Clinical Immunology (2011) 141, 328–337

available at www.sciencedirect.com

Clinical Immunology

www.elsevier.com/locate/yclim

Administering human adipose-derived mesenchymal stem cells to prevent and treat experimental arthritis

Bin Zhou [a], Jingdong Yuan [b], Yixuan Zhou [a], Maher Ghawji Jr. [a], Yun-Ping Deng [c], Austin J. Lee [a], Albert J. Lee [a], Usha Nair [a], Andrew H. Kang [a], David D. Brand [a], Tai June Yoo [a,*]

[a] Department of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163, USA
[b] Department of Urology, Wuhan First Hospital, Wuhan, Hubei 430030, China
[c] Department of Anatomy & Neurobiology, University of Tennessee Health Science Center, Memphis, TN 38163, USA

Received 17 June 2011; accepted with revision 26 August 2011
Available online 2 September 2011

KEYWORDS
Adipose-derived mesenchymal stem cells;
Collagen-induced arthritis;
Regulatory T cells

Abstract Rheumatoid arthritis is a chronic autoimmune disease and affecting approximately 1% of the population. Human adipose-derived mesenchymal stem cells (hASCs) were recently found to suppress effector T cell and inflammatory responses and, thus, to have beneficial effects in various autoimmune diseases. In this study, we examined whether hASCs could play a protective and/or therapeutic role in collagen-induced arthritis (CIA). We showed that hASCs both prevented and treated CIA by significantly reducing the incidence and severity of experimental arthritis. We further demonstrated that treatment with hASCs inhibited the production of various inflammatory mediators, decreased antigen-specific Th1/Th17 cell expansion, and induced the production of anti-inflammatory cytokine interleukin-10. Moreover, hASCs could induce the generation of antigen-specific Treg cells with the capacity to suppress collagen-specific T cell responses. Published by Elsevier Inc.

1. Introduction

Rheumatoid arthritis (RA) is a symmetric and polyarticular arthritis associated with significant functional disability and decreased quality of life. The disease affects primarily the small diarthrodial joints of the hands and feet [1,2]. RA is a chronic autoimmune disorder caused by the breakdown of tolerance that ultimately leads to the activation of autoreactive T cells against joint components and subsequent

Abbreviations: BM-MSC, bone marrow-derived MSC; CIA, collagen-induced arthritis; CII, type II collagen; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; hASC, human adipose-derived mesenchymal stem cells; MSC, mesenchymal stem cells; PBS, phosphate-buffered saline; RA, rheumatoid arthritis; Treg, regulatory T.

* Corresponding author at: University of Tennessee Health Science Center, 956 Court Avenue, Room E332, Memphis, TN 38163, USA. Fax: +1 901 448 5735.
E-mail address: tyoo@uthsc.edu (T.J. Yoo).

1521-6616/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.clim.2011.08.014

irreversible destruction of joint architecture [1–3]. The initial stages of RA involve multiple steps, which can be divided into two main phases: the induction of autoimmunity to collagen-rich joint components, and subsequently the evolution of a destructive inflammatory process [1–3]. RA is mediated by both autoreactive Th1 and Th17 cells, which can enter joint tissues and release pro-inflammatory cytokines and chemokines. These mediators can also promote macrophage and neutrophil infiltration and activation in joint tissues [1–3]. Consequently, infiltrating inflammatory cells can produce excessive levels of free radicals, cytokines, and extracellular matrix-degrading enzymes, which could worsen RA [1–3]. Therefore, inhibiting the activation of inflammatory and autoimmune responses is an appropriate strategy to treat RA. On the other hand, the induction and maintenance of immunological self-tolerance depends on the Treg-mediated homeostatic deletion or suppression of self-reactive clones in the periphery during adult life. Therefore, enhancing the frequency and immunosuppressive function of Treg cells may provide an important therapeutic strategy for RA [3–6].

Mesenchymal stem cells (MSCs) are subsets of adult stem cells and are multipotent progenitor cells that reside in virtually all tissues, including bone marrow (BM), umbilical cord blood, dermis, and adipose tissue. These cells can differentiate into cells of the mesenchymal lineage like bone, fat, and cartilage [7–9]. Therefore, they have been proposed as attractive candidates for cell-based tissue repair. In addition to their regenerative potential, MSCs have immunomodulatory properties and can act by suppressing many functions of immune cells in a major histocompatibility complex independent manner [7–9]. Thus, use of MSCs as immunosuppressant agents is suitable for the therapy of autoimmune diseases. For many years, bone marrow-derived MSCs (BM-MSCs) were considered the major source of stem cells for tissue engineering applications. In addition to their therapeutic use in repairing damaged tissues, BM-MSCs have been described as potent immunomodulators in various immune disorders, including inhibition of dendritic cell maturation, T cell proliferation and B cell function. [3,10–14]. However, harvesting BM-MSCs is extremely painful for patients and yields low numbers of cells, and their potential clinical applications require large numbers of cells for infusion, which in most cases are not available [13–16].

When compared with BM-MSCs, human adipose-derived mesenchymal stem cells (hASCs) are equally capable of differentiating into cells and tissues of mesodermal origin [15–19]; hASCs are capable of forming cartilage, bone, and muscle, as well as fat. Abundant numbers of hASCs can be derived from lipoaspirate, the waste product of liposuction surgery. Compared with BM-MSCs, hASCs are more easily cultured and grow more rapidly. They can also be cultured longer than BM-MSCs before becoming senescent [15–19]. Moreover, recent reports have demonstrated that hASCs share some of the immunomodulatory properties that characterize BM-MSCs [17–19] and exert profound immunomodulatory properties and protective effects on acute graft versus host disease and experimental arthritis. All these qualities make hASCs a useful source for innovative therapeutic treatment of autoimmune diseases. Our results showed that hASC treatment has both prophylactic and therapeutic effects. Notably, the suppression of collagen-induced arthritis (CIA) by hASCs was associated with an induction of $CD25^+CD4^+Foxp3^+$ Treg cells secreting IL-10 that could suppress the in vivo induced Th1 and inflammatory responses in an in vitro coculture assay.

2. Materials and methods

2.1. Mice

Female DBA/1 LacJ mice (Jackson Laboratory, Bar Harbor, ME) were used in this study. All mice were maintained in groups of five in polycarbonate cages and fed standard rodent chow (Ralston Purina Co., St. Louis, MO) and water ad libitum. The environment was specific pathogen-free, and sentinel mice were tested routinely for mouse hepatitis and Sendai viruses. Mice were maintained in the animal facility at the University of Tennessee Health Science Center, according to the institutional guidelines for animal care and use. These studies were approved by the Institutional Animal Care and Use Committee of the University of Tennessee.

