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Adoptive Transfer of Human Gingiva-derived Mesenchymal Stem Cells Ameliorates Collagen-induced Arthritis via Suppression of Th1 and Th17 Cells and Enhancement of Regulatory T Cell Differentiation

Abstract

Objective Current approaches offer no cures for rheumatoid arthritis (RA). Accumulating evidence has revealed that manipulation of bone marrow-derived mesenchymal stem cells (BM-MSCs) may have the potential to control or even prevent RA, but BM-MSC-based therapy faces many challenges, such as limited cell availability and reduced clinical feasibility. This study in mice with established collagen-induced arthritis (CIA) was undertaken to determine whether substitution of human gingiva-derived mesenchymal stem cells (G-MSCs) would significantly improve the therapeutic effects. Methods CIA was induced in DBA/1J mice by immunization with type II collagen and Freund's complete adjuvant. G-MSCs were injected intravenously into the mice on day 14 after immunization. In some experiments, intraperitoneal injection of PC61 (anti-CD25 antibody) was used to deplete Treg cells in arthritic mice. Results Infusion of G-MSCs in DBA/1J mice with CIA significantly reduced the severity of arthritis, decreased the histopathology scores, and down-regulated the production of inflammatory cytokines (interferon- γ and interleukin-17A). Infusion of G-MSCs also resulted in increased levels of CD4+CD39+FoxP3+ cells in arthritic mice. These increases were noted early after infusion in the spleens and lymph nodes, and later after infusion in the synovial fluid. The FoxP3+ Treg cells that were increased in frequency mainly consisted of Helios-negative cells. When Treg cells were depleted, infusion of G-MSCs partially interfered with the progression of CIA. Pretreatment of G-MSCs with a CD39 or CD73 inhibitor significantly reversed the protective effect of G-MSCs on CIA. Conclusion The role of G-MSCs in controlling the development and severity of CIA mostly depends on CD39/CD73 signals and partially depends on the induction of CD4+CD39+FoxP3+ Treg cells. G-MSCs provide a promising approach for the treatment of autoimmune diseases. Copyright © 2013 by the American College of Rheumatology.

Keywords

5'-Nucleotidase, Animals, Antigens, CD, Apyrase, Arthritis, Experimental, Cell Differentiation, Female, Gingiva, GPI-Linked Proteins, Humans, Immunotherapy, Adoptive, Mesenchymal Stem Cell Transplantation, Mesenchymal Stromal Cells, Mice, Mice, Inbred DBA, Signal Transduction, T-Lymphocytes, Regulatory, Th1 Cells, Th17 Cells, 5' nucleotidase, beta1 integrin, CD25 antibody, CD39 antigen, collagen type 2, endoglin, Freund adjuvant, gamma interferon, Hermes antigen, interleukin 10, interleukin 13, interleukin 17, interleukin 4, interleukin 5, lymphocyte antibody, transcription factor FOXP3, tumor necrosis factor alpha, unclassified drug, adoptive transfer, animal experiment, animal model, animal tissue, arthritis, article, CD4+ CD39+ FoxP3+ T lymphocyte, controlled study, cytokine production, disease severity, down regulation, female, gingiva, gingiva derived mesenchymal stem cell, histopathology, human, human cell, in vitro study, lymph node, lymphocyte differentiation, lymphocyte proliferation, mesenchymal stem cell, mouse, nonhuman, priority journal, regulatory T lymphocyte, signal transduction, spleen, synovial fluid, T cell depletion, T lymphocyte, Th1 cell, Th17 cell

Disciplines

Dentistry | Periodontics and Periodontology

Comments

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Adoptive Transfer of Human Gingiva-Derived Mesenchymal Stem Cells Ameliorates Collagen-Induced Arthritis via Suppression of Th1 and Th17 Cells and Enhancement of Regulatory T Cell Differentiation

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Objective. Current approaches offer no cures for rheumatoid arthritis (RA). Accumulating evidence has revealed that manipulation of bone marrow-derived mesenchymal stem cells (BM-MSCs) may have the potential to control or even prevent RA, but BM-MSC-based therapy faces many challenges, such as limited cell availability and reduced clinical feasibility. This study in mice with established collagen-induced arthritis (CIA) was undertaken to determine whether substi-

tution of human gingiva-derived mesenchymal stem cells (G-MSCs) would significantly improve the therapeutic effects.

Methods. CIA was induced in DBA/1J mice by immunization with type II collagen and Freund's complete adjuvant. G-MSCs were injected intravenously into the mice on day 14 after immunization. In some experiments, intraperitoneal injection of PC61 (anti-CD25 antibody) was used to deplete Treg cells in arthritic mice.

Results. Infusion of G-MSCs in DBA/1J mice with CIA significantly reduced the severity of arthritis, decreased the histopathology scores, and down-regulated the production of inflammatory cytokines (interferon- γ and interleukin-17A). Infusion of G-MSCs also resulted in increased levels of CD4+CD39+FoxP3+ cells in arthritic mice. These increases were noted early after infusion in the spleens and lymph nodes, and later after infusion in the synovial fluid. The FoxP3+ Treg cells that were increased in frequency mainly consisted of Helios-negative cells. When Treg cells were depleted, infusion of G-MSCs partially interfered with the progression of CIA. Pretreatment of G-MSCs with a CD39 or CD73 inhibitor significantly reversed the protective effect of G-MSCs on CIA.

Conclusion. The role of G-MSCs in controlling the development and severity of CIA mostly depends on CD39/CD73 signals and partially depends on the induction of CD4+CD39+FoxP3+ Treg cells. G-MSCs provide a promising approach for the treatment of autoimmune diseases.

Rheumatoid arthritis (RA) is a symmetric polyarticular arthritis that primarily affects the small di-

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arthrodial joints (1). Clinical drug development for treatment of RA has progressed slowly. Currently, only ~50% of RA patients show a response to treatment with most of the available products, such as tumor necrosis factor (TNF) inhibitors, interleukin-1 (IL-1) antagonists, and anti-IL-6 receptor antibody. None of these treatments are curative for RA (1). Therefore, novel approaches to cure this disease are sorely needed.

Mesenchymal stem cells (MSCs) can exhibit immunomodulatory effects. They inhibit T cell proliferation in mixed lymphocyte cultures, prolong skin allograft survival, and decrease graft-versus-host disease (GVHD) when cotransplanted with hematopoietic stem cells (2). These properties make them well-suited to serve as a candidate for a new approach in the prevention and treatment of allograft rejection, GVHD, and other autoimmune diseases. Bone marrow-derived MSCs (BM-MSCs) have been considered as a potential strategy in clinical cell therapy; however, there are some drawbacks and limitations to their clinical feasibility, such as the difficulty in obtaining sufficient cell numbers for therapeutic use.

Findings of a recent study confirmed that gingiva-derived MSCs (G-MSCs), a population of stem cells that exists in the human gingival tissue (3), have several advantages over BM-MSCs. G-MSCs are easy to isolate, are homogeneous, and proliferate more rapidly than BM-MSCs (4). In addition, G-MSCs display stable morphologic and functional characteristics at higher passage numbers and are not tumorigenic (4). Although G-MSCs demonstrate beneficial effects in preventing experimental colitis (3) and mitigating chemotherapy-induced oral mucositis (5), utilization of G-MSCs for the treatment of autoimmune arthritis and other immune diseases has not been explored.

Recent studies have demonstrated that adoptive transfer of MSCs can up-regulate CD4+CD25+FoxP3+ Treg cells in vivo (6,7). Treg cells play an important role in the prevention and control of experimental autoimmune arthritis, an animal model that shares many of the features of RA (8,9). However, it is less clear what role Treg cells may play in the suppressive effect of MSCs on immune responses. Deaglio and colleagues demonstrated that the coexpression of CD39 (NTPDase1) and CD73 (ecto-5'-nucleotidase) in Treg cells contributes to their inhibitory function (10). CD39 promotes the hydrolysis of ATP and ADP to generate AMP, which is then hydrolyzed by CD73 to adenosine. ATP is an important signaling molecule involved in many biologic processes, including immune responses. Although MSCs are known to express CD73, it is unclear whether they also express CD39, and it remains

to be determined whether either of these ectoenzymes participates in the immunoregulatory function of MSCs.

