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Treatment of Experimental Arthritis by Inducing Immune Tolerance With Human Adipose-Derived Mesenchymal Stem Cells

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Objective. **Rheumatoid arthritis (RA) is a chronic autoimmune disease caused by loss of immunologic self tolerance and characterized by chronic joint inflammation. Adult mesenchymal stem cells (MSCs) were recently found to suppress effector T cell responses and to have beneficial effects in various immune disorders. The purpose of this study was to examine a new therapeutic strategy for RA based on the administration of human adipose-derived MSCs (AD-MSCs).**

Methods. **DBA/1 mice with collagen-induced arthritis were treated with human AD-MSCs after disease onset, and clinical scores were determined. Inflammatory response was determined by measuring the levels of different mediators of inflammation in the joints and serum. The Th1-mediated autoreactive response was evaluated by determining the proliferative response and cytokine profile of draining lymph node cells stimulated with the autoantigen. The number of Treg cells and the suppressive capacity on self-reactive Th1 cells were also determined.**

Results. **Systemic infusion of human AD-MSCs significantly reduced the incidence and severity of experimental arthritis. This therapeutic effect was mediated by down-regulating the 2 deleterious disease components: the Th1-driven autoimmune and inflammatory responses. Human AD-MSCs decreased the production of various inflammatory cytokines and chemokines, decreased antigen-specific Th1/Th17 cell expansion, and induced the production of antiinflammatory interleukin-10 in lymph nodes and joints. Human AD-MSCs also induced de novo generation of antigen-specific CD4+CD25+FoxP3+ Treg cells with the capacity to suppress self-reactive T effector responses.**

Conclusion. **Human AD-MSCs emerge as key regulators of immune tolerance by inducing the generation/activation of Treg cells and are thus attractive candidates for a cell-based therapy for RA.**

Rheumatoid arthritis (RA) is an autoimmune disease caused by loss of immunologic self tolerance that leads to chronic inflammation in the joints and subsequent cartilage destruction and bone erosion. The crucial process underlying disease initiation is the induction of autoimmunity to collagen-rich joint components; later events evolve a destructive inflammatory process (1). Progression of the autoimmune response implies the development of autoreactive Th1 and Th17 cells, their entry into the joint tissues, and their release of proinflammatory cytokines and chemokines, which promote macrophage and neutrophil infiltration and activation. Excessive levels of mediators of inflammation, such as cytokines, free radicals, and extracellular matrix–degrading enzymes, produced by infiltrating inflammatory cells play a critical role in joint damage (1). A desirable therapeutic approach would be to prevent the activation of inflammatory and autoimmune compo-

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Drs. González, Büscher, and Delgado are coinventors with Cellerix SA on a patent application for the use of adipose-derived mesenchymal stem cells in autoimmunity. The study reported herein is part of that patent application.

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nents of the disease. On the other hand, failures in the function of the Treg cell compartment can contribute to the development of RA, and enhancing the function of this compartment may also represent a therapeutic strategy (2–6).

Mesenchymal stem cells (MSCs) are mesoderm-derived cells that reside in the stroma of solid organs and function as precursors of nonhematopoietic connective tissues. Besides their capacity to differentiate into mesenchymal and nonmesenchymal cell lineages (7,8) and their potential clinical application for the repair of damaged tissues, several recent studies have shown that bone marrow–derived MSCs (BM-MSCs) regulate the immune response, including in vitro inhibition of T cell proliferation, B cell function, and dendritic cell maturation (9–13). Some researchers have reported the use of BM-MSCs to treat allograft rejection and acute graft-versus-host disease as well as to alleviate experimental autoimmune encephalomyelitis, collagen-induced arthritis (CIA), and autoimmune myocarditis (11,14–20). However, the specific molecular and cellular mechanisms involved in the immunoregulatory activity of BM-MSCs remain a subject of controversy.

A critical issue for the clinical translation of BM-MSCs in autoimmunity is that their therapeutic use requires large quantities of cells for infusion, which in most cases, are not available. Human MSCs obtained from subcutaneous adipose tissue (AD-MSCs) have recently emerged as an attractive alternative source of MSCs for cell therapy (7,8,10,21). Large amounts of human AD-MSCs can be easily obtained from lipoaspirates from healthy donors and rapidly expanded in vitro to generate a clinically effective dosage, and recent studies have reported that human AD-MSCs share some of the immunomodulatory properties that characterize the BM-MSCs (18,22,23). Importantly, we recently found that human AD-MSCs exert profound suppressive responses on collagen-reactive T cells from RA patients through various ways (24). Here, we report our findings of the therapeutic effect of human AD-MSCs in experimental arthritis and their capacity to restore immune tolerance and to inhibit the inflammatory response in vivo.

MATERIALS AND METHODS

Induction and treatment of CIA. Human and murine AD-MSCs were obtained essentially as described previously (8,18). Animal experimental protocols were approved by the Ethics Committee of the Spanish Council of Scientific Research. DBA/1 mice (7–10 weeks old; The Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously with 200 μg of chicken type II collagen (CII; Sigma, Madrid, Spain) emulsified in Freund's complete adjuvant (CFA) containing 200 μg of *Mycobacterium tuberculosis* H37Ra, and then given subcutaneous booster injections with 100 μg of CII in CFA.

Mice were monitored for signs of arthritis onset based on paw swelling and clinical scores (25). Paw swelling was assessed by measuring the mean thickness of both hind paws with 0–10-mm calipers. Clinical arthritis was scored on a scale of 0–3, where 0 = no swelling, 1 = slight swelling and erythema, 2 = pronounced edema, and 3 = joint rigidity. Each limb was graded, and the grades were summed to yield the arthritis score for each animal (maximum possible score 12 per animal).

Treatment was begun after the onset of disease, when arthritis had become well established (arthritis score >2) or severe (arthritis score >4). Mice with CIA were injected intraperitoneally each day for 5 days with phosphate buffered saline (PBS; control) or with PBS containing 10^6 human AD-MSCs, allogeneic murine AD-MSCs (from C57BL/6 mice), or syngeneic murine AD-MSCs (from DBA/1 mice). Alternatively, mice with established CIA (arthritis score 2) were injected once intraarticularly into 1 of the affected joints with 100 μl of PBS containing 10^6 human AD-MSCs. Alternatively, other mice received a single intraperitoneal injection of 10^6 human AD-MSCs at disease onset (day 22; arthritis score 0.5). In addition, 10^6 serum-starved, dead (viability <96%), human AD-MSCs, human dermal fibroblasts, or human skeletal myoblasts (the latter 2 cell lines from Lonza, Barcelona, Spain) were injected intraperitoneally each day for 5 days into mice with CIA (arthritis score >2).

For adoptive transfer experiments, draining lymph node (DLN) cells obtained on day 35 postimmunization from untreated or human AD-MSC–treated arthritic mice were purified and stimulated in vitro with inactivated CII (10 μg/ml) for 72 hours. After stimulation, CD4+ T cells (>97% CD4+) were isolated, depleted of CD25+ cells (>99% CD4+CD25−) by immunomagnetic selection (Miltenyi Biotec, Madrid, Spain), and injected (5×10^6 cells) intravenously into mice with CIA on day 24 after immunization. For in vivo CD25+ T cell depletion (>98% CD25+), DBA/1 mice were treated intravenously with 1 mg of anti-CD25 antibody (clone PC61) 3 days before immunization with CII.

Cytokine, autoantibody, and myeloperoxidase (MPO) activity determinations. Protein extracts were isolated from paws obtained on day 35, by homogenization (50 mg tissue/ml) in 50 m*M* Tris HCl, pH 7.4, with 0.5 m*M* dithiothreitol and proteinase inhibitor cocktail (10 μg/ml). Serum samples were also collected on day 35. Cytokine and chemokine levels in the serum and joint protein extracts were determined by sandwich enzyme-linked immunosorbent assays (ELISAs) using capture/biotinylated detection antibodies obtained from BD PharMingen (Madrid, Spain). Synovial neutrophil infiltration was monitored by measuring MPO activity in paws collected on day 35 (26). Serum levels of anti-CII IgG, IgG1, and IgG2a antibodies were determined by ELISA (25).