2.2. Induction and assessment of CIA

Female DBA/1 mice of 8 weeks age were used in the present study. Chicken type II collagen (CII) was prepared according to the methods of Miller [20]. The CII was dissolved in 10 mM acetic acid (4 mg/ml overnight at 4 °C) and emulsified with an equal volume of CFA (Difco Laboratories, Detroit, MI) containing 4 mg/ml of H37Ra *Mycobacterium tuberculosis* (Difco). CIA was induced as described previously [21] with a single, subcutaneous injection of 100 μg CII/50 μl emulsion into the base of the tail on day 0. Mice were monitored daily for signs of arthritis and assessed using a visual scoring system [21]. Each paw was scored on a graded scale of 0–4, where 0 = no evidence of erythema and swelling, 1 = mild swelling with erythema, 2 = significant joint swelling, 3 = severe swelling and digit deformity, and 4 = maximal swelling with ankylosis. Each limb was graded, with a maximum possible score of 16 per mouse. The clinical score was determined daily for each mouse for up to 42 days. In addition, paw swelling was evaluated by measuring the mean thickness of both hind paws with 0–10-mm calipers.

2.3. Treatment protocols

hASCs were provided by RNL Life Science, Inc., Korea [22]. In brief, human adipose tissues were obtained by liposuction and were digested with collagenase I (1 mg/ml) under gentle agitation for 60 min at 37 °C. The digested tissues were filtered through a 100-μm nylon sieve to remove cellular debris and were centrifuged at 470 *g* for 5 min to obtain a pellet. The pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen)-based media containing 0.2 mM ascorbic acid and 10% fetal bovine serum (FBS). The cell suspension was re-centrifuged at 470 *g* for 5 min to collect cell pellet. The cell fraction was cultured overnight at 37 °C/5% CO_2 in DMEM-based media containing 0.2 mM ascorbic acid and 10% FBS. After 24 h, the cell adhesion was checked under an inverted microscope, and nonadherent cells were removed by washing with phosphate-buffered saline

(PBS). The cell medium was changed to Keratinocyte-SFM (Invitrogen)-based media containing 0.2 mM ascorbic acid, 0.09 mM calcium, 5 ng/ml rEGF, and 5% FBS. Aliquots of the hAdMSCs are then tested for cell viability and fungal, bacterial, endotoxin, and mycoplasma contamination as demanded before further use. To evaluate the preventive potential of hASCs against the development of CIA, mice received 100 μl of PBS containing 2×10^6 hASCs intravenously on days −9, −7, and −4 and then challenged with CII on day 0. The therapeutic treatment started after the onset of disease, when arthritis had become well established (arthritis score >2). Mice with CIA received 2×10^6 hASCs, 2×10^6 Jurkat cells (ATCC, Manassas, VA), or PBS intravenously on days 26, 28, and 32 after CII immunization.

2.4. Determining CII antibodies from mice sera by ELISA

Serum levels of anti-CII IgG and IgG subclasses, IgG1and IgG2a, were evaluated by ELISA, as previously described [23]. In brief, Maxisorb 96-well plates (Nunc, Roskilde, Denmark) were coated with 100 μl of CII (5 μg/ml in 0.1 M sodium carbonate buffer, pH 9.7) overnight at 4 °C. After blocking with 2% BSA in PBS for 2 h at 37 °C, sera were added in serial dilutions in 2% BSA/PBS and incubated overnight at 4 °C. The plates were then washed and incubated with biotin-conjugated goat anti-mouse IgG, IgG1, or IgG2a, followed by adding avidin–peroxidase, and subsequently were developed using avidin-biotinylated enzyme complex. The reaction was stopped with 2.5 N sulfuric acid, and optical density at 490–650 nm was measured.

2.5. In vitro cytokine production and lymphocyte proliferation

Single-cell suspensions of spleens were obtained 42 days postimmunization, and cells (2×10^5 cells/well) were cultured in 96-well flat-bottomed plates (Costar, Corning, NY) in RPMI 1640 medium supplemented with 5% FCS (Invitrogen, Carlsbad, CA), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and 10 U penicillin/streptomycin and stimulated with different concentrations of heat-inactivated CII. Positive control wells contained 2 μg/ml anti-mouse CD3 (BD Biosciences, San Jose, CA), and negative control wells contained only PBS. Supernatants were harvested after 48 h and stored at −70 °C for cytokine array. Proliferation assays were evaluated at 72 h by determining bromodeoxyuridine-substituted DNA incorporation (Roche, Indianapolis, IN). To investigate the suppressive activity of hASCs in vitro, 2×10^5 splenocytes isolated from mice with CIA at the peak of disease were stimulated with 10 μg/ml of CII in the presence of 2×10^4 hASCs. Proliferative responses and cytokine production were then evaluated. Some cocultures of splenocytes with hASCs were treated with anti-IL-10 Ab (10 μg/ml; BD PharMingen, San Jose, CA).

2.6. Milliplex protein array system

Protein extracts [3] were isolated from paws on day 42 by homogenization (50 mg tissue/ml) in 50 mM Tris HCl, pH 7.4, with 0.5 mM dithiothreitol and proteinase inhibitor cocktail (10 μg/ml). Serum samples were also collected on day 42. The levels of cytokines and chemokines in serum, culture supernatants, and protein extracts were determined by a multiplex cytokine bead array system-MILLIPLEX Mouse Cytokine/Chemokine 22-plex assay (Millipore, St. Charles, MO) according to the manufacturer's instructions. The reaction mixture was read using the Bio-Plex protein array reader, and data were analyzed with the Bio-Plex Manager software program in the Rheumatic Disease Research Core Center, Veterans Affairs Medical Center (Memphis, TN).

2.7. Flow cytometry analysis

To determine the frequency of Treg cells in vivo, flow cytometry was performed on freshly isolated splenocytes by using a Treg detection kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The $CD4^+CD25^+Foxp3^+$-expressing T cells were identified by staining splenocytes with PE-labeled anti-CD4 and APC-labeled anti-CD25. For intracellular staining of Foxp3, cells were fixed and permeabilized before incubation with FITC-labeled anti-mouse Foxp3. For all the markers evaluated in this study, appropriate isotype-matched control antibodies were used to determine nonspecific staining. Labeled cells were washed with PBS, and a minimum of 10,000 cells was analyzed from each sample by flow cytometry with an LSR II (BD Biosciences). The percentage of Treg cells was determined after gating on $CD4^+$ T cells and using FlowJo software (Tree Star, Ashland, OR).

