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This information is current as of March 5, 2022.

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J Immunol 2013; 191:1073-1081; Prepublished online 26 June 2013;

doi: 10.4049/jimmunol.1203535

<http://www.jimmunol.org/content/191/3/1073>

Supplementary Material <http://www.jimmunol.org/content/suppl/2013/06/26/jimmunol.1203535.DC1>

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Myeloid-Derived Suppressor Cells Play Crucial Roles in the Regulation of Mouse Collagen-Induced Arthritis

Wataru Fujii,* Eishi Ashihara,[†] Hideyo Hirai,[‡] Hidetake Nagahara,* Naoko Kajitani,* Kazuki Fujioka,* Ken Murakami,* Takahiro Seno,*[§] Aihiro Yamamoto,* Hidetaka Ishino,* Masataka Kohno,* Taira Maekawa,[‡] and Yutaka Kawahito*

Myeloid-derived suppressor cells (MDSCs) are of myeloid origin and are able to suppress T cell responses. The role of MDSCs in autoimmune diseases remains controversial, and little is known about the function of MDSCs in autoimmune arthritis. In this study, we clarify that MDSCs play crucial roles in the regulation of proinflammatory immune response in a collagen-induced arthritis (CIA) mouse model. MDSCs accumulated in the spleens of mice with CIA when arthritis severity peaked. These MDSCs inhibited the proliferation of CD4⁺ T cells and their differentiation into Th17 cells in vitro. Moreover, MDSCs inhibited the production of IFN- γ , IL-2, TNF- α , and IL-6 by CD4⁺ T cells in vitro, whereas they promoted the production of IL-10. Adoptive transfer of MDSCs reduced the severity of CIA in vivo, which was accompanied by a decrease in the number of CD4⁺ T cells and Th17 cells in the draining lymph nodes. However, depletion of MDSCs abrogated the spontaneous improvement of CIA. In conclusion, MDSCs in CIA suppress the progression of CIA by inhibiting the proinflammatory immune response of CD4⁺ T cells. These observations suggest that MDSCs play crucial roles in the regulation of autoimmune arthritis, which could be exploited in new cell-based therapies for human rheumatoid arthritis. *The Journal of Immunology*, 2013, 191: 1073–1081.

The existence of a population of immature myeloid cells suppressing T cell immune responses was first suggested >20 years ago in mouse cancer models (1). Many studies have since revealed the importance of these cells, which are now widely referred to as myeloid-derived suppressor cells (MDSCs) (2). MDSCs are of myeloid origin, are able to suppress T cell responses, and are characterized by the coexpression of the myeloid differentiation Ags Gr-1 and CD11b in mice (3). A human counterpart of MDSCs is most commonly defined as CD11b⁺CD33⁺CD14[−]HLA-DR[−] (4, 5) or Lin[−]CD11b⁺CD33⁺HLA-DR[−] (6) subsets. MDSCs in mice can be divided into two major sub-

sets, Ly6G⁺Ly6C^{low} granulocytic MDSCs and Ly6G[−]Ly6C^{high} monocytic MDSCs, according to the expression of the cell surface Ags Ly6G and Ly6C (both recognized by the Gr-1 Ab) (3). The ability of MDSCs to suppress T cell responses is dependent on two enzymes that are involved in the metabolism of L-arginine: arginase 1 and inducible NO synthase (iNOS) (7, 8).

MDSCs were first characterized and studied in tumor-bearing mice (3, 9, 10) and cancer patients (4, 5, 9), and they were reported to promote tumor progression by suppressing anti-tumor immunity by T cells. MDSCs have since been found to accumulate in response to infectious disease (11, 12) and traumatic stress (13). MDSCs generated from bone marrow cells can prevent graft-versus-host disease (14). Furthermore, MDSCs accumulate in models of several autoimmune diseases, including multiple sclerosis (15–18), inflammatory bowel disease (19), uveoretinitis (20), alopecia areata (21), and type 1 diabetes (22). MDSCs accumulating in autoimmune diseases possess the potential of suppressing immune responses of T cells, but the roles of MDSCs in autoimmune diseases remain controversial. There are few reports pertaining to the role of MDSCs in experimental autoimmune arthritis. It was recently reported that MDSCs in mice with proteoglycan-induced arthritis suppress T cell proliferation through a mechanism dependent on dendritic cells in vitro (23); however, the roles of MDSCs in autoimmune arthritis in vivo remain unclear.

Rheumatoid arthritis (RA) is a common multiple articular disease that is caused by various immune disorders. For example, disruptions in tolerance to self-Ags can lead to abnormalities, such as the recognition of citrullinated Ags by B and T cells (24). As for myeloid cells in RA, not only macrophages, which contribute considerably to inflammation and joint destruction (25), but also neutrophils have been reported to trigger inflammation through releasing protease and immune mediators (26). Little is known about the possibility of the existence of a myeloid cell population that can suppress inflammation and autoimmunity in RA.

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Received for publication December 27, 2012. Accepted for publication May 29, 2013.

This work was supported in part by the Shimizu Foundation for Immunological Research Grant for 2011 (to E.A.); a research grant from the Japan Rheumatism Foundation Information Center (to Y.K.); research grants from the Yasuda Medical Foundation, the Fujiwara Foundation, and the Takeda Science Foundation (to H.H.); a research grant from the Kobayashi Foundation for Cancer Research (to T.M.); a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to E.A., H.H., T.M., and Y.K.); the Global Centers of Excellence (COE) Program "Center for Frontier Medicine" from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T.M.); and by National Cancer Center Research and Development Fund Grant 23-A-23 (to T.M.).

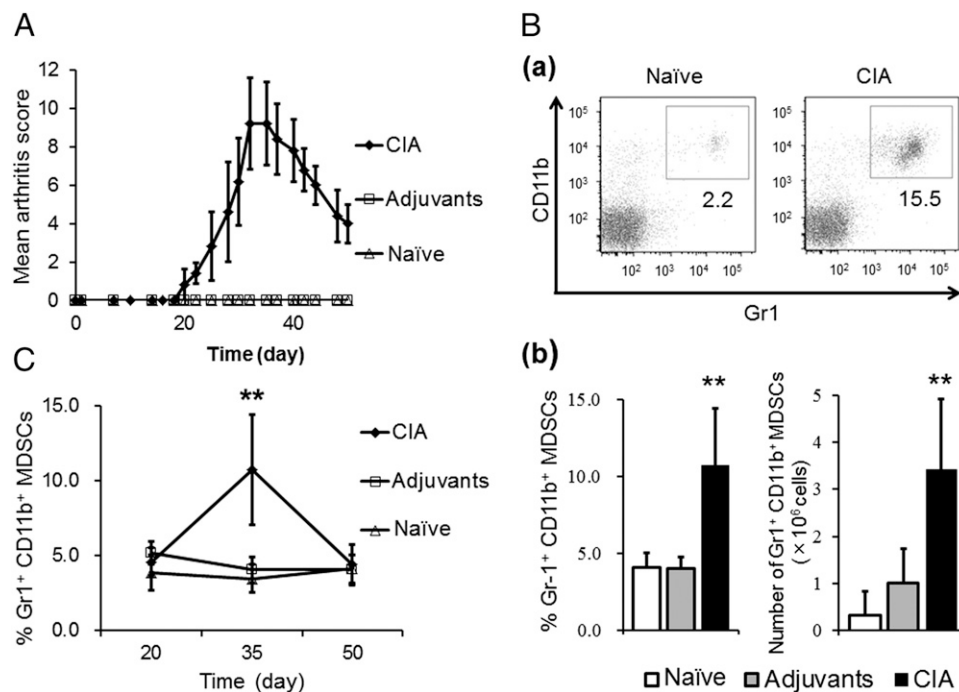
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The online version of this article contains supplemental material.

