Cell Therapy Using Allogeneic Bone Marrow Mesenchymal Stem Cells Prevents Tissue Damage in Collagen-Induced Arthritis

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Objective. Mesenchymal stem cells (MSCs) are precursors of tissue of mesenchymal origin, but they also have the capacity to regulate the immune response by suppressing T and B lymphocyte proliferation in a non–major histocompatibility complex–restricted manner. Use of MSCs as immunosuppressants in autoimmune diseases has been proposed and successfully tested in animal models. We explored the feasibility of using allogeneic MSCs as therapy for collagen-induced arthritis, a mouse model for human rheumatoid arthritis.

Methods. DBA/1 mice were immunized with type II collagen in Freund’s complete adjuvant, and some of the animals received an intraperitoneal injection of allogeneic MSCs.

Results. A single injection of MSCs prevented the occurrence of severe, irreversible damage to bone and cartilage. MSCs induced hyporesponsiveness of T lymphocytes as evidenced by a reduction in active proliferation, and modulated the expression of inflammatory cytokines. In particular, the serum concentration of tumor necrosis factor α was significantly decreased. MSCs exerted their immunomodulatory function by educating antigen-specific Tregs.

Conclusion. Our results suggest an effective new therapeutic approach to target the pathogenic mechanism of autoimmune arthritis using allogeneic MSCs. However, further studies are required before these results can be translated to clinical settings.

Although mesenchymal tissue and the immune system have different functions (skeletal support and defense of the organism, respectively), they share some characteristics, such as heterogeneity of their cellular components and the molecular mechanisms regulating their interactions (1). Experimental evidence suggests that bone development and hematopoiesis are strictly intertwined processes; not only do hematopoietic precursor cells reside close to endosteal surfaces, but osteoprogenitor cells produce many factors essential for the survival, renewal, and maturation of hematopoietic stem cells (HSCs), which are precursors of the cell components of the immune system (1,2). Osteoprogenitors are part of the very heterogeneous population of mesenchymal stem cells (MSCs), which reside primarily in the bone marrow but can be found in other tissue (e.g., fat) and are capable of self-renewal and multilineage differentiation (3,4). Under appropriate stimulation, MSCs undergo orthodox differentiation into the 3 mesenchymal lineages: adipocytes, chondrocytes, and osteoblasts (3,4). MSCs may also be induced experimentally to undergo unorthodox differentiation, i.e., neural and myogenic cells (5–7).

The heterogeneity of the MSC population is reflected by the absence of a unique, specific molecular marker. MSCs derived from different tissues express developmental markers of mesenchymal, endothelial, and hematopoietic tissues (8–10), but they also produce molecules directly involved in regulation of the immune response, such as the costimulatory molecule CD28, the inhibitory molecules programmed death ligand 1 (PDL-1) and PDL-2, and an array of different cyto-
kines (11,12). Through these molecules, MSCs can regulate the immune response. It has been shown that MSCs suppress in vitro proliferation of T and B lymphocytes, triggered by cellular stimuli, nonspecific mitogenic stimuli, or antigenic peptides. Immunologic restriction appears not to be involved, indicating that third-party nonhistocompatible cells can be used to suppress lymphocyte activation (11,13,14). In vitro MSC-mediated immunoregulation also involves differentiation of alloantigen-induced dendritic cells activating CD4+ and CD25+ T cell subsets (15). The immunoregulatory function of MSCs has also been observed in in vivo settings. In humans, treatment with MSCs improved the outcome of allogeneic transplantation by promoting hematopoietic engraftment and limiting graft-versus-host disease (GVHD) (16,17), while in animal models, MSCs were successfully used to ameliorate experimental autoimmune encephalomyelitis (18) and prevent the recurrence of autoimmunity in lupus-prone mice (19).