Assessment of T cell autoreactive response in mice with CIA. Single-cell suspensions (10^6 cells/ml) from DLNs and synovial membranes from the knee joints were obtained 30

days postimmunization as described previously (25) and stimulated in RPMI medium with different concentrations of heat-inactivated CII. Cell proliferation was determined at 72 hours by measuring bromodeoxyuridine-substituted DNA incorporation (Roche, Madrid, Spain) and cytokine contents in culture supernatants at 48 hours by ELISAs. For intracellular cytokine analysis, DLN and synovial cells were stimulated with CII (10 μg/ml) and monensin (1.33 μM) for 8 hours, stained with peridinin chlorophyll A protein (PerCP)–conjugated anti-CD4 at 4°C, fixed/permeabilized with Cytofix/Cytoperm solution (BD Biosciences), stained with fluorescein isothiocyanate (FITC)–conjugated and phycoerythrin (PE)–conjugated anticytokine-specific monoclonal antibodies, and the CD4+ population was analyzed by flow cytometry using a FACSCalibur instrument with CellQuest software (both from BD Biosciences).

To examine the suppressive activity of human AD-MSCs in vitro, 10^5 DLN cells or 10^5 synovial cells isolated from the knee of arthritic mice at the peak of disease were stimulated with 10 μg/ml of CII in the presence of 2×10^4 human AD-MSCs. Proliferation and cytokine production were then determined. Some cocultures of DLN cells with human AD-MSCs and synovial cells with human AD-MSCs were treated with indomethacin (20 μM; Sigma) or with anti–interleukin-10 (anti–IL-10) or anti–transforming growth factor β (anti-TGFβ) antibodies (10 μg/ml; BD PharMingen).

To determine cell contact dependence of the suppressive response, 10^5 DLN cells or 10^5 synovial cells were seeded in the lower insert of a Transwell system (0.4-μm pore size; Millipore, Madrid, Spain), and 2×10^4 human AD-MSCs were placed in the upper well, and proliferation and cytokine levels in the lower compartment were determined after 96 hours or 48 hours, respectively. Alternatively, DLNs were cultured in conditioned medium from 10^5 human AD-MSCs that had been stimulated for 24 hours with tumor necrosis factor α (TNFα; 20 ng/ml) or interferon-γ (IFNγ; 200 ng/ml).

Determination of Treg cell suppressive activity. T cells (>98% CD3+) were isolated from DLNs obtained from PBS-treated and human AD-MSC–treated mice with CIA, using magnetic bead–labeled anti-CD3 monoclonal antibodies (Miltenyi Biotec). To determine the suppressive capacity of human AD-MSC–induced Treg cells, autoreactive T cells (4×10^5/well) from CIA mouse DLNs were stimulated with spleen antigen-presenting cells (10^5/well), CII (10 μg/ml), and with DLN T cells (5×10^4) isolated from untreated or human AD-MSC–treated mice with CIA. After 72 hours, the proliferation of autoreactive T cells was assayed.

To determine the suppressive activity of murine AD-MSCs in vitro, DLNs (10^5) isolated at the peak of CIA were cocultured with different numbers of allogeneic murine AD-MSCs, and proliferation and cytokine contents were determined. After 4 days, viable T cells ($T_{AD\text{-}MSCs}$) were isolated from the DLN/murine AD-MSC cocultures using immunomagnetic anti-CD3 beads and rested for 2 days in complete RPMI medium containing IL-2 (20 units/ml). $T_{AD\text{-}MSCs}$ (2×10^4) were then added to 10^5 CII-activated DLNs from mice with CIA, and proliferation was determined. $T_{AD\text{-}MSCs}$ (5×10^6) were also injected intravenously into arthritic mice 2 days after disease onset, and signs of arthritis were scored daily.

To determine the number of Treg cells in vivo, spleen, DLN, and knee joint synovial cells isolated on days 28–30 postimmunization were stained with PerCP-conjugated anti-CD4 and FITC-conjugated anti-CD25 (2.5 μg/ml; BD PharMingen), fixed as described above, and incubated with PE-conjugated anti-FoxP3 monoclonal antibody (0.5 μg/sample; eBioscience, San Diego, CA) diluted in PBS containing 1% bovine serum albumin and 0.5% saponin. Cells were then analyzed by flow cytometry.

Statistical analysis. All results are expressed as the mean ± SD. The Mann-Whitney U test was used to compare nonparametric data for statistical significance. P values less than 0.05 were considered significant.

RESULTS

Decreased severity of experimental arthritis following treatment with human AD-MSCs. We recently described the immunosuppressive activity of human AD-MSCs in vitro on cells from RA patients (24). In the present study, we investigated the potential therapeutic action of human AD-MSCs in an experimental model of murine arthritis that shares a number of clinical, histologic, and immunologic features with RA. Although a single injection of human AD-MSCs at the onset of disease completely abolished the progression of arthritis, administration of human AD-MSCs to mice with established arthritis (arthritis score >2) progressively attenuated the severity of CIA and decreased the percentages of mice with arthritis, as compared with the untreated mice (Figure 1A).

Human AD-MSC treatment in mice with severe CIA (arthritis score >4) slightly ameliorated the clinical signs, although it failed to reduce the incidence of arthritis (Figure 1A). A single intraarticular injection of human AD-MSCs was sufficient to moderately reduce arthritis in all joints (Figure 1A). Because of the higher therapeutic efficacy of daily intraperitoneal administration for 5 days on established arthritis, all further experiments were performed using this treatment protocol.

The therapeutic effect was specific to viable human AD-MSCs because dead human AD-MSCs failed to prevent the progression of arthritis (Figure 1B). Moreover, other human stromal cells, such as myoblasts and dermal fibroblasts, which show potent immunoregulatory effects in vitro (27), showed no significant therapeutic effects in mice with CIA as compared with the effects of human AD-MSCs (Figure 1B), although these differences could be the result of using commercially available fibroblasts (likely of high passage numbers) rather than freshly isolated cells.

