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Human adipose-derived mesenchymal stem cells reduce inflammatory and T cell responses and induce regulatory T cells in vitro in rheumatoid arthritis

E Gonzalez-Rey,1 M A Gonzalez,2,3 N Varela,4 F O’Valle,5 P Hernandez-Cortes,6 L Rico,1 D Büscher,1 M Delgado4

ABSTRACT

Objectives: Adult mesenchymal stem cells were recently found to suppress effector T cell and inflammatory responses and have emerged as attractive therapeutic candidates for immune disorders. In rheumatoid arthritis (RA), a loss in the immunological self-tolerance causes the activation of autoreactive T cells against joint components and subsequent chronic inflammation. The aim of this study is to characterise the immunosuppressive activity of human adipose-derived mesenchymal stem cells (hASCs) on collagen-reactive T cells from patients with RA.

Methods: The effects of hASCs on collagen-reactive RA human T cell proliferation and cytokine production were investigated, as well as effects on the production of inflammatory mediators by monocytes and fibroblast-like synoviocytes from patients with RA.

Results: hASCs suppressed the antigen-specific response of T cells from patients with RA. hASCs inhibited the proliferative response and the production of inflammatory cytokines by collagen-activated CD4 and CD8 T cells. In contrast, the numbers of IL10-producing T cells and monocytes were significantly augmented upon hASC treatment. The suppressive activity of hASCs was cell-to-cell contact dependent and independent. hASCs also stimulated the generation of FoxP3 protein-expressing CD4+CD25+ regulatory T cells, with the capacity to suppress collagen-specific T cell responses. Finally, hASCs downregulated the inflammatory response and the production of matrix-degrading enzymes by synovial cells isolated from patients with RA.

Conclusions: The present work identifies hASCs as key regulators of immune tolerance, with the capacity to suppress T cell and inflammatory responses and to induce the generation/activation of antigen-specific regulatory T cells.

Adaptive immune response is carried out by a complex biological network of cells and molecular signals that rely on the discrimination between self and non-self antigens to protect the organism against a broad range of infectious agents while avoiding the development of misguided or excessive immune reactions, such as autoimmune and allergic reactions. The induction and maintenance of immunological self-tolerance seems to depend on the deletion of self-reactive clones during development and the generation/activation of regulatory T cells (Tr) in the periphery during adult life. Understanding the mechanisms and factors involved in the generation or activation of Tr cells is a major challenge in immunology and medicine.

The initial stages of rheumatoid arthritis (RA) involve multiple steps, which can be divided into two main phases: initiation and establishment of autoimmune to collagen-rich joint components, and later events associated with the evolving immune and inflammatory responses. Certain therapeutic approaches address the self-reactive T cell component of RA, complementing existing therapies directed toward diminishing the inflammatory response. However, current therapies fail to completely arrest disease progression. In this sense, enhancement of the function of the Tr component could represent a valuable therapeutic strategy for RA.

Mesenchymal stem cells (MSCs) are resident mesoderm-derived stromal cells that function as precursors of non-hematopoietic connective tissues with capacity to differentiate into mesenchymal and non-mesenchymal cell lineages. Apart from their potential clinical application in tissue repair, bone marrow-derived MSCs (BM-MSCs) have been recently described as potent immunomodulators in various immune disorders. Apart from BM-MSCs, other major sources of human MSCs are umbilical cord blood, peripheral blood and adipose tissue. Based on their accessibility, human adipose-derived MSCs (hASCs) have lately emerged as attractive alternatives for cell therapy. MSCs exert profound immunomodulatory properties and protective effects on acute graft versus host disease and experimental arthritis. Here, we characterise the immunosuppressant activity of hASCs on collagen-reactive T cells and synovial cells from patients with RA.