The activation and ending of the immune response are tightly regulated by a set of connections involving regulatory and suppressive cytokines and cells (20–22). When this network is not well balanced and the immune response remains abnormally induced, a pathologic autoimmune reaction occurs. Autoimmune diseases are the result of interactions between genes and environmental factors and can be experimentally induced in susceptible animals. Collagen-induced arthritis (CIA) is an experimental autoimmune disease that can be elicited in susceptible strains of rodents (rats and mice) and nonhuman primates by immunization with type II collagen (CII), the major constituent protein of articular cartilage. Following immunization in the animals, an autoimmune polyarthritis develops that shares several clinical and histologic features with rheumatoid arthritis (RA). Susceptibility to CIA in rodents, and to RA in humans, is linked to the class II molecules of the major histocompatibility complex (MHC), and the immune response to CII is characterized by both the stimulation of collagen-specific T cells and the production of high titers of specific antibodies (23).

Given the success of cell therapy with MSCs for the treatment of GVHD in humans (17), and for some autoimmune conditions in animal models (18,19), we explored the feasibility of using MSCs as immunosuppressant agents in therapy for CIA, and also examined the mechanisms by which MSC-mediated immunosuppression occurs in in vivo settings.

MATERIALS AND METHODS

Mice. DBA/1 (H-2b haplotype) and C57Bl/10 (H-2b haplotype) mice, 6–8 weeks old, were purchased from Charles River (Calco, Italy). Green-fluorescent protein (GFP)–transgenic mice of the C57Bl/6 background (H-2b haplotype) were kindly donated by Dr. Giuliana Ferrari (Telethon Institute for Gene Therapy, San Raffaele Hospital, Milan, Italy) (for more information, see the following Web site: http://jaxmice.jax.org).

Mice were bred and maintained at the institution’s animal facility of the National Institute for Cancer Research, Genoa, Italy. The care and use of the animals were in compliance with laws of the Italian Ministry of Health and the guidelines of the European Community.

Cells. MSCs were isolated from male and female C57Bl/10 and GFP-transgenic mice. Bone marrow cells were collected by flushing the cells out of femurs and tibiae with cold phosphate buffered saline (PBS). Cells were cultured at a concentration of 10 × 10^6 nucleated cells per 10-cm Petri dish in Coon’s modified Ham’s F-12 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Gibco, Milan, Italy), 1% glutamine, and 1% penicillin–streptomycin (standard medium). No cytokines were added at any stage. Cultures (stage P0) were incubated at 37°C in a 5% CO2 atmosphere. After 3 days, nonadherent cells were removed. When they reached 80% confluency in the dish, adherent cells were trypsinized (0.05% trypsin–EDTA at 37°C for 15 minutes) and expanded (stage P1).

Randomly chosen MSCs from individual male or female animals were pooled and tested for their capacity to form colonies and for their ability to undergo differentiation into chondrocytes, osteocytes, and adipocytes, as well as the MSC immunophenotype, as previously described (11). Only pools of MSCs showing the potential for full differentiation into mesenchymal lineages were selected and used for the experiments. More than 20 different cell pools were used in the in vitro and in vivo tests.

After GFP-transgenic cell pools were killed, spleen cells were obtained by mechanical shedding and collected in RPMI 1640 medium (Sigma, Milan, Italy) supplemented with 2-mercaptoethanol, glutamine, nonessential amino acids, sodium pyruvate, antibiotics (Sigma), and 1% normal mouse serum.

CIA induction and clinical scoring. We immunized male and female DBA/1 mice by injecting an emulsion of 100 μg of murine acid-soluble CII (Sigma) dissolved in 0.1N acetic acid and mixed with 50 μl Freund’s complete adjuvant (CFA) (Sigma) at the base of the tail. An immunization boost was given on day 21, by injecting 50 μg of murine acid-soluble CII dissolved in 0.1N acetic acid and mixed with 25 μl CFA at the base of the tail.

A first regimen of immunosuppressive cell therapy with MSCs was tested by cotreating, at the beginning of the experiment (day 0), DBA/1 mice with CII as described above and with an intraperitoneal injection of 100 μl of a cell suspension containing 5 × 10^6 allogeneic MSCs from C57Bl/10 or GFP-transgenic mice. As a control, another group of CII-immunized mice was cotreated with a cell suspension of 5 × 10^6 allogeneic splenocytes from GFP-transgenic mice in a volume of 100 μl, injected intraperitoneally.