Abbreviations used in this article: CIA, collagen-induced arthritis; DLN, draining lymph node; iNOS, inducible NO synthase; MDSC, myeloid-derived suppressor cell; RA, rheumatoid arthritis; Treg, regulatory T cell.

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FIGURE 1. Gr-1⁺CD11b⁺ MDSCs accumulate in the spleens of CIA mice. **(A)** Clinical arthritis scores of CIA, adjuvants, and naive mice between day 0 and day 50. Means \pm SD are shown. **(B)** Levels of Gr-1⁺CD11b⁺ MDSCs in the spleens of CIA, adjuvants, and naive mice at the peak of arthritis (day 35) were investigated by flow cytometry. **(Ba)** Representative dot plots of CIA and naive mice. Gray squares represent Gr-1⁺CD11b⁺ MDSCs and numbers indicate the percentage of Gr-1⁺CD11b⁺ MDSCs in living splenocytes. **(Bb)** Percentages and absolute numbers of Gr-1⁺CD11b⁺ MDSCs in the spleens. Means \pm SD are shown ($n = 5/\text{group}$). **(C)** Percentage of Gr-1⁺CD11b⁺ MDSCs in the spleens of CIA, adjuvants, and naive mice. Means \pm SD are shown ($n = 5/\text{group}$). Data are representative of three independent experiments. $^{**}p < 0.01$, compared with naive mice.



The object of this study was to elucidate the roles of MDSCs in auto-immune arthritis. We used collagen-induced arthritis (CIA) mouse models, one of the animal models of RA that are widely used to elucidate

the pathogenesis of RA and to develop strategies for RA therapies (27, 28). We show that MDSCs inhibit the proinflammatory immune response of CD4⁺ T cells and thereby play regulatory roles in mouse CIA.

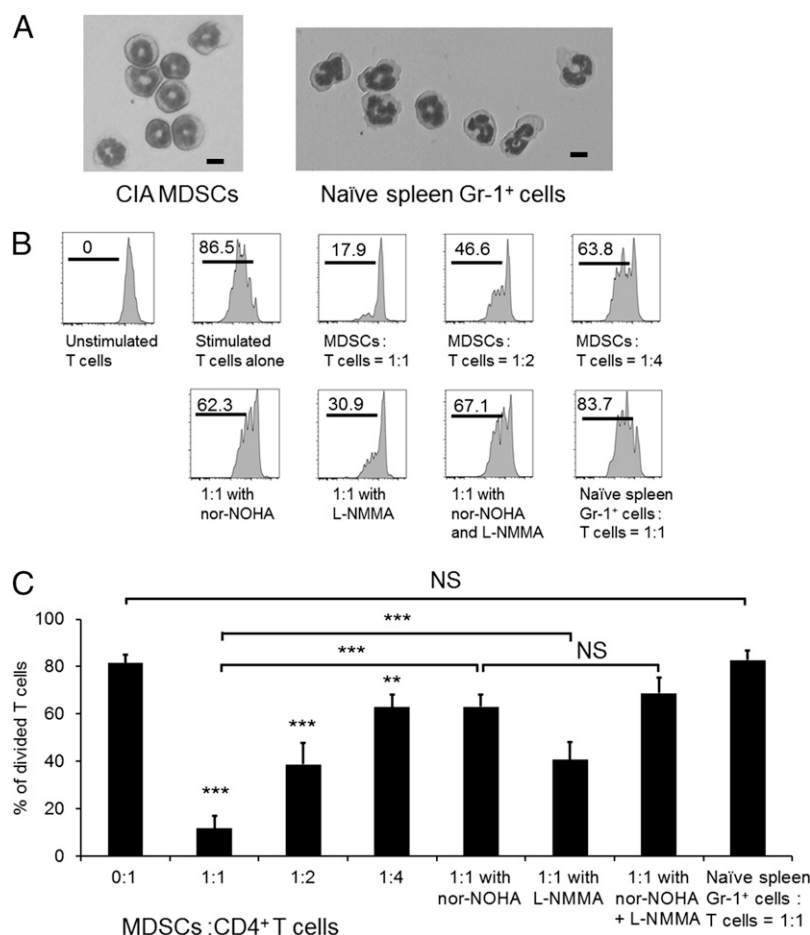


FIGURE 2. MDSCs inhibit the proliferation of CD4⁺ T cells mainly through the activity of arginase. **(A)** Cytopsin preparations of Gr-1⁺CD11b⁺ MDSCs isolated from splenocytes of CIA mice (left panel) and naive Gr-1⁺ cells isolated from naive mice (right panel) stained with Wright-Giemsa stain. Scale bar, 10 μm . Original magnification, $\times 400$. **(B)** CFSE-labeled CD4⁺ T cells stimulated with anti-CD3/anti-CD28 Abs and IL-2 were cultured alone or cocultured with MDSCs at the indicated ratios for 5 d. Cells were cultured in the presence of *N*^G-monomethyl-L-arginine (L-NMMA; iNOS inhibitor) and/or *N*^w-hydroxy-nor-L-arginine (nor-NOHA; arginase inhibitor) as indicated. Flow cytometry data show the dilution of CFSE dye in CD4⁺ T cells. Numbers represent the percentage of divided CD4⁺ T cells. **(C)** Mean \pm SD percentages of divided CD4⁺ T cells are shown ($n = 6/\text{group}$). Data are representative of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with stimulated CD4⁺ T cells cultured alone unless indicated otherwise.

Materials and Methods

Animals

Male DBA/1 mice at 7–8 wk old were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). Mice were maintained in specific pathogen-free conditions. Animal experiments were approved by the Animal Research Committee, Graduate School of Medical Science, Kyoto Prefectural University of Medicine.

Induction and assessment of CIA

DBA/1 mice received 200 μ g bovine type II collagen in Freund's complete adjuvant by intradermal injection on day 0 as a first immunization. Collagen (200 μ g) in Freund's incomplete adjuvant was given by intradermal injection on day 21 as a second immunization. Collagen and adjuvants were purchased from Chondrex (Redmond, WA). Signs of arthritis in mice were monitored according to the swelling and redness of each limb, the severity of which was separately scored by two independent researchers in a blinded manner and then the mean score was calculated as follows: 0, normal; 1, mild; 2, moderate; 3, severe. The severity scores of each limb were totaled, giving a maximum score of 12 per mouse. This combined score was designated the "arthritis score."