METHODS

Cell isolation

To obtain hASCs, liposapirates obtained from adipose tissue from healthy subjects were digested with 0.075% type I collagenase (37°C, 30 min) and erythrocyte depleted with 160 mM ClNH4. Cells (2–5×10^3/cm²) were expanded in complete medium (Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine and 1% penicillin/streptomycin) at 37°C and 5% CO₂, changing the culture medium every 3–4 days. A total of 6 different samples with population doublings 6–9 were used in the study. These cells showed a fibroblast-like morphology and differentiation capacity to the adipocytic and osteocytic lineages, and expressed the phenotype HLA-DR + CD3 CD14 + CD18 CD31– CD34+ CD40–.
Peripheral blood mononuclear cells (PBMCs) isolated from 22 patients with RA (5 men and 17 women) by density sedimentation on Ficoll–Hypaque gradients were immediately used for culture or separated immunomagnetically into T cells and non-T cells using anti-CD3 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Non-T cells were used as antigen presenting cells (APCs). All patients were of Spanish origin, met the American College of Rheumatology 1987 revised classification criteria for RA,11 had clinically active synovitis and received inflammatory drugs. T cell proliferative response to type II collagen (CII) in these patients with RA was assayed as previously,9 and only 12 of the 22 PBMC samples responded positively to CII. T cell proliferative responses were considered in the absence of antigen). Cytokine levels in culture supernatants were determined by sandwich ELISAs using capture/biotinylated detection Abs from BD Pharmingen (Erembodegem, Belgium) following the manufacturer’s recommendations. To identify whether hASCs selectively affected the production of cytokines on T cell subsets, intracellular cytokine contents were determined in CD4+ and CD8+ T cells from patients with RA. hASCs and RA PBMCs were stimulated with CII (400 μg/ml) for 14 h, in the presence of monensin (1.33 μM, Sigma) for the last 6 h, and then stained with peridinin chlorophyll protein (PerCP) anti-CD4 or fluorescein isothiocyanate (FITC) anti-CD8 mAbs at 4°C, washed, fixed/permeabilised with Cytofix/Cytoperm solution (Becton Dickinson, Erembodegem, Belgium), stained with PE-conjugated anti-cytokine-specific mAbs (BD Pharmingen).

### Cytokine determination

Cytokine levels in culture supernatants were determined by sandwich ELISAs using capture/biotinylated detection Abs from BD Pharmingen (Erembodegem, Belgium) following the manufacturer’s recommendations. To identify whether hASCs selectively affected the production of cytokines on T cell subsets, intracellular cytokine contents were determined in CD4+ and CD8+ T cells from patients with RA. hASCs and RA PBMCs were stimulated with CII (400 μg/ml) for 14 h, in the presence of monensin (1.33 μM, Sigma) for the last 6 h, and then stained with peridinin chlorophyll protein (PerCP) anti-CD4 or fluorescein isothiocyanate (FITC) anti-CD8 mAbs at 4°C, washed, fixed/permeabilised with Cytofix/Cytoperm solution (Becton Dickinson, Erembodegem, Belgium), stained with PE-conjugated anti-cytokine-specific mAbs (BD Pharmingen).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characterisation of the human adipose-derived mesenchymal stem cells (hASCs) used in the study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker</td>
<td>Percentage, mean (SD)</td>
</tr>
<tr>
<td>HLA-ABC</td>
<td>92 (5)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>CD3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>CD13</td>
<td>94 (4)</td>
</tr>
<tr>
<td>CD14</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>CD18</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>CD29</td>
<td>92 (3)</td>
</tr>
<tr>
<td>CD31</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>CD34</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>CD40</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>CD44</td>
<td>94 (5)</td>
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</tbody>
</table>