In the present study, we demonstrate that G-MSCs can significantly attenuate inflammatory arthritis in an experimental collagen-induced arthritis (CIA) model. The therapeutic effects of G-MSCs appear to be mainly dependent on CD39/CD73 signals. We also found that their effects are dependent, at least in part, on the in vivo induction and expansion of Treg cells, a cell type that has been recognized as playing an important role in controlling autoimmunity (11–14). These results imply that manipulation of G-MSCs may provide a promising therapeutic approach for the treatment of patients with RA and other autoimmune diseases.

MATERIALS AND METHODS

Mice. DBA/1J mice (all female, ages 8–10 weeks) were obtained from The Jackson Laboratory. C57BL/6 mice transfected with a green fluorescent protein (GFP)-FoxP3 reporter construct (C57BL/6-FoxP3^{gfp} mice) were generously provided by Dr. Talil Chatilla (University of Southern California, Los Angeles). DBA/1J-FoxP3^{gfp} mice were produced by backcrossing C57BL/6-FoxP3^{gfp} mice with DBA/1J mice for 8–10 generations. All experiments using mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Southern California.

Induction of arthritis. Bovine type II collagen (CII) was extracted and purified from bovine articular cartilage according to established protocols. CII was emulsified with an equal volume of Freund's complete adjuvant (CFA) containing 4 mg/ml heat-denatured *Mycobacterium* (Chondrex). DBA/1J mice or DBA/1J-FoxP3^{gfp} mice were immunized via intradermal injection at the base of the tail with 50 μ l of emulsion (100 μ g CII/mouse). To determine intervention effects, mice received a single intravenous injection of 2×10^6 G-MSCs on day 14 after immunization. Alternatively, a similar dose of human dermal fibroblasts (a cell line from American Type Culture Collection) was injected intravenously as a control. To deplete CD4+CD25+FoxP3+ Treg cells, mice were treated intraperitoneally (IP) with 0.25 mg of anti-CD25 antibody (clone PC61) 7 days after CII immunization.

Evaluation for clinical arthritis. Clinical signs of arthritis were evaluated every 2–3 days after immunization to determine arthritis incidence. Each paw was evaluated and scored individually for severity of arthritis using a previously described scoring system (scale 0–4) (15–17). The scores for each paw were summed to yield a total arthritis severity score per mouse, with a maximum score of 16 for each animal. Each paw score was judged as follows: 0 = no signs of arthritis, 1 = mild swelling confined to the tarsal bones or ankle joint, 2 = mild swelling extending from the ankle to the tarsal bones, 3 = moderate swelling extending from the ankle to the metatarsal joints, and 4 = severe swelling encompassing the ankle, foot, and digits, or ankylosis of the limb.

Histopathologic evaluation of the joints. After the mice were killed on day 60, the hind limbs were collected. Following routine fixation, decalcification, and paraffin em-

bedding, tissue sections were prepared and stained with hematoxylin and eosin. All slides were evaluated by investigators who were blinded with regard to the experimental conditions. The extent of synovitis, pannus formation, and bone/cartilage destruction was determined using a graded scale, as follows: grade 0 = no signs of inflammation, 1 = mild inflammation with hyperplasia of the synovial lining without cartilage destruction, 2–4 = increasing degrees of inflammatory cell infiltration and cartilage/bone destruction.

Flow cytometric analysis. Ice-cooled single-cell suspensions were prepared from trypsinized G-MSC cultures, G-MSCs cocultured with mouse T cells, or mouse lymphoid organs. For G-MSC phenotype identification, we used antibodies directed against human CD11b, CD29, CD45, CD73, CD86, CD90, class II major histocompatibility complex (MHC), or isotype-matched control IgG (all from BD PharMingen), and against human CD31, CD34, CD44, CD105, class I MHC, or isotype-matched IgG (all from eBioscience). Antibodies against CD4 (RM4-5), interferon- γ (IFN γ), IL-4, and IL-17 were from eBioscience. Antibodies to Helios and CD39 were from Biolegend. Synovial fluid from 2 knee joints of each mouse with arthritis was collected and flushed out using 10 ml phosphate buffered saline via a 25-gauge needle. This method usually yields $1\text{--}6 \times 10^4$ cells from normal mice and $3\text{--}10 \times 10^4$ cells from arthritic mice. For evaluation of mouse Treg cells *in vivo*, results were obtained on a BD FACSCalibur flow cytometer and analyzed using FlowJo.

Cytokine analysis. T cells were isolated from the spleens and draining lymph nodes of arthritic mice on day 60 after CII immunization, and then stimulated *in vitro* with phorbol myristate acetate (PMA) (50 ng/ml) and ionomycin (500 ng/ml) for 5 hours, with brefeldin A (10 μ g/ml) (all from Calbiochem) for 4 hours, and intracellular expression of IL-4, IL-17, IFN γ , TNF α , IL-2, and IL-10 was analyzed by flow cytometry.

Murine naive CD4+ T cell differentiation *in vitro*. Naive CD4+CD25⁻CD62L+ T cells were purified from the spleens of DBA/1 mice via magnetic isolation (Miltenyi Biotec). G-MSCs were cocultured with naive CD4+CD25⁻CD62L+ T cells (1:25) during their *in vitro* differentiation into T helper cells. G-MSCs were allowed to adhere to the plate overnight before coculture. Naive CD4 cells were stimulated with anti-CD3 (2 μ g/ml) and anti-CD28 (2 μ g/ml) antibodies (both from Biolegend) in the presence of irradiated (30 cGy) syngeneic non-T cells, along with cytokines for Th1, Th2, or Th17 cell polarization differentiation, as previously described (18). After 3 days in culture, differentiated cells were restimulated with PMA and ionomycin for 5 hours and brefeldin A for 4 hours. The expression of IFN γ , IL-4, and IL-17 was then measured by flow cytometry.

***In vitro* suppression assays.** To examine the suppressive activity of G-MSCs *in vitro*, mouse splenic T cells, which were isolated from DBA/1J mice using nylon wool, or splenic CD4+CD25⁻ cells, which were isolated from DBA/1J mice using magnetic isolation, were stimulated with anti-CD3 antibody (0.025 μ g/ml) and irradiated (30 cGy) antigen-presenting cells (APCs). G-MSCs were plated in triplicate in 96-well plates and allowed to adhere to the plate overnight. The ratio of G-MSCs to mouse CD4+CD25⁻ T cells ranged from 1:1 to 1:200. Cells were cultured for 3 days and 1 μ Ci/well of ³H-thymidine was added for the last 18 hours of culture, as previously reported (19).

To assess the possibility that G-MSCs may induce mouse T cell death, CD4+CD25⁻ T cells labeled with 5,6-carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) were stimulated with soluble anti-CD3 antibody (0.025 μ g/ml) along with irradiated non-T cells as APCs (1:1). A gradient of G-MSCs was added to the CD4+CD25⁻ T responder cells at a ratio of 1:1–1:200, and suppression of cycling CFSE-labeled CD4+CD25⁻ T cells was assessed on the gate of CFSE-labeled CD4+ cells negative for 7-aminoactinomycin D (7-AAD).

To determine the dependence of the suppressive function of G-MSCs on cell contact, a Transwell system was used. Briefly, these experiments were performed in 24-well Transwell plates with 0.4- μ m pore membranes (Corning Costar). Mouse CD4+CD25⁻ cells (1×10^6) and irradiated APCs (1×10^6) were seeded to the upper compartment of the chamber, while G-MSCs (2×10^5) were seeded to the lower compartment. Cells were cultured in the presence of anti-CD3 antibody for 72 hours and analyzed as described above.