Flow cytometric analysis

A single-cell suspension was prepared from mouse spleens, and RBCs were removed with lysing buffer (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's protocol. After Fc receptor blocking using a rat 2.4G2 purified mAb, rat anti-mouse mAbs against Gr-1 (RB6-8C5), CD4 (RM4-5), B220 (RA3-6B2) (all from eBioscience, San Diego, CA), Ly6G (1A8), Ly6C (AL-21), CD11b (M1/70) (all from BD Pharmingen, San Diego, CA), a goat anti-mouse polyclonal Ab against IL-4R α (R&D Systems, Minneapolis, MN), and the isotype-matched controls (from R&D Systems and BD Pharmingen) were used to stain specific surface Ags. Dead cells were excluded with propidium iodide staining. For intracellular cytokine staining, 1×10^6 cells were cultured at 37°C for 5 h in RPMI

1640 media (Wako, Osaka, Japan) containing PMA/ionomycin in the presence of GolgiPlug (all from BD Biosciences). Cells were stained for intracellular cytokines using an intracellular cytokine staining kit containing rat anti-mouse mAbs against IL-17A (TC11-18H10.1), IFN- γ (XMG1.2), IL-2 (JES6-5H4), IL-10 (JES6-16E3), TNF- α (MP6-XT22) (all from BD Pharmingen), Foxp3 (FJK-16s; eBioscience), a sheep anti-mouse polyclonal Ab against arginase 1 (R&D Systems), and the isotype-matched controls (from R&D Systems and BD Pharmingen). Flow cytometry was performed with a FACSCanto II flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Isolation of MDSCs and CD4⁺ T cells

MDSCs or naive Gr-1⁺ cells were isolated from the single-cell suspension prepared from the spleens of CIA or naive mice, respectively, using a biotinylated mAb against Gr-1, streptavidin magnetic beads, and MidMACS columns (all from Miltenyi Biotec, Auburn, CA), according to the manufacturer's protocol (purity >98%). To remove all cells except for CD4⁺ T cells, the CD4⁺ T cells were isolated from the single-cell suspension prepared from the spleens of naive mice using a biotinylated Ab mixture and anti-biotin magnetic beads (all from Miltenyi Biotec), in accordance with the manufacturer's protocol (purity >95%).

Cytospin preparation

Cytospins were obtained by centrifuging 5×10^4 cells on microscope slides and stained with a Diff-Quik kit (Sysmex, Kobe, Japan), a modified Wright–Giemsa staining system. Cell morphology was examined by microscopic evaluation of stained cells using a DMBA210 microscope (Shimadzu Rika, Tokyo, Japan) equipped with Motic Images Plus 2.2s software (Shimadzu Rika).

Proliferation assay

Isolated CD4⁺ T cells were incubated with CFSE (eBioscience) at a final concentration of 2.5 μ M, according to the manufacturer's protocol. Cells

FIGURE 3. MDSCs alter the production of cytokines by CD4⁺ T cells. CD4⁺ T cells stimulated with anti-CD3/anti-CD28 Abs were cultured alone or were cocultured with MDSCs at a ratio of 1:1 for 3 d. **(A)** The concentrations of various cytokines in the culture supernatant were determined by ELISA. Mean concentrations \pm SD of each cytokine are shown ($n = 5$ /group). * $p < 0.05$, ** $p < 0.01$. **(B)** Cells were stained for CD4 and the indicated intracellular cytokines. Representative dot plots are shown. Gray squares indicate the gated CD4⁺ cells that were positive for the indicated cytokine, and numbers indicate the percentage of these cells. Data are representative of three independent experiments.

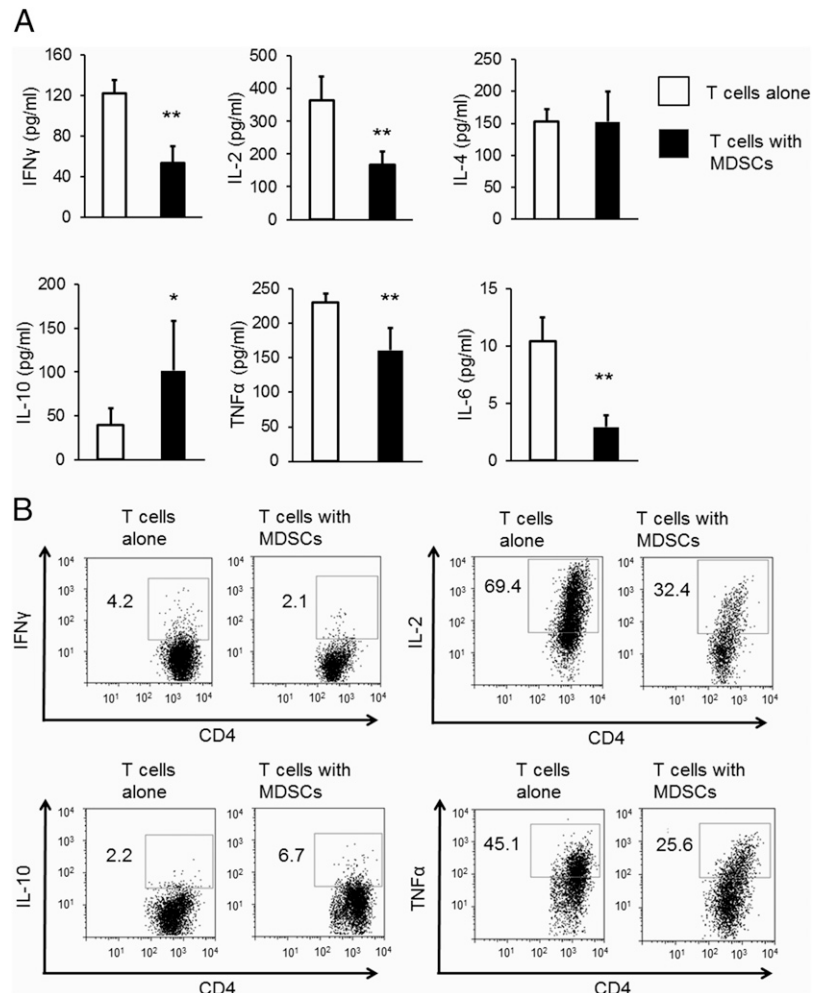
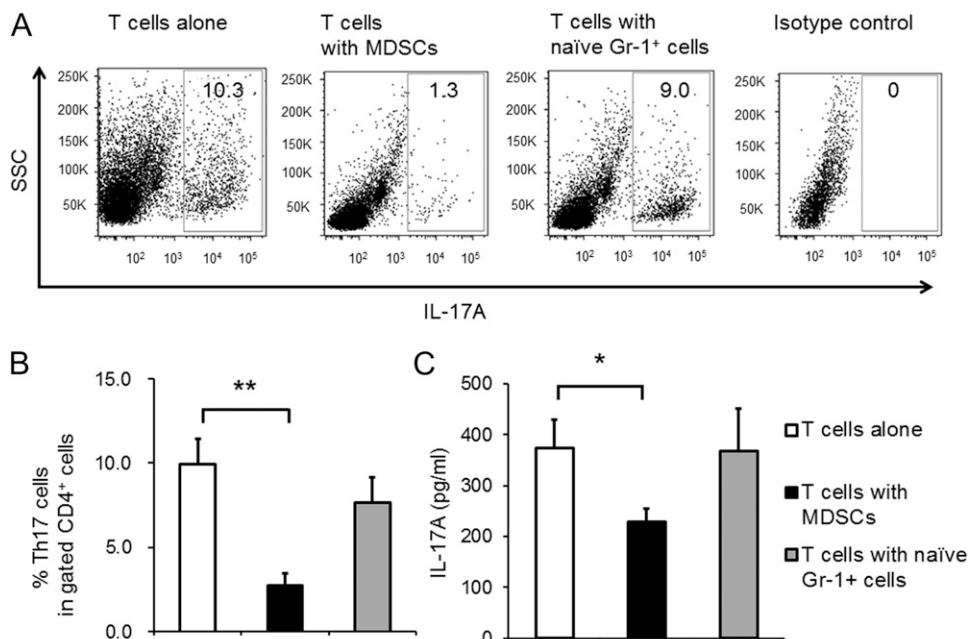