Cells.
A second regimen of immunosuppressive cell therapy with MSCs was tested by treating CII-immunized DBA/1 mice with an intraperitoneal injection of 100 μl of a cell suspension containing 5 × 10^6 allogeneic MSCs from C57Bl/10 or GFP-transgenic mice at the moment of the boost (day 21). At the end of the experiments (day 42), we killed the animals and collected lymph nodes, splenocytes, peritoneum specimens, and limbs for further studies.

Clinical scores were assessed on a 4-point scale combining clinical signs, such as limb redness, swelling, and deformities, and histologic characteristics of joint tissues, particularly synovial infiltration and cartilage or bone destruction. The scoring system was as follows: 0 = no damage, 1 = limb redness without histologic lesions, 2 = limb swelling without histologic lesions, 3 = limb deformities with reversible histologic lesions, and 4 = limb deformities accompanied by permanent histologic lesions such as bone or cartilage erosions. Each limb of a mouse was scored separately, and the averaged score for each animal was calculated, using a modification of the method described by Thornton et al (24).

**Delayed-type hypersensitivity (DTH) response.** Seven days after the boost (day 28), the DTH response of immunized animals was tested by treating mice with a suspension of 10 μl of CII administered intradermally into the pinna of one ear. The other ear was injected similarly, but with medium (0.1 N acetic acid diluted in PBS). Ear swelling was measured 48 hours later with a spring-loaded micrometer.

**Histologic and immunohistochemical analyses.** Formalin-fixed limbs were decalcified and paraffin-embedded using standard histologic techniques. Serial 4-μm sections were cut and stained with hematoxylin and eosin to examine morphologic features and assess the histologic arthritis score.

To detect the presence of allogeneic MSCs from C57Bl/10 or GFP-transgenic mice, immunohistochemical analysis using an anti-class I H-2b monoclonal antibody (clone AF6-88.5; BD Pharmingen, Milan, Italy) or rabbit polyclonal anti-GFP antibody (Molecular Probes, Leiden, The Netherlands) was performed on limbs, peritoneum sections, and spleens collected at different time points during the experiment (days 3, 7, 11, and 42).

Primary antibody staining was followed by treatment with biotinylated goat anti-mouse or anti-rabbit secondary antibody (Vector, Burlingame, CA) and peroxidase-conjugated streptavidin (BD Pharmingen) using 3-amino-9-ethylcarbazole substrate (Sigma), generating a red precipitate. Images were captured by transmitted-light microscopy using a Zeiss Axiolab 200M microscope equipped with a Zeiss AxioCam MRc color chilled 3CCD camera (Zeiss, Wetzlar, Germany).

**Separation of Tregs.** At the end of the experiment (day 42), we collected spleens from CII-immunized DBA/1 mice that were treated or untreated with allogeneic MSCs. Spleen cells were obtained by mechanical shedding, collected in complete RPMI medium, and pooled within each experimental group. We first isolated T lymphocytes using a Pan T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) by depletion of non–T cells, then we isolated CD4^+ ,CD25^+ T lymphocytes (Tregs) from this CD4^+ cell–enriched population using a CD4^+ ,CD25^+ Regulatory T Cell Isolation Kit (Miltenyi Biotec). Cells were then counted and examined by flow cytometry to evaluate the expression of CD4, CD25, and CD27.

To test the antigen specificity of CD4^+ ,CD25^+ Tregs, another set of DBA/1 mice was immunized with 100 μg of bovine serum albumin (BSA) (Sigma) dissolved in 0.1 N acetic acid and mixed with 50 μl CFA (Sigma). Some of the animals were treated with 5 × 10^6 allogeneic MSCs from GFP-transgenic mice. Tregs were isolated using the CD4^+ ,CD25^+ Regulatory T Cell Isolation Kit (Miltenyi Biotec), as described above.