In some experiments, mouse CD4+CD25⁻ T cells were cocultured with G-MSCs (1:25) and stimulated with anti-CD3 antibody (0.025 μ g/ml) in the presence of soluble factors, including a CD39 inhibitor (100 μ M sodium polyoxotungstate 1 [POM-1]; Tocris Bioscience), CD73 inhibitor (100 μ M α,β -methylene ADP [APCP]; Sigma-Aldrich), selective A_{2A} adenosine receptor competitive antagonist (25 μ M SCH58261; Tocris Bioscience), selective A_{2B} adenosine receptor antagonist (10 μ M alloxazine; Sigma-Aldrich), heme oxygenase 1 (HO-1) inducer (50 ng/ml hemin; Sigma-Aldrich), selective HO-1 inhibitor (50 ng/ml zinc protoporphyrin IX; Frontier Scientific), selective cyclooxygenase 1 (COX-1) inhibitor (20 μ M indomethacin; Sigma-Aldrich), indoleamine 2,3-dioxygenase (IDO) inhibitor (500 μ M L-1-methyltryptophan; Sigma-Aldrich), nitric oxide (NO) synthase inhibitor (1 mM L-N^G-nitroarginine methyl ester hydrochloride; Sigma-Aldrich), selective COX-2 inhibitor (10 μ M NS398; Tocris Bioscience), anti-transforming growth factor β (anti-TGF β) antibody (10 μ g/ml; BD PharMingen), or anti-IL-10 receptor antibody (anti-IL-10R) (10 μ g/ml; R&D Systems). Proliferation was determined with ³H-thymidine incorporation.

Statistical analysis. For comparison of treatment groups, we performed unpaired *t*-tests (Mann-Whitney), paired *t*-tests, and one-way or two-way analysis of variance (where appropriate). Comparisons of percentages were done using the chi-square test. All statistical analyses were performed using GraphPad Prism software (version 4.01). *P* values less than 0.05 were considered significant.

RESULTS

Suppression of mouse T cell proliferation and differentiation by G-MSCs through CD39/CD73 signals.

We and other investigators have recently shown that G-MSCs display immunomodulatory properties similar to those of human BM-MSCs, including inhibition of the activation and proliferation of human T cells (3,4,20,21). To determine whether G-MSCs have immunosuppressive effects on mouse CD4+ T lymphocytes in response to T cell receptor stimulation *in vitro*, we cocultured these cells and found that the G-MSCs inhibited the

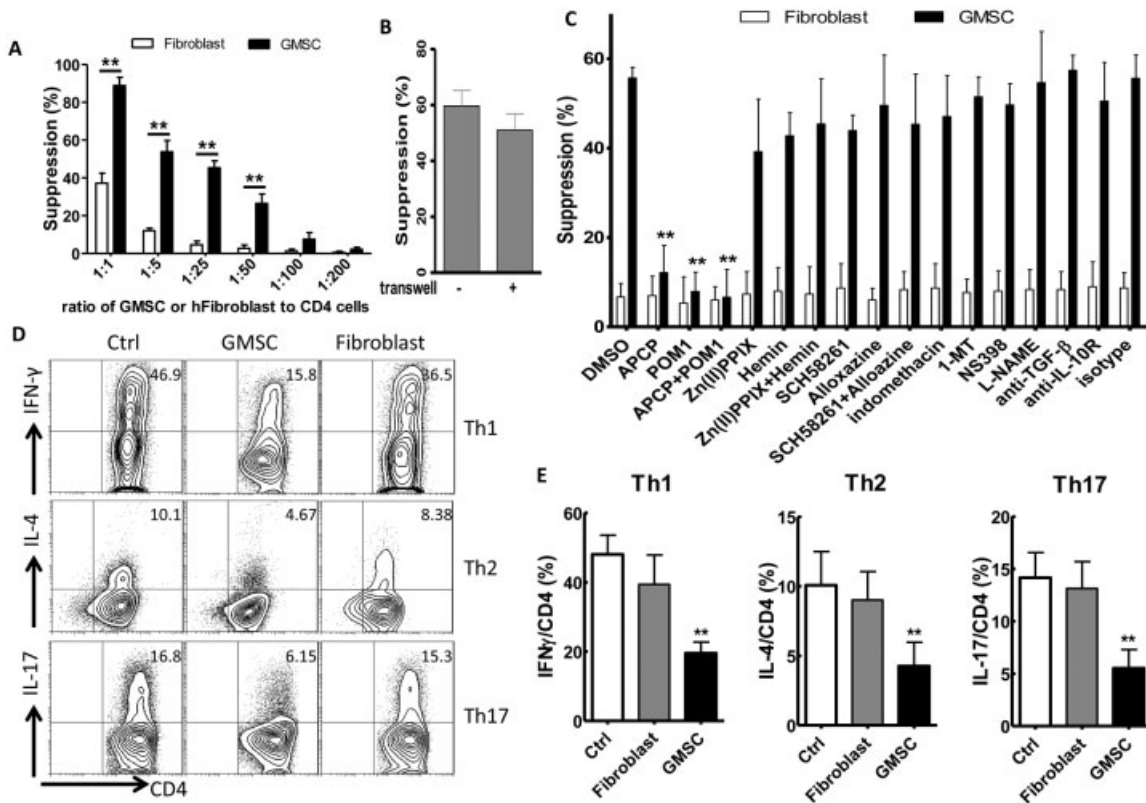


Figure 1. Gingiva-derived mesenchymal stem cells (G-MSCs) inhibit the proliferation and differentiation of mouse CD4⁺ T lymphocytes in vitro. **A** and **B**, Mouse CD4⁺ T cells labeled with 5,6-carboxyfluorescein succinimidyl ester (CFSE) were cocultured with gradient doses of G-MSCs (or a human fibroblast cell line [hFibroblast] as control) and stimulated with soluble anti-CD3 and irradiated non-T cells for 72 hours. Suppression of CD4⁺ T cell proliferation was analyzed by flow cytometry (gated on live cells, defined as CFSE-labeled CD4⁺ T cells negative for 7-aminoactinomycin D) (**A**). Transwell assays were used to assess suppression of CD4⁺ T cell proliferation in cultures without or with G-MSCs (1:5 ratio of G-MSCs to CD4⁺ T cells) (**B**). **C**, CFSE-labeled mouse CD4⁺ T cells were cocultured with G-MSCs or human fibroblasts (1:25 ratio of G-MSCs or fibroblasts to CD4⁺ T cells) in the presence of soluble factors (α,β -methylene ADP [APCP], sodium polyoxotungstate 1 [POM-1], zinc protoporphyrin IX [Zn(II)PPIX], heme oxygenase 1 inducer [hemin], SCH58261, alloxazine, indomethacin, L-1-methyltryptophan [1-MT], NS398, L-N^G-nitroarginine methyl ester hydrochloride [L-NAME], anti-transforming growth factor β [anti-TGF β], or anti-interleukin-10 receptor [anti-IL-10R]; DMSO and isotype used as controls), and suppression of CD4⁺ T cell proliferation was determined. **D** and **E**, Naive CD4⁺ T cells were cultured without (control) or with G-MSCs or human fibroblasts (1:25 ratio of G-MSCs or fibroblasts to CD4⁺ T cells) for 3 days under Th1, Th2, or Th17 cell-polarization conditions. Expression of intracellular cytokines (interferon- γ [IFN γ], IL-4, and IL-17) in each T helper cell subset was analyzed by flow cytometry (representative data shown) (**D**), with results also expressed quantitatively (**E**). Bars show the mean \pm SEM of 3 separate experiments. In **A**, **C**, and **E**, ** = $P < 0.001$ versus control group.

proliferation of mouse CD4⁺CD25⁻ T cells in a dose-dependent manner (Figure 1A) (see also Supplementary Figures 1A and B, available on the *Arthritis & Rheumatism* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.37894/abstract>). Coculture of the mouse T cells with human fibroblast cells as a control showed significantly less suppression than G-MSCs in vitro (Figure 1A). In experiments using a Transwell system in which G-MSCs and CD4⁺CD25⁻ T cells were physically separated, G-MSCs alone still inhibited mouse T cell proliferation (Figure 1B) (see also Supplementary Figure 1A), which suggests that the soluble factors secreted by G-MSCs could have a main role in the suppressive function of G-MSCs.