hASCs were incubated with saturating amounts of different monoclonal antibodies conjugated with phycoerythrin, fluorescein isothiocyanate or allophycocyanin against various surface markers and analysed by flow cytometry. Results are the mean (SD) of the percentage of positive cells for a given phenotypic marker (n = 5–7). HLA, human leukocyte antigen.
Human adipose-derived mesenchymal stem cells (hASCs) deactivate type II collagen (CII)-reactive peripheral blood mononuclear cells (PBMCs) from patients with rheumatoid arthritis (RA). (A) PBMCs (10^5) isolated from 22 patients with RA were stimulated with phytohaemagglutinin (PHA) in the absence or presence of hASCs (2 × 10^4). Proliferation was determined after 4 days culture. Interferon (IFN)γ content in the supernatant was determined after 72 h. *p < 0.001 vs PHA-stimulated PBMCs. (B) Upper panel: PBMCs (10^5) isolated from 12 patients with RA were stimulated with CII in the absence or presence of different numbers of allogeneic hASCs. Bottom panel: T cells isolated from 12 patients with RA were stimulated with CII and syngeneic antigen presenting cells (APCs) in the absence or presence of different numbers of allogeneic hASCs, and proliferation determined after 4 days of culture. *p < 0.001 vs CII-stimulated PBMCs/T cells in the absence of hASCs. (C) Control experiments measuring the proliferation in the presence of CII of 12 CII-responder RA PBMCs (column 1), 10 CII-non-responder RA PBMCs (column 2), PBMCs from healthy subjects (column 3), hASCs alone (column 4), mytomycin-treated hASCs (column 5), mytomycin-treated APCs (column 6), CII-responder RA PBMCs plus hASCs (column 7), and CII-responder RA PBMCs plus mytomycin-treated hASCs (column 8). PBMCs from 22 patients with RA cultured in the absence of CII were used to determine the basal proliferative response (column 9). (D) PBMCs from eight patients with RA were stimulated with CII, and hASCs added at different times after initiation of cultures. Proliferation was determined after 5 days culture. Results are expressed as percentage of inhibition of proliferation of CII-activated PBMCs. (E) PBMCs isolated from eight patients with RA were stimulated with CII in the absence or presence of hASCs, and proliferation determined 96 h later. Cytokine contents in the supernatants were determined after 72 h. When indicated (bracket, column 6), CII-activated PBMCs were separated from hASCs by a semipermeable membrane in transwell plates, and cytokine content and proliferation determined. Alternatively, PBMCs were activated with CII in conditioned medium (CM) collected from tumour necrosis factor (TNF)α/IFNγ-stimulated hASC cultures. *p < 0.001 vs CII-activated PBMCs, #p < 0.001 vs PBMC alone, p < 0.001 vs CII-activated PBMC–hASC cocultures. (F) PBMCs isolated from eight patients with RA were stimulated with CII in the absence or presence of hASCs. After 14 h, cells were analysed for CD4 or CD8 and intracellular IFNγ and TNFα expression by flow cytometry. *p < 0.001 vs CII-activated PBMC.
complete medium supplemented with IL2 (20 U/ml), and added at different ratios in a secondary culture to 10^5 PBMCs stimulated with CII (400 μg/ml) or with anti-CD3 Ab (5 μg/ml). To investigate the antigen-dependent specificity these cells, the secondary cultures were performed with 10^5 TASC and 2 × 10^5 tetanus toxin (TT)-primed RA PBMCs in the presence of CII (400 μg/ml) or TT (1 μg/ml, AbD Serotec, Oxford, UK).

**Statistical analysis**

All results are expressed as mean (SD) and the Mann–Whitney U test was used to compare non-parametric data for statistical significance.

**RESULTS**

hASCs inhibit the activation of CII-specific CD4 and CD8 T cells from patients with RA

Recent studies have characterised the phenotype of hASCs and their immunoregulatory effects on T cells. In this study, we investigated the ability of hASCs to inhibit the activation of PBMCs isolated from patients with RA. hASCs significantly inhibited the secretion of IFNγ and the proliferation of PBMCs stimulated with PHA, a polyclonal mitogen (fig 1A). Importantly, hASCs dose-dependently decreased the proliferative response of PBMCs isolated from patients with RA that responded positively to CII, a major component of the hyaline cartilage (fig 1B, upper panel). A similar suppressive effect on the proliferative response was observed when allogeneic hASCs were added to cultures of CII-activated T cells and APCs (fig 1B, bottom panel). hASCs did not affect the survival and apoptosis of CII-reactive T cells (not shown). Noteworthy from a therapeutic point of view, delayed addition of hASCs significantly inhibited CII-stimulated T cell proliferation (fig 1D).

