Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T cell unresponsiveness

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Short Title: Regulatory cells induced by mesenchymal stem cells

Abstract

Infusion of either embryonic or mesenchymal stem cells prolongs the survival of organ transplants derived from stem cell donors and prevents graft-versus-host-disease (GVHD). An in-depth mechanistic understanding of this tolerization phenomenon could lead to novel cell-based therapies for transplantation. Here we demonstrate that while human mesenchymal stem cells (hMSC) can promote superantigen-induced activation of purified T cells, addition of antigen-presenting cells (APC; either monocytes or dendritic cells) to the cultures inhibits the T cell responses. This contact- and dose-dependent inhibition is accompanied by secretion of large quantities of IL-10 and aberrant APC maturation, which can be partially overridden by the addition of factors that promote APC maturation (i.e., LPS or anti-CD40 mAb). Thus, our data support an immunoregulatory mechanism wherein hMSC inhibit T cells indirectly by contact-dependent induction of regulatory APC with T cell suppressive properties. Our data may reveal a physiological phenomenon whereby the development of a distinct APC population is regulated by the tissue cellular microenvironment.
Introduction

Mesenchymal stem cells (MSC) are multipotential non-hematopoietic progenitor cells of the adult marrow capable of differentiating into various lineages of the mesenchyme \(^1\). Although present at a low frequency in adult bone marrow, MSC can be isolated and further replicate as undifferentiated cells \textit{in vitro}. These cells are characterized by the absence of hematopoietic markers (such as CD45\(^-\) and CD34\(^-\)) and the expression of a specific pattern of adhesion molecules (such as CD106\(^+\) and CD105\(^+\)). Under appropriate conditions MSC have the potential to differentiate to chondrocytes, tenocytes, skeletal myocytes, neurons, and cells of visceral mesoderm \(^2\).

There are several provocative links between stem cells and the induction of specific immune tolerance. More than one stem cell type has been associated with immune tolerance induction, including embryonic, hematopoietic and mesenchymal stem cells \(^3-7\). These stem cells have been successfully employed for tolerance induction in a variety of rodent and large animal studies. A previous report \(^8\), suggests that MSC are not only able to evade allogeneic immune system, but they can also suppress immune responses directed against third party cells, even inducing tolerance towards other tissues of the same origin when transplanted following intravenous infusion of MSC. This and other studies have further demonstrated that MSC inhibit T cell activation \textit{ex vivo} \(^8-11\). A recent case report has suggested that systemic infusion of haploidentical mesenchymal stem cells suppressed a grade 4 graft-versus-host-disease (GVHD) in a bone marrow transplanted 9-year-old child \(^12\). Nevertheless, the underlying mechanism for this tolerizing phenomenon, including the involved target cells, is not yet known.

Tolerance induction in the periphery is believed to be critical for the prevention of autoimmunity and maintenance of immune homeostasis. Central tolerance has been classically ascribed to clonal deletion of self-reactive T cells in the thymus upon interaction with self-antigens. However, central tolerance is incomplete, since not all self-antigens gain access to the thymus and several self-reactive lymphocytes escape central deletion. Over the past several years there is growing evidence supporting this notion, revealing
subpopulations of cells representing different arms of the immune system, as potential regulators of the immune system. These specific groups include T cell subtypes (such as CD4+CD25+ T cells), as well as unique fraction of dendritic cells (DC) described as semi-mature DC, all of whom were shown to possess immune modulating properties. Therefore, 'sentinels' in the periphery of the body are essential to maintain tolerance as well as immunity. These tolerogenic effectors, while constitutively active in autoimmunity prevention, may play a pivot role in maternal-fetal non-rejection, as well as in immune evasion of tumors and metastases.