2.8. In vitro suppression assay

Isolation of mouse $CD4^+$, $CD4^+CD25^+$, and $CD4^+CD25^-$ T cells was performed by using a mouse Treg isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, $CD4^+$ T cells were first enriched by negative selection (depleting CD8a, CD11b, CD45R, CD49b, and Ter-119-positive cells) with MACS. The $CD4^+$ T cells were incubated with magnetic beads conjugated with an anti-CD25 mAb to separate $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subpopulations. The purity of the resulting T cell subpopulations was higher than 95% by flow cytometry. To determine the suppressive capacity of hASC-induced Treg cells, proliferation assays were performed in triplicate by culturing $CD4^+CD25^-$ cells (responder, 5×10^4 from splenocytes of CIA mice), $CD4^+CD25^+$ T cells (suppressor, 5×10^4 from splenocytes of CII-immunized mice treated with either hASCs or PBS) in 96-well plates with irradiated APCs (5×10^4 from splenocytes of normal DBA/1 mice) for 72 h at 37 °C in complete medium. Cultures were stimulated by CII (100 μg/ml), and some cocultures were treated with anti-IL-10 Ab (10 μg/ml; BD PharMingen). After 72 h, the proliferation of self-reactive T cells was evaluated by measuring bromodeoxyuridine-substituted DNA incorporation.

2.9. Statistical analysis

Data were analyzed using ANOVA or Student's *t*-test to compare differences between the treatment groups.

3. Results

3.1. Ability of hASCs to prevent and suppress CIA

In the present study, we examined the potential protective and therapeutic effects of hASCs in mice with CIA, which is an experimental model of human RA and also the most widely used animal model of RA. The CIA model shares many clinical, histological, and immunologic features, as well as genetic linkage with the human counterpart. It is typically used to investigate mechanisms of autoimmunity, to study inflammation and new anti-arthritic treatments. Mice were examined daily for signs of developing arthritis. The severity of arthritis was evaluated using a visual scoring system and a 0–10-mm caliper.

Severe arthritis developed in CII-immunized mice treated with PBS or Jurkat cells (Fig. 1A). The onset of disease was evident from day 20 after CII immunization (Fig. 1A), and maximum arthritis scores were evident by day 32 (Fig. 1A). The total incidence of arthritis in those positive control mice reached 100% by day 24 (Fig. 1A), thus exhibiting the

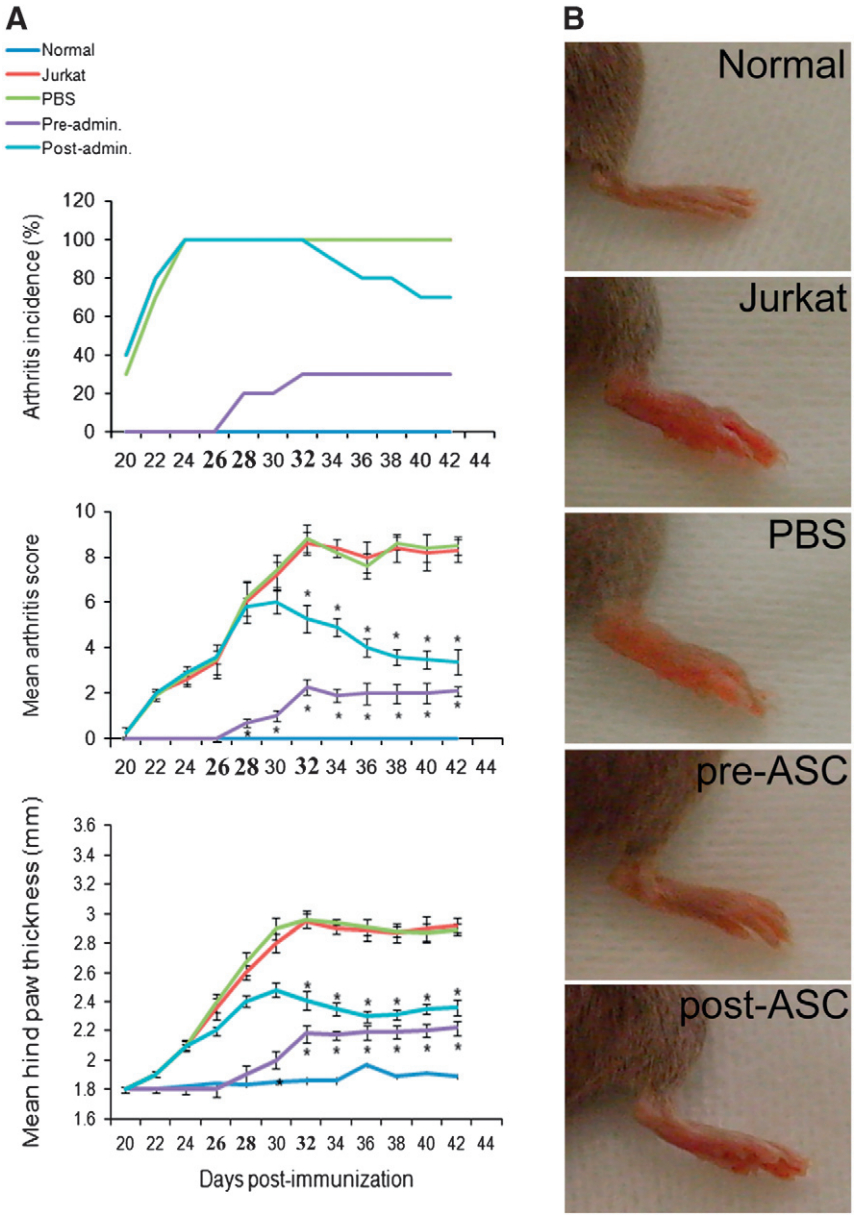


Figure 1 Treatment with hASCs prevents and suppresses CIA. DBA/1 mice were immunized with 100 μg of CII in CFA subcutaneously on day 0. CII-immunized mice were treated with hASCs, Jurkat cells, or PBS only intravenously, according to preventive (days −9, −7, and −4) or suppressive (days 26, 28, and 32) treatment protocols. Severity of arthritis (A) was evaluated by assigning a score of 0–4 per paw based on the degree of inflammation in each limb, with 0 = no arthritis and 4 = severe arthritis, for a maximum possible score of 16 per mouse. In addition, arthritis severity (A) was also assessed by measuring hind paw thickness. Photographs at the right (B) show representative examples of the paw swelling in normal mice, PBS or Jurkat cell-treated mice, or human AD-MSCs-treated mice. Values are the mean ± SD of 10 mice per group. *=$P<0.001$ versus Jurkat cell controls.

classical features of a CIA model. However, mice which received prophylactic treatment with hASCs administered on days −9, −7, and −4 before disease induction, continued observation for 42 days showed that hASCs markedly decreased the incidence of arthritis, with 70% of the treated mice free of clinical arthritis at the end of the observation period. (Fig. 1A). The appearance of clinical signs was delayed in mice that received hASCs (day 28) compared with those that received PBS (day 20) or Jurkat cells (day 20) (Fig. 1A). Even in the mice in which disease did eventually develop, the mean arthritis score and hind paw volume were markedly lower in the mice that received hASCs than in the mice treated with PBS or Jurkat cells (Fig. 1A). We noted that arthritis occurred later and the disease was less severe in treated mice.