To explore the mechanisms responsible for G-MSC-mediated suppression of T cell proliferation, we analyzed several potential candidates. To this end, we found that G-MSCs inhibited mouse T cell proliferation via a process that is dependent on CD73 and CD39 signals. We also observed that the TGF β , IDO, and prostaglandin E₂ (PGE₂) pathways were not involved (Figure 1C) (see also Supplementary Figure 1C, available on the *Arthritis & Rheumatism* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.37894/abstract>). As a control, we used a human epidermal fibroblast cell line that is also differentiated from MSCs (22), to determine whether any fibroblast cell could mediate this suppression. We observed that human epidermal fibro-

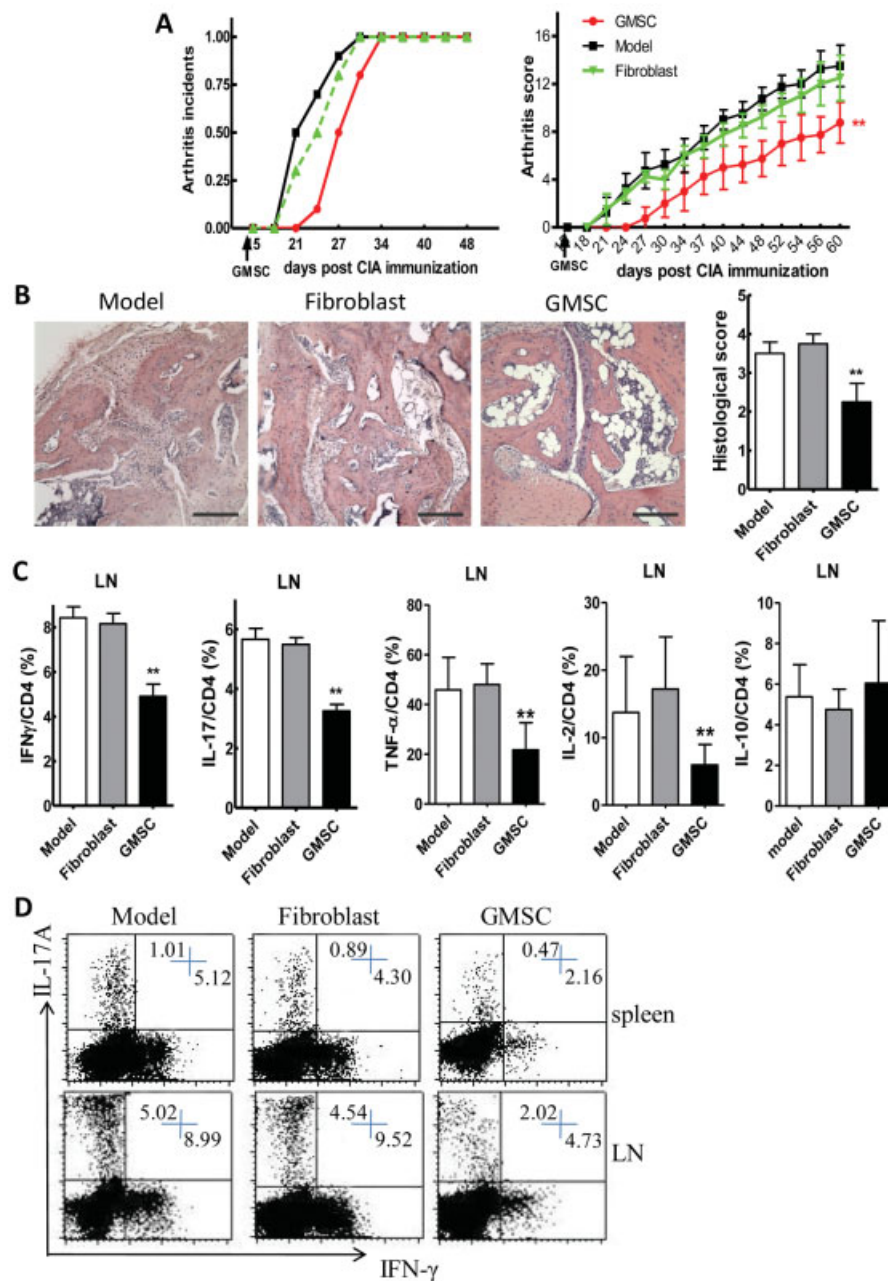


Figure 2. G-MSCs protect against collagen-induced arthritis (CIA) in DBA/1J mice when administered before the onset of inflammation. DBA/1J mice were immunized with type II collagen emulsified with Freund's complete adjuvant. On day 14 after immunization, 2×10^6 G-MSCs (obtained from different donors and used after 2–6 passages) or human skin fibroblasts were injected into the mice via the tail vein. **A**, Incidence of arthritis and arthritis severity scores were determined at various time points after immunization. **B**, Ankle joint sections were stained with hematoxylin and eosin 60 days after the primary immunization and evaluated for the histopathologic features of synovitis, pannus, and erosion (representative results shown; bars = 100 μ m) (left), with results expressed quantitatively as the histopathology score (right). **C**, Expression of cytokines, including IFN γ , IL-17, tumor necrosis factor α (TNF α), IL-2, and IL-10, on CD4+ cells was determined in the draining lymph nodes (LNs) of mice with CIA. In **A–C**, bars show the mean \pm SEM of 6 mice per group from 1 of 2 independent experiments. ** = $P < 0.01$ versus the other groups. **D**, Representative flow cytometry data show the expression of IFN γ and IL-17 gated on CD4+ cells in the spleens and draining LNs of mice with CIA. The model group comprises untreated arthritic mice as controls. See Figure 1 for other definitions.

blasts did not inhibit T cell proliferation in vitro (Figure 1C), even though these fibroblasts expressed CD73

but did not express CD39 (see Supplementary Figure 2, available on the *Arthritis & Rheumatism* web site at