**FoxP3 expression analysis.** Total RNA was isolated from CD4^+ ,CD25^+ T lymphocytes, pooled within each experimental group, using an RNaseasy Mini Kit (Qiagen, Milan, Italy). RNA was treated with DNase (RNase-Free DNase Set; Qiagen) to avoid contamination of genomic DNA. We used a SuperScript II First-Strand Synthesis System (Invitrogen, San Diego, CA) to synthesize complementary DNA. Real-time polymerase chain reaction (PCR) was performed using FoxP3-specific primers (forward 5’-CTCACCCCCACCTACAGGCC-3’; reverse 5’-GGCACCTCACAGTGAGCT-3’) and probe (5’-FAM-TCTCCAGGACAGAACACTTCA TGCAT-XTP-3’). Gene expression levels were measured as the ratio of expression values and internal GAPDH (Rodent GAPDH Control Reagents [VIC-labeled]; Applied Biosystems, Monza, Italy).

**Proliferation assay.** T lymphocytes were isolated from the spleens of untreated or MSC-treated immunized DBA/1 mice, by negative selection using the Pan T Cell Isolation Kit (Miltenyi Biotec). In proliferation and blocking assays, responder T lymphocytes (5 × 10^5 cells/well) were plated in a round-bottomed 96-well plate (Corning Life Sciences, Peto, Italy). We examined the proliferation of DBA/1 T cells in response to stimulation with 0.2 μM phytohemagglutinin (Sigma), 10 μg murine CII (Sigma), or 10 μg BSA (Sigma) in a final volume of 200 μl/well. Cell proliferation was tested in the presence or absence of 5 × 10^5 γ-irradiated allogeneic MSCs/well or 5 × 10^5 Tregs/well, derived from CII- or BSA-immunized mice that were untreated or treated with allogeneic MSCs.

Proliferation of Tregs in response to stimulation with CII in the presence or absence of interleukin-2 (IL-2) was tested by plating 5 × 10^5 Tregs/well in a round-bottomed 96-well plate (Corning) and stimulating them with 10 μg of murine CII (Sigma), 33 units of IL-2 (PeproTech, Rocky Hill, NJ), or both in a final volume of 200 μl/well. The cultures were incubated for 60 hours and pulsed with 3H-thymidine (1.0 μCi/well) (Amersham Biosciences, Cologno Monzese, Italy) for the last 18 hours. The cells were harvested, and cell proliferation was evaluated by counting thymidine uptake, measured as counts per minute.

**Cytokine profile.** We tested IL-2, IL-4, IL-10, and interferon-γ (IFNγ) concentrations in sera from blood of MSC-treated and untreated immunized mice, using a mouse Th1/Th2 ELISA Ready-SET-Go! kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions. Serum concentrations of TNFα were also measured, using a mouse TNFα ELISA Ready-SET-Go! kit (eBioscience) according to the manufacturer’s instructions.

Concentrations of IL-2, IL-4, IL-10, IFNγ, and TNFα in supernatants of CII-primed T lymphocytes in vitro rechallenged with CII were also assessed. Responder T lymphocytes
(5 × 10⁵) were plated in each well of a round-bottomed 96-well plate and stimulated with 10 μg of murine CII (Sigma) in the presence or absence of 5 × 10⁵ γ-irradiated allogeneic MSCs/well in a final volume of 200 μl. Supernatants were collected and tested using mouse Th1/Th2 ELISA Ready-SET-Go! and mouse TNFα ELISA Ready-SET-Go! kits (eBioscience) according to the manufacturer’s instructions.

Concentrations of TNFα were also measured in supernatants of MSCs, cultured at a concentration of 5 × 10⁵/well and treated or untreated with 10 μg of murine CII (Sigma) in a final volume of 200 μl/well, using a mouse Th1/Th2 ELISA Ready-SET-Go! kit and a mouse TNFα ELISA Ready-SET-Go! kit (eBioscience) according to the manufacturer’s instructions. Reactions were read at 450 nm, obtained values were normalized with given standard solutions, and concentrations (pg/100 μl) were calculated.

**Statistical analysis.** A disease score was assessed for each treated animal, as previously described. The results of 5 independent immunization experiments were compiled, and the statistical significance of observed differences among experimental groups was calculated by nonparametric Mann-Whitney U test. A 2-tailed t-test was used to evaluate the statistical significance of observed differences between DTH response values (calculated as Δmm), the frequency of CD4+, CD25+, CD27+ T lymphocytes (calculated by flow cytometric analysis of pooled spleen cell populations), FoxP3 expression data (obtained using real-time PCR after calculation of the averaged gene:internal GAPDH ratio), serum or supernatant concentrations of IL-2, IFNγ, IL-4, IL-10, and TNFα (measured as pg/100 μl in 4 independent experiments), and cell proliferation rates (measured as counts per minute).