Taken together, these studies on MSC with their potential immunoregulatory activities create a compelling case for further pursuing stem cells as cellular tolerogens in the periphery. Given the uniqueness and potential importance of this observation, in this study we explore the mechanism underlying the immunoregulatory properties of human MSC (hMSC) and their ability to inhibit T cell activation. Our data have uncovered a unique immunoregulatory mechanism wherein human mesenchymal stem cells (hMSC) induce regulatory antigen-presenting cells (APC) that in turn inhibit T cell activation.
Materials and Methods

Cells
Cells were purified from the venous blood of healthy donors. Either, CD4+, CD8+ T cells or CD14+ cells were isolated by negative selection using the RosetteSep™ enrichment cocktail (StemCell Technologies, Vancouver, Canada). hMSC were obtained from discarded bone tissues from patients undergoing total hip replacement surgeries, under approval of Hadassah Medical Center Helsinki Ethics Committee following an informed consent. The hMSC were separated from other bone-marrow residing cells by plastic adherence, and were then grown under tissue culture conditions, as previously described (10,11). The cells were maintained in a low-glucose DMEM medium supplemented with 10% heat-inactivated fetal calf serum, 2mM glutamine, and penicillin/streptomycin (Biological Industries, Beit-Haemek, Israel). For DC generation, CD14+ cells were plated in RPMI 1640 (Biological Industries) containing 1% autologous plasma, 0.1µg/ml IL-4 and 0.1 µg/ml GM-CSF (PeproTech, Rocky Hill, NJ). Every 2 days 0.3 ml were removed and 0.5 ml media containing plasma and cytokines were added. By day 7, >90% of the cells were CD14- and CD11c+. In order to activate DC, LPS (1µg/ml; Sigma, St. Louis, MO) was added to the cells for additional 24h.

Cytokine production
Cultures containing 5x10^4 CD4+ or CD8+ T cells, and the indicated numbers of monocytes or DC in the absence or presence of the superantigen SEB (Sigma), and in the absence or presence of hMSC, were plated in individual wells of flat-bottom 96-well plates (Corning, Corning, NY). Cells were stimulated for 72 h and conditioned media were collected. In some experiments either anti-CD40 (Pharmingen, BD Biosciences, San Jose, CA), anti TGF-β (R&D Systems, Minneapolis, MN) or anti IL-10 receptor (R&D Systems) antibodies were added. IFN-γ, IL-5, TNF-α, TGF-β (R&D Systems), IL-1β, IL-12 and IL-10 (Diaclone, Besancon, France) levels in the conditioned media were assayed by ELISA.
Flow cytometry of cell surface markers
CD4+ T cells were cultured with SEB-loaded monocytes for 72 h in the absence or presence of hMSC. CD86 (Pahrmingen), HLA-DR, CD14 (IQ Products, Groningen, Netherlands) and CD11c (Dako, Glostrup, Denmark) expression was measured by direct immunofluorescence using conjugated antibodies (R&D Systems), and the immunostained cells (1x10^4 cells/sample) were analyzed on a FACS Calibure flow cytometer (Becton Dickinson, San Jose, CA) using the Cell Quest software. The data were calculated as the mean fluorescence intensity.

Proliferation assay
CD4+ T cells and either monocytes or DC or whole PBMC were cultured in flat-bottom 96-well plates (Corning) at 1 x 10^5 cells in 0.2 ml volume per well in triplicate. Cultures were pulsed with [3H]-methyl-thymidine (Amersham-Pharmacia Biotech, Buckinghamshire, England) and was added for the last 18 h. Cells were harvested onto glass-fiber filter paper (Schleicher & Schull, Dassel, Germany), dried, and the incorporated 3H analyzed with liquid scintillation counter (Wallac, Gaithersburg, MD). Data points are expressed as mean cpm of triplicate samples.
Results

Several previous studies have demonstrated that MSC have immunoregulatory effects on T cells. These studies create a compelling case for further pursuing stem cells as cellular tolerogens. Given the uniqueness and potential importance of this observation, in this study we explore the mechanism underlying the immunoregulatory properties of human MSC (hMSC) and their ability to inhibit T cell activation. To this end, hMSC were derived from discarded bone tissues obtained from patients undergoing total hip replacement. The hMSC were separated from other bone-marrow residing cells by plastic adherence, and then were grown under tissue culture conditions as previously described, and tested for their ability to inhibit T cell activation, as measured by cytokine secretion and T cell proliferation.