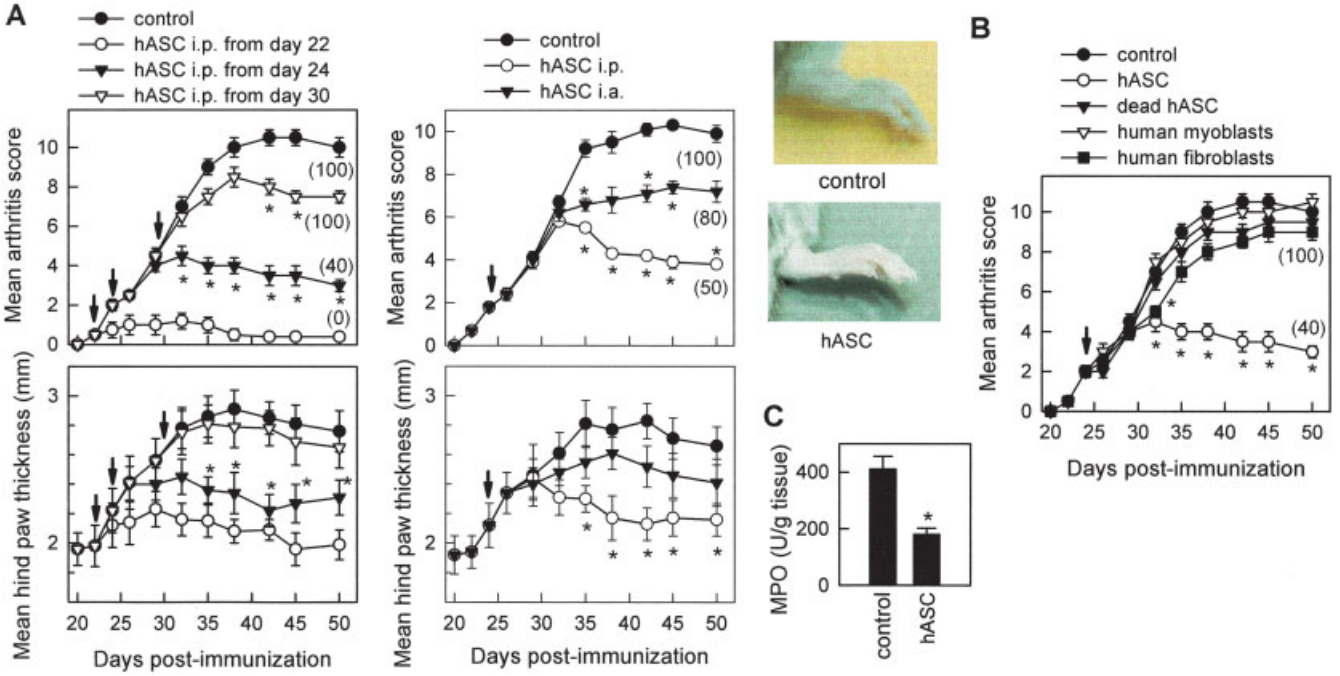

Figure 1. Decreased severity of collagen-induced arthritis (CIA) after treatment with human adipose-derived mesenchymal stem cells (AD-MSCs). **A,** Mice with established CIA were injected intraperitoneally (IP) with phosphate buffered saline (PBS; control) or with 10^6 human AD-MSCs (hASC) daily for 5 days starting on day 24 or day 30 or were injected intraperitoneally with a single dose of human AD-MSCs on day 22 (arrows in left panels). Alternatively, mice with CIA were injected intraarticularly (IA) with a single dose of human AD-MSCs into 1 of the affected joints (arrow in right panels). Arthritis severity was assessed by clinical scoring and measurement of hind paw thickness. Photographs at the right show representative examples of the paw swelling in PBS-treated control or human AD-MSC–treated arthritic mice. **B,** Mice with established CIA were injected intraperitoneally with 10^6 dead human AD-MSCs, human fibroblasts, or human myoblasts daily for 5 days starting on day 24 (arrow). Arthritis severity was assessed by clinical scoring. **C,** Myeloperoxidase (MPO) activity was determined by measuring neutrophil infiltration into joints obtained on day 35 from mice with CIA. Numbers in parentheses in **A** and **B** represent the percentage of mice with established arthritis (score >2 on day 40). Values are the mean ± SD of 8–11 mice per group in **A**, 8–10 mice per group in **B**, and 5 mice per group in **C**. $* = P < 0.001$ versus control.

Consistent with the striking reduction in paw inflammation, the joints of mice treated with human AD-MSCs showed decreased neutrophil infiltration, as measured by MPO activity (Figure 1C).

Down-regulation of the inflammatory response in CIA following treatment with human AD-MSCs. We next investigated the mechanisms underlying the decrease in severity of CIA following administration of human AD-MSCs. We first evaluated the effect on the production of mediators of inflammation that are mechanistically linked to joint inflammation. Human AD-MSC injection significantly reduced protein expression of various inflammatory cytokines and chemokines (RANTES and macrophage inflammatory protein 2), while it increased expression of the antiinflammatory cytokine IL-10, in the joints of mice with CIA (Figure 2A). The broad antiinflammatory activity of human AD-MSCs in the inflamed joint was accompanied by down-regulation of the systemic inflammatory response (Figure 2A).

Moreover, human AD-MSCs inhibited the production of proinflammatory mediators by synovial cells isolated from mice with CIA and restimulated with CII in vitro (Figure 2B). Transwell experiments showed that this inhibitory effect was both cell-to-cell contact–dependent and cell-to-cell contact–independent (Figure 2C). The involvement of soluble factors was supported by the fact that IL-10 blocking or prostaglandin E_2 (PGE_2) inhibition partially reversed the inhibitory effect of human AD-MSCs on the production of TNFα and IFNγ by CII-activated synovial cells (Figure 2C). Interestingly, blockade of TGFβ1 did not affect human AD-MSC–mediated suppression (Figure 2C).

Down-regulation of the Th1-mediated autoreactive response in CIA following treatment with human AD-MSCs. In RA and CIA, progression of the autoimmune response involves the development of autoreactive Th17 cells (producing IL-17) and Th1 cells (producing IFNγ and TNFα), their entry into the joint tissues, and their subsequent recruitment of inflamma-