FIGURE 4. MDSCs suppress differentiation of CD4⁺ T cells into Th17 cells. CD4⁺ T cells (1×10^6) isolated from the spleens of naive mice were cultured alone or cocultured with MDSCs (1×10^6) or naive Gr-1⁺ cells (1×10^6) in Th17-skewing conditions for 6 d. Cells were stained for CD4 and IL-17A. **(A)** Representative dot plot of gated CD4⁺ cells. Gray squares indicate CD4⁺/CD17A⁺ Th17 cells, and numbers indicate the percentage of Th17 cells in the gated CD4⁺ cells. **(B)** Percentage of Th17 cells in gated CD4⁺ T cells. Means \pm SD are shown. **(C)** Concentration of IL-17A in the culture supernatant determined by ELISA. Means \pm SD are shown ($n = 3$ /group). Data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$.



were suspended in RPMI 1640 media containing 2 mM L-glutamine, 1% penicillin/streptomycin, and 10% FCS. CFSE-labeled CD4⁺ T cells (2×10^5) suspended in 100 μ l media were cultured in a 96-well flat-bottom plate with or without purified MDSCs. To proliferate CD4⁺ T cells, cells were cultured in the presence of 30 U/ml recombinant mouse IL-2 (Wako) and 2×10^5 Mouse T-Activator CD3/CD28 Dynabeads (Life Technologies, Carlsbad, CA). In some experiments, the arginase inhibitor *N*^ω-hydroxy-nor-L-arginine (Merck KGaA, Darmstadt, Germany) and/or the iNOS inhibitor *N*^G-monomethyl-L-arginine (Merck KGaA) were added to a final concentration of 0.5 mM. After 5 d of culture, the level of CD4⁺ T cell proliferation was investigated by measuring CFSE fluorescence by flow cytometry.

Measurement of cytokine levels

To measure cytokine levels in vitro, 8×10^4 purified CD4⁺ T cells were suspended in 100 μ l RPMI 1640 media containing 2 mM L-glutamine, 1% penicillin/streptomycin, and 10% FCS. Cells were cultured in a 96-well flat-bottom plate with or without 8×10^4 purified MDSCs. To activate CD4⁺ T cells, 8×10^4 Mouse T-Activator CD3/CD28 Dynabeads were added. After 3 d of culture, the levels of cytokines released into the culture supernatant were measured using ELISA kits for TNF- α , IL-10 (both from Life Technologies), IFN- γ , IL-4, and IL-6 (all from eBioscience), according to the manufacturers' protocols. To assess cytokine levels in vivo, the levels of TNF- α and IL-6 were measured in serum collected from CIA mice on day 45 using ELISA kits.

Th17 cell differentiation

CD4⁺ T cells (1×10^6) isolated from the single-cell suspension prepared from the spleens of naive mice were stimulated for 6 d in 24-well plates with or without 1×10^6 purified MDSCs or naive Gr-1⁺ cells. To stimulate differentiation into Th17 cells, CD4⁺ T cells were cultured in RPMI 1640 media containing 2 mM L-glutamine, 1% penicillin/streptomycin, 10% FCS, 1×10^6 Mouse T-Activator CD3/CD28 Dynabeads, 20 ng/ml recombinant mouse IL-6 (R&D Systems), 2.5 ng/ml recombinant human TGF- β 1 (Wako), and 10 μ g/ml cytokine neutralizing Abs against IFN- γ , IL-4, and IL-12 (all from BioLegend, San Diego, CA). After 6 d of culture, the media were removed, cells were stimulated again, and intracellular cytokine staining analysis was performed as described above. The culture supernatant was collected and ELISA for IL-17A (eBioscience) was performed according to the manufacturer's protocol.

Adoptive transfer

For adoptive transfer experiments, Gr-1⁺CD11b⁺ MDSCs were isolated from the single-cell suspension prepared from the spleens of CIA mice on day 35. These cells (2×10^6) were transferred to CIA mice through the tail vein on day 0 and day 21, during the first and second immunizations with collagen and adjuvants, respectively. PBS was injected as a control. In a separate experiment, MDSCs isolated from the spleens of CIA mice or

naive Gr-1⁺ cells isolated from the spleens of naive mice were transferred to CIA mice only on day 25 to investigate the therapeutic effects of MDSCs on established arthritis.

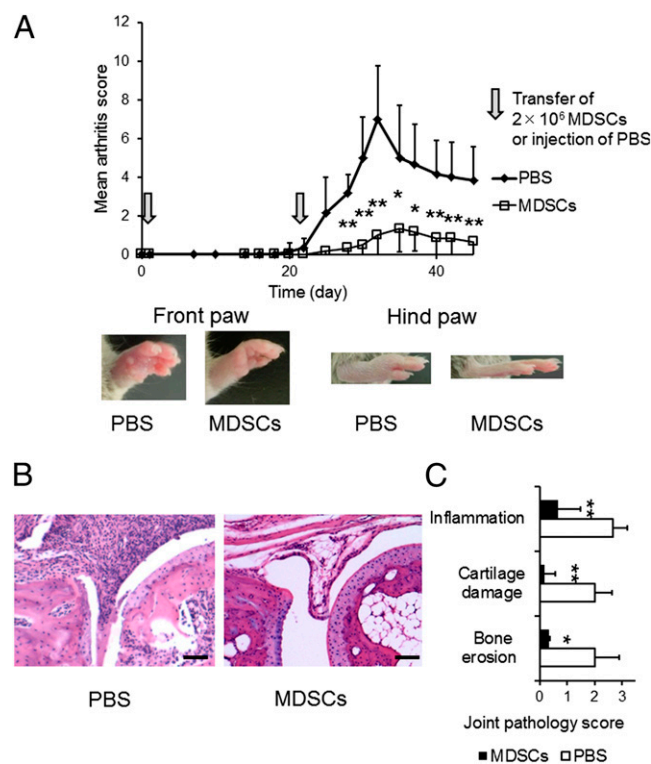


FIGURE 5. Adoptive transfer of MDSCs reduces the severity of CIA. **(A)** Clinical arthritis scores of CIA mice that were injected with PBS or received MDSCs. Means \pm SD are shown (top panels) ($n = 6$ /group). Paws of representative mice from each group are shown (bottom panels). * $p < 0.05$, ** $p < 0.01$. **(B)** Histopathology of hind paws of CIA mice on day 45. H&E-stained sections of joints from CIA mice that were injected with PBS (left panel) or received MDSCs (right panel) are shown. Scale bars, 100 μ m. Original magnification, $\times 100$. **(C)** Histopathological scores of inflammation, cartilage damage, and bone erosion in CIA on day 45 that were injected with PBS or received MDSCs. Means \pm SD are shown ($n = 6$ /group). Data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

In vivo depletion of MDSCs

For a depletion assay, 0.2 mg anti-Gr-1 mAb (RB6-8C5) or rat IgG2b isotype control (both from Bio X Cell, West Lebanon, NH) were i.p. administered to CIA mice every 3 d from day 35 after the first immunization.