**RESULTS**

Prevention of severe tissue damage in CII-immunized mice by MSCs. Male and female DBA/1 mice bearing the mouse MHC H-2<sup>b</sup> haplotype were immunized with CII emulsified in CFA, to generate CIA. In this model, male DBA/1 mice are described as being susceptible to disease induction, while female mice fail to develop severe arthritis but show some of the immunopathogenic features of disease (25) and thus served as a negative disease control. In our experiments,
all 27 immunized male DBA/1 mice (100%) showed signs of CIA, with a mean ± SD disease score of 2.64 ± 1.17, while 10 (71.42%) of 14 female mice showed significantly milder signs of disease (mean ± SD disease score 1.21 ± 1.13; *P = 0.0006) (Figure 1A), indicating that the vast majority of immunized male animals developed irreversible bone or cartilage erosions in at least 1 joint, while female mice showed only reversible signs of synovial inflammation without permanent tissue damage.

Because data from the literature have shown that the immunosuppressant function of MSCs is exerted in a non–MHC-restricted manner (11,13,14,17), and that allogeneic MSCs might be more exploitable in clinical settings (17,26), we used allogeneic MSCs from C57Bl/10 mice (MHC H-2b haplotype) or GFP-transgenic mice (MHC H-2b haplotype) as immunosuppressant agents for CIA. We first intraperitoneally injected MSCs in both male and female mice at the time of immunization (day 0). The incidence (17 [70.83%] of 24 mice) and severity of disease were significantly reduced in MSC-treated male mice (mean ± SD disease score 0.59 ± 0.59) compared with the incidence and severity in mice that were immunized but not treated with MSCs (*P < 0.0001), while MSC treatment did not cause any significant change in disease severity in 6 (85.71%) of 7 female mice (mean ± SD disease score 1.15 ± 0.87) compared with female mice that did not receive treatment with MSCs (*P = 0.9109) (Figure 1A).

Immunosuppression appeared to be a specific property of MSCs, because when allogeneic spleen cells were used for treatment of CIA, disease was exacerbated in all 6 treated animals (mean ± SD disease score 3.32 ± 0.47; *P < 0.0001) (Figure 1A). To establish whether cell therapy with osteoprogenitor cells was also efficient when the pathogenic mechanisms of the disease were established, we intraperitoneally injected MSCs in CII-immunized male DBA/1 mice at the moment of the boost (day 21). At that time, the mice already showed clinical signs of disease, such as redness and/or swelling of the joints, corresponding to a clinical score of 1–2 (Figure 1B). Treatment with MSCs prevented exacerbation of the disease; at the end of the experiment, only 7 (70%) of 10 animals treated with MSCs at the moment of the boost showed signs of disease, with a mean ± SD disease score of 1.05 ± 1.29; the incidence and disease score were significantly lower than the incidence and disease score of mice that were immunized but did not receive treatment with MSCs (*P = 0.001) and were comparable with the incidence and disease score of animals that received MSC therapy at the time of immunization (*P = 0.1590).
Figure 3. Cytokine profiles. A, Serum concentrations of interleukin-2 (IL-2), interferon-γ (IFNγ), IL-4, IL-10, and tumor necrosis factor α (TNFα) at the end of the experiment, analyzed by enzyme-linked immunosorbent assay. Results are representative of 3 independent experiments. B, Supernatant concentrations of IL-2, IFNγ, IL-4, and IL-10 produced by type II collagen (CII)–primed T lymphocytes stimulated in vitro with CII in the presence or absence of mesenchymal stem cells (MSCs). The TNFα concentration was measured in supernatants of cultures of CII-primed T lymphocytes challenged in vitro with the immunizing antigen and cultured in the presence or absence of allogeneic MSCs. The expression profile of TNFα secreted by MSCs cultured in the presence or absence of CII is also shown. Results are representative of 3 independent experiments. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. * = P < 0.03.
conditions ($P = 0.0005$, $P = 0.0219$, $P = 0.0019$, respectively) (Figure 2).