Moreover, hASCs downregulated the production of the T helper (Th)1 cytokines IFNγ and TNFα, as well as IL17 by CII-reactive T cells, whereas induced the secretion of the anti-inflammatory cytokine IL10 (fig 1E). Probably because they lack...
class II MHCs (table 1), allogeneic ASCs did not contribute to T cell proliferation in the absence of antigenic stimulation (fig 1E). Intracellular cytokine staining in CD4+ and CD8+ T cells analysed from the total PBMC population indicated that hASCs inhibited the production of IFNγ and TNFα in both T cell subpopulations, while increasing the number of IL10-producing CD4 and CD8 T cells (fig 1F). hASC-mediated inhibition of CII-induced proliferation and IFNγ, IL17 and TNFα production was partially reversed when PBMCs and hASCs were separated in transwell plates by a semipermeable membrane (fig 1E), suggesting a cell-to-cell contact dependence. The partial involvement of soluble factors produced by hASCs on their immunosuppressive activity was supported by the fact that conditioned media from TNFα/IFNγ-stimulated hASCs suppressed CII-induced PBMC activation (fig 1E).

hASCs deactivate the inflammatory response of monocytes and synovial cells from patients with RA
Interestingly, we found that the hASC-induced production of IL10 by CII-reactive PBMCs was entirely cell-to-cell contact dependent (fig 1E). An initial cell contact between PBMCs and hASC seems to be enough to induce the secretion of IL10, even in the absence of CII stimulation (fig 1E). It has been recently demonstrated that BM-MSCs induce monocytes and dendritic cells (DCs) in a cell contact-dependent manner to secrete high IL10 amounts.6 Therefore, we investigated whether hASCs stimulated IL10 production by monocytes. Monocytes isolated from patients with RA were LPS activated in the absence or presence of allogeneic hASCs. Neither monocytes nor hASCs produced significant IL10 levels when cultured alone, even in the presence of LPS; however, hASC–monocyte cocultures produced high amounts of IL10 through a cell to cell contact-dependent mechanism (fig 2A). Intracellular IL10 determination in the two cell populations showed that, once they enter into contact, IL10 expression is induced in hASCs and monocytes (fig 2A). The involvement of IL10 was confirmed by the partial reversal of the inhibitory activity of hASCs on CII-induced RA T cell activation by neutralising anti-IL10 Abs (fig 2B).

We next investigated the capacity of hASCs to regulate the inflammatory response of the resident cells of the synovial membrane. hASC treatment of RA FLS cultures decreased the production of TNFα and PGE2, but not IL6, and reduced
Human adipose-derived mesenchymal stem cells (hASCs) induce the generation of antigen-specific regulatory T (Tr) cells in the peripheral blood mononuclear cells (PBMCs) of patients with rheumatoid arthritis (RA). A. PBMCs isolated from patients with RA were stimulated with type II collagen (CII) in the absence (control) or presence of hASCs. After 4 days, T cells (Tcontrol and TASC) were isolated from cocultures, rested, and added at

**Figure 4**

A. PBMCs isolated from patients with RA were stimulated with type II collagen (CII) in the absence (control) or presence of hASCs. After 4 days, T cells (Tcontrol and TASC) were isolated from cocultures, rested, and added at

B. Proliferation (A450) of T cells in response to CII stimulation with or without hASCs.

C. Interleukin-17 (IL-17) levels in the supernatants of T cells with or without hASCs.

D. Flow cytometry analysis of the expression of FoxP3 and CTLA4 in T cells with or without hASCs.

E. Proliferation (A450) of T cells with CD25^high and CD25^intermediate subsets in response to CII stimulation with or without hASCs.
hASCs induce antigen-specific Tr in PBMCs from patients with RA