Histopathology

For histological analysis, mice were sacrificed on day 45 and paws were collected and fixed in 4% buffered formaldehyde. Tissues were then paraffin embedded, sectioned, and stained with H&E. Images were acquired with a DMBA210 microscope equipped with Motic Images Plus 2.2s software. Histopathological changes were separately scored by two independent researchers in a blinded manner and then mean score was calculated using three parameters: 1) infiltration of inflammatory cells was scored based on the level of inflammatory cells in the synovial cavity and synovial tissue; 2) cartilage destruction was scored based on the appearance of dead chondrocytes and the loss of articular cartilage; and 3) bone erosion was scored based on the erosion of cortical bone. Each parameter was graded on a scale of 0–3 where 0 represents no change and 3 represents severe change.

Statistical analysis

The Student unpaired *t* test was used to analyze parametric data, and the Mann–Whitney *U* test was used to analyze clinical and histological CIA scores. The *p* values < 0.05 were considered statistically significant.

Results

MDSCs accumulate in the spleens of CIA mice

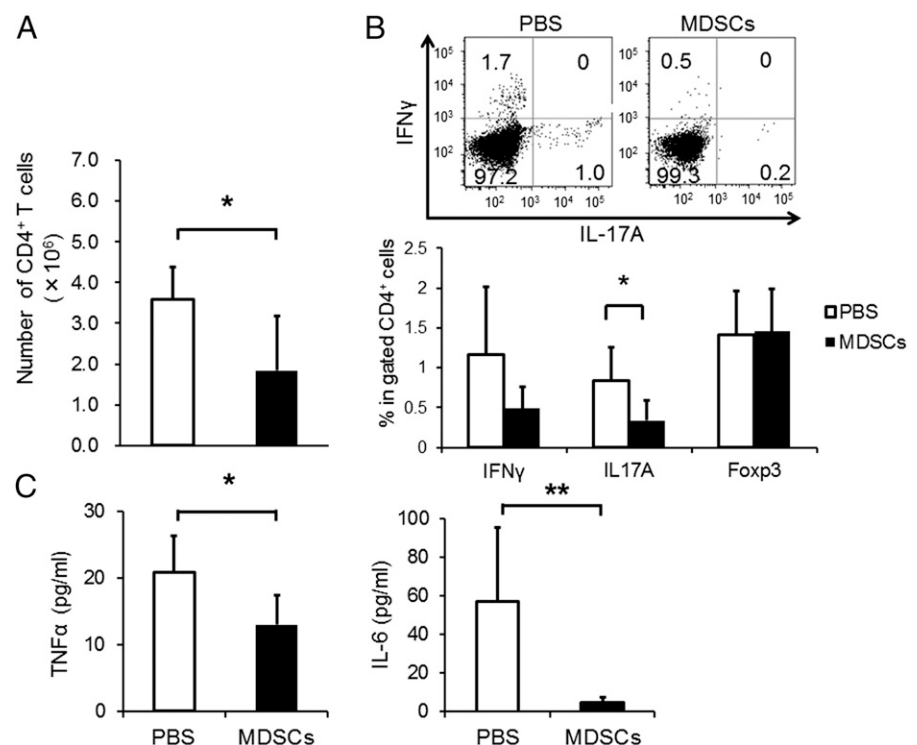
The accumulation of CD11b⁺Gr-1⁺ MDSCs in the spleens of CIA mice was investigated. Mice immunized with collagen and adjuvants first developed signs of swelling and redness in the paws on day 20, after which mean arthritis score rapidly increased, peaked, and then gradually decreased (Fig. 1A). Mice were injected with adjuvants alone (adjuvants mice) or were not injected (naive mice) as controls. Neither adjuvants mice nor naive mice showed any signs of swelling or redness in the paws. To analyze the accumulation of MDSCs, the spleens were collected on days 20, 35, and 50, when mean arthritis scores began to increase, peaked, and decreased in CIA mice, respectively. MDSCs significantly accumulated in the spleens of CIA mice on day 35 compared with

those of naive mice (Fig. 1B), and these were mainly CD11b⁺Ly6G⁺Ly6C^{low} granulocytic MDSCs (Supplemental Fig. 1). It was confirmed that these cells were negative for B220, a B cell lineage marker (Supplemental Fig. 2). Alternatively, MDSCs did not significantly accumulate in the spleens of CIA mice on day 20 or on day 50 compared with those of naive mice (Fig. 1C). MDSCs did not significantly accumulate in the spleens of adjuvants mice throughout the time course compared with those of naive mice (Fig. 1B, 1C). These results indicate that MDSCs accumulate in the spleens of CIA mice at the peak of arthritis progression, independently of adjuvant administration.

MDSCs suppress CD4⁺ T cell proliferation

To investigate the functions of MDSCs in CIA mice, magnetic cell sorting was used to isolate CD4⁺ T cells from the spleens of naive mice and MDSCs from the spleens of CIA mice on day 35. All Gr-1⁺ cells isolated were Gr-1⁺CD11b⁺ MDSCs (Supplemental Fig. 3A), which had ring-shaped nuclei, similar to immature mouse neutrophils (Fig. 2A). More than 97% of these cells were CD11b⁺Ly6G⁺Ly6C^{low} granulocytic MDSCs, according to flow cytometric analysis (Supplemental Fig. 3B). To examine the effect of MDSCs on CD4⁺ T cell proliferation, MDSCs were mixed with CD4⁺ T cells at different ratios, and CD4⁺ T cell proliferation was evaluated by a CFSE dye dilution assay. CD4⁺ T cell proliferation stimulated with anti-CD3/anti-CD28 Abs and IL-2 was significantly suppressed when MDSC/CD4⁺ T cell ratios of 1:1 to 1:4 were used; however, proliferation was maximally suppressed at a ratio of 1:1 (Fig. 2B, 2C). The suppression of CD4⁺ T cell proliferation by MDSCs was abrogated following the addition of an arginase inhibitor or an iNOS inhibitor (Fig. 2B, 2C). The MDSC suppressive function was more effectively restored by treatment with the arginase inhibitor than by treatment with the iNOS inhibitor, and there was no additive effect when these two inhibitors were used in combination. These results indicate that MDSCs inhibit CD4⁺ T cell proliferation mainly through the activity of arginase and, to a lesser extent, through the activity of iNOS.