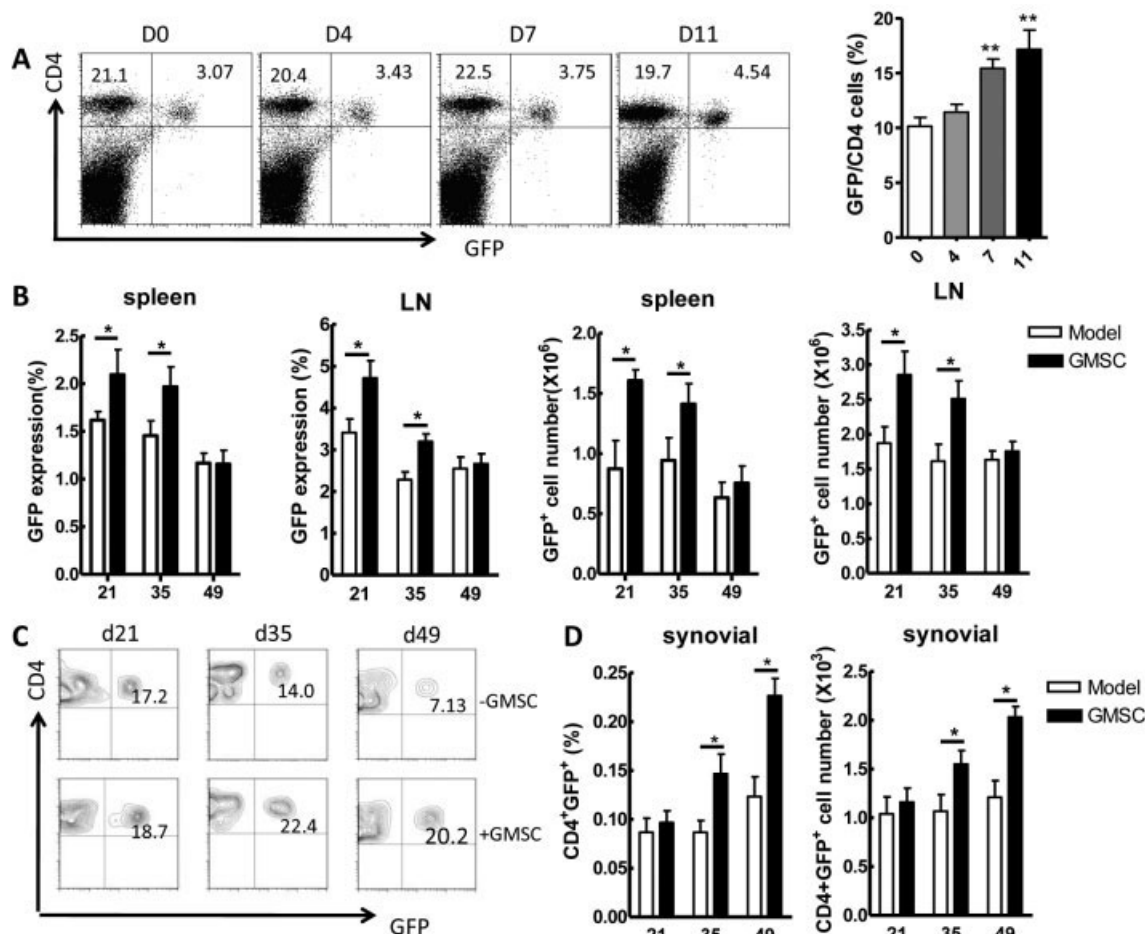


Figure 3. Gingiva-derived mesenchymal stem cells (G-MSCs) induce Treg cells in vivo. **A**, Naive DBA/1J-FoxP3^{gfp} mice were injected intravenously with G-MSCs (2×10^6). CD4⁺FoxP3⁺ (green fluorescent protein–positive [GFP⁺]) Treg cells were counted in the spleens by flow cytometry (left), with results also expressed quantitatively (right), on days 0, 4, 7, and 11 after G-MSC injection. Representative data from 1 of 2 separate experiments are shown. Bars show the mean \pm SEM of 5 mice per group. ** = $P < 0.01$ versus day 0. **B–D**, DBA/1J-FoxP3^{gfp} mice were immunized with type II collagen emulsified with Freund's complete adjuvant. G-MSCs (2×10^6) were injected into the mice via the tail vein on day 14 after immunization. Percentages (left) and absolute numbers (right) of FoxP3⁺ Treg cells were determined in the spleens and lymph nodes (LNs) of mice with collagen-induced arthritis on days 21, 35, and 49 after immunization (**B**). In the joint synovial fluid of arthritic mice treated without (model) or with G-MSCs, the frequency of CD4⁺FoxP3⁺ cells was determined by flow cytometry (representative results from 1 of 2 separate experiments shown) (**C**), and the frequency and total numbers of CD4⁺FoxP3⁺ cells were assessed quantitatively (**D**). Bars in **B** and **D** show the mean \pm SEM of 6 mice per group. * = $P < 0.05$.

<http://onlinelibrary.wiley.com/doi/10.1002/art.37894/abstract>).

To rule out the possibility that human-derived gingival cells might kill murine T cells as a way to nonspecifically suppress T cell responses, we labeled murine T cells with CFSE and measured the inhibition of proliferation (CFSE dilution) of T responder cells by gating on CFSE-labeled CD4⁺7-AAD⁻ live cells. We observed that in cultures with a G-MSC-to-T responder cell ratio of 1:25, there was 50% suppression of CD4⁺ T cell proliferation (Figure 1A), suggesting that cell killing was not the mechanism involved. Furthermore,

G-MSCs, but not fibroblasts, also significantly inhibited the differentiation of mouse Th1, Th2, and Th17 cells in vitro (Figures 1D and E).

Decreased severity of experimental arthritis following treatment with G-MSCs. To determine the immunomodulatory role of G-MSCs in the context of autoimmune arthritis, we relied on the CIA model. We observed a significant delay in disease onset and a decrease in arthritis severity scores following a single injection of G-MSCs on day 14 after CII/CFA immunization (Figure 2A). Histologic and quantitative analyses of the whole ankle joints demonstrated a significant

decrease in synovitis, pannus formation, and destruction of bone and cartilage in G-MSC-treated mice compared with controls (Figure 2B). Because mouse skin fibroblasts have been shown to suppress the inflammatory response in a mouse model of autoimmune arthritis (23), we chose human skin fibroblasts as a control for the human-derived gingival stem cells. The human skin fibroblasts exhibited no protective effect in this mouse CIA model (Figures 2A and B).

Down-regulation of the inflammatory responses in CIA following treatment with G-MSCs. We next investigated the mechanisms underlying the decrease in severity of CIA following administration of G-MSCs. Injection of G-MSCs into the tail vein of mice with CIA significantly reduced the percentage of cells secreting the proinflammatory cytokines IFN γ , IL-17, and TNF α in the draining lymph nodes (Figure 2C). G-MSC-treated mice also produced consistently lower percentages of Th1 and Th17 cells (Figures 2C and D). In addition, G-MSC treatment decreased the production of IL-2 from mouse CD4⁺ effector T cells, but did not significantly change IL-10 production (Figure 2C). In contrast, cells producing Th2-type cytokines (IL-4, IL-5, and IL-13) were almost undetectable in this model, and G-MSC treatment did not alter their levels (results not shown).

Promotion of Treg cells in CIA following treatment with G-MSCs. Several studies have indicated that Treg cells confer significant protection against CIA by decreasing the activation and joint homing of autoreactive Th1 cells, and by inhibiting osteoclastogenesis (9,24–26). To determine the relationship between G-MSCs and Treg cells *in vivo*, we first infused G-MSCs into naive DBA/1J-Foxp3^{gfp} mice. As shown in Figure 3A, treatment with G-MSCs significantly increased the frequency of CD4⁺FoxP3⁺ cells in the spleens and lymph nodes 1 week after injection in these mice. The Treg cell frequency reached a peak on day 11 after G-MSC infusion. However, the levels of Treg cells returned to baseline values 2 weeks after G-MSC injection in naive mice (results not shown).

We next investigated the dynamic effects of Treg cells in mice with CIA, again using FoxP3^{gfp} mice on the DBA/1J background. Consistent with previous findings showing that G-MSC treatment increased the expression of FoxP3 in the inflamed colon tissue of mice with Dextran sulfate sodium-induced experimental colitis (3), our results revealed that G-MSCs were also able to induce Treg cell responses in mice with CIA (Figure 3B). The percentage of cells expressing FoxP3 in the spleens and draining lymph nodes was significantly increased at 1 week and 3 weeks after G-MSC injection.

However, the increased FoxP3⁺ cell frequency in the spleens and draining lymph nodes gradually declined to levels that were similar to those in the control group by 5 weeks following cell infusion (Figure 3B). Interestingly, we began to observe a significant up-regulation of FoxP3⁺ cell frequency in the synovial fluid of mice with CIA 3 weeks after G-MSC infusion, although this increase was not observed in the early stages (Figures 3C and D).

Increased levels of induced Treg (iTreg) but not natural Treg (nTreg) cells following treatment with G-MSCs. A study has recently revealed that expression of Helios, an Ikaros transcription factor family member, might distinguish thymus-derived nTreg cells from iTreg cells (27–29). In our assessment of the phenotypes of increased FoxP3⁺ cells in G-MSC-treated mice with CIA, we found that the majority of the expanded Treg cell population was Helios negative (Figure 4A). Similarly, in the synovial fluid, most of the FoxP3⁺ cells did not express Helios (results not shown), suggesting that G-MSC treatment may induce the generation of new iTreg cells rather than the expansion of endogenous nTreg cells in CIA.

Given that a population of CD4⁺CD39⁺ cells, comprising TGF β -producing FoxP3[–]CD39⁺CD4⁺ T cells and IL-10-producing FoxP3⁺CD39⁺CD4⁺ T cells, has been shown to have a regulatory function in the murine CIA model (30), we sought to investigate whether CD4⁺CD39⁺ T cells were affected by G-MSC treatment in our CIA model. We found that there was no alteration of the percentages and total numbers of CD4⁺CD39⁺ T cells after G-MSC treatment (Figure 4B). CD4⁺FoxP3⁺ Treg cells expressed higher levels of CD39 than did CD4⁺FoxP3[–] non-Treg cells, whereas the expression of CD39 on Helios-positive Treg cells and Helios-negative Treg cells was the same (see Supplementary Figure 3, available on the *Arthritis & Rheumatism* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.37894/abstract>). Nonetheless, G-MSC treatment increased the CD39-positive population in CD4⁺FoxP3⁺ cells, while it suppressed CD39 expression in CD4⁺FoxP3[–] cells (Figures 4C and D). Taken together, these findings likely indicate that G-MSC treatment can selectively induce the Helios-negative CD4⁺FoxP3⁺CD39⁺ Treg cell subset in CIA.