We then evaluated whether hASCs had an effect when injected after arthritis was already established (arthritis score >2). Groups of mice were treated with PBS, hASCs, or Jurkat cells on days 26, 28, and 32 after CII immunization. As shown in Fig. 1A, even after the onset of arthritis, hASCs progressively attenuated the severity of CIA and hind paw volume of the arthritic mice as compared to the PBS- and Jurkat cell-treated arthritic mice (Fig. 1A). Strikingly, at the end of the treatment time, 30% of the arthritic mice completely recovered (Fig. 1A).

Representative photographs of the hind paws from normal mice, PBS- or Jurkat cell-treated mice, or hASC-treated mice in both treatment protocols are shown in Fig. 1B, respectively. Clinical assessment revealed severe edema and/or erythema in the joints of the PBS and Jurkat cell-treated CIA mice (Fig. 1B) compared with the joints of normal mice (Fig. 1B). In contrast, hASC treatment in either protocol markedly inhibited edema and/or erythema of the arthritic joints (Fig. 1B). These results suggest both the preventive and suppressive potential for hASCs in CIA.

3.2. Protective and therapeutic hASCs treatment downregulates the inflammatory response in CIA

To evaluate the possible mechanisms that could be responsible for the prophylactic or therapeutic effects of hASCs, we assessed changes in the production of inflammation mediators, which are mechanistically linked to joint inflammation. We measured the expression of pro- and anti-inflammatory cytokines and chemokines in the joint extracts and serum by cytokine array.

First, joint extracts from CIA mice treated on days −9, −7, and −4 with hASCs or PBS were isolated 42 days after CII immunization and prepared for cytokine assay. Strikingly, assays conducted 47 days after the last injection of hASCs still revealed markedly reduced levels of inflammation mediators compared with the levels in CIA mice treated with PBS. hASC injection markedly decreased protein expression of various inflammatory mediators, including IFN-γ, TNF-α, IL-1α, IL-1β, IL-6, IL-12, IL-15, IL-17, MCP-1, Rantes, and KC, while it promoted expression of the anti-inflammatory cytokine IL-10 in the joints of CIA mice (Table 1).

To evaluate the ability of hASC treatment to suppress the ongoing immune process, CIA mice were administered PBS or hASCs on days 26, 28, and 32 after CII immunization, and joint extracts were isolated 10 days after the last treatment

Table 1 Inhibition of inflammatory mediators (pg/ml) in joint extracts of CIA mice by protective and therapeutic hASCs treatments.

	TNFα	IL-6	IL-12	IFNγ	MCP-1	Rantes	IL-1α	IL-1β	IL-10	IL-17	KC	IL-15
PBS	190.6±34	1551±283.3	177.8±19.6	388.8±48.4	22633.3±1396.1	4020.4±425.2	168.3±13.7	20±2.3	20.75±5.4	6434.3±728.6	789.6±157.9	118.7±15.4
Pre-admin.	31.6±5.1*	249±31.6*	42.5±3.7*	140.4±21.6*	15246.6±2950*	2165±547.9*	44.8±10.9*	10.6±2.6*	32.7±0.6^	520.3±103.7*	305±56.3*	48.3±12*
Post-admin.	19.5±5.5*	150.2±50.6*	29.6±8.7*	54±11.8*	8509.2±1363*	1163.3±278.8*	30.4±11.4*	6.4±1.5*	168.6±19.8^	103.7±49.2*	232.2±26.6*	25.7±9*

Values are the mean±SD of 5 mice per group.
*=$P<0.001$; ^=$P<0.003$ versus PBS controls.

Table 2 Inhibition of inflammatory mediators (pg/ml) in serum of CIA mice by both protective and therapeutic approaches.

	IL-6	MCP-1	Rantes	IL-1α	IL-17	KC
PBS	361.5±100.8	22.3±1.5	306±93.2	399.7±40.3	53±17.6	1203.8±483.9
Pre-admin	105.7±55.8*	11.3±1.2*	220.3±48.7^	170.7±32.9*	19.7±3.1^	1007.6±475.3^
Post-admin.	70.8±7.3*	10±0*	150.1±44.7^	138.5±46.3*	14.7±4.1^	388±124^

The data represent the means±SD (n=5).
*=$P<0.005$; ^=$P<0.01$ versus PBS controls.

with hASCs. hASC injection markedly decreased protein expression of various inflammatory mediators, including IFN-γ, TNF-α, IL-1α, IL-1β, IL-6, IL-12, IL-15, IL-17, MCP-1, Rantes, and KC compared with the levels in CIA mice treated with PBS. hASC-treated mice showed an expression level of the anti-inflammatory cytokine IL-10 in the joints of CIA mice (Table 1), substantially above that detected in mice treated prophylactically with hASCs (Table 1).

Levels of pro-inflammatory cytokines in the serum were measured on day 42 after immunization with CII for the prophylactic treatment on days −9, −7, and −4 and the therapeutic treatment on days 26, 28, and 32, respectively. Consistent with joint swelling, IL-1α, IL-6, IL-17, MCP-1, Rantes, and KC in the PBS-treated CIA mice were systemically overproduced in the serum (Table 2). In contrast, markedly low serum levels of IL-1α, IL-6, IL-17, MCP-1, Rantes, and KC were seen in the CIA mice treated either prophylactically with hASCs or therapeutically with hASCs (Table 2). Therefore, these data demonstrated the broad anti-inflammatory activity of hASCs in the inflamed joint together with the suppression of the systemic inflammatory response.

3.3. Protective and therapeutic hASC treatment downregulates the Th1-mediated autoreactive response in CIA

RA has been described as both autoreactive Th1 cell- (producing IFN-γ and TNF-α) and Th17 cell-(producing IL-17) regulated disease, which can enter joint tissues and release inflammatory mediators. These mediators can also promote macrophage and neutrophil infiltration and activation in joint tissues, thereby worsening RA [1–3]. We examined the possible immunomodulating effect of hASCs on T cell priming and differentiation in vivo by evaluating the recall response to CII in isolated splenocytes from hASC-treated or PBS-treated CIA mice in vitro.

We showed that the T cell proliferative response to CII from hASC-treated mice, even 47 days after the last injection of hASCs (on day −4 before CII immunization), still revealed a markedly decreased stimulation index compared to mice treated with PBS (Fig. 2A). To evaluate the ability of hASC treatment to suppress the ongoing inflammatory process, mice with CIA were treated with PBS or hASCs on days 26, 28, and 32 after CII immunization, and splenocytes isolated 10 days after the last treatment with the hASCs were evaluated for proliferative responses to CII. T cells from hASC-treated mice exhibited a markedly reduced stimulation index compared to that in cells from PBS-treated mice (Fig. 2A).