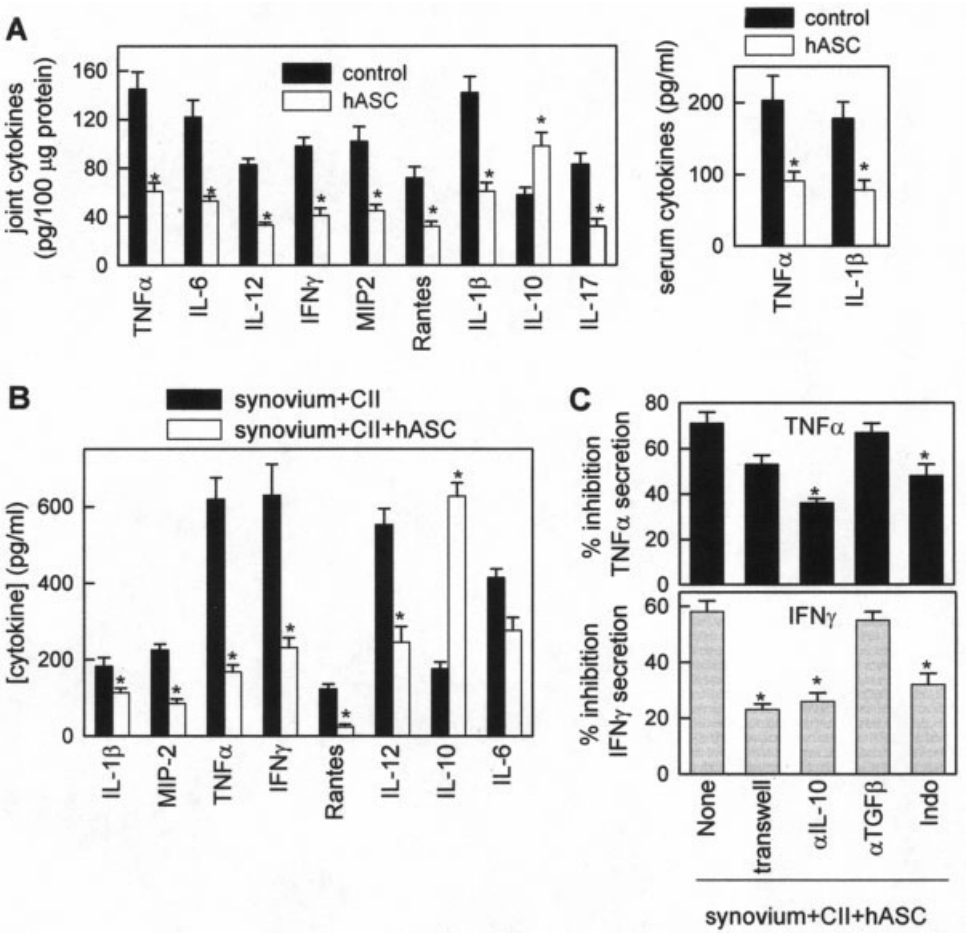

Figure 2. Inhibition of inflammatory responses in mice with collagen-induced arthritis (CIA) after treatment with human adipose-derived mesenchymal stem cells (AD-MSCs). Mice with established CIA were injected intraperitoneally with phosphate buffered saline (PBS; control) or with 10^6 human AD-MSCs (hASC) daily for 5 days starting on day 24. **A,** Levels of tumor necrosis factor α (TNFα), interleukin-6 (IL-6), IL-12, interferon-γ (IFNγ), macrophage inflammatory protein 2 (MIP-2), RANTES, IL-1β, IL-10, and IL-17 in the joints (left) and TNFα and IL-1β in the sera (right) obtained on day 35. **B,** Inhibition of the in vitro inflammatory response of synovial cells from mice with CIA by human AD-MSCs. Synovial cells isolated on day 35 from mice with CIA were stimulated with chicken type II collagen (CII) in the absence (control) or presence of human AD-MSCs. After 24 hours, cytokine and chemokine contents in supernatants were determined. **C,** Deactivation of synovial cells by human AD-MSCs through both cell-to-cell contact–dependent and cell-to-cell contact–independent mechanisms. Synovial cells isolated from the knees of mice with CIA were stimulated with CII plus human AD-MSCs in the absence (none) or presence of neutralizing antibodies against IL-10 (αIL-10), neutralizing antibodies against transforming growth factor β1 (TGFβ1 [αTGFβ]), or indomethacin (Indo), or CII-activated synovial cells were separated from human AD-MSCs in Transwells. Inhibition of TNFα and IFNγ was then determined. Values are the mean and SD of 6–8 mice per group in **A** and 6 mice per group in **B** and **C**. $* = P < 0.001$ versus control in **A** and **B** and versus cocultures of synovium/human AD-MSCs in **C**.

tory cells (1). DLN cells from mice with CIA showed marked CII-specific proliferation as well as effector T cells producing high levels of IL-17 and Th1-type cytokines (IFNγ, IL-2, and TNFα) and low levels of Th2-type cytokines (IL-4 and IL-10) (Figure 3A). In contrast, DLN cells from human AD-MSC–treated mice proliferated much less and produced low levels of Th1 and Th17 cytokines (Figure 3A). The Th2-type cytokine IL-4 was not significantly affected, whereas the regulatory cytokines IL-10 and TGFβ1 were increased

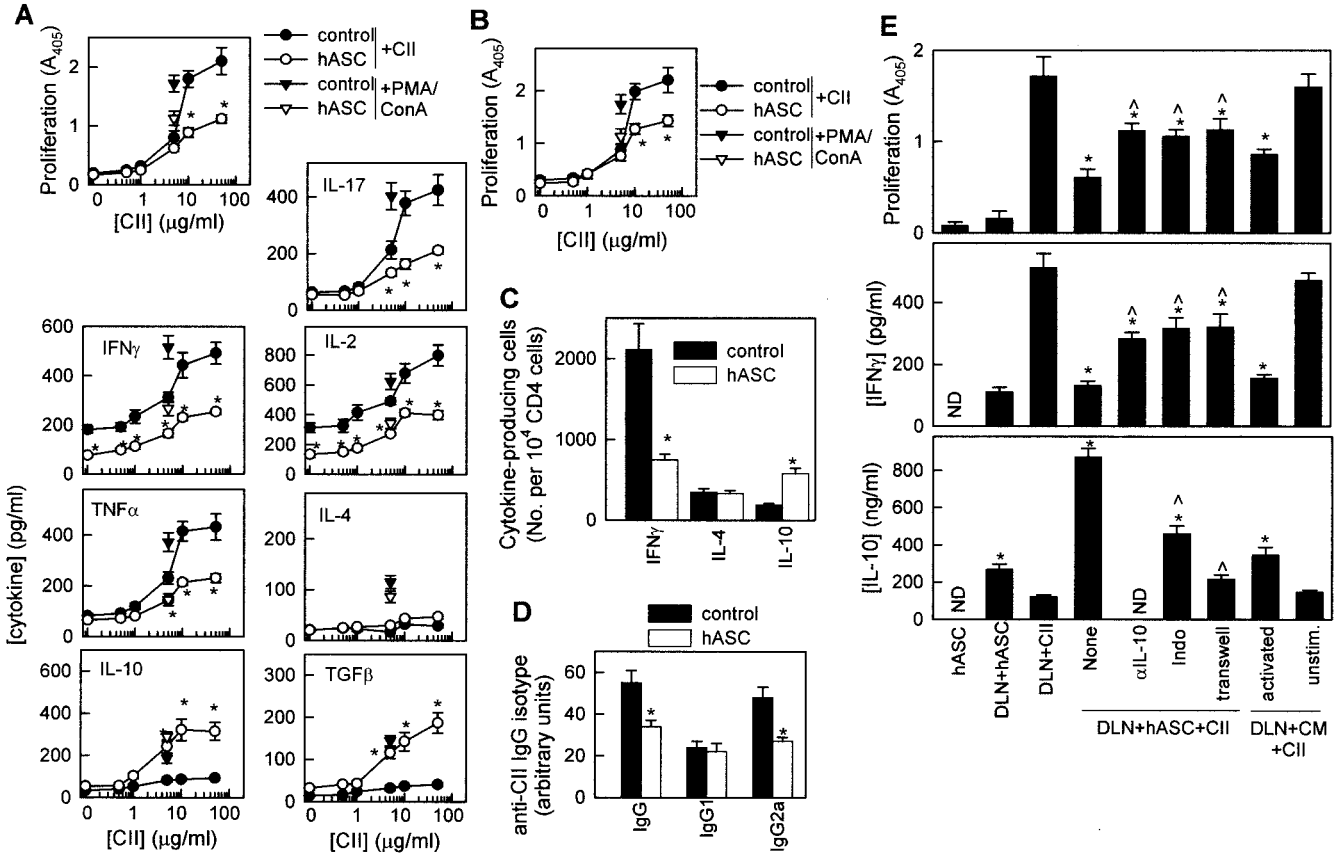

Figure 3. Inhibition of the Th1-mediated response in CIA after treatment with human AD-MSCs. Mice with established CIA were injected intraperitoneally with PBS (control) or human AD-MSC (hASC) daily for 5 days starting on day 24. **A,** Proliferative response and cytokine production by draining lymph node (DLN) cells isolated on day 30 and stimulated in vitro with different concentrations of CII. Phorbol myristate acetate (PMA) plus concanavalin A (Con A) was used to assess nonspecific stimulation. Treatment with human AD-MSCs did not significantly affect the proliferative response or cytokine production by DLN cells induced by anti-CD3 monoclonal antibody (data not shown). **B,** CII-specific proliferative response of synovial membrane cells isolated from the knee joints of PBS-treated (control) or human AD-MSC–treated mice with CIA stimulated in vitro for 48 hours with CII. **C,** Number of CII-specific cytokine-producing CD4+ T cells in DLNs from PBS-treated (control) or human AD-MSC–treated mice with CIA restimulated in vitro with CII. **D,** Levels of CII-specific IgG, IgG1, and IgG2a in sera collected on day 35 from PBS-treated (control) or human AD-MSC–treated mice with CIA. **E,** Proliferation and concentrations of IFNγ and IL-10 in DLN cells obtained at the peak of disease from mice with CIA. Proliferation (measured after 72 hours) and levels of IFNγ and IL-10 (measured after 48 hours) were determined in DLN cells stimulated with or without CII in the presence or absence of human AD-MSCs; in cocultures of DLN cells/human AD-MSCs treated with anti–IL-10 antibodies (αIL-10), indomethacin, or separated in a Transwell system; and in DLN cells activated with CII in conditioned medium (CM) collected from cultures of human AD-MSCs (unstimulated [unstim.]) or of human AD-MSCs activated with TNFα plus IFNγ. Values are the mean ± SD of 5 mice per group in **A**, 3 mice per group in **B**, 5 mice per group in **C**, 8–12 mice per group in **D**, and 8 mice per group in **E**. $* = P < 0.001$ versus control in **A–D** and versus CII-activated DLNs in **E**; $\wedge = P < 0.001$ versus cocultures of CII-activated DLNs/human AD-MSCs in **E**. ND = not detected (see Figure 2 for other definitions).

(Figure 3A). We observed similar effects on synovial cells (Figure 3B).