Numerous sources indicate that Tr cells play a critical role in the maintenance of self-immune tolerance in RA. The observed downregulation of the CII-reactive Th1 response, together with the elevated levels of the regulatory cytokine IL10, prompted us to investigate the suppressive activity of T cells generated in the presence of hASCs (TASC) on the activation of effector T cells from patients with RA. In contrast to T control cells, TASC cells suppressed the proliferation of and IFNγ production by CII-activated RA PBMCs (fig 4A). This effect was significantly reversed by anti-IL10 Abs (fig 4B). As expected, IL2 bypassed the suppressive activity of TASC cells (fig 4B). This suppressive effect was antigen specific, because TASC neither inhibited the proliferative response of RA PBMCs activated with anti-CD3 Abs (fig 4A) or prevented TT-induced activation in TT-primed RA PBMCs (fig 4C). Flow cytometry analysis of TASC cells showed increased percentages of CD4+CD25high T cells expressing the Tr markers/mediators CTLA4 and FoxP3 protein (fig 4D). Addition of the different T cell populations generated by hASCs to CII-activated T cell cultures demonstrated that the suppressive activity of the TASC cells mainly resides in the CD4+CD25high T cell population (fig 4E).

DISCUSSION

A desirable therapeutic approach for RA should prevent inflammatory and autoimmune components of the disease. In this study, we demonstrated that adult mesenchymal stem cells isolated from adipose tissue exert profound suppressive responses on CII-reactive T cells from patients with RA in various ways: they suppress CII-induced T cell proliferation, inhibit the production of inflammatory cytokines by CD4 Th1 and CD8 Tc1 cells, stimulate the production of the anti-inflammatory suppressive cytokine IL10 by monocytes and T cells and induce the generation of antigen-specific Tr cells. Moreover, hASCs inhibit the production of inflammatory factors by activated synovial cells involved in the destruction of cartilage and bone in RA.

Cell-based therapy for autoimmune disease with autologous haematopoietic stem cell transplants was initiated 12 years ago on over 700 patients with various severe autoimmune diseases. Although this treatment was quite encouraging for systemic lupus erythematosus, systemic sclerosis and multiple sclerosis, achieving stable remission, most patients with RA showed only transient responses. This failure was most likely due to low dose conditioning regimens, resulting in too low a level of immunosuppression. Based on their “immune privileged” status, allogeneic healthy MSCs emerged as optimal substitutes in this therapy, resulting in more function seen in clinical settings. In agreement with our data, human BM-MSCs deactivated CII-reactive T cells isolated from blood and synovial fluid of allogeneic patients with RA. However, an important issue for clinical translation of MSCs in RA is that their therapeutic use requires large quantities of cells for infusion. In this sense, large amounts of hASCs can be easily obtained from liposprats of allogeneic healthy donors and rapidly expanded in vitro to generate a clinically effective dosage.

Before clinical translation, we must corroborate that hASCs maintain the immunosuppressive activity observed in vitro after their infusion in vivo. To this end, Djouad et al reported that an immortalised murine cell line derived from BM-MSCs did not confer any beneficial effect in a model of collagen-induced arthritis, while showing potent immunosuppressive actions in vitro, suggesting that an inflammatory milieu could influence the immunoregulatory properties of MSCs. However, using the same experimental model, Augello et al described how the injection of primary allogeneic murine BM-MSCs at the disease onset significantly ameliorated arthritis signs. This discrepancy could reside in the use of cell lines versus primary MSCs. Supporting the beneficial effect of MSCs in vivo, we recently demonstrated that hASCs and murine ASCs provided a highly effective therapy for experimental arthritis by strikingly reducing the two deleterious components of the disease (ie, the Th1-mediated autoimmune and inflammatory responses). Of importance from a therapeutic point of view is the fact that hASCs efficiently ameliorated the clinical signs in mice with established arthritis. This correlates with the present work, showing the capacity of delayed administration of hASCs to still suppress CII-reactive RA PBMCs in ongoing cultures.