Moreover, production of Th17 cytokines IL-17 and proinflammatory Th1 cytokines including IFN-γ, TNF-α, and IL-2 was significantly lower in cultures of splenocytes from mice treated by both protective and therapeutic experimental approaches with hASCs than from mice treated with PBS (Fig. 2A). Importantly, hASCs dramatically stimulated the production of the regulatory cytokines IL-10 and TGF-β1 (Fig. 2A) by CII-activated T cells, whereas the Th2-type cytokine IL-4 was not significantly affected by either treatment (Fig. 2A).

Thus, our findings indicated that administering hASCs to CIA mice in both the prophylactic and therapeutic regimens was associated with strong immunomodulating effects on the priming of CII-specific $CD4^+$ T cells. The results were skewing activated CD4 T cells toward lower activity of Th1 and Th17 effector cells but increasing activity of the anti-inflammatory cytokines IL-10 and TGF-β1, suggesting that this treatment may generate IL-10/TGF-β-secreting Treg cells.

Antibodies (Abs) directed against collagen-rich joint tissue are thought to drive RA and CIA progression through their activation of FcγR- and complement-dependent pathways [3,24,31].Thus, anticollagen antibody titers were assessed at the end of each treatment protocol. Administering hASCs (protective approach) was able to prevent the induction of CII-specific IgG, especially autoreactive IgG2a Abs (Fig. 2B). Furthermore, hASCs also elicited the IgG-suppressing effect in the therapeutic regimen. Administration of hASCs (therapeutic approach) decreased serum levels of CII-specific IgG by 70%, particularly autoreactive IgG2a Abs (Fig. 2B). No significant difference was observed in serum anti-CII IgG1 levels in the different groups of mice (data not shown).

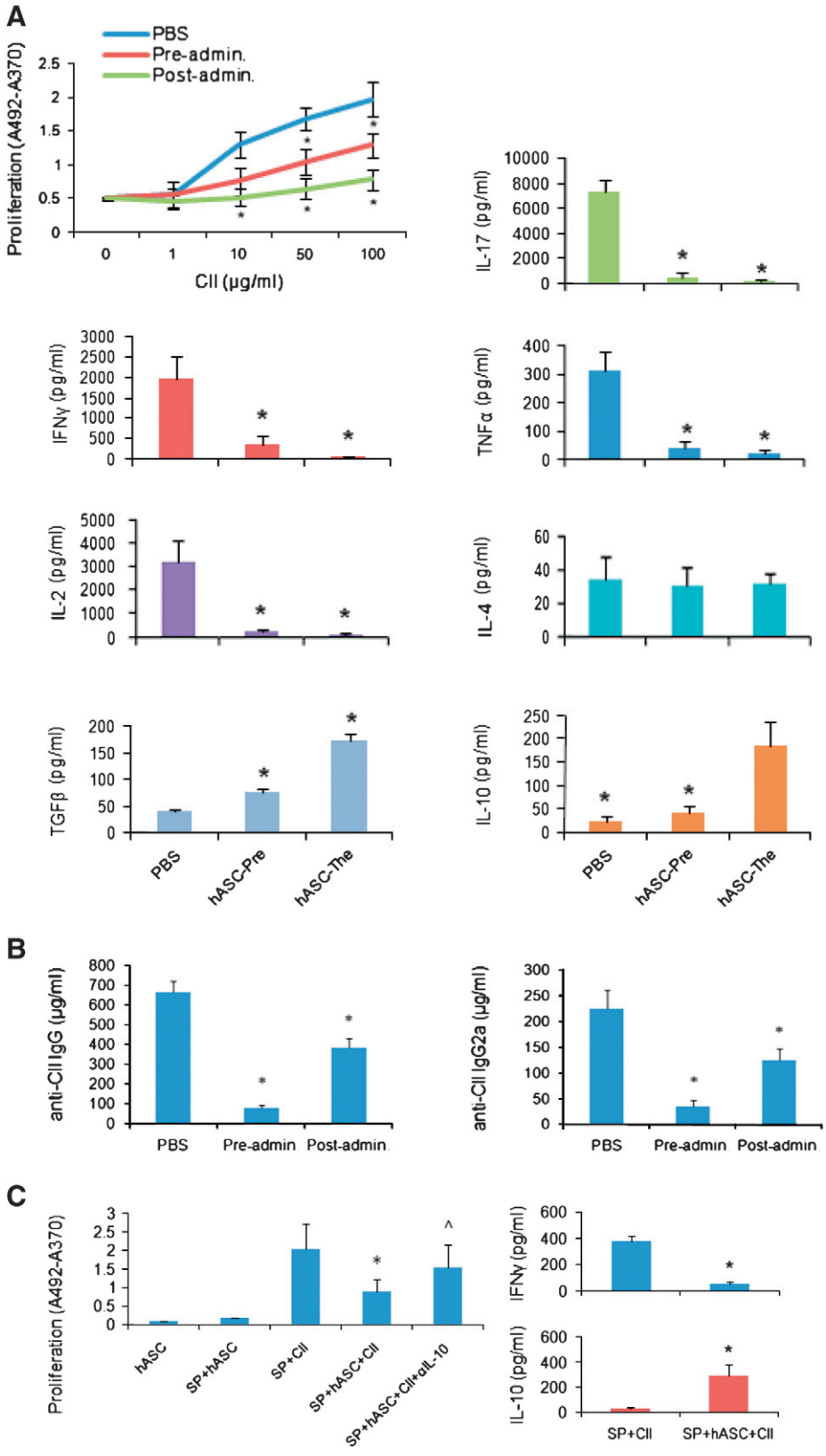
To examine whether or not hASCs can directly deactivate effector T cells, we cocultured autoreactive T cells from CIA mice and hASCs. From this we found that hASCs clearly resulted in a suppression of the proliferative responses of CII-specific T cells, and this effect was significantly reversed by anti-IL-10 Ab (Fig. 2C). Moreover, hASCs inhibited the production of IFN-γ and stimulated the expression of IL-10 by CII-activated T cells (Fig. 2C). These results suggest that hASCs could inhibit the Th1 responses and recruit Treg cells.

3.4. Protective and therapeutic hASC treatment recruits antigen-specific Treg cells in CIA

Previous studies have proven that Treg cells play a critical role in maintaining peripheral homeostasis and establishing controlled immune response [3]. Our data indicating a downregulation of autoreactive Th1 response and increased levels of regulatory cytokine IL-10 encouraged us to hypothesize the involvement of CII-specific Treg cells during in vivo treatment. Therefore, we compared the proportion and suppressive activity of Treg cells among experimental and

control groups, in view of the critical role of Treg cells in restraining autoaggressive T cells in experimental settings.