Determination of the intracellular expression of these cytokines in sorted CD4 T cells showed that human AD-MSCs decreased the number of TNFα/IFNγ-producing Th1 cells and increased the number of IL-10–producing CD4 T cells in DLNs (Figure 3C). This suggests that administration of human AD-MSCs to mice with CIA partially inhibited the differentiation of autoreactive/inflammatory Th1 cells and generated IL-10–secreting Treg cells.

The tolerogenic effect of human AD-MSCs was antigen-specific, since treatment with human AD-MSCs did not affect proliferation and cytokine production by anti-CD3–stimulated spleen cells (data not shown), although DLN cells and synovial cells from mice treated

with human AD-MSCs were hyporesponsive with respect to proliferation and cytokine production upon stimulation with phorbol myristate acetate plus concanavalin A (Figures 3A and B). However, T cell priming was not significantly affected by human AD-MSCs in mice with CIA, since the delayed-type hypersensitivity response was positively recalled by CII in both PBS-treated and human AD-MSC–treated mice, although human AD-MSCs slightly reduced the CII-induced ear swelling (mean ± SD change 0.11 ± 0.02 mm in PBS-treated mice versus 0.08 ± 0.02 mm in human AD-MSC–treated mice). This suggests that human AD-MSC treatment deactivated tissue-specific autoreactive Th1 cell clones.

Antibodies directed against collagen-rich joint tissue are involved in the pathogenesis of RA and CIA (28). Administration of human AD-MSCs resulted in reduced serum levels of CII-specific IgG, particularly autoreactive IgG2a antibodies (Figure 3D).

To investigate whether human AD-MSCs directly deactivated autoreactive Th1 cells, human AD-MSCs were cocultured with DLN cells from mice with CIA. Human AD-MSCs suppressed CII-induced T cell proliferation and IFNγ production (Figure 3E). Moreover, human AD-MSCs dramatically increased IL-10 production by CII-activated DLN cells, although it only slightly elevated IL-10 secretion in unstimulated DLN cells (Figure 3E), which emphasizes the antigen specificity of this response. The effects of human AD-MSCs on T cell proliferation and IFNγ production were partially dependent on cell-to-cell contact and IL-10 and PGE_2 production (Figure 3E). Accordingly, conditioned medium from activated, but not naive, human AD-MSCs significantly inhibited CII-stimulated DLN cells (Figure 3E). However, human AD-MSC–induced IL-10 production by DLN cells was primarily cell-to-cell contact dependent and was partially dependent on PGE_2 production (Figure 3E).

Promotion of the emergence of antigen-specific Treg cells in CIA following treatment with human AD-MSCs. Several studies have indicated that Treg cells confer significant protection against CIA by decreasing the activation and joint homing of autoreactive Th1 cells (3–6). The observed down-regulation of the self-reactive Th1 response, together with the elevated levels of regulatory cytokines, prompted us to investigate the involvement of CII-specific Treg cells in the in vivo immunosuppressive activity of human AD-MSCs.

Mice with CIA treated with human AD-MSCs had significantly higher numbers of CD4+CD25+ FoxP3+ Treg cells in both DLNs and synovium than did control mice with CIA (Figure 4A). Similarly, human AD-MSC treatment slightly increased the number of circulating CD4+CD25+FoxP3+ Treg cells in peripheral blood (mean ± SD 7.8 ± 0.6% and 11.9 ± 0.9% in PBS-treated and human AD-MSC–treated mice with CIA, respectively; $P < 0.01$). T cells isolated from human AD-MSC–treated mice with CIA function as suppressive Treg cells, since they inhibited in a dose-dependent manner the proliferation of syngeneic T cells in response to CII (Figure 4B). This suppressive effect was antigen-specific, since T cells from human AD-MSC–treated mice with CIA failed to inhibit the ovalbumin-induced proliferation of T cells from ovalbumin-immunized mice (data not shown).

We also assessed the ability of these cells to affect CIA. Two days after the onset of CIA, mice were treated with CD4 T cells isolated from arthritic mice that had been treated with PBS ($CD4_{control}$) or human AD-MSCs ($CD4_{AD\text{-}MSCs}$). In contrast to $CD4_{control}$ treatment, $CD4_{AD\text{-}MSC}$ treatment prevented arthritis progression (Figure 4C). Depletion of CD25+ cells from $CD4_{AD\text{-}MSCs}$ significantly abolished this therapeutic effect (Figure 4C). These results demonstrate that injection of human AD-MSCs during arthritis development induces the generation and/or activation of CII-specific CD4+CD25+FoxP3+ Treg cells that efficiently suppress autoreactive CD4 T cells, presumably by producing IL-10 and TGFβ1.

CD4+CD25+ Treg cells can be generated peripherally from CD4+CD25− T cells (2,29). To determine whether the human AD-MSC–induced increase in CD4+CD25+ Treg cells during CIA was due to expansion of the existing, naturally occurring CD4+CD25+ Treg cells or to Treg cells newly generated from CD4+CD25− T cells, mice with CIA were depleted of CD4+CD25+ T cells before injection with human AD-MSCs. As expected, CD25+ T cell depletion prior to CIA induction resulted in early onset of disease and more severe disease than in the controls (Figure 4D). CD25+ T cell depletion did not affect the beneficial effect of human AD-MSCs (Figure 4D). Importantly, human AD-MSC treatment recovered the number of splenic and DLN CD4+CD25+FoxP3+ T cells in CD25-depleted mice at the peak of CIA (Figure 4D), suggesting that human AD-MSCs could induce the peripheral generation of CD4+CD25+ Treg cells from the CD4+CD25− compartment.

Protection against CIA and generation of Treg cells following treatment with syngeneic and allogeneic murine AD-MSCs. In order to investigate whether the beneficial effect of human AD-MSCs that we observed was a consequence of treatment in a xenogeneic system, CIA was induced in DBA/1 mice and then treated with