**Effect of MSC treatment on cytokine production in immunized mice.** At the end of the experiment, we assessed serum concentrations of IL-2, IL-4, IL-10, IFNγ, and TNFα. In parallel, we checked the cytokine expression profiles in supernatants of CII-primed T lymphocytes that were rechallenged in vitro with the immunizing antigen, in the presence or absence of allogeneic MSCs.

As a result, we observed that serum concentrations of IL-2 were not modified by the effect of MSCs (Figure 3A), while MSCs tended to decrease serum concentrations of IFNγ, IL-4, and IL-10 (Figure 3A). These data paralleled the results obtained in an analysis of supernatants of lymphocyte cultures, in which the presence of MSCs did not affect IL-2 concentrations, tended to reduce IFNγ and IL-4 concentrations, and significantly lowered concentrations of IL-10 ($P = 0.0084$) (Figure 3B).

TNFα is a pivotal cytokine in the pathogenesis of RA (27), and the effect of MSCs on TNFα production during CIA appeared to be an extremely complex phenomenon. The serum concentration of TNFα was significantly decreased in immunized mice treated with allogeneic MSCs (mean ± SD TNFα concentration in MSC-treated mice $37 ± 45$ pg/100 μl versus $145 ± 21$ pg/100 μl in untreated mice; $P = 0.0197$) (Figure 3A). In contrast, secretion of TNFα by in vitro–stimulated CII-primed T lymphocytes was significantly increased when they were cocultured with allogeneic MSCs (mean ± SD concentration $686 ± 109$ pg/100 μl) compared with
TNFα production in the absence of MSCs (mean ± SD concentration 414 ± 156 pg/100 μl; P = 0.029) (Figure 3B). When we tested whether MSCs would produce TNFα if they were cultured in the presence of CII, we surprisingly observed that adding CII to the culture medium induced the secretion of significantly higher amounts of TNFα (mean ± SD concentration 627 ± 28 pg/100 μl; P = 0.0001) (Figure 3B).

Role of MSC viability in immunosuppression of CIA. We tracked the fate of MSCs in the recipient organism by the detection of H-2b–positive or fluorescence-labeled MSCs isolated from GFP-transgenic mice and injected in CIA-immunized mice. At the end of the experiment, H-2b–positive, green-fluorescent cells were not detectable by immunohistochemistry in the joints of MSC-treated mice, suggesting that injected MSCs did not restore tissue integrity by mechanisms of tissue repair (data not shown [28,29]).

At the end of the experiment, H-2b–positive, green-fluorescent cells were not evident at the site of injection, in the peritoneum, or in secondary lymphoid organs, such as the spleen (data not shown); however, we were able to detect these cells at intermediate time points during the course of the disease (Figure 4). Briefly, MSCs were found to colonize the peritoneum throughout the first week after the initiation of treatment (Figure 4). At the same time, they possibly started to circulate through the bloodstream, ending in the spleen, where they were found as cell ghosts 7 day after treatment (Figure 4). At day 11, both the peritoneum and spleen were negative (Figure 4).

Mediation of MSC action by antigen-specific Tregs. To explore the possibility that the in vivo immunosuppressant action of MSCs was mediated by activation of a cascade of different cell types, i.e., Tregs, we compared the frequency of peripheral Tregs characterized by the CD4+,CD25+,CD27+,FoxP3+ phenotype (30) within spleen cell populations from mice immunized with CII with the frequency in spleen cells from mice immunized with CII and treated with MSCs at the beginning of the experiment. We observed that CD4+,CD25+,CD27+ T lymphocytes represented a mean ± SD of 5.39 ± 4.01% (range 1.19–9.57%) of the spleen cell populations of immunized and MSC-treated mice, while they were virtually absent in the spleens of mice receiving only immunization with CII (mean ± SD frequency 0.19 ± 0.21% [range 0–0.37%]; P = 0.0412) (Figure 4).