FIGURE 6. Adoptive transfer of MDSCs in CIA mice decreases the level of CD4⁺ T cells and Th17 cells in DLNs and the level of proinflammatory cytokines in serum. **(A)** Number of CD4⁺ T cells in DLNs of a single mouse in CIA mice on day 45 that were injected with PBS or received MDSCs. Means \pm SD are shown (*n* = 6/group). **p* < 0.05. **(B)** Representative dot plots (top panel) and the percentage of IFN- γ ⁺, IL-17A⁺, and Foxp3⁺ cells of gated CD4⁺ T cells from DLNs of CIA mice that were injected with PBS or received MDSCs (bottom panel). Means \pm SD are shown (*n* = 6/group). **p* < 0.05. **(C)** Serum concentration of TNF- α (left panel) and IL-6 (right panel) in CIA mice that were injected with PBS or received MDSCs. Means \pm SD are shown (*n* = 6/group). Data are representative of three independent experiments. **p* < 0.05, ***p* < 0.01.



To exclude the possibility that the suppression of CD4⁺ T cell proliferation by MDSCs was simply due to overcrowding in the culture plates and was not due to the suppressive effect of MDSCs, CD4⁺ T cells were cocultured with naive Gr-1⁺ cells from the spleens of naive mice, which were considered to be mature neutrophils because of their mature segmented nuclei (Fig. 2B, 2C). The culture conditions were the same as those used in MDSC and CD4⁺ T cell coculture experiments. In contrast to MDSCs, naive Gr-1⁺ cells had no suppressive effect on CD4⁺ T cell proliferation (Fig. 2B, 2C). We further investigated the differences between MDSCs and nonsuppressive naive Gr-1⁺ cells. MDSCs had slightly higher expression of IL-4R α , which is reportedly associated with the suppressive function of MDSCs (3, 29), and higher expression of arginase 1 than naive Gr-1⁺ cells (Supplemental Fig. 4).

MDSCs alter the production of cytokines by CD4⁺ T cells

Next, the effects of MDSCs on the production of cytokines associated with CIA by effector CD4⁺ T cells were investigated using ELISA. The levels of IFN- γ , IL-2, TNF- α , and IL-6 in the culture supernatant of CD4⁺ T cells stimulated with anti-CD3/anti-CD28 Abs were significantly lower when cells were cocultured with MDSCs than when cultured alone, whereas the level of IL-10 increased and the level of IL-4 was not altered (Fig. 3A). None of these cytokines was detected in the culture supernatant when MDSCs were cultured alone (data not shown). It is possible that the decrease of these cytokines was due to their utilization by MDSCs rather than a block in their production by T lymphocytes. To investigate this, intracellular cytokine staining assay was performed, which revealed that the expression of IFN- γ , IL-2, and TNF- α in CD4⁺ T cells was lower when cells were cocultured with MDSCs than when they were cultured alone, whereas the expression of IL-10 was increased (Fig. 3B). These results indicate that MDSCs suppressed the production of proinflammatory cytokines and promoted the production of the anti-inflammatory cytokine IL-10 by CD4⁺ T cells.

MDSCs inhibit differentiation of CD4⁺ T cells into Th17 cells

Next, the effects of MDSCs on CD4⁺ T cell differentiation were investigated. Specifically, differentiation of CD4⁺ T cells into CD4⁺IL-17A⁺ Th17 cells was probed, a distinct CD4⁺ T cell phenotype that has been reported to exacerbate human RA (30) and mouse CIA (31, 32). When CD4⁺ T cells were cultured in Th17-skewing conditions, the number of Th17 cells generated was lower when cells were cocultured with MDSCs or than when cultured alone (Fig. 4A, 4B). Furthermore, the level of IL-17A in the culture supernatant was significantly lower when CD4⁺ T cells were cocultured with MDSCs than when cultured alone (Fig. 4C). In contrast, none of these effects was observed when CD4⁺ T cells were cultured with naive Gr-1⁺ cells isolated from the spleens of naive mice (Fig. 4). These results indicate that MDSCs inhibit Th17 cell differentiation.

Adoptive transfer of MDSCs reduces the severity of CIA

These in vitro experiments indicate that MDSCs inhibit the proinflammatory response by CD4⁺ T cells; thus, it was hypothesized that MDSCs have regulatory roles in CIA. To investigate this hypothesis, MDSCs isolated from the spleens of CIA mice at the peak of arthritis were adoptively transferred to CIA mice days 0 and 21, during the first and second immunizations, respectively. The clinical courses of CIA mice with or without MDSC transfer were compared. The mean arthritis scores of CIA mice were significantly lower following adoptive transfer of MDSCs than after PBS injection of CIA (Fig. 5A). Adoptive transfer of MDSCs markedly reduced several histopathological features of arthritis,

namely inflammation, cartilage damage, and bone erosion (Fig. 5B, 5C). These improvements in the severity of CIA were accompanied by a significant decrease in the number of CD4⁺ T cells and the percentage of Th17 cells in the draining lymph nodes (DLNs) (Fig. 6A, 6B). The percentage of CD4⁺IFN- γ ⁺ Th1 cells in the DLNs of CIA mice tended to be lower following adoptive transfer of MDSCs than after PBS injection; however, the difference was not statistically significant. The percentage of CD4⁺Foxp3⁺ regulatory T cells (Tregs) was similar in the DLNs of CIA mice that received MDSCs or PBS. Serum cytokine levels of TNF- α and IL-6 in CIA mice were significantly lower following adoptive transfer of MDSCs than after PBS injection (Fig. 6C).

These results suggest that the MDSCs play regulatory roles in CIA. This may be due to MDSCs suppressing CD4⁺ T cell proliferation, differentiation of CD4⁺ T cells into Th17 cells, and proinflammatory cytokine production by CD4⁺ T cells. However, because CIA mice received MDSCs before CIA developed, it is possible that MDSCs do not modulate established arthritis but rather prevent the pathogenesis of CIA. To investigate the therapeutic effect of MDSCs on established arthritis, MDSCs or control naive Gr-1⁺ cells were transferred to CIA mice only on day 25, after the onset of arthritis. The severity of arthritis in CIA mice was lower following adoptive transfer of MDSCs than after PBS injection (Fig. 7); however, the reduction of the severity of ar-