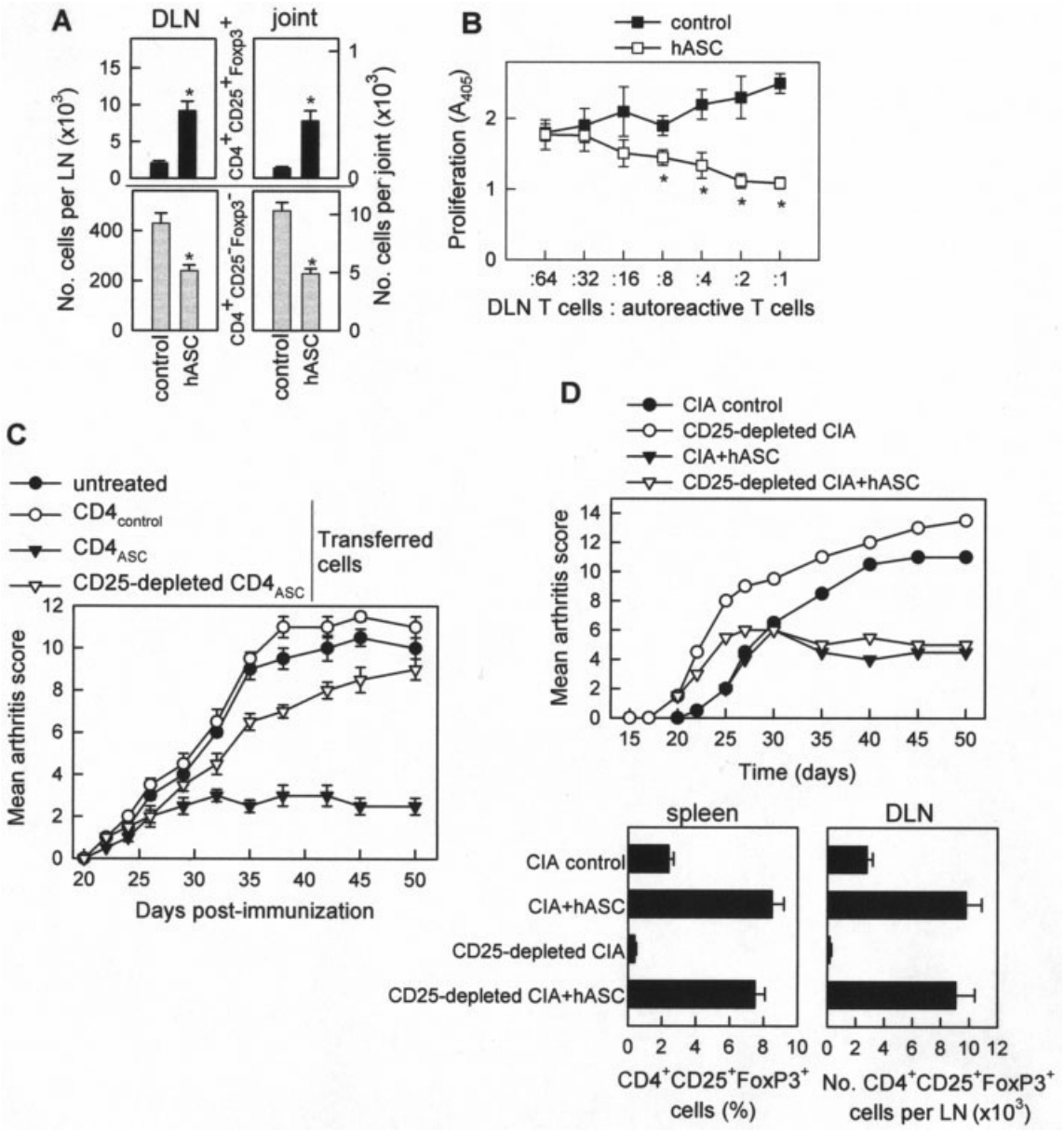

Figure 4. Induction of regulatory CD4+CD25+ T cells in CIA after treatment with human AD-MSCs. Mice with established CIA were injected intraperitoneally with PBS (control) or with human AD-MSCs (hASC) daily for 5 days starting on day 24. **A,** Numbers of CD4+CD25+FoxP3+ and CD4+CD25−FoxP3− cells per lymph node (LN) and per synovial membrane (joint) isolated on day 30, as determined by flow cytometry. **B,** Proliferative response of autoreactive T cells from mice with CIA. Cells were cocultured at different ratios (1:64 to 1:1) with draining LN (DLN) T cells isolated from PBS-treated (control) or human AD-MSC–treated mice with CIA and stimulated with CII and splenic antigen-presenting cells. **C,** Arthritis severity in mice with CIA treated with CD4+ DLN cells. CD4+ DLN cells isolated from PBS-treated ($CD4_{control}$) or human AD-MSC–treated ($CD4_{AD\text{-}MSCs}$ [$CD4_{ASC}$]) mice with CIA were stimulated in vitro with CII and then injected into arthritic mice 2 days after disease onset. Some samples were depleted of CD25+ cells before transfer. Differences were significant ($P < 0.001$) for PBS-treated controls versus $CD4_{AD\text{-}MSC}$ –treated cells after day 32 and for $CD4_{AD\text{-}MSC}$–treated cells versus CD25-depleted $CD4_{AD\text{-}MSC}$–treated cells after day 35. **D,** Arthritis severity (top), percentage of CD4+CD25+FoxP3+ T cells in the spleen (bottom left), and numbers of CD4+CD25+FoxP3+ T cells per LN (bottom right) in mice with CIA. CD25+ T cells were depleted in vivo in DBA/1 mice 10 days before CII immunization, and treatment with human AD-MSCs was started on day 22 postimmunization. CD4+CD25+FoxP3+ T cells were determined on day 28. Values are the mean ± SD of 6 mice per group in **A** and **B**, 4–6 mice per group in **C**, 4–7 mice per group in **D** top, and 4 mice per group in **D** bottom (left and right). $* = P < 0.01$ versus control in **A** and **B**. See Figure 2 for other definitions.

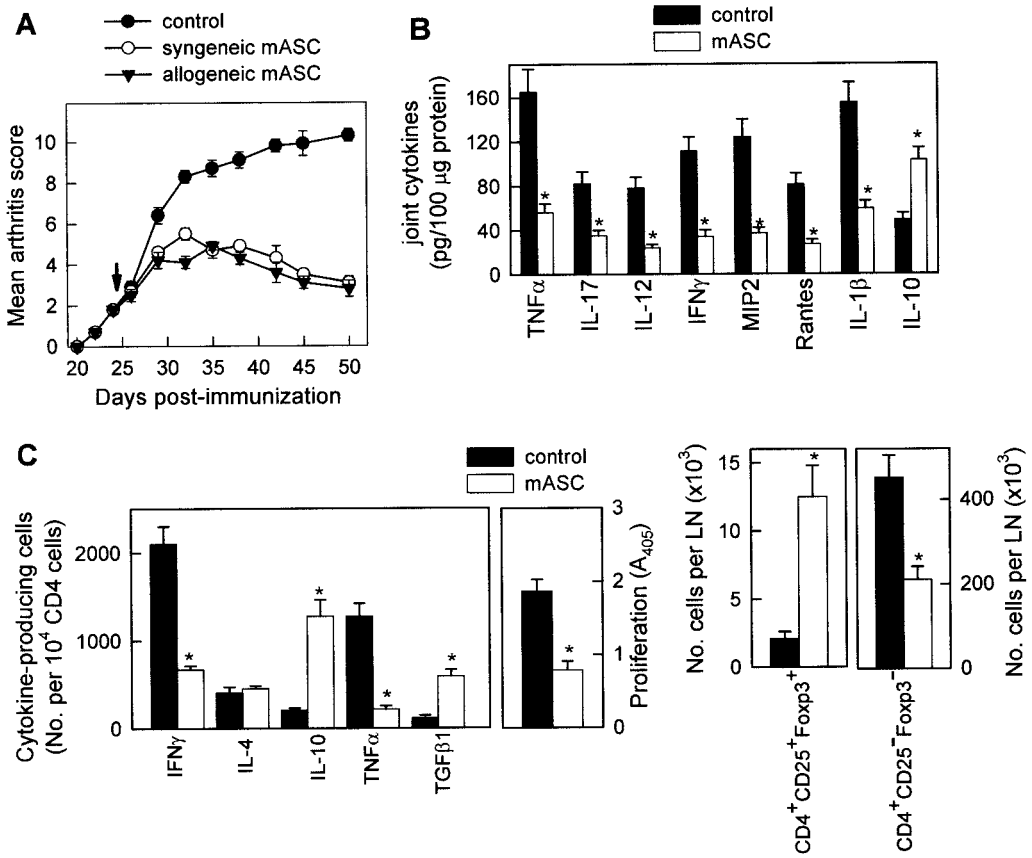

Figure 5. Protection against CIA and induction of Treg cells after treatment with syngeneic and allogeneic murine AD-MSCs. Mice with established CIA were injected intraperitoneally with PBS (control) or with allogeneic (from C57BL/6 mice [**A–C**]) or syngeneic (from DBA/1 mice [**A**]) murine AD-MSCs (mASC) daily for 5 days starting on day 24. **A,** Arthritis severity in mice with CIA, as assessed by clinical scoring. Differences were significant ($P < 0.001$) versus controls beginning on day 32. **B,** Cytokine contents in joint extracts isolated on day 35 from PBS-treated (control) or allogeneic murine AD-MSC–treated mice with CIA. **C,** Proliferation and intracellular cytokine expression in CII-activated CD4+ cells from draining lymph nodes (DLNs) (left), and numbers of CD4+CD25+FoxP3+ and CD4+CD25−FoxP3− cells per lymph node in DLNs obtained on day 35, as determined by flow cytometry (right). Values are the mean ± SD of 8–10 mice per group in **A**, 4–6 mice per group in **B**, and 5 mice per group in **C**. $* = P < 0.001$ versus controls in **B** and versus mice with CIA in **C**. See Figure 2 for other definitions.

murine AD-MSCs isolated from C57BL/6 (allogeneic) or DBA/1 (syngeneic) mice. Both allogeneic and syngeneic murine AD-MSCs efficiently protected against CIA (Figure 5A) and reduced the inflammatory response in the joint (Figure 5B). Moreover, treatment with murine AD-MSCs diminished the number of autoreactive TNFα/IFNγ-secreting T cells, while increasing the number of regulatory IL-10/TGFβ1–producing T cells and CD4+CD25+FoxP3+ T cells (Figure 5C).