We also assessed the expression rate of the forkhead transcription factor FoxP3, a molecular marker characterizing cells with an immunoregulatory function (31). FoxP3 was expressed at significantly higher levels in CD4+,CD25+ T lymphocytes than in CD4+,CD25− cells isolated from splenocytes of both MSC-untreated and MSC-treated immunized mice (mean ± SD relative expression rates 6.8 ± 0.3 versus 1.1 ± 0.2 and 8 ± 0.4 versus 0.7 ± 0.1, respectively; P < 0.0001 for both comparisons) (Figure 5). Moreover, Tregs from immunized mice treated with MSCs expressed significantly higher levels of messenger RNA for FoxP3 (mean ± SD relative expression rate 8 ± 0.4) than immunized but untreated mice (relative expression rate 6.8 ± 0.3; P =
0.002). In contrast, CD4+CD25+ T lymphocytes from mice that were not treated with MSCs expressed higher levels of FoxP3 (mean ± SD relative expression rate 1.1 ± 0.2) than CD4+CD25− T lymphocytes from MSC-treated mice (relative expression rate 0.7 ± 0.1; P = 0.0124).

Proliferation and blocking assays showed that Tregs isolated from the splenocytes of immunized and MSC-treated mice were antigen specific, being able to suppress the proliferation of primed lymphocytes in response to stimulation with CII (Figure 6). In these experiments, T lymphocytes from immunized mice were rechallenged in vitro with the specific antigen CII that recalled a significant cell proliferation (P = 0.0044). CII-induced lymphocyte proliferation was not blocked by Tregs from immunized mice that were not treated with MSCs (P = 0.1196), but it was significantly inhibited by the presence of Tregs from MSC-treated immunized mice (P = 0.0053) (Figure 6).

In order to countercheck the antigen specificity of MSC-educated Tregs, we immunized DBA/1 mice with BSA, treating some of the animals with allogeneic MSCs. We isolated Tregs from MSC-treated animals and performed proliferation assays, using CD4+ lymphocytes from BSA-treated animals as responder cells. These cells did not proliferate when stimulated with CII (P = 0.4222 versus basal proliferation), but they proliferated when stimulated with BSA (P = 0.0113 versus basal proliferation). Cell proliferation was significantly reduced when Tregs from BSA-immunized and MSC-treated animals were added (P = 0.0366) but not when Tregs from CII-immunized and MSC-treated animals were added (P = 0.3338) (Figure 6).

CD4+CD25+CD27+,FoxP3+ Tregs from immunized and MSC-treated mice did not proliferate when cultured in the presence of CII, but they actively proliferated in the presence of IL-2 (P < 0.0001), and proliferation tended to increase in the presence of IL-2 plus CII (P < 0.0001) (Figure 6).

**DISCUSSION**

Cell therapy for autoimmune disease began several years ago when a consensus statement on use of HSC transplantation in the treatment of severe autoimmune disease was published (32,33). However, a broader application of stem cell therapy requires better understanding of how the use of adult stem cells can modify the autoimmune process, interfering with hyperactivation of the host’s immune responses.

Recent characterization of MSCs and their role in hematopoiesis (2) and immune modulation (34) suggests their potential use for cell therapy (35,36). In animal models, autologous MSCs have been successfully used to ameliorate experimental autoimmune encephalitis (18), and therapy with allogeneic osteoprogenitor cells prevented recurrence of autoimmunity in lupus-prone mice (19). Data in the literature showed that the immunosuppressant function of MSCs is not MHC restricted (11,13,14,17), and that allogeneic MSCs might be more functional in clinical settings (17,26). Therefore, we used allogeneic MSCs from completely MHC-mismatched mice for the treatment of CIA.

A single intraperitoneal injection of allogeneic MSCs given at the moment of immunization with CII was sufficient to prevent the occurrence of bone and cartilage erosions in the joints of immunized mice. These are permanent, irreversible lesions corresponding to the
most severe grade of disease and are never observed in MSC-treated mice. The observation that the DTH response to the immunizing antigen was preserved, albeit reduced, in MSC-treated mice indicates that priming of T lymphocytes occurred.

Together, the absence of severe tissue injuries and the presence of a positive DTH response suggest that MSCs might act by inhibiting the activation and proliferation of tissue-specific autoreactive T cell clones. Furthermore, we determined whether the immunosuppressive function of MSCs could also be in effect when the mechanisms of disease are already established, by injecting allogeneic MSCs on day 21, together with the immunization boost, when the mice already showed signs of disease. In this setting, MSCs were able to prevent the occurrence of severe lesions in the joints of immunized mice.