In vivo role of Treg cells in the suppressive effect of G-MSCs on CIA. Because we had observed that Treg cells play an important role in controlling CIA progression, we next investigated whether the effects of G-MSC treatment are dependent on the function of Treg cells. To determine this, Treg cells were depleted in arthritic mice by IP administration of PC61, an anti-CD25 anti-

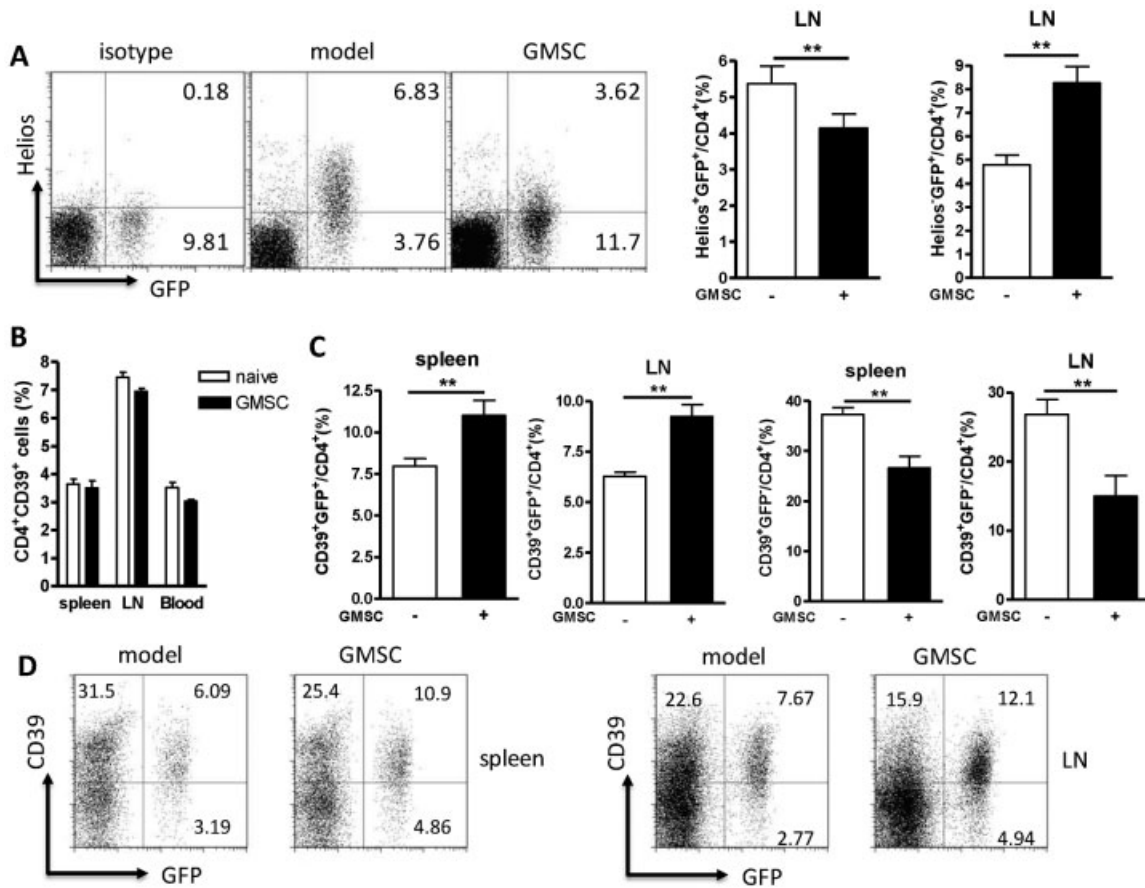


Figure 4. Gingiva-derived mesenchymal stem cells (G-MSCs) increase the frequency of induced Treg cells (most being CD4⁺ T cells positive for FoxP3, positive for CD39, and negative for Helios), but not natural Treg cells, in the collagen-induced arthritis model. DBA/1J-FoxP3^{flp} mice were immunized with type II collagen and Freund's complete adjuvant, and 2×10^6 G-MSCs were injected into the mice via the tail vein on day 14 after immunization. Mice were killed after 1 week. Each experiment included 5 mice per group, and the experiment was performed twice. **A**, The number of Helios-expressing cells and FoxP3-expressing (green fluorescent protein–positive [GFP⁺] cells) was assessed by flow cytometry (gated on CD4⁺ T cells) (representative results shown; isotype used as control) (left), with results also expressed quantitatively (right), in the draining lymph nodes (LNs) of arthritic mice treated without (model) or with G-MSCs. **B**, The frequency of CD4⁺CD39⁺ cells was assessed in the spleens, LNs, and blood of naive or G-MSC–treated arthritic mice. **C**, The frequencies of CD4⁺FoxP3⁺CD39⁺ (left) or CD4⁺FoxP3⁻CD39⁺ (right) cells were assessed in the spleens and LNs of arthritic mice treated without or with G-MSCs. **D**, The frequency of CD39⁺FoxP3⁺ cells (gated on CD4⁺ cells) was assessed by flow cytometry in the spleens (left) and LNs (right) of arthritic mice treated without (model) or with G-MSCs. Bars in **A–C** show the mean \pm SEM of 5 mice per group. ** = $P < 0.01$.

body. Levels of CD4⁺FoxP3⁺ Treg cells in the spleens decreased significantly 1 week after PC61 administration. Treg cell depletion was maintained for 4 weeks, and thereafter starting at 5 weeks after PC61 treatment, the Treg cell levels began to be restored (Figure 5A).

We then determined the role of Treg cells in the G-MSC–mediated suppression of CIA. Treatment with PC61 resulted in an accelerated incidence of arthritis (Figure 5B), significantly increasing arthritis severity scores (Figure 5B), and severe histologic damage to the joint (Figure 5C). G-MSC injection in combination with

PC61 treatment delayed the onset and reduced the severity of arthritis when compared to PC61 treatment alone (Figure 5B). However, compared to G-MSC treatment alone, the combination of G-MSC and PC61 treatment led to a significantly greater severity of arthritis (Figure 5B), suggesting that the depletion of Treg cells in vivo partially attenuated the protective effect of the G-MSCs. Similarly, combined treatment with G-MSCs and PC61 produced lower frequencies of Th1 and Th17 cells as compared to treatment with PC61 alone, and produced significantly higher percentages of