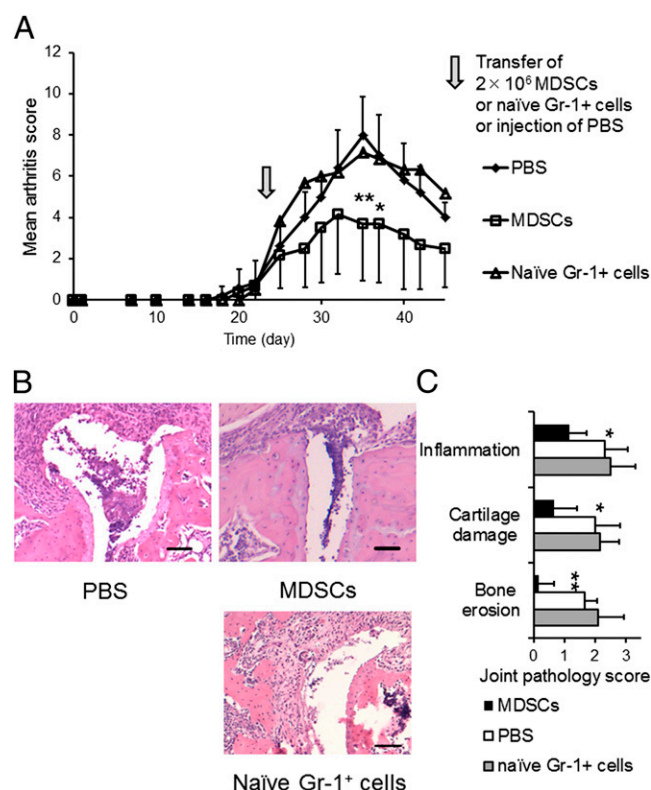
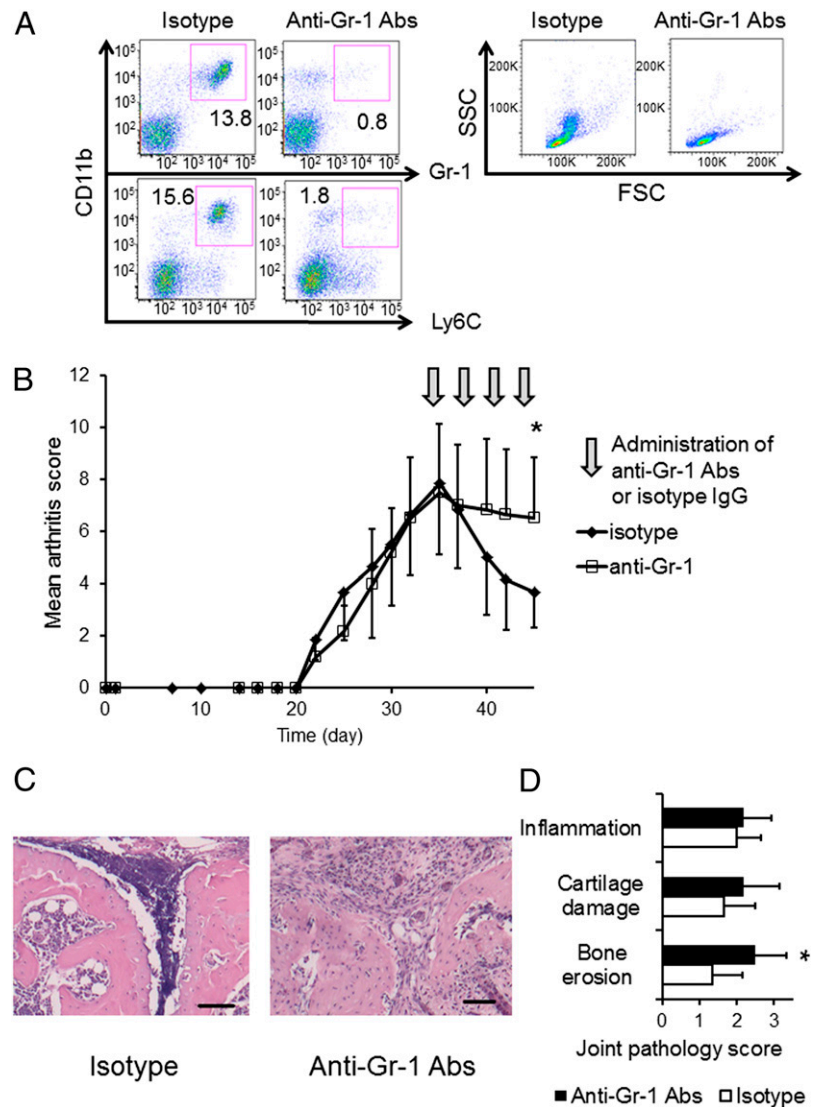


FIGURE 7. Adoptive transfer of MDSCs reduces the severity of established CIA. (A) Clinical arthritis scores of CIA mice that were injected with PBS or that received MDSCs or naive Gr-1⁺ cells on day 25. Means \pm SD are shown ($n = 6$ /group). * $p < 0.05$, ** $p < 0.01$. (B) Histopathology of hind paws of CIA mice on day 45. H&E-stained sections of joints from CIA mice that were injected with PBS (left panel) or that received MDSCs (upper right panel) or naive Gr-1⁺ cells (lower right panel) on day 25 are shown. Scale bars, 100 μ m. Original magnification, $\times 100$. (C) Histopathological scores of inflammation, cartilage damage, and bone erosion in CIA on day 45 that were injected with PBS or that received MDSCs or naive Gr-1⁺ cells on day 25. Means \pm SD are shown ($n = 6$ /group). Data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

FIGURE 8. Depletion of MDSCs abrogates the spontaneous improvement of CIA. **(A)** Representative dot plots of CIA mice that were i.p. administrated neutralizing Abs against Gr-1 or rat isotype IgG2b are shown. Red squares represent Gr-1⁺CD11b⁺ (upper left panels) and Ly6C⁺CD11b⁺ MDSCs (lower left panels), and numbers indicate the percentage of these cells in living splenocytes. Analysis of living splenocytes by forward and side scatter is shown in the right panels. **(B)** Clinical arthritis scores of CIA mice that were i.p. administrated neutralizing Abs against Gr-1 or rat isotype IgG2b every 3 d from day 35 after the first immunization. Means \pm SD are shown ($n = 6$ /group). * $p < 0.05$. **(C)** Histopathology of hind paws of CIA mice on day 45. H&E-stained sections are shown of joints from CIA mice that were i.p. administrated rat isotype IgG2b (left panel) or with neutralizing Abs against Gr-1 (right panel) every 3 d from day 35 on after first immunization. Scale bars, 100 μ m. Original magnification, $\times 100$. **(D)** Histopathological scores of inflammation, cartilage damage, and bone erosion in CIA on day 45 that were administrated neutralizing Abs against Gr-1 or rat isotype IgG2b. Means \pm SD are shown ($n = 6$ /group). Data are representative of three independent experiments. * $p < 0.05$.



thrititis was not as marked as when mice received MDSCs prophylactically. In contrast, naive Gr-1⁺ cells isolated from the spleens of naive mice had no therapeutic effect (Fig. 7). These results indicate that MDSCs prevent the pathogenesis of arthritis and reduce the severity of established arthritis.

Depletion of MDSCs abrogates the spontaneous improvement of CIA

Furthermore, to validate the regulatory roles of MDSCs in CIA, MDSCs were depleted using neutralizing Abs against Gr-1 (33). Rat isotype IgG2b was used as a control. Depletion of MDSCs in the spleens of CIA mice was confirmed by flow cytometry using anti-Gr-1 or anti-Ly6C Abs, as well as analysis of forward scatter/side scatter to exclude the possibility that the affinity of the anti-Gr-1 Ab was impaired by the neutralizing Ab that recognizes the same epitope (Fig. 8A). Depletion of MDSCs did not increase the severity of CIA; however, the spontaneous improvement of CIA was abrogated (Fig. 8B–D). These results indicate that MDSCs that accumulate in CIA play roles in the mechanism that underlies spontaneous improvement after the peak of the disease.