A first report of cell therapy for CIA using mesenchymal cell lines was not encouraging, showing that the use of these cells was not beneficial in curing arthritis, and that the inflammatory milieu in the injured tissues might reverse their immunomodulatory effect through activation of the TNFα inflammatory pathway (37). The discrepancy between those results and ours could be attributed to several factors. The main difference is that Djouad et al used an immortalized mesenchymal cell line (37), while we used primary cultures of mouse MSCs obtained after the first in vitro passage (11). The mesenchymal cell line used by Djouad et al was not efficient in blocking the antigen-specific immune response in in vivo settings, despite the fact that its inhibitory activity was preserved in vitro (37). Moreover, when those investigators added TNFα to cocultures of splenocytes and immortalized mesenchymal cells, in vitro mimicking the inflammatory milieu occurring during CIA, they reverted the immunosuppressant activity of MSCs.

Our data, however, depicted a much more complex scenario, in which MSCs themselves produced high levels of TNFα when cultured in the presence of CII, in vitro mimicking the antigen-specific immunizing conditions; even if MSCs produced the same high amounts of TNFα also in the in vivo setting, the final effect of their action was an overall significant decrease in the TNFα concentration in sera from cured animals. This might suggest that TNFα specifically secreted by MSCs targeted a particular type of cells bearing TNF receptor I, leading to a paradoxical antinflammatory action (38), or that the activity of TNFα eventually produced by MSCs would be, in the long run, counterbalanced by the function of other MSC-educated immunomodulatory cells or factors. Therefore, it is possible that MSCs lose the ability to “educate” immunosuppressant effectors during the immortalization process, preserving their in vitro inhibitory activity, or that the TNFα produced by the host as an antitumor response to the immortalized cell line (39) could block the antiproliferative cascade initiated in vivo by cells of mesenchymal origin.

The use of MSCs for cell therapy greatly relies on their capacity to engraft and survive long-term in the appropriate target organs (40), contributing to the repair of injured tissues (28,29). Their plasticity and capacity to undergo orthodox and unorthodox differentiation processes allowed the use of MSCs, alone or combined with biomaterials, for repair of tissues, such as bone (41), heart (42,43), kidney (44), lung (45,46), and brain (47). However, in our experiments we found that MSC viability was not required for their long-term immunosuppressant action; MSCs were, in fact, detectable in the recipient for no more than 10 days after treatment. During this time lag, they were able to “educate” other cells to inhibit the pathogenic immune reaction.

We observed that the in vitro proliferation rate of T lymphocytes isolated from MSC-treated mice was significantly lower than the proliferation rate of T cells from immunized mice that did not receive MSCs. This result was evident either under basal conditions or when the proliferation was recalled by a mitogenic stimulus or by challenge with the immunizing antigen. The observation that serum concentrations of the different cytokines were lower in MSC-treated animals strengthened the idea that MSCs down-regulated activation of the pathogenic immune mechanism leading to tissue damage.

Tregs play an important role in the prevention of autoimmunity, and it has been demonstrated that they modulate the severity of CIA (48,49). We found that MSC treatment in immunized mice induced proliferation of antigen-specific clones of Tregs with a CD4+,CD25+,CD27+,FoxP3+ phenotype, suggesting that the immunosuppressive activity of MSCs could be prolonged by the action of Treg clones that can be activated by an antigen-specific stimulus.

Current therapy for RA is directed toward diminishing the inflammatory response and treating the sequelae of uncontrolled inflammation. To date, it has not been possible to prevent the disease or to completely arrest the disease process through medical therapy (50). Our results represent an effective new therapeutic approach to target the pathogenic mechanism of autoimmune arthritis using adult stem cells, although further studies are required before these results can be translated to the clinical setting.
AUTHOR CONTRIBUTIONS

Dr. Pennesi 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. Augello, Pennesi.

Acquisition of data. Augello, Tasso, Negrini.

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Statistical analysis. Augello, Tasso, Pennesi.

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