Administration of hASCs (both protective and therapeutic experimental approaches) resulted in markedly higher percentages of $CD4^+CD25^+Foxp3^+$ Treg cells in splenocytes than PBS control mice with CIA had (Fig. 3A): mean ± SD 7.6 ± 1.1%, 12.2 ± 1.2%, and 14.1 ± 1.7% in PBS-treated, hASC-treated mice with protective, and therapeutic approaches,



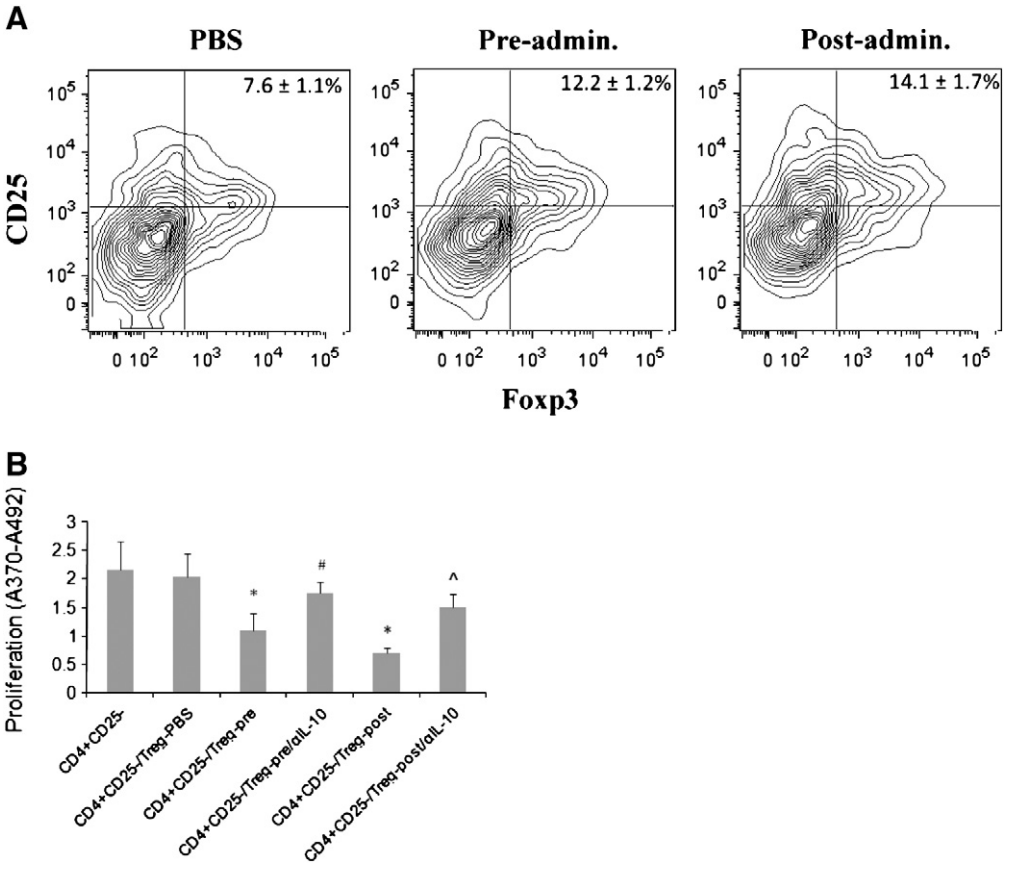


Figure 3 Induction of Treg cells with suppressive functions in CIA by protective and therapeutic hASCs. Mice were injected i.v. with PBS or hASCs on days −9, −7, and −4 (prophylactic protocol) or on days 26, 28, and 32 (therapeutic protocol). A, Analysis of $CD4^+CD25^+Foxp3^+$ cells. To evaluate the proportion of Treg cells in the experimental groups, splenocytes were stained with antibodies to mouse CD4, CD25, and Foxp3, and the labeled cells in the live lymphocyte gate from each sample were analyzed by flow cytometry. B, The suppressive capacity of hASC-induced Treg cells. $CD4^+CD25^+$ T cells were purified by magnetic antibody cell sorting from spleen of CII-immunized mice treated with either hASCs or PBS. Responder $CD4^+CD25^-$ T cells were isolated from CIA mice. $CD4^+CD25^-$ T cells alone or together with $CD4^+CD25^+$ T cells were cultured with autologous irradiated APCs and CII (100 μg/ml), some cocultures treated with anti-IL-10 Ab. After 72 h, the proliferation of autoreactive T cells was assayed by measuring bromodeoxyuridine-substituted DNA incorporation. Values are the means ± SD of five mice per group. *=$P<0.001$ versus cocultures of CII-activated $CD4^+CD25^-$ T cells/Treg-PBS cells stimulated with CII. #=$P<0.01$ versus cocultures of CII-activated $CD4^+CD25^-$ T cells/Treg-Pre cells stimulated with CII; ^=$P<0.005$ versus cocultures of CII-activated $CD4^+CD25^-$ T cells/Treg-post cells stimulated with CII.

respectively; $P<0.01$ and $P<0.001$. Importantly, administration of hASCs (both protective and therapeutic experimental approaches) had a significantly higher number of cells with $Foxp3^+$ expression (13.2 ± 5.4% and 14.6 ± 5.3%, respectively) compared to the PBS control mice (7.7 ± 1.2%, $P<0.01$ and $P<0.001$, respectively).

Moreover, we examined the suppressive activity of CII-specific Treg cells generated in the presence of hASCs on

Figure 2 Inhibition of the Th1-mediated response in CIA mice by protective and therapeutic hASC treatments. Mice were injected i.v. with PBS or hASCs on days −9, −7, and −4 (prophylactic protocol) or on days 26, 28, and 32 (therapeutic protocol). A, Proliferation and cytokine production by splenocytes isolated on day 42 and stimulated with experimental antigen in vitro. Proliferative responses were evaluated at 72 h by measuring bromodeoxyuridine-substituted DNA incorporation. Data are shown as the mean incorporation ± SD of triplicate cultures and represent three separate experiments with similar results. A total of 2×10^6 splenocytes per well were cultured for 48 h in the presence of CII 100 μg/ml. Supernatants were harvested and measured by cytokine array (the expected range is from 0 to 10,000 pg/ml). The data represent the means ± SD (n = 5). *=$P<0.001$ versus PBS controls. B, Levels of CII-specific Abs in sera collected on day 42 from PBS-treated or hASC-treated CIA mice. The data represent the means ± SD (n = 5). *=$P<0.001$ versus PBS controls. C, Proliferative response and production of IFN-γ and IL-10 in splenocytes isolated at the peak of disease from CIA mice. Proliferation and production of IFN-γ and IL-10 were evaluated in splenocytes stimulated with or without CII in the presence or absence of hASCs in some cocultures of splenocytes/hASCs treated with anti-IL-10 antibodies (αIL-10). Values are the mean ± SD of five mice per group. *=$P<0.001$ versus CII-activated splenocytes stimulated with CII; ^=$P<0.005$ versus cocultures of CII-activated splenocytes/hASCs stimulated with CII.

the activation of autoreactive T cells obtained from CIA mice. $CD4^+CD25^+$ Treg cells from CIA mice treated with PBS failed to suppress the proliferation of autologous $CD4^+CD25^-$ effector T cells (Fig. 3B). However, $CD4^+CD25^+$ Treg cells isolated from hASC-treated mice in both the protective and therapeutic experimental approaches could suppress the proliferative response of $CD4^+CD25^-$ effectors (Fig. 3B) as compared with PBS-treated mice. Those effects were significantly reversed by anti-IL-10 Ab (Fig. 3B). Thus, administering hASCs could be inducing Treg cells secreting IL-10, which suppresses the self-reactive T cells.

