Transforming Growth Factor β–Transduced Mesenchymal Stem Cells Ameliorate Experimental Autoimmune Arthritis Through Reciprocal Regulation of Treg/Th17 Cells and Osteoclastogenesis

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Objective. Bone marrow–derived mesenchymal stem cells (MSCs) can prevent various autoimmune diseases. We examined the therapeutic potential of transforming growth factor β (TGFβ)–transduced MSCs in experimental autoimmune arthritis, using an accepted animal model of collagen-induced arthritis (CIA).

Methods. DBA/1J mice with CIA were treated with syngeneic TGFβ-induced MSCs, whereas control mice received either vehicle or MSCs alone. Arthritis severity was assessed by clinical and histologic scoring. TGFβ-transduced MSCs were tested for their immunosuppressive ability and differential regulation in mice with CIA. T cell responses to type II collagen were evaluated by determining proliferative capacity and cytokine levels. The effects of TGFβ-transduced MSCs on osteoclast formation were analyzed in vitro and in vivo.

Results. Systemic infusion of syngeneic TGFβ-transduced MSCs prevented arthritis development and reduced bone erosion and cartilage destruction. Treatment with TGFβ-transduced MSCs potently suppressed type II collagen–specific T cell proliferation and downregulated proinflammatory cytokine production. These therapeutic effects were associated with an increase in type II collagen–specific CD4+FoxP3+ Treg cells and inhibition of Th17 cell formation in the peritoneal cavity and spleen. Furthermore, TGFβ-transduced MSCs inhibited osteoclast differentiation.

Conclusion. TGFβ-transduced MSCs suppressed the development of autoimmune arthritis and joint inflammation. These data suggest that enhancing the immunomodulatory activity of MSCs and modulating T cell–mediated immunity using gene-modified MSCs may be a gateway for new therapeutic approaches to clinical rheumatoid arthritis.

Rheumatoid arthritis (RA) is an autoimmune disease caused by chronic joint inflammation, with subsequent cartilage destruction and bone erosion. Although the precise etiology of RA remains unclear, proinflammatory cytokines and autoreactive T cells are crucial in its pathogenesis. Th1 cell and/or Th17 cell activation has been implicated in the development of cell-mediated autoimmune arthritis (1,2). In contrast, Th2 cells and/or Treg cells are protective in RA and in animal models of collagen-induced arthritis (CIA) (3,4).

Transforming growth factor β (TGFβ) is a multifunctional cytokine that regulates cell proliferation, differentiation, and migration, and wound healing and tissue repair (5). It also plays critical roles in the induction of FoxP3+ Treg cells (6,7), interleukin-6 (IL-6)–induced differentiation of Th17 cells (8), and Treg cell homeostasis (9). However, TGFβ must be activated to exert its regulatory effects, and there is controversy regarding the exact mechanism(s) involved.

Treg cells that express the transcription factor FoxP3 play a critical role in controlling autoimmune responses and maintaining peripheral tolerance (10,11).
Deficiencies in Treg cell function have been identified in autoimmune diseases including RA (12), psoriasis (13), and myasthenia gravis (14); these studies suggest that Treg cells control autoimmunity. Th17 cells, a new subset of CD4+ T helper cells, may play a central role in the pathology of autoimmune disease. Genetic deletion of IL-17 in mice inhibited the development of CIA (15). Increased levels of IL-17 have been detected in RA synovial fluid (16) and synovial tissue memory T cells (17).

Mesenchymal stem cells (MSCs) are multipotent progenitor cells. They can be isolated from a number of adult tissues capable of giving rise to adipogenic, osteogenic, and chondrogenic lineages, and have the ability to differentiate to lineages of mesenchymal tissues, including bone, cartilage, and adipose tissues (18). These cells have potent immunosuppressive and antiinflammatory effects, through direct cell–cell contact or by secreting soluble factors, and have the potential for clinical application in the repair of damaged tissue. MSCs also suppress T cell proliferation induced by alloantigens or mitogens (19–21). Because of their immunomodulatory effects, MSCs were proposed as a treatment of acute graft-versus-host disease after allogeneic stem cell transplantation (22) as well as experimental encephalomyelitis (23) and diabetes (24). However, the specific mechanisms involved in the immunoregulatory activity of bone marrow MSCs remain unknown, and the therapeutic effects of MSCs have varied in different CIA models (25,26).

We used adenoviral TGFβ gene transfer to mouse bone marrow MSCs to achieve more potent therapeutic efficacy for autoimmune arthritis. We observed that systemic delivery of TGFβ-transduced MSCs reduced disease severity, osteoclastogenesis, and bone erosion. Moreover, TGFβ-transduced MSCs regulated type II collagen–reactive T cell responses and proinflammatory cytokines by inducing type II collagen–specific Treg cells and inhibiting Th17 cell differentiation. These data demonstrate the ability to provide effective treatment in established arthritis models through systemic administration of TGFβ-transduced MSCs. Thus, these MSCs may represent a new and powerful approach to the treatment of RA and other autoimmune diseases.

**MATERIALS AND METHODS**

**Mice.** Male DBA/1J mice (The Jackson Laboratory) were maintained in a specific pathogen–free environment and fed standard laboratory mouse chow (Ralston Purina). The mice were treated according to the regulations of the Catholic Ethics Committee of the Catholic University of Korea, which conform to the National Institutes of Health guidelines.

**Collagens.** Professor Andrew Kang (University of Tennessee) provided bovine type II collagen. Type II collagen was extracted in its native form from the articular cartilage of fetal calves and purified as described previously (27).

**Isolation and culture of mouse bone marrow–derived MSCs.** Bone marrow cells were collected by flushing mouse femurs and tibias with medium. The cell immunophenotypes were persistently positive for stem cell antigen 1, CD44, and CD29 but were negative for CD11b, CD34, and CD45 after more than 9 passages (data not shown).

**Adenoviral expression vectors for TGFβ and transduction conditions.** The Eco RI/Sal I fragment of pMX eGFP-hTGFβ (containing, sequentially, the full human TGFβ gene, EMCV-IRES, and enhanced green fluorescent protein [EGFP]) and the Xho I/Sal I fragment of pMX IRES-eGFP (containing EMCV-IRES and EGFP [28]) were subcloned into the adenovirus loxP vector (29). Briefly, recombinant adenovirus loxP vectors were cotransfected into Cre-expressing 293 cells with psi5 adenoviral DNA, using calcium phosphate precipitation. Primary supernatants containing recombinant adenoviruses were plaque purified and amplified on 293 cells. MSCs were generated as described above and transduced with adenoviral human TGFβ or adenoviral GFP at multiplicities of infection of 100. The expression of EGFP was analyzed by flow cytometry.

**In vitro Treg cell conversion assay.** MSCs or TGFβ-transduced MSCs were cultured with sorted CD4+CD25− T cells using the Vantage FACSorter (BD Biosciences) (the ratio of MSCs to CD4+CD25− T cells was 1:10). After 3 days, the cell surface was stained with CD4, CD25, and FoxP3 antibodies.

**Induction and treatment of CIA.** CIA was induced as described previously (30). Briefly, DBA/1J mice received an injection into the base of the tail with 100 µg of type II collagen emulsified in Freund’s complete adjuvant. Two weeks later, they received booster injections of 100 µg of type II collagen in Freund’s incomplete adjuvant. Clinical signs of arthritis in the wrist and ankle joints were assessed visually, as reported previously (31). Seven weeks after the primary immunization, mice received intraperitoneal injections of 1 × 10^6 MSCs or TGFβ-transduced MSCs. Control mice received intraperitoneal injections of an equal volume of phosphate buffered saline (PBS) at the same time points.

**Histologic evaluation of CIA.** A histologic analysis was conducted to determine the extent of joint damage. Mouse joint tissues were fixed in 10% paraformaldehyde, decalcified in EDTA bone decalcifier, and paraffin-embedded. The sections were then dehydrated using xylene and dehydrated through an alcohol gradient. Endogenous peroxidase activity was quenched with methanol/3% H2O2. Sections were routinely stained with hematoxylin and eosin, Safranin O–fast green, and toluidine blue O, and were evaluated in a blinded manner, as described previously (32).

Immunohistochemical analysis was performed using the Vectastain ABC kit (Vector). Tissues were incubated first with the primary anti-GFP antibody (Abcam) overnight at 4°C, a biotinylated secondary linking antibody, and finally a streptavidin–peroxidase complex for 1 hour. The final color product was developed using aminoethylcarbazole (Dako). Sections were counterstained with hematoxylin, and samples were photographed with an Olympus photomicroscope.

**Measurement of cytokine- and type II collagen–specific IgG titers.** Concentrations of IL-10, IL-17, tumor necrosis factor α (TNFα), IL-6, and IL-21 in cell culture
supernatants and serum were measured using a Duoset sandwich enzyme-linked immunosorbent assay (ELISA; R&D Systems). Serum levels of anti-type II collagen IgG, IgG1, and IgG2a antibodies were measured using a commercially available ELISA kit (Bethyl Laboratories).

**Proliferation assay.** For proliferation analysis, cells were pulsed with 1 μCi 3H-thymidine (GE Healthcare) per well for the final 8 hours of the 72-hour culture period. Finally, 3H-thymidine incorporation was determined using a liquid beta-scintillation counter (Beckman).

**Flow cytometric analysis.** Mononuclear cells were immunostained with various combinations of the following fluorescence-conjugated antibodies: CD25, CD4, CCR7, FoxP3, IL-17, CTLA-4, and glucocorticoid-induced tumor necrosis factor receptor (GITR). These cells were also intracellularly stained with the following antibodies: CTA-4 (BD Biosciences), IL-17, and FoxP3 (eBioscience). Before intracellular staining, the cells were restimulated for 4 hours with 25 ng/ml of phorbol myristate acetate and 250 ng/ml of ionomycin in the presence of Golgistop (BD Biosciences). Intracellular staining was conducted using an intracellular staining kit (eBioscience) according to the manufacturer’s protocol. Flow cytometric analysis was performed on a FACSCalibur cytometer (BD Biosciences).

**Labeling with 5,6-carboxyfluorescein succinimidyl ester (CFSE).** Mononuclear cells isolated from mice spleens were washed once in 0.1% bovine serum albumin (BSA) in PBS and labeled with 1 μM of 5 mM CFSE (Invitrogen) at a density of 10^7 cells/ml in 0.1% BSA in PBS for 10 minutes at 37°C in the dark. CFSE-labeled cells were stimulated with anti-CD3 monoclonal antibody (1 µg/ml) and type II collagen (40 µg/ml) for 3 days in 24-well plates (1 × 10^6 cells/ml). Flow cytometry was used to assess CFSE fluorescence.

**Real-time reverse transcription–polymerase chain reaction (RT-PCR).** Total RNA was extracted using TRIzol LS reagent (Invitrogen). Total RNA (2 µg) was reverse transcribed at 50°C for 2 minutes, followed by 60°C for 30 minutes. Quantitative PCR was performed using the FastStart DNA Master SYBR Green I kit and a LightCycler 480 detection system (both from Roche), as specified by the manufacturer. The crossing point was defined as the maximum of the second derivative from the fluorescence curve. Negative controls were included and contained all elements of the reaction mixture except template DNA. For quantification, we report relative expression levels of specific genes obtained according to the 2^{-ΔΔCt} method and used the β-actin housekeeping gene for normalization.

The following gene-specific primers were used: for CCR1, forward 5'-CCATCCACAGGAGAACATGA-3’ and reverse 5’-GCCCATTTTTGTATTC-3’; for CCR4, forward 5’-ATCGTGACCGGATTTCC-3’ and reverse 5’-GACGGGTATTAAGGCAGACGTGA-3’; for CCR7, forward 5’-TTCTGCCAAGATGAGTCCACGTCGAT-3’ and reverse 5’-ACAGGAAAGACGTGAAGC-3’; for CXCR4, 5’-AGCATGAGGAAATGACCACATC-3’ and reverse 5’-GATGATGAATGCGGAACTC-3’; for tartrate-resistant acid phosphatase (TRAP), forward 5’-TCTTGGTCGAAAAAGAAGT-3’ and reverse 5’-ACATGGCCACACCGGCTTC-3’; for RANK, forward 5’-CACAGCAGAGGACATCAG-3’ and reverse 5’-CAGTGAAATCTTCCACACAG-3’; for NFATc1, forward 5’-CGGGAAGAGATGTGCTGT-3’ and reverse 5’-TTGGACGGGGCTGGTTAT-3’; for matrix metalloproteinase 9 (MMP-9), forward 5’-CTGGTACCAACAGGAGTTACGGCCT-3’ and reverse 5’-GGAGTGATTTGGAGGACATAGTTG-3’; for calcitonin receptor (CTR), forward 5’-CAGCTTTGACACAGCAGAAG-3’ and reverse 5’-AGCAGAACAGACAGCAG-3’; for β-integrin, forward 5’-CTGTGCGTGACATCAAAG-3’ and reverse 5’-GGATGATATGGACAGCCTTAC-3’; and for β-actin, forward 5’-GAAAATCGTGGCTGACATCAAAG-3’ and reverse 5’-TGATGTTTCATGGATGCCACAG-3’.

**Osteoclastogenesis.** Murine osteoclasts were generated as described previously, with minor modifications (33). Briefly, bone marrow cells from normal mice were cultured overnight, and nonadherent bone marrow cells were harvested and cultured with MSCs or TGFβ-transduced MSCs in the presence of 10 ng/ml of macrophage colony-stimulating factor (M-CSF) and 20 ng/ml of RANKL. TRAP staining was then performed. TRAP-positive cells with ≥3 nuclei were defined as osteoclasts, and the number of osteoclasts was counted. To investigate osteoclast differentiation in vivo, we isolated bone marrow cells from mice with CIA treated with either PBS, MSCs, or TGFβ-transduced MSCs and cultured them with 10 ng/ml of M-CSF and 20 ng/ml of RANKL for 3 days.

**Statistical analysis.** Data are presented as the mean ± SEM. Statistical analyses were performed using SPSS software version 10.0 for Windows. The intergroup analysis was performed using a Mann-Whitney U test. P values less than 0.05 were considered significant.

**RESULTS**

**Regulatory function following injection of TGFβ-transduced MSCs.** We determined immunologic regulatory features to characterize the TGFβ-transduced MSCs and compare them with MSCs. MSCs suppress T cell proliferation activity in vitro (19). To determine whether TGFβ-transduced MSCs also suppress T cell proliferative responses in vivo, we examined proliferative responses to CD3 in cells isolated from mouse spleens or lymph nodes after injection of TGFβ-transduced MSCs or MSCs. CD3-induced proliferation of wild-type lymphocytes was sharply inhibited by both TGFβ-transduced MSCs and MSCs (Figure 1A), although TGFβ-transduced MSCs did so more efficiently (Figure 1A), suggesting more potent immunosuppression.

Experimental evidence indicates that human adipose tissue–derived MSCs induce de novo generation of Treg cells from peripheral CD4+CD25−T cells (34). To investigate the time kinetics of generation of Treg cells by TGFβ-transduced MSCs in vivo, we performed a fluorescence-activated cell sorting analysis of the Treg cell populations in mouse spleens, mesenteric lymph nodes (LNs), draining LNs, and the peritoneal cavity.
following injection of TGFβ-transduced MSCs or MSCs. Interestingly, the number of Treg cells in the peritoneal cavity increased substantially in mice that received an injection of TGFβ-transduced MSCs compared with the number in wild-type or MSC-treated mice. Kinetics studies suggested that Treg cell expansion after injection peaked on day 5 and then decreased and approached background levels by approximately day 20 (Figure 1B). However, the absolute number of CD4+CD25+ Treg cells in mesenteric LNs and draining LNs increased on day 30 in mice treated with TGFβ-transduced MSCs.

We investigated whether TGFβ-transduced MSCs or MSCs could induce naive CD4+CD25− T cells to differentiate into CD4+CD25+ FoxP3+ T cells. As shown in Figure 1C, the percentage of CD4+CD25+ T cells was higher in CD4+CD25− T cells cocultured with TGFβ-transduced MSCs than in those cocultured with MSCs. These findings indicate
that TGFβ-transduced MSCs have strong immunologic regulatory functions.

**Effect of MSCs and TGFβ-transduced MSCs on the development and severity of CIA.** Treg cells suppress activation of the immune system and prevent organ-specific autoimmunity and inflammation (4,35). To investigate whether Treg cell populations expanded by treatment with TGFβ-transduced MSCs could be used to inhibit the induction of T cell–mediated autoimmune arthritis, we examined whether a single intraperitoneal injection of TGFβ-transduced MSCs would impair induction of CIA. Treatment with MSCs delayed the onset of clinical symptoms, but severe disease eventually developed in all mice. Following delivery in vivo, TGFβ-transduced MSCs induced a significant reduction in both the incidence and severity of clinical arthritis (Figure 2A), indicating a strong therapeutic effect. The hind paw joints of mice were histologically assessed 5 weeks after administration of the primary immunization. The joints of mice treated with TGFβ-transduced MSCs showed decreased destruction of the articular cartilage, bone synovial hyperplasia, and infiltration by inflammatory cells (Figure 2B).

Serum anti-type II collagen IgG levels were
measured by ELISA to determine whether treatment with TGFβ-transduced MSCs was associated with a change in the humoral immune response to type II collagen. Type II collagen–specific IgG and IgG2a levels increased and the IgG1 level decreased in placebo-treated mice and MSC-treated mice compared with TGFβ-transduced MSC–treated mice. In contrast, in TGFβ-transduced MSC–treated mice, the type II collagen–specific IgG and IgG2a levels decreased and the IgG1 level increased at 8 weeks, but then declined by 10 weeks (Figure 2C). Eight weeks following the primary immunization, production of IL-6, TNFα, and IL-21 was significantly lower, and IL-17 production was also lower (although not significantly so) in serum from TGFβ-transduced MSC–treated mice compared with MSC–treated mice and placebo–treated mice (Figure 2D). These results suggest that TGFβ-transduced MSCs have a strong therapeutic effect in terms of the severity of CIA.

**Regulation of type II collagen–specific responses by TGFβ-transduced MSCs.** To determine whether the reduction in severity of CIA in mice treated with TGFβ-

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**Figure 3.** Inhibition of the inflammatory response by TGFβ-transduced MSCs in mice with collagen-induced arthritis (CIA). A, Proliferative response by lymph nodes (LNs) from MSC-treated mice, TGFβ-transduced MSC–treated mice, and placebo–treated mice (n = 5 per group) 8 weeks after administration of the primary immunization. LN cells were cultured with or without type II collagen (CII). Ovalbumin (OVA) and phytohemagglutinin (PHA) were used as negative and positive controls, respectively. B, Concentrations of interleukin-6 (IL-6), tumor necrosis factor α (TNFα), IL-17, and IL-10 in culture supernatants of unstimulated (Nil), type II collagen–stimulated, or OVA–stimulated LN cells obtained from each group of mice, as measured by enzyme-linked immunosorbent assay. Values in A and B are the mean ± SEM stimulation index (SI; ratio of cpm with antigen to cpm without antigen). C, Fluorescence of 5,6-carboxyfluorescein succinimidyl ester (CFSE)–labeled LN cells obtained from each group of mice. Cells were cultured with type II collagen plus anti-CD3 monoclonal antibody for 72 hours, followed by flow cytometry. Dot plots are representative of 1 of 3 experiments. The numbers shown in the boxes represent the percentage of double-positive cells. Graph shows the mean ± SEM ratio of CFSE-positive FoxP3-positive cells among proliferative CD4+ cells, which was calculated as the ratio of CFSE-positive/FoxP3-positive cells to CFSE-positive CD4+ T cells. * = P < 0.05 by Student’s t-test. PBS = phosphate buffered saline (see Figure 1 for other definitions).
transduced MSCs was attributable simply to a defective T cell response to type II collagen, we examined type II collagen–specific T cell responses and cytokine production in mice spleenocytes 8 weeks after administration of the primary immunization. Following type II collagen–specific stimulation, the suppressive effect was more prominent in splenic T cells from mice treated with TGFβ-transduced MSCs. There was no difference in type II collagen–specific T cell proliferation between placebo-treated mice and MSC-treated mice (Figure 3A). Compared with type II collagen–stimulated T cells obtained from placebo-treated mice, those obtained from MSC-treated mice produced similar or slightly higher levels of IL-6, IL-17, and TNFα, whereas those from TGFβ-transduced MSC–treated mice showed markedly decreased production of these inflammatory cytokines. Interestingly, IL-10 production in TGFβ-transduced MSC–treated mice was significantly higher than that in placebo-treated mice and MSC-treated mice (Figure 3B).

Although TGFβ-transduced MSCs inhibited type II collagen–specific effector CD4+ T cell proliferation under T cell receptor–stimulated conditions, they induced an increase in the proliferation of FoxP3+ T cells in response to type II collagen compared with controls (Figure 3C). Thus, spleens from mice treated with TGFβ-transduced MSCs had more FoxP3+ cells and a

Figure 4. Treg cell expansion by TGFβ-transduced MSCs in vivo. Mice with collagen-induced arthritis (CIA; n = 6 per group) received intraperitoneal injections of 1 × 10^6 MSCs or TGFβ-transduced MSCs 7 weeks after administration of the primary immunization. Control mice (n = 6) received intraperitoneal injections of an equal volume of phosphate buffered saline (PBS) at the same time points. A and B, Expansion of Treg cells (A) and total number of FoxP3-positive cells (B) in the peritoneal cavity, draining lymph nodes, mesenteric lymph nodes, and spleens on day 7. The numbers shown in the boxes in A represent the percentage of double-positive cells. C, Increased percentage of CD25, CTLA-4, glucocorticoid-induced tumor necrosis factor receptor (GITR), and CCR7 in Treg cells from mice treated with TGFβ-transduced MSCs relative to Treg cells from MSC-treated mice or placebo-treated mice, as analyzed by flow cytometry. Gray histograms represent cells stained with isotype-matched control monoclonal antibody. D, In vitro suppression of CD4+CD25− T cells (effectors) by fluorescence-activated cell–sorted CD4+CD25+ Treg cells (suppressors; 1:10 ratio) isolated from the peritoneal cavity (shaded bars) and spleens (open bars) of each mouse 7 days after treatment. The proliferation of effectors was measured by incorporation of 3H-thymidine. Values in B and D are the mean ± SEM and are representative of 3 independent experiments. * = P < 0.05. Nil = not stimulated; PC = peritoneal cavity; SP = spleen; CII = type II collagen (see Figure 1 for other definitions).
higher ratio of type II collagen–reactive proliferative FoxP3+ cells to CD4+ cells (Figure 3C). Our results indicate that the resistance of TGFβ-transduced MSC–treated mice to arthritis was due primarily to a reduction in type II collagen–specific T cell responses and inflammatory cytokine levels during CIA induction.

**Induction of Treg cells by TGFβ-transduced MSCs in mice with CIA.** To investigate other mechanisms of suppression, we examined Treg cell populations in the peritoneal cavities, draining LNs, mesenteric LNs, and spleens of mice 1 week after administration of the primary immunization. Expanded Treg cells were observed in the mesenteric LNs and spleens of mice that received TGFβ-transduced MSCs compared with placebo-treated mice and MSC-treated mice (for mesenteric LNs, mean ± SD 7.1 ± 0.75% versus 6.06 ± 0.29% and 6.11 ± 0.44%, respectively; for spleen, 2.6 ± 0.1% versus 1.59 ± 0.09% [P < 0.05] and 2.26 ± 0.15%, respectively). Expanded Treg cells were especially prominent in the peritoneal cavities of mice treated with TGFβ-transduced MSCs (mean ± SD 4.6 ± 0.4% versus 0.5 ± 0.1% and 0.79 ± 0.1% in placebo-treated mice and MSC-treated mice, respectively; P < 0.05).

Treg cell expansion in each organ of MSC-treated mice was not significantly different from that in mice treated with placebo. These findings applied to both the percentage (Figure 4A) and the total number of Treg cells (Figure 4B). Treatment with TGFβ-transduced MSCs expanded Treg cells in the peritoneal cavity and spleen 7 days postinjection. Such treatment
Figure 6. Migration into inflamed joints and inhibition of osteoclast differentiation by TGFβ-transduced MSCs. A, Immunohistochemical analysis of joint tissue 1 week after injection of MSCs or TGFβ-transduced MSCs expressing enhanced green fluorescent protein (GFP); an anti-GFP monoclonal antibody was used for cell detection. Arrows indicate GFP-positive cells. Graphs show the gene expression profile of MSCs (open bars) and TGFβ-transduced MSCs (solid bars), as analyzed using real-time polymerase chain reaction (PCR). Values for the relative expression of each gene were determined as described in Materials and Methods. B, Survival of tartrate-resistant acid phosphatase (TRAP)–stained mononuclear cells (MNCs) in the presence of TGFβ-transduced MSCs and MSCs. Bone marrow cells (BMCs) were cultured in the presence of monocyte colony-stimulating factor (M-CSF) and RANKL (R) with MSCs or TGFβ-transduced MSCs. Cells were fixed and stained for TRAP. TRAP-positive MSCs with ≥3 nuclei were counted as osteoclasts. C, Real-time PCR analysis of osteoclast-associated markers. Relative gene expression was normalized against the gene expression levels of stimulated M-CSF alone (1 = M-CSF, 2 = M-CSF plus RANKL, 3 = M-CSF plus RANKL plus MSCs, 4 = M-CSF plus RANKL plus TGFβ-transduced MSCs). D, Osteoclast differentiation induced by 2 doses of RANKL in BMCs derived from mice treated with TGFβ-transduced MSCs. BMCs isolated from 3 groups of mice were cultured in the presence of M-CSF with increasing concentrations of RANKL. Cells were fixed and stained for TRAP. Graphs in B and D show the number of TRAP-positive MNCs/well. Bars show the mean ± SEM results of 3 experiments, each of which was performed in triplicate. Original magnification × 400 in A; × 100 in B and D. * = P < 0.05. CIA = collagen-induced arthritis; Cat K = cathepsin K; MMP-9 = matrix metalloproteinase 9 (see Figure 1 for other definitions).
appeared to increase the percentage (Figure 4C) and mean fluorescence intensity (data not shown) of molecules crucial to suppression, such as CD25, CTLA-4, GITR, and CCR7 relative to Treg cells from MSC-treated mice or placebo-treated mice. Interestingly, Treg cells in the peritoneal cavity expressed a stronger Treg phenotype than that in the spleens of TGFβ-transduced MSC–treated mice. Furthermore, the expanded Treg cells in the peritoneal cavity and spleens of mice treated with TGFβ-transduced MSCs had a more potent suppressive effect than those of placebo-treated or MSC-treated mice in response to type II collagen stimulation (Figure 4D). These data suggest that TGFβ-transduced MSCs induce local and systemic type II collagen–reactive Treg cells and suppressive activity.

**Reciprocal regulation of Treg cells and Th17 cells by TGFβ-transduced MSCs in mice with CIA.** Although TGFβ is essential for FoxP3 induction in peripheral T cells, recent studies also demonstrated an indispensable role for TGFβ and IL-6 in the generation of Th17 cells (36). Thus, we next examined the possibility that TGFβ-transduced MSCs may contribute to Treg cell induction and inhibit the development of Th17 cells. The data were consistent with our hypothesis that CD4+ T cell activation in the presence of type II collagen induces FoxP3+ Treg cell differentiation in both the spleen and peritoneal cavity (mean ± SEM 12.7 ± 0.07% and 14.35 ± 0.2%, respectively; P < 0.05), and that up-regulated IL-17–producing CD4+ T cells are abolished after type II collagen stimulation in both the spleen and peritoneal cavity (mean ± SEM 2.5 ± 0.48% and 2.89 ± 0.1%, respectively; P < 0.05) in TGFβ-transduced MSC–treated mice compared with controls (Figures 5A and B). However, unlike the reduction in the number of Th17 cells induced in mice treated with TGFβ-transduced MSCs, there was a significant increase in the frequency of IL-17–producing T cells in the presence of type II collagen in placebo-treated and MSC-treated mice (Figures 5A and B). Additionally, the FoxP3+/IL-17+ cell ratio was significantly higher in both the spleens and peritoneal cavities of mice treated with TGFβ-transduced MSCs (Figure 5C). Inhibition of IL-17 by TGFβ-transduced MSCs was associated with increased FoxP3 expression, suggesting that TGFβ-transduced MSCs reciprocally affect both Treg cell induction and Th17 cell differentiation.

**Inhibition of osteoclast differentiation by TGFβ-transduced MSCs.** Recent evidence suggests that systemically transplanted MSCs selectively migrate to sites of injury and inflammation, where they are involved in tissue repair (17). To determine the potential of these MSCs to migrate into inflamed joints, we compared chemokine receptors in TGFβ-transduced MSCs with those in MSCs. Expression of the CCR1, CCR4, CCR7, and CXCR4 gene transcripts was greater in TGFβ-transduced MSCs (Figure 6A). Both TGFβ-transduced MSCs and MSCs migrated into the joints after transfer (Figure 6A), localizing mainly in the sublining region of the synovial membrane and bone marrow. These data suggest that systemic delivery of TGFβ-transduced MSCs up-regulated chemokine receptor expression and increased the migratory capacity of MSCs.

Histologic analysis showed that TGFβ-transduced MSCs reduced bone erosion and cartilage destruction in the arthritic joints of mice with CIA (Figure 2B). We therefore examined whether migration of TGFβ-transduced MSCs into the joints affected osteoclast differentiation. First, we determined whether TGFβ-transduced MSCs affected osteoclastogenesis in vitro. To determine whether MSCs could inhibit osteoclastogenesis, bone marrow–derived monocytes cultured for 10 days in the presence of M-CSF and RANKL were cocultured with TGFβ-transduced MSCs or MSCs. Approximately half as many TRAP-positive mononuclear cells survived in the presence of TGFβ-transduced MSCs compared with the number that survived in the presence of MSCs (Figure 6B). In addition, the levels of the osteoclast-related markers TRAP, RANK, NF-ATc1, MMP-9, CTR, β3 integrin, and cathepsin K mRNA decreased significantly following coculture with TGFβ-transduced MSCs compared with coculture with MSCs (Figure 6C).

To identify the effects of migrating MSCs or TGFβ-transduced MSCs on osteoclastogenesis in vivo, we compared the osteoclastogenic capacity of bone marrow isolated from mice with CIA treated with MSCs or TGFβ-transduced MSCs. Osteoclast differentiation induced by 2 doses of RANKL in vitro was decreased in nonadherent bone marrow cells derived from mice treated with TGFβ-transduced MSCs, suggesting that TGFβ-transduced MSCs in the bone marrow either decrease the osteoclast progenitor pool or decrease their sensitivity to RANKL (Figure 6D). We also examined osteoprotegerin (OPG) expression in TGFβ-transduced MSCs and MSCs, using RT-PCR. TGFβ-transduced MSCs showed greater expression of the OPG gene transcript compared with MSCs (data not shown). These data indicate that TGFβ-transduced MSCs have a negative effect on RANKL-mediated osteoclastogenesis.

**DISCUSSION**

MSCs are increasingly being used to treat autoimmune and systemic inflammatory diseases. Although
considerable progress has been made in understanding the mechanisms by which MSCs exert immunomodulatory functions in autoimmune disease, these mechanisms are still not fully understood. A balance of Treg cells and Th17 cells may be critical for maintaining immune tolerance and for the treatment of autoimmune diseases. In the present study, we demonstrated a novel mechanism of action: use of TGFβ-transduced MSCs to control progression of CIA by regulating Treg/Th17 cells. We showed that MSCs, when given systemically after disease onset, reduce both inflammatory and antigen-reactive T cell responses. Interestingly, we observed that infused TGFβ-transduced MSCs migrated to inflamed joints and inhibited osteoclast differentiation. We believe that these results could help shape future clinical strategies for the treatment of autoimmune diseases.

The immunomodulatory and reparative anti-inflammatory properties of MSCs have been evaluated in an experimental autoimmune disease model. Previous studies using bone marrow MSCs in the CIA model have generated conflicting results. Although Augello et al (25) reported that a single injection of MSCs had a therapeutic effect on disease onset by decreasing serum levels of proinflammatory cytokines, another study showed a negative effect of MSCs on CIA, because MSCs alone did not inhibit proinflammatory cytokines such as TNFα (26). The mechanisms underlying the beneficial effect described by Augello et al need to be explored further, because no convincing increase in the number of Treg cells was observed in vivo, despite in vitro evidence of T cell inhibition by MSCs.

In an attempt to augment the therapeutic efficacy of MSCs in suppressing RA, we developed genetically modified MSCs using an adenoviral vector encoding TGFβ. Compared with MSCs, TGFβ-transduced MSCs exerted more potent antiproliferative activity and induced Treg cell generation in vitro (Figure 1). These data suggest that TGFβ-transduced MSCs enhanced therapeutic efficacy in the CIA model.

We demonstrated that compared with systemic administration of MSCs, systemic administration of TGFβ-transduced MSCs had greater long-lasting effects in established CIA. We investigated changes in the cytokine secretion profiles induced by these cells to understand the mechanisms involved in the observed TGFβ-transduced MSC–mediated immunosuppressive effects. Mice with CIA treated with TGFβ-transduced MSCs showed lower production of proinflammatory cytokines. The secretion of TNFα decreased, and the secretion of IL-10 increased in the supernatant of the mixed lymphocyte reaction, suggesting that TGFβ-transduced MSC–mediated suppressive activity in vitro is partly attributable to soluble mediators. Antigen-induced IL-10 secretion is involved in the induction of specific T cell tolerance (37).

Polyclonal or antigen-specific activated TGFβ-induced Treg cells are potent suppressors of both organ-specific (38) and systemic autoimmune diseases (39). There is growing evidence that the immunosuppressive effects of bone marrow MSCs are associated with CD4+CD25+FoxP3+ Treg cell expansion (40,41). However, it has also been reported that bone marrow MSCs ameliorate autoimmune enteropathy in vivo, an effect that is independent of Treg cells (42). We observed that, compared with mice with CIA treated with placebo or MSCs, mice with CIA treated with an intra-peritoneal injection of TGFβ-transduced MSCs had a significantly higher percentage and absolute number of Treg cells in the peritoneum and spleen and a smaller increase in the mesenteric LNs (Figure 4). Treatment with TGFβ-transduced MSCs expanded Treg cells in both the peritoneum and spleen 7 days after the injection, producing a Treg phenotype with potent suppression of type II collagen–reactive effector T cells. We also observed that depletion of Treg cells from TGFβ-transduced MSCs substantially abolished the therapeutic effects on arthritis (data not shown). Thus, the expanded Treg cells prevented the proliferation and trafficking of pathogenic effector T cells, resulting in an absence of inflammatory arthritis.

Our results suggest that expanding Treg cells using TGFβ-transduced MSCs in vivo could play a key role in the maintenance of peripheral tolerance. Interestingly, treatment with TGFβ-transduced MSCs resulted in increased FoxP3 levels with a concomitant decrease in IL-17 production (Figure 5). We propose that treatment with TGFβ-transduced MSCs enhanced FoxP3+ Treg cells and reduced serum levels of IL-6, TNFα, and IL-17. We observed a dramatic increase in the ratio of Treg cells to IL-17–expressing effector T cells following antigen stimulation in mice treated with TGFβ-transduced MSCs compared with MSC-treated mice. Reciprocal regulation of Treg/Th17 cells could control the pathogenesis, onset, and progression of CIA.

Although TGFβ is well known for its immunosuppressive properties, it also promotes angiogenesis (43) and inflammation (44). Previous studies have shown not only that TGFβ synergizes with TRANCE to induce osteoclast-like cells from bone marrow precursors and monocytes, but also that osteoclast-like cell formation is abolished by recombinant soluble TGFβ receptor type II (45). In contrast, TGFβ is a potent inhibitor of osteoclast-
like cell formation in human bone marrow cultures (46). Bone marrow MSCs have the capacity to differentiate into bone and cartilage and thus have attracted interest as potential therapeutic tools for tissue repair. Several reports have suggested that TGFβ-transduced MSCs improve cartilage repair (47). We demonstrated that TGFβ-transduced MSCs negatively regulate RANKL-mediated osteoclastogenesis, using in vitro and in vivo analyses (Figure 6). This inhibitory effect was related to MSC migration to inflamed joints. The antiosteoclastogenic effect of TGFβ-transduced MSCs is likely related to OPG expression.

The mechanisms by which MSCs are recruited to tissues and cross the endothelial cell layer are not yet fully understood, but it is probable that chemokines and their receptors are involved. It is thus likely important that TGFβ-transduced MSCs expressed high levels of the chemokine receptor CXCR4, and that the ligand stromal cell–derived factor 1 is produced by synovial fibroblasts in joints. Treatment with TGFβ-transduced MSCs that express CCR1, CCR4, and CCR7 may result in MSC engraftment and improvements in injured tissue. Thus, we suggest that TGFβ-transduced MSCs may be useful for recruiting MSCs to inflamed tissues.

In conclusion, therapy with TGFβ-transduced MSCs is a highly beneficial method for specifically suppressing the immune response. We demonstrated that a systemic infusion of genetically modified TGFβ-transduced MSCs effectively ameliorated autoimmune arthritis through reciprocal regulation of Treg/Th17 cells. Our observations explain the beneficial effects of TGFβ-transduced MSCs effectively ameliorated autoimmune arthritis through reciprocal regulation of Treg/Th17 cells.

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. Dr. S-G. Cho 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.


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