As described previously, hMSC failed to elicit proliferation or secretion of interferon gamma (IFN-γ) when co-cultured with unmatched peripheral blood mononuclear cells (PBMC; data not shown). Moreover, while significant levels of IFN-γ were detected in the conditioned medium of PBMC stimulated with either phytohemagglutinin (PHA) or the superantigen Staphylococcal Enterotoxin B (SEB), the addition of hMSC significantly decreased the level of IFN-γ secretion (data not shown). This inhibitory activity of hMSC (as determined by the inhibition of IFN-γ or proliferation) directly correlated with the number of stem cells in the culture, reaching up to 95% inhibition of the response (Fig. 1A) and was independent of the concentration of stimulus (PHA; data not shown) or SEB (Fig 1B). hMSC inhibitory activity was retained following γ-irradiation, but not after paraformaldehyde-fixation (data not shown) as previously observed. As was previously reported, there is cell:cell contact dependence of hMSC-mediated inhibition based on the absence of inhibition when hMSC were replaced with their conditioned media (data not shown), or when PBMC and hMSC were on opposite sides of a transwell membrane (Fig. 1C). Interestingly, IFN-γ secretion as well as proliferation could be partially restored in hMSC-containing cultures by adding either LPS or anti-CD40 mAb (Fig. 1D). Given that triggering APC such as monocytes with either LPS or anti-CD40 mAb is known to promote
APC maturation, this finding suggests that hMSC may somehow interfere with normal APC maturation, thereby indirectly attenuating T cell activation.

Monocyte involvement in hMSC-mediated tolerance induction was tested by titrating increasing numbers of monocytes into co-cultures of hMSC and CD4^+ T cells in the presence of SEB. In control co-cultures, CD4^+ T cells were not activated in the absence of monocytes, verifying the purity of the CD4^+ T cell preparations, and addition of purified CD14^+ cells (monocytes) increased IFN-γ secretion in a dose-dependent manner (Fig. 2A, left panel). While considerable levels of IFN-γ secretion were detected when CD4^+ T cells were co-cultured with hMSC in the absence of monocytes, the addition of monocytes to these hMSC-containing co-cultures significantly inhibited IFN-γ secretion in a dose-dependent manner (Fig. 2A, left panel). Both T cells proliferation (Fig 2A, middle panel) and IL-5 secretion (Fig 2A, right panel) exhibited similar monocyte dose-dependent inhibition, as seen earlier with IFN-γ. Similar results were also obtained when CD8^+ T cells were used instead of CD4^+ T cells (Fig 2B). Of note, this suppressive effect was observed in both, autologous or allogeneic monocytes (data not shown). Taken together these data suggest that monocytes play a pivotal role in mediating hMSC inhibitory activity, and that the inhibitory signal from the hMSC directed towards the APC is contact-dependent (as exemplified in repeated transwell experiments where PBMC were replaced by purified CD4^+ T cells and monocytes- data not shown).

These experiments employed monocytes as APC, showing that they can be conditioned by hMSC to inhibit T cells. Given that dendritic cells (DC) are prototypical professional APC, we questioned whether DC can be similarly conditioned by hMSC. To this end, we repeated the experiments described above, using monocyte-derived DC in place of monocytes. Peripheral monocytes were cultured with GM-CSF and IL-4 for 7 days according to a standard protocol, and their subsequent ability to inhibit T cell activation in the presence of hMSC, was tested. Titrating the number of DC added to the hMSC co-cultures inhibited IFN-γ secretion and T cell proliferation in a dose-dependent manner. Thus, DC parallel monocytes in their effects on T cell activation in this hMSC-induced immunoregulatory system. Furthermore, we tested the possibility that this hMSC-elicited inhibitory property of DC was somehow dependent upon a DC maturation state at the time of hMSC contact. DC that had been grown with GM-CSF and IL-4 for 7 days were then
exposed to LPS for one more day, prior to washing and adding them to hMSC co-cultures. Inhibition of T cell activation in these co-cultures was significantly reduced as compared to DC that had not been treated with LPS (data not shown). This LPS priming, reversed the inhibitory phenotype, presumably by overriding hMSC-mediated block of DC maturation.

We next tested the hypothesis that hMSC influence APC maturation, somehow locking them into an immature or semi-mature phenotype that correlates with T cell inhibition. Therefore, we molecularly profiled hMSC-conditioned APC, comparing them to non-conditioned APC as controls, looking at both secreted cytokines for which APCs are the main producers using ELISA, and surface molecules by immunofluorescence and flow cytometry.

To this end, monocytes were co-cultured with CD4+