4. Discussion

Because no specific therapeutic strategies exist to treat RA, we tested the efficacy of hASCs, an innovative therapeutic tool for RA, against CIA in mice as the experimental model of autoimmune disease. Here we showed that administration of hASCs before disease onset markedly decreased the incidence of arthritis, ameliorated clinical signs, and hindered damage to the joints. Most importantly, administration of hASCs after disease entered an irreversible clinical course, displayed a true therapeutic effect for CIA.

Thus, our data demonstrated that hASC treatment has both prophylactic and therapeutic effects.

A number of possible mechanisms exist for the efficacy of hASCs on the downregulation of inflammatory and T cell responses in vitro and in vivo. As in the case in RA, CIA is primarily an autoimmune disease of the joints, the result of both autoreactive Th1 and Th17 cell-mediated responses to collagen-rich joint antigens [1–3]. Our data proved that administering hASCs by both protective and therapeutic approaches to mice with or without established CIA significantly suppressed the proliferation of CII-specific T cells and the production of the Th1-type and Th17-type cytokines. The inhibition of IFN-γ- and IL-17-producing cells might be the result of a direct effect on autoreactive T cells, because coculturing autoreactive T cells and hASCs clearly resulted in an inhibition of T-cell proliferation and IFN-γ production. Therefore, this observation emphasizes the direct inhibition role of hASCs on CII-reactive T cells from CIA mice.

Moreover, administering hASCs significantly increased the production of IL-10. IL-10 is not only a major anti-inflammatory cytokine, it is also an important regulatory factor for Treg cells, which play a critical role in the homeostatic regulation of the autoreactive T cell repertoire and the induction of peripheral tolerance in vivo [25,26]. Thus, the upregulation of CII-specific IL-10 production suggests the possibility that hASCs may induce IL-10-producing Treg cells [25–30] that suppress the responses of CII-reactive T cells in CIA mice. We, therefore, evaluated the possibility that this suppression was mediated by the recruitment of Treg cells in vivo. We found a significantly elevated percentage of $CD4^+CD25^+Foxp3^+$ cells from arthritic mice exposed to hASCs compared to the PBS control groups. Also, these hASCs-induced Treg cells significantly inhibited the proliferative response of autoreactive T cells in vitro, and these effects were significantly abrogated by anti-IL-10 Ab. Therefore, hASC treatment could induce IL-10-producing CII-specific $CD4^+CD25^+Foxp3^+$ Treg cells in mice with CIA and mediate T cell tolerance and protection against joint antigens.

In addition, our data demonstrated that administering hASCs significantly diminished the production of a wide range of inflammatory mediators in joint and systemic. hASC treatment suppressed the production of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-1α, IL-1β, IL-6, IL-12, IL-15, and IL-17 in the joint and increased levels of the anti-inflammatory cytokine IL-10. Furthermore, hASC treatment reduced chemokines, including MCP-1, Rantes, and KC, associated with the absence of inflammation in the joints of mice treated with hASCs. Several possibilities need to be investigated for this anti-inflammatory effect of hASCs. Prior studies [3,31] suggest at least three possibilities. First, the decrease in inflammatory mediators could correlate with the breakdown of cell infiltration in joints. Second, hASCs also directly impair the inflammatory response of CII-activated synovial cells from mice with CIA. Third, hASCs could directly downregulate the production of various inflammatory mediators by monocytes/macrophages and synovial cells.

Our data are consistent with the report from Augello et al. [32], they have demonstrated that administration of allogeneic bone marrow mesenchymal stem cells is capable of suppressing both the development of CIA and ongoing arthritis. Moreover, MSCs can induce hyporesponsiveness of T lymphocytes as evidenced by a reduction in active proliferation, and modulated the expression of inflammatory cytokines. In particular, MSCs exerted their immunomodulatory function by educating antigen-specific Treg cells. However, the main difference is that Augello et al. [32] used allogeneic bone marrow mesenchymal stem cells and have found that MSC treatment in immunized mice induced proliferation of antigen-specific clones of Treg cells 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, while we used adipose-derived mesenchymal stem cells to induce the antigen-specific $CD4^+CD25^+Foxp3^+$ Treg cells with the capacity to suppress autoreactive T cells, and the suppress in a IL-10 dependent manner. In fact, the production of the immune regulator IL-10 has been considered as one of their major therapeutic benefits of injected MSCs [33].

In summary, the present study demonstrated that hASC treatment, beyond its protective properties, also displays a therapeutic potential and suggests that hASCs may provide a promising approach for treating RA. Mechanistically, our results indicated that the hASCs could suppress a wide spectrum of inflammatory mediators and inhibit the Th1/Th17 cell expansion through the generation of IL-10–producing Treg cells with the capacity to suppress autoreactive T cells, thereby maintaining self-tolerance.

Conflict of interest statement

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by RNL Life Science, Inc, Korea.

References

[1] C. Fournier, Where do T cells stand in rheumatoid arthritis? Joint Bone Spine 72 (6) (2005 Dec) 527–532.

[2] G.S. Firestein, Evolving concepts of rheumatoid arthritis, Nature 423 (2003) 356–361.

[3] M.A. González, E. Gonzalez-Rey, L. Rico, D. Büscher, M. Delgado, Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells, Arthritis Rheum. 60 (4) (2009 Apr) 1006–1019.

[4] S. Sakaguchi, T. Yamaguchi, T. Nomura, M. Ono, Regulatory T cells and immune tolerance, Cell 133 (2008) 775–787.

[5] M.E. Morgan, R. Flierman, L.M. van Duivenvoorde, H.J. Witteveen, W. van Ewijk, J.M. van Laar, R.R. de Vries, R.E. Toes, Effective treatment of collagen-induced arthritis by adoptive transfer of $CD25^+$ regulatory T cells, Arthritis Rheum. 52 (2005) 2212–2221.

[6] J.M. van Amelsfort, J.A. van Roon, M. Noordegraaf, K.M. Jacobs, J.W. Bijlsma, F.P. Lafeber, L.S. Taams, Proinflammatory mediator-induced reversal of $CD4^+CD25^+$ regulatory T cell-mediated suppression in rheumatoid arthritis, Arthritis Rheum. 56 (3) (2007 Mar) 732–742.

[7] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.

[8] Z.J. Liu, Y. Zhuge, O.C. Velazquez, Trafficking and differentiation of mesenchymal stem cells, J. Cell. Biochem. 106 (6) (2009 Apr 15) 984–991.