Increasing numbers of murine AD-MSCs progressively inhibited the proliferative response of, and the production of IFNγ by, CII-activated DLN cells isolated from mice with CIA and stimulated the production of IL-10 and TGFβ1 (Figure 6A). This suggests that murine AD-MSCs were able to suppress Th1 responses and to induce T cells with regulatory functions. Therefore, we evaluated the suppressive activity of CD4 T cells generated in the presence of murine AD-MSCs ($T_{AD\text{-}MSCs}$) on the activation of effector CD4 T cells isolated from mice with CIA. $T_{AD\text{-}MSCs}$, but not control T cells, suppressed the proliferation of CII-activated DLN cells, and this effect was significantly reversed by anti–IL-10 and/or anti-TGFβ1 antibodies (Figure 6B). As expected, the addition of IL-2 to cocultures bypassed the suppressive activity of $T_{AD\text{-}MSCs}$ on effector T cells (Figure 6B). $T_{AD\text{-}MSCs}$ maintained their suppressive

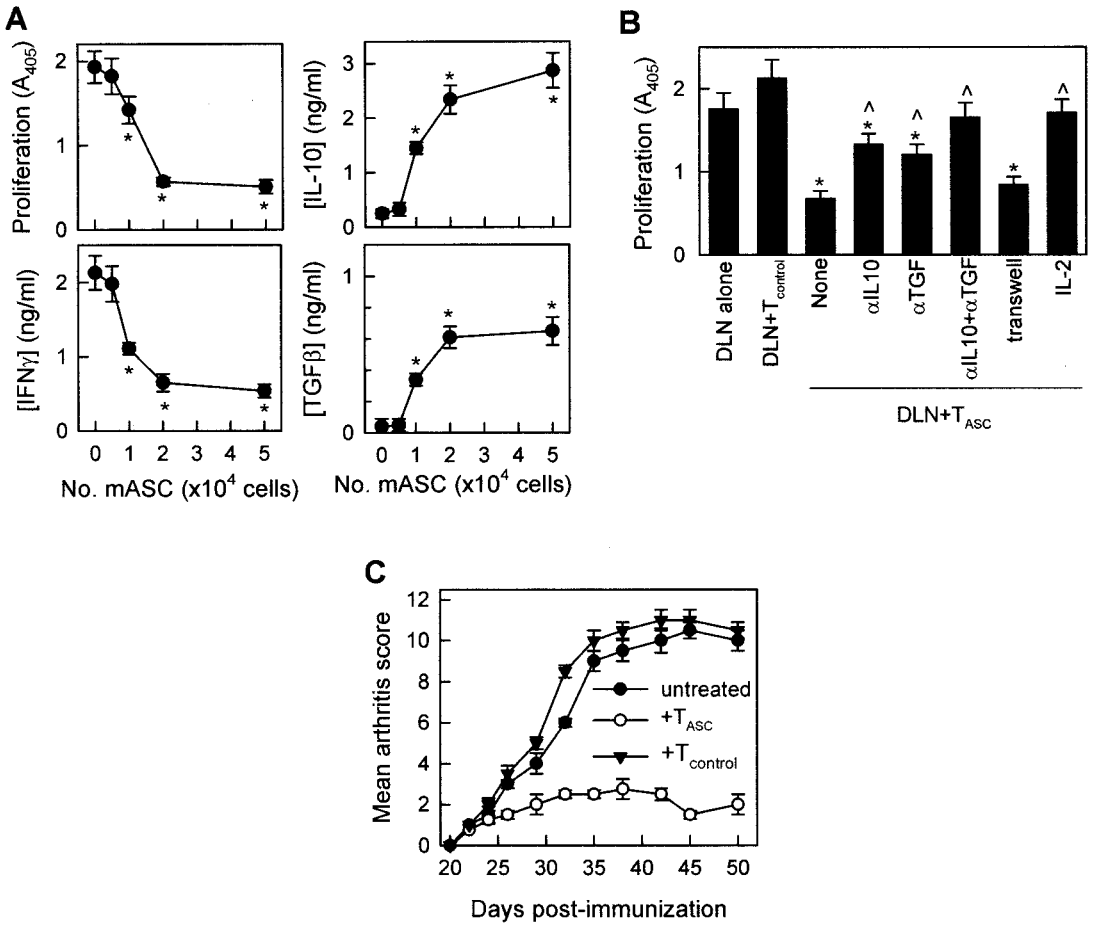

Figure 6. Induction of Treg cells with suppressive functions after treatment with murine AD-MSCs. **A,** Proliferation (measured after 4 days) and cytokine production (measured after 48 hours) by draining lymph node (DLN) cells from mice with CIA cocultured with different numbers of allogeneic murine AD-MSCs (mASC) and activated with CII. **B,** Proliferation of CII-activated DLN cells from mice with CIA cocultured with T cells isolated from DLNs from mice with CIA ($T_{control}$); with T cells isolated from cocultures of DLN cells/murine AD-MSCs ($T_{AD\text{-}MSCs}$ [T_{ASC}]); with $T_{AD\text{-}MSCs}$ and neutralizing antibodies to IL-10 (αIL-10) and/or TGFβ (αTGFβ); with $T_{AD\text{-}MSCs}$ separated in Transwells; and with $T_{AD\text{-}MSCs}$ and IL-2. **C,** Arthritis severity, as assessed by clinical scoring, in mice with CIA treated with $T_{control}$ or $T_{AD\text{-}MSCs}$ 2 days after disease onset. PBS-treated mice with CIA were used as controls. Values are the mean ± SD of 5 mice per group in **A,** 8 mice per group in **B,** and 5 mice per group in **C.** $* = P < 0.001$ versus DLN cells CII-stimulated without murine AD-MSCs in **A** and without $T_{AD\text{-}MSCs}$ in **B;** $\hat{} = P < 0.001$ versus $T_{AD\text{-}MSC}$–treated DLNs (none) in **B.** Differences were significant ($P < 0.001$) for controls versus $T_{AD\text{-}MSC}$ treatment after day 28 in **C.** See Figure 2 for other definitions.

activity in vivo, since the injection of $T_{AD\text{-}MSCs}$, but not control T cells, into mice with CIA prevented the progression of the disease (Figure 6C).

DISCUSSION

In this study, we examined a novel cell-based therapeutic strategy for RA using adult mesenchymal stem cells isolated from adipose tissue. Human AD-MSCs provided a highly effective therapy for CIA in mice, strikingly reducing the 2 deleterious components of the disease, the autoimmune and inflammatory responses. As a consequence, human AD-MSC treatment reduced the frequency of arthritis, ameliorated symptoms, and prevented joint damage. The beneficial effect of AD-MSCs on CIA was not restricted to a xenogeneic system, since both syngeneic and allogeneic murine AD-MSCs were as efficient as human AD-MSCs at ameliorating the clinical signs of arthritis. From a ther-

apeutic point of view, it is noteworthy that the delayed administration of human AD-MSCs was able to ameliorate ongoing CIA, which fulfills an essential prerequisite for an antiarthritic agent. Moreover, initial treatment with human AD-MSCs prevented recurrence of disease. Our findings also suggest that the immunosuppressive action of AD-MSCs is not restricted to the major histocompatibility complex and that the infused AD-MSCs are immunotolerated by the host, which is very convenient for future clinical application of these cells in RA.