Several studies have reported that BM-MSCs and ASCs of murine origin downregulate the inflammatory and T cell responses in vitro and in vivo, involving very diverse mechanisms. Our data demonstrate the participation of cell to cell contact and soluble factors in the deactivation of T cells by hASCs, where IL10 seems to play a critical role. IL10 involvement in the immunosuppressive action of BM-MSCs has been previously demonstrated. Interleukin-10 is a powerful anti-inflammatory cytokine that directly inhibits the production of inflammatory cytokines by CD4 Th1 cells, regulates the expression of T cell co-stimulatory molecules and dampens immune responses. However, recent studies have shown that human MSCs do not express IL10 by themselves, but this cytokine can be induced from bone marrow mesenchymal stem cells by T cells, monocytes and other inflammatory cells. Therefore, MSCs can be considered as a potential source of IL10 for the immunosuppressive effect.

Supporting the beneficial effect of MSCs in vivo, we recently demonstrated that hASCs and murine ASCs provided a highly effective therapy for experimental arthritis by strikingly reducing the two deleterious components of the disease (ie, the Th1-mediated autoimmune and inflammatory responses). Of importance from a therapeutic point of view is the fact that hASCs efficiently ameliorated the clinical signs in mice with established arthritis. This correlates with the present work, showing the capacity of delayed administration of hASCs to still suppress CII-reactive RA PBMCs in ongoing cultures.

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could act through different suppressive mechanisms. Alternatively, hASCs could be interfering with the function of the APCs and subsequent T cell activation. However, the fact that hASCs suppressed cell proliferation induced by a T cell mitogen argues against this hypothesis.

By contrast, we demonstrated that the induction of IL10-secreting T cells with antigen-specific regulatory capacity could significantly contribute to the suppressive activity of hASCs on RA T cells. hASC-induced Tr cells specifically suppress CII-driven responses, but not other responses to unrelated antigens, such as TT, or to polyclonal activation. Phenotypic characterisation of these cells showed that hASCs induced the emergence of CD4+CD25+ cells, expressing the transcription factor FoxP3, a phenotypic Tr marker, and the immunosuppressive molecule CTLA4.3 As described for other inducible Tr,5 IL10 seems to play a crucial role in the suppressive capacity of the hASC-induced Tr. Other authors previously reported that BM-MSCs directly induce CD4 and CD8 Tr functions in allogeneic systems.22 23 Although our study could be the first describing the induction of antigen-specific suppressive T cells by human MSCs of any origin, other reports described the capacity of murine MSCs to suppress antigen-specific T cell responses,24 25 and human MSCs to inhibit anti-antigen-driven T cell activation.26 Regarding the mechanism involved, it is still unknown whether hASCs act directly on T cells inducing de novo generation of Tr or the expansion of existing naturally occurring Tr. We recently described that hASCs can convert in the periphery of arthritic animal antigen-primed CD4+CD25− cells to CD4+CD25+ Tr,27 supporting de novo generation of Tr by hASCs. TGFβ1 and IL10 have been critically involved in the peripheral generation of CD4+CD25+ Tr.1 Whereas TGFβ1 was not significantly augmented in the hASCs and RA PBMCs cocultures, the production of IL10 did dramatically increase. Therefore, the ASC-induced IL10 secretion could participate in the Tr induction. Alternatively, BM-MSCs alter the differentiation and activation of DCs, inducing the generation of tolerogenic DCs with capacity to generate Tr.21 The hypothesis of induction of APCs with tolerogenic activity by hASCs emerges as an attractive mechanism that needs further investigation, and it will partially explain the antigen-specific nature in the action of the hASC-induced Tr.

We envision that once the benefits and risks associated with the injection of hASCs are well defined, the use of hASCs opens new therapeutic perspectives for a cell-based therapy of autoimmune/inflammatory diseases. The capacity of hASCs to regulate various inflammatory mediators, and suppress Th1-type responses through the generation of Tr, might offer a therapeutic advantage over existing therapies directed against a single mediator. However, because RA is of unknown aetiology and CII is not absolutely established as an autoantigen,4 26 27 precautions should be taken before considering translatable clinical application of hASCs to RA from our results.

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Competing interests: This work is part of a patent application by Cellnex. MAG and MB are inventors in this patent application. Since Cellnex SA and/or the inventors and/or their Institutes stands to profit from this work, the authors have a conflict of interest in this capacity, which hereby has been officially disclosed.

Extended report

REFERENCES