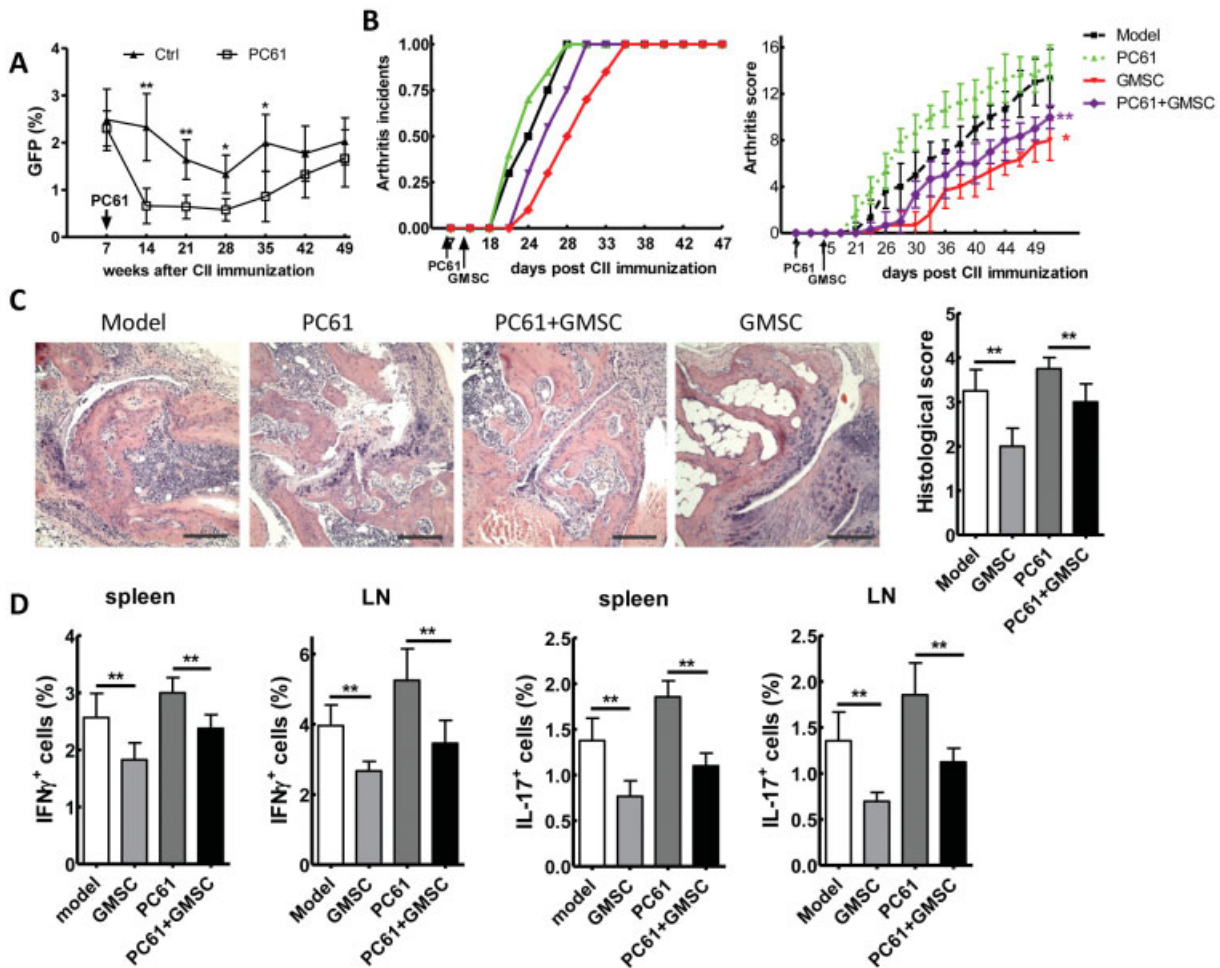


Figure 5. G-MSCs attenuate the inflammation of arthritis, which partially depends on the effects of Treg cells. **A**, DBA/1J-Foxp3^{flp} mice were immunized with type II collagen (CII) and Freund’s complete adjuvant (CFA). On day 7, mice were injected intraperitoneally (IP) with PC61 (anti-CD25 monoclonal antibody; 250 μ g/mouse) or control phosphate buffered saline. **A**, CD4+FoxP3+ (green fluorescent protein–positive [GFP+]) cells were counted in the spleens at various time points after immunization. Each experiment included 5 mice per group, and the experiment was performed twice. * = $P < 0.05$; ** = $P < 0.01$ versus the PC61 treatment group. **B–D**, DBA/1J mice were immunized with CII and CFA, followed by IP injection of PC61 on day 7 and/or intravenous infusion of 2×10^6 G-MSCs on day 14. Incidence of arthritis and arthritis severity scores were determined in the mice at various time points after immunization (**B**). Tarsal joint sections were stained with hematoxylin and eosin and evaluated for the histopathologic features of synovitis, pannus, and erosion (representative results shown; bars = 200 μ m) (left), with results expressed quantitatively as the histopathology score (right) (**C**). Frequencies of IFN γ ⁺ cells (left) and IL-17⁺ cells (right) were determined in the spleens and draining lymph nodes (LNs) of arthritic mice (**D**). Bars in **B–D** show the mean \pm SEM of 6 mice per group from 1 of 2 separate experiments. Model indicates untreated arthritic mice. ** = $P < 0.01$. See Figure 1 for other definitions.

IFN γ -producing and IL-17–producing cells as compared to treatment with G-MSC alone (Figure 5D).

Taken together, these findings suggest that G-MSC treatment in mice can still protect against arthritis after Treg cell depletion. However, the therapeutic effect of G-MSCs was significantly less pronounced under conditions of Treg cell depletion compared to conditions in which Treg cells remain intact. Thus, the G-MSC–mediated immunoregulatory func-

tion in vivo may be attributed to Treg cells, but could also be attributed to other mechanisms.

Dependency of G-MSC–mediated suppression of CIA on CD39 and CD73 signals. We next sought to identify the kinds of mechanisms involved in the functions of G-MSCs. Consistent with previous studies (31,32), we showed that G-MSCs were homogeneously positive for the mesenchymal markers CD29, CD44, and CD105, negative for the hematopoietic markers CD34

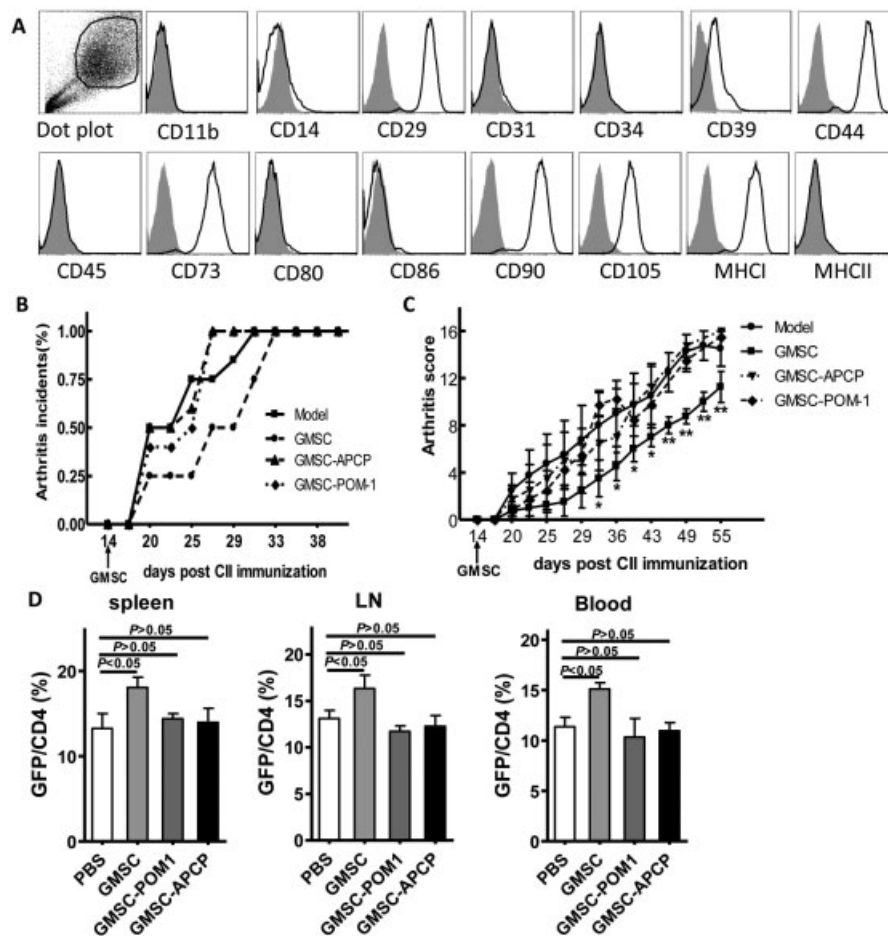


Figure 6. G-MSCs attenuate the inflammatory responses in mice with collagen-induced arthritis via CD39 and/or CD73 signals. **A**, Expression of G-MSC surface proteins was assessed by flow cytometry. Fifth-passage G-MSCs were stained with the indicated antibodies. MHCI = class I major histocompatibility complex. **B–D**, DBA/1J mice were immunized with type II collagen (CII) and Freund's complete adjuvant. On day 14 after immunization, 2×10^6 G-MSCs, which were pretreated overnight without or with α,β -methylene ADP or sodium polyoxotungstate 1 (each $100 \mu\text{M}$), was injected intravenously into the mice. Incidence of arthritis (**B**) and arthritis severity scores (**C**) were determined at various time points after immunization. * = $P < 0.05$; ** = $P < 0.01$ versus untreated arthritic mice (model). The frequency of FoxP3+ (green fluorescent protein-positive [GFP+]) cells was determined by flow cytometry in the spleens, lymph nodes (LNs), and blood of mice at 1 week after treatment (**D**). Bars show the mean \pm SEM of 6 mice per group from 1 of 2 separate experiments. PBS = phosphate buffered saline (see Figure 1 for other definitions).

and CD45 and the endothelial cell marker CD31, and negative for the costimulating molecule CD86 (Figure 6A). Furthermore, positivity for CD39 and positivity for CD73 were also found to be phenotypic characteristics of G-MSCs (Figure 6A).

