**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 before and after gemcitabine treatment. The results shown represent three independent experiments. (F) Effect of gemcitabine treatment on il1r1, il1r2, and il17a gene expression levels in the spleen (left) and lymph node (LN, right). Relative mRNA levels were measured by quantitative real-time RT-PCR and normalized to β-actin gene in the PBS group (***p < 0.001, GEM versus PBS). (G) Reduced expression of il1b gene in the spleen after gemcitabine treatment. Gene expression of il1b was determined by quantitative real-time RT-PCR and normalized to actin gene in the PBS group. Results shown represent three independent experiments.
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 administered 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-1$\beta$ 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-1$\beta$-independent fashion (41). Although our studies support the notion that IL-1$\beta$ represents a major factor involved in MDSC-facilitated Th17 polarization, other IL-1$\beta$-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-1$\beta$ production from MDSCs, suggesting that MDSC-associated IL-1$\beta$ 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-1$\beta$ production disrupts this effect. This result strongly supports the critical role of MDSC-derived IL-1$\beta$ 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$^+$ (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 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$^+$Ly6G$^{hi}$ or CD11b$^+$Gr-1$^{lo}$ 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-1$^{hi}$ cells, represented mostly by G-MDSCs (29, 30). Intriguingly, we have shown that CD11b$^+$Gr-1$^{lo}$ cells promote Th17 polarization more efficiently than CD11b$^+$Gr-1$^{hi}$ 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