There are several potential mechanisms for the effect of human AD-MSCs on the effector phase of CIA. Similar to RA, CIA is characterized by joint inflammation, owing to the Th1 and Th17 cell-mediated responses to cartilage antigens and joint components (1). Our results demonstrate that administration of human AD-MSCs to mice with established CIA results in a decreased CII-specific T cell–mediated response. Treatment with human AD-MSCs down-regulated the proliferative response and the expression of the Th1-type and Th17-type cytokines in DLN cells. The inhibition of Th1/Th17 responses might be the result of a direct action on cells of the DLN and the joint, since synovial cells and DLN cells obtained from animals treated with human AD-MSCs were refractory in vitro to Th1 restimulation by mitogens or challenge with antigens. Accordingly, human AD-MSCs directly inhibited the in vitro activation of CII-reactive T cells from arthritic mice. However, priming of the T cells was not significantly affected by human AD-MSC treatment, since the delayed-type hypersensitivity response to the immunizing antigen was preserved. These results suggest that the suppression of the Th1 response by human AD-MSCs is not the result of a general, nonspecific immunosuppressive activity by these cells, but rather, it is induced by a robust tissue-specific mechanism.

In contrast to the effect on Th1-type cytokines, treatment with human AD-MSCs increased the production of IL-10, but not IL-4, in DLN and synovial CD4 T cells. This does not support the notion of involvement of a predominant Th2 response. Aside from its role as an antiinflammatory factor (30), IL-10 is a signature cytokine for Treg cells, which play a key role in the control of self-antigen–reactive T cells and the induction of peripheral tolerance in vivo (2,31). Therefore, human AD-MSCs could induce IL-10–secreting Treg cells in arthritic mice that suppress effector T cells against joint antigens. Indeed, human AD-MSC treatment of mice with CIA increased the percentage of CD4+CD25+FoxP3+ T cells in DLNs and joints. In addition, CD4 cells isolated from mice treated with human AD-MSCs showed profound immunosuppressive activities on CII-specific effector T cells and important therapeutic actions on CIA. These effects were significantly abrogated when the CD4 cells were depleted of CD25+ cells. Moreover, DLN cells from arthritic mice exposed to murine AD-MSCs in vitro acquired a Treg cell–like phenotype, characterized by intrinsically low proliferative capacity, low IFNγ secretion, and high production of IL-10 and TGFβ1. These AD-MSC–induced Treg cells potently suppressed the proliferative response of self-reactive T cells in vitro, and once transferred to arthritic mice, they fully prevented the progression of disease. Therefore, the induction of IL-10/TGFβ–producing CD4+CD25+ Treg cells by human AD-MSCs in CIA could explain why delayed administration of human AD-MSCs inhibited events in the inflammatory phase of established arthritis following the activation/differentiation of antigen-specific effector Th1 cells.

Our results are consistent with those of a recent study showing that the injection of murine BM-MSCs into mice with CIA prevented disease progression by inducing the emergence of antigen-specific Treg cells (17). However, the mechanisms involved in the generation/activation of Treg cells by MSCs were not addressed in that study. Whether AD-MSCs act directly on T cells to induce the generation or expansion of CD4+CD25+ Treg cells remains to be established. The present study demonstrates that in the periphery of arthritic mice, human AD-MSCs could convert antigen-primed CD4+CD25− cells to CD4+CD25+ Treg cells, which apparently, could migrate to the joints and induce local suppression of the self-reactive response. Because IL-10 has been found to be critical in the peripheral generation of CD4+CD25+ Treg cells (32), the observed AD-MSC–induced increase in IL-10 could participate in the induction of Treg cells.

In addition, we found that human AD-MSCs directly induced anergic T cells, and induction of T cell anergy is a prerequisite for the generation of Treg cells in the periphery (33,34). Therefore, human AD-MSCs could act as tolerogenic cells, directly generating anergic T cells with regulatory functions. Indeed, BM-MSCs were found to directly induce CD4 and CD8 Treg cell functions (35,36). Alternatively, some studies have suggested that BM-MSCs could alter the differentiation and activation of dendritic cells, inducing the generation of dendritic cells with tolerogenic functions (37,38). Whether human AD-MSCs share this capacity with BM-MSCs is still unknown, but the generation of Treg

cells by human AD-MSCs through the induction of tolerogenic dendritic cells emerges as an attractive mechanism that needs further investigation.

Our findings also indicate that human AD-MSCs strongly reduced joint and systemic inflammation with efficiency similar to or even greater than that of some of the most potent immunosuppressive drugs. Human AD-MSCs exerted their effect by down-regulating the production of a wide range of mediators involved in the pathogenic inflammatory response that leads to joint damage. Reduction of chemokines could partly explain the absence of inflammatory infiltrates in the synovium of mice treated with human AD-MSCs. Besides, human AD-MSC treatment down-regulated the production of the proinflammatory/cytotoxic cytokines TNFα, IFNγ, IL-6, IL-17, IL-1β, and IL-12 by synovial immune cells and increased the levels of the antiinflammatory cytokine IL-10. The decrease in mediators of inflammation could be the consequence of a diminished cell infiltration in the joint. However, the fact that human AD-MSCs directly impaired the inflammatory response of CII-activated synovial cells from arthritic mice is evidence against this idea.

The possibility still exists that this antiinflammatory effect is a consequence of the deactivation of the Th1-driven inflammatory response by human AD-MSCs. However, several findings suggest a certain capacity of human AD-MSCs to switch off the inflammatory response of monocyte/macrophages and synovial cells, independently of their action on T cells. Human AD-MSCs directly down-regulated the production of mediators of inflammation by activated synovial cells. Moreover, human AD-MSCs have been shown to inhibit the secretion of various inflammatory cytokines by monocytes isolated from RA patients and macrophages from mice with colitis (24,39). Several studies have also shown that BM-MSCs and AD-MSCs of murine origin are able to down-regulate systemic and local inflammatory responses (11,14–18). However, the specific molecular mechanisms involved are not yet fully understood.

Our data suggest the involvement of both cell-to-cell contact and soluble factors in the deactivation of synovial cells by human AD-MSCs. Similar to BM-MSCs (23,40–42), PGE_2 and IL-10 seem to play a critical role in the suppressive effect of human AD-MSCs. Importantly, human AD-MSCs express various Toll-like receptors and become activated in response to different inflammatory mediators and bacterial products (43). Therefore, it is in this inflammatory milieu where human AD-MSCs can show the most potent antiinflammatory activity.

Part of the beneficial effect of human AD-MSCs on CIA could lie in their potential capacity to repair the damaged joint tissue via differentiation (7,8). Efficient engraftment is a prerequisite for this stromal cell–based therapy. However, we found that viability of human AD-MSCs was not required for their long-term immunosuppressive actions, since human AD-MSCs were detectable in the recipient for more than 5–7 days after injection (data not shown). During this time period, it seems that the cells were able to exert their immunosuppressive activity and to instruct other cells to inhibit the pathogenic immune reaction.

In conclusion, our findings support the use of human AD-MSCs in the intensive treatment of RA. The improvement in arthritis scores seen in AD-MSC–treated mice compares favorably with that achieved with other therapies (44). The capacity of human AD-MSCs to regulate a wide spectrum of inflammatory mediators, together with the suppression of Th1-type responses through the generation of Treg cells, might offer a therapeutic advantage over neutralizing antibodies directed against a single mediator. In addition, the maintenance of tolerance by IL-10–secreting CD4+CD25+ FoxP3+ Treg cells could be related to the resistance to disease recurrence observed in mice treated with human AD-MSCs. Finally, although transplantation of allogeneic or xenogeneic MSCs represents experimental, but not physiologic conditions, these results support a possible physiologic role of tissue-resident MSCs in the maintenance of peripheral T cell tolerance.

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AUTHOR CONTRIBUTIONS

Dr. Delgado had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. González, Gonzalez-Rey, Büscher, Delgado.
Acquisition of data. Gonzalez-Rey, Rico, Delgado.
Analysis and interpretation of data. Gonzalez-Rey, Delgado.
Manuscript preparation. González, Delgado.
Statistical analysis. Gonzalez-Rey.

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