As noted earlier (Figure 1B), we observed that G-MSCs inhibited mouse T cell proliferation via CD39 and CD73 signals. It has been reported that pretreatment of G-MSCs with the COX inhibitor indomethacin will significantly reverse the inhibitory effect of G-MSCs on dendritic cell differentiation and cytokine secretion in vitro, and that these cells will lose their capacity to attenuate contact hypersensitivity after injection into

mice in vivo (21). Moreover, overnight treatment with indomethacin can maintain these effects on G-MSCs for at least 1 week (21). Previous observations of CD39 and CD73 expression levels in mouse T cells (33,34) suggest that it is not feasible to treat mice with CD39 and CD73 inhibitors because of their pleiotropic effects. Therefore, in order to determine whether CD39 and/or CD73 signals are involved in the mechanism of G-MSC protection against CIA in mice, we pretreated G-MSCs with $100 \mu\text{M}$ POM-1 (a CD39 inhibitor) or $100 \mu\text{M}$ APCP (a CD73 inhibitor) overnight in vitro, and then injected these cells into arthritic mice.

Following pretreatment with POM-1 and APCP,

the expression of CD39 and CD73 in G-MSCs was significantly reduced. The cell avidity of G-MSCs did not change following APCP and POM-1 treatment (see Supplementary Figure 4, available on the *Arthritis & Rheumatism* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.37894/abstract>). Furthermore, blocking the activity of CD39 or CD73 in G-MSCs significantly reversed the protective effect of G-MSCs on the progression of CIA, by inducing an accelerated incidence of arthritis (Figure 6B) and increased severity of arthritis (Figure 6C), suggesting that both CD39 and CD73 are important in the G-MSC-mediated control of T cell-mediated diseases.

We next investigated the role of CD39 and CD73 signals in the G-MSC-mediated up-regulation of Treg cells in vivo. To address this, we treated G-MSCs with the CD39 inhibitor and CD73 inhibitor overnight, and then infused G-MSCs into mice with CIA. At 1 week following infusion, the FoxP3+ Treg cell frequency in the G-MSC-treated group was identical to that in the control group (Figure 6D), indicating that pretreatment of G-MSCs with CD39 or CD73 inhibitors results in the abrogation of their ability to increase Treg cell frequencies and to protect against the progression of CIA.

DISCUSSION

MSCs represent a variety of stromal cells that have the potential to self-renew and differentiate. The immunosuppressive function of MSCs is ascribed to their inhibitory effects on T cells and other immune cells, including dendritic cells and natural killer cells in vitro (35,36). It has been reported that human BM-MSCs strongly suppress T lymphocyte proliferation via mechanisms that do not involve the induction of T cell apoptosis, but are likely due to the production of soluble factors, including hepatocyte growth factor (37), PGE₂ (38), HO-1 (39), TGFβ₁, and NO (7). In this study, we observed that G-MSCs suppress mouse T cell responses, and that cell contact is not necessary for this suppression, suggesting that soluble factors are involved in this mechanism. However, to our knowledge, this is the first study to show that G-MSCs inhibit mouse T cell proliferation via CD39 and/or CD73 signals, but not via IL-10, NO, IDO, PGE₂, or TGFβ₁, in vitro. The species used as a source of cells may have led to the different results.

Extracellular and/or immediate pericellular accumulation of adenosine, released by damaged cells as an indicator of trauma and cell death, elicits immunosuppressive cellular responses that are mediated through several type 1 purinergic (adenosine) receptors, includ-

ing the A_{2A} adenosine receptor (10,40). The activation of A_{2A}-mediated signals can attenuate T cell-mediated experimental colitis by suppressing the expression of proinflammatory cytokines in a manner independent of both IL-10 and TGFβ (41). Recent studies show that coexpression of CD39 and CD73 on CD4+CD25+ FoxP3+ Treg cells, which catalyzes the sequential generation of adenosine by degradation of extracellular ATP/ADP to 5'-AMP (CD39) and conversion of 5'-AMP to adenosine (CD73), leads to strong down-regulation of T cell proliferation and a decreased secretion of proinflammatory cytokines (10,34,40). Herein, we demonstrated that G-MSCs express CD39 and CD73, thus supporting the generation of adenosine and thereby promoting strong immunosuppression of effector T cells in vitro and in vivo. G-MSCs not only can promote the up-regulation of FoxP3+ Treg cells and the possible migration of these cells in inflammatory disease in vivo, but also may share part of the mechanisms of immunosuppressive functions indirectly via adenosine.

G-MSCs may directly or indirectly suppress CIA. Since G-MSCs express CD39 and CD73 and since both 5'-AMP and adenosine have a potent immunosuppressive activity, it is reasonable to expect that G-MSCs will suppress CIA in a CD39- or CD73-dependent manner. However, G-MSCs may also promote Treg cells through CD39 and CD73 signaling, since pretreatment of G-MSCs with CD39 or CD73 inhibitors abrogated G-MSC-mediated Treg cell up-regulation. We have demonstrated that the suppressive effect of G-MSCs on CIA is dependent, at least in part, on Treg cells, supporting the theory that G-MSCs exert their immunosuppressive function via direct suppression of inflammatory cell responses and via an indirect immunoregulatory function, involving increased induction of Treg cells.

Multiple studies have shown that the immunoregulatory function of MSCs is associated with up-regulated expression of Treg cells in vivo (6,7,42). Recently, a population of CD4+CD39+ T cells was identified as having a regulatory function in the CIA model. This subset is composed of TGFβ-producing FoxP3-CD39+CD4+ T cells and IL-10-producing FoxP3+CD39+CD4+ T cells, each of which plays an important role in autoimmune diseases (30). Our results suggest that G-MSCs selectively promote the production of the FoxP3+CD39+CD4+ Treg cell subset in naive mice and in the proinflammatory CIA disease model. Although it is arguable whether Helios can distinguish nTreg from iTreg cells, our data suggest that Helios-

negative FoxP3+CD39+ T cells are a newly identified cell population that may be induced in CIA.

Although the frequency of Treg cells was increased temporarily in naive mice, it is notable that treatment with G-MSCs sustained the increased expression of CD39+FoxP3+ Treg cells in CIA. It is unknown whether the inflammatory environment affects the function of G-MSCs. Interestingly, whereas the increased Treg cell frequency in the spleens and lymph nodes gradually declined, the increased frequencies of FoxP3+ cells were observed in the synovial fluid of mice with CIA 3 weeks after G-MSC treatment. As MSCs may have difficulty in obtaining access to the joints, it is possible that soluble factors secreted by G-MSCs may regulate Treg cell induction in the joints or promote the increased frequency of Treg cells in the periphery, resulting in Treg cell migration into the synovial fluid in CIA.

Thus, in this study, we have demonstrated, for the first time, that G-MSCs can inhibit T cell responses and T cell-mediated diseases via CD39/CD73 signals. Our findings suggest that G-MSCs exert their immunoregulatory functions in the CIA model directly and/or indirectly. Moreover, G-MSCs promote the induction of CD39+FoxP3+ Treg cells, and these cells play a role in the G-MSC-mediated suppression of CIA. These findings further support the notion that G-MSCs, a unique population of MSCs with functional similarities to BM-MSCs, are a promising cell source for stem cell-based therapies for inflammatory diseases and stem cell transplantation.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. He and Zheng had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Huang, He, Le, Zheng.

Acquisition of data. Chen, Su, Lin, Guo, Wang, Zhang.

Analysis and interpretation of data. Chen, Lin, Guo, Brand, Ryffel, Huang, Liu.

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