Discussion

As for MDSCs in autoimmune arthritis, it was recently reported that MDSCs produced during proteoglycan-induced arthritis suppress

T cell proliferation in vitro (23); however, the roles of MDSCs in autoimmune arthritis in vivo are unknown. In this study, we demonstrated that MDSCs play regulatory roles in autoimmune experimental arthritis. MDSCs in the spleens of CIA mice suppressed CD4⁺ T cell proliferation and proinflammatory cytokine production in response to Ag-nonspecific stimulation. MDSCs in several tumor models were reported to suppress Ag-specific T cell

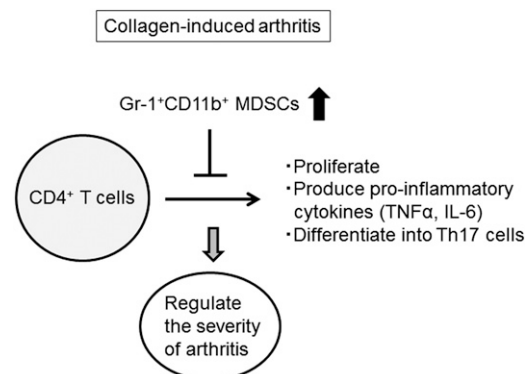


FIGURE 9. Working model to explain the roles of MDSCs in CIA mouse models.

proliferation (3); however, MDSCs in autoimmune disease models were reported to suppress T cell proliferation even in response to Ag-nonspecific stimulation (17), which is consistent with our results. It is possible that the mechanisms of MDSCs suppressing T cell functions differ between tumors and autoimmune diseases.

MDSCs in cancer patients are one of main immunosuppressive factors that promote tumor progression (4, 5); however, the functions of MDSCs in patients with autoimmune diseases remain unclear. Conflicting studies have reported that MDSCs decrease (17, 19) and increase (16, 18) the severity of autoimmune diseases in animal models. MDSCs may have different functions in different conditions. Because CD4⁺ T cells are essential for the pathogenesis of CIA (34), we hypothesized that MDSCs have a disease regulatory role in CIA. Indeed, adoptive transfer of MDSCs reduced the severity of CIA, and depletion of MDSCs abrogated the spontaneous improvement of CIA. T cells are thought to be involved in the pathogenesis of human RA (35), and T cell-targeted therapies are clinically used for RA (36, 37). Therefore, the suppressive effects of MDSCs on CD4⁺ T cells could be exploited in novel therapies for RA.

Th17 cells, a distinct subset of CD4⁺ T cells, play an important role in host defense against specific pathogens, potentially induce autoimmunity and tissue inflammation (38), and have been reported to exacerbate autoimmune arthritis (30–32). Because little is known about the effects of MDSCs on Th17 cells, we investigated whether MDSCs affect Th17 cell differentiation. Th17 cell differentiation was suppressed when CD4⁺ T cells were cocultured with MDSCs in vitro, and adoptive transfer of MDSCs to CIA mice reduced the proportion of Th17 cells in the DLNs. In contrast, a previous study reported that MDSCs promote Th17 cell differentiation (18). The previous study used a disease model of experimental autoimmune encephalomyelitis and monocytic MDSCs, whereas the present study focused on CIA and used granulocytic MDSCs; these differences may underlie the discrepancies between the two studies.

Conflicting studies report that MDSCs contribute to the generation of Tregs (39, 40) and impair Treg differentiation (41). In the present study, adoptive transfer of MDSCs to CIA mice did not affect the percentage of Tregs in DLNs. The effects of MDSCs on Tregs need to be clarified by future studies.

Adoptive transfer of MDSCs reduced the severity of arthritis in CIA mice. However, MDSCs spontaneously accumulated in the spleens of CIA mice at the peak of the disease. We hypothesize that MDSCs accumulate in the spleens of CIA mice at the peak of arthritis and reduce the severity of CIA via a negative feedback mechanism (Fig. 9). Indeed, arthritis scores peaked and then spontaneously decreased in CIA mice following proliferation of MDSCs. MDSCs may play a role in the mechanism(s) responsible for the spontaneous improvement of CIA. This hypothesis was validated by depletion of MDSCs, which abrogated the spontaneous improvement of CIA (Fig. 8).

It was reported that the suppressive effect of MDSCs in malignant tumors is due to monocytic MDSCs rather than to granulocytic MDSCs (42). However, it was also reported that granulocytic MDSCs have suppressive functions in autoimmune disease models (17). It is possible that the phenotypes of MDSCs responsible for suppressing T cell functions differ between tumors and autoimmune diseases. Recently, it was reported that neutrophils have regulatory roles in the infection (43, 44) and in tumor models (45). Because the cell surface Ags (Gr-1 and CD-11b) and the morphology of granulocytic MDSCs are similar to neutrophils, we further investigated the difference between MDSCs in CIA mice and neutrophils in naive mice. Granulocytic MDSCs had slightly higher expression of IL-4R α and higher expression of arginase 1

than did naive neutrophils. These differences might be associated with the different functions of these cells. However, the differences were only marginal and further studies are required to further elucidate how granulocytic MDSCs differ from neutrophils. It is possible that MDSCs are a distinct myeloid cell population or a population of neutrophils that have regulatory functions in pathologic conditions.

The stimuli that induce MDSC proliferation during CIA remain unknown; however, many factors are reported to induce MDSC proliferation in tumor models. The level of TNF- α is elevated in CIA (46) and human RA (47), and TNF- α is a key cytokine in the development of these diseases. Moreover, TNF- α induces MDSC proliferation in s.c. tumor models (48) and chronic inflammation models (49). The level of GM-CSF is elevated in experimental inflammatory arthritis (50), and GM-CSF is critical for the proliferation of MDSCs in cancer (51). MDSCs may accumulate in response to increased level of TNF- α and/or GM-CSF in CIA.

In conclusion, this study shows that MDSCs play crucial roles in the regulation of CIA, a mouse model of autoimmune arthritis, by suppressing the proinflammatory immune responses of CD4⁺ T cells. Although further investigation is needed to determine whether the observed results in CIA mice are replicated in human RA, these results could be used to develop novel cell-based therapies for human RA.

Acknowledgments

We thank Drs. Yasuo Miura, Hisayuki Yao, Satoshi Yoshioka, Yoshihiro Hayashi, Akihiro Tamura, and Asumi Yokota in the Department of Transfusion Medicine and Cell Therapy of Kyoto University Hospital for valuable discussions. We also thank Satoko Adachi in the Department of Molecular Gastroenterology and Hepatology of Kyoto Prefectural University of Medicine for excellent technical assistance. We are grateful to Dr. Yoshinori Marunaka and Mariko Ohta in the Department of Molecular Cell Physiology of Kyoto Prefectural University of Medicine for valuable advice.

Disclosures

The authors have no financial conflicts of interest.

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