[9] P.A. Zuk, M. Zhu, H. Mizuno, J. Huang, J.W. Futrell, A.J. Katz, P. Benhaim, H.P. Lorenz, M.H. Hedrick, Multilineage cells from human adipose tissue: implications for cell-based therapies, Tissue Eng. 7 (2001) 211–228.

[10] A. Nasef, N. Ashammakhi, L. Fouillard, Immunomodulatory effect of mesenchymal stromal cells: possible mechanisms, Regen. Med. 3 (4) (2008 Jul) 531–546.

[11] D. Noël, F. Djouad, C. Bouffi, D. Mrugala, C. Jorgensen, Multipotent mesenchymal stromal cells and immune tolerance, Leuk. Lymphoma 48 (7) (2007 Jul) 1283–1289.

[12] F. Djouad, C. Bouffi, S. Ghannam, D. Noël, C. Jorgensen, Mesenchymal stem cells: innovative therapeutic tools for rheumatic diseases, Nat. Rev. Rheumatol. 5 (7) (2009 Jul) 392–399.

[13] S. Glennie, I. Soeiro, P.J. Dyson, W. Lam, F. Dazzi, Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells, Blood 105 (2005) 2821–2827.

[14] R. Wehner, D. Wehrum, M. Bornhäuser, S. Zhao, K. Schäkel, M.P. Bachmann, U. Platzbecker, G. Ehninger, E.P. Rieber, M. Schmitz, Mesenchymal stem cells efficiently inhibit the proinflammatory properties of 6-sulfo LacNAc dendritic cells, Haematologica 94 (8) (2009 Aug) 1151–1156.

[15] M. Locke, J. Windsor, P.R. Dunbar, Human adipose-derived stem cells: isolation, characterization and applications in surgery, ANZ J. Surg. 79 (4) (2009) 235–244.

[16] C. Bouffi, F. Djouad, M. Mathieu, D. Noël, C. Jorgensen, Multipotent mesenchymal stromal cells and rheumatoid arthritis: risk or benefit? Rheumatology (Oxford) 48 (10) (2009 Oct) 1185–1189.

[17] R. Yañez, M.L. Lamana, J. García-Castro, I. Colmenero, M. Ramírez, J.A. Bueren, Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versus-host disease, Stem Cells 24 (11) (2006 Nov) 2582–2591.

[18] S. Beyth, Z. Borovsky, D. Mevorach, M. Liebergall, Z. Gazit, H. Aslan, E. Galun, J. Rachmilewitz, Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness, Blood 105 (5) (2005 Mar 1) 2214–2219.

[19] B. Puissant, C. Barreau, P. Bourin, C. Clavel, J. Corre, C. Bousquet, C. Taureau, B. Cousin, M. Abbal, P. Laharrague, L. Penicaud, L. Casteilla, A. Blancher, Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells, Br. J. Haematol. 129 (1) (2005 Apr) 118–129.

[20] E.J. Miller, Isolation and characterization of a collagen from chick cartilage containing three identical alpha chains, Biochemistry 10 (9) (1971 Apr 27) 1652–1659.

[21] D.D. Brand, K.A. Latham, E.F. Rosloniec, Collagen-induced arthritis, Nat. Protoc. 2 (5) (2007) 1269–1275.

[22] J.C. Ra, I.S. Shin, S.H. Kim, S.K. Kang, B.C. Kang, H.Y. Lee, Y.J. Kim, J.Y. Jo, E.J. Yoon, H.J. Choi, E. Kwon, Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans, Stem Cells Dev. 20 (8) (2011 Aug) 1297–1308.

[23] L.K. Myers, J.M. Stuart, A.H. Kang, A CD4 cell is capable of transferring suppression of collagen-induced arthritis, J. Immunol. 143 (1989) 3976–3980.

[24] J.L. Ellsworth, N. Hamacher, B. Harder, K. Bannink, T.R. Bukowski, K. Byrnes-Blake, S. Underwood, C. Oliver, K.S. Waggie, C. Noriega, L. Hebb, M.W. Rixon, K.E. Lewis, Recombinant soluble human FcgammaR1A (CD64A) reduces inflammation in murine collagen-induced arthritis, J. Immunol. 182 (11) (2009 Jun 1) 7272–7279.

[25] M. Battaglia, C. Gianfrani, S. Gregori, M.G. Roncarolo, IL-10-producing T regulatory type 1 cells and oral tolerance, Ann. N. Y. Acad. Sci. 1029 (2004 Dec) 142–153.

[26] M.K. Levings, R. Bacchetta, U. Schulz, M.G. Roncarolo, The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells, Int. Arch. Allergy Immunol. 129 (4) (2002 Dec) 263–276.

[27] C.M. Lloyd, C.M. Hawrylowicz, Regulatory T cells in asthma, Immunity 31 (3) (2009 Sep 18) 438–449.

[28] C. Prevosto, M. Zancolli, P. Canevali, M.R. Zocchi, A. Poggi, Generation of $CD4^+$ or $CD8^+$ regulatory T cells upon mesenchymal stem cell-lymphocyte interaction, Haematologica 92 (2007) 881–888.

[29] Y.P. Li, S. Paczesny, E. Lauret, S. Poirault, P. Bordigoni, F. Mekhloufi, O. Hequet, Y. Bertrand, J.P. Ou-Yang, J.F. Stoltz, P. Miossec, A. Eljaafari, Human mesenchymal stem cells license adult $CD34^+$ hemopoietic progenitor cells to differentiate into regulatory dendritic cells through activation of the Notch pathway, J. Immunol. 180 (2008) 1598–1608.

[30] R. Maccario, M. Podestà, A. Moretta, A. Cometa, P. Comoli, D. Montagna, L. Daudt, A. Ibatici, G. Piaggio, S. Pozzi, F. Frassoni, F. Locatelli, Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of $CD4^+$ T-cell subsets expressing a regulatory/suppressive phenotype, Haematologica 90 (4) (2005 Apr) 516–525.

[31] E. Gonzalez-Rey, M.A. Gonzalez, N. Varela, F. O'Valle, P. Hernandez-Cortes, L. Rico, D. Büscher, M. Delgado, Human adipose mesenchymal cells reduce inflammatory and T cell responses and induce regulatory T cells in vitro in rheumatoid arthritis, Ann. Rheum. Dis. 69 (1) (2010 Jan) 241–248.

[32] A. Augello, R. Tasso, S.M. Negrini, R. Cancedda, G. Pennesi, Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis, Arthritis Rheum. 56 (4) (2007 Apr) 1175–1186.

[33] C. Holladay, K. Power, M. Sefton, T. O'Brien, W.M. Gallagher, A. Pandit, Functionalized scaffold-mediated interleukin 10 gene delivery significantly improves survival rates of stem cells in vivo, Mol. Ther. 19 (5) (2011 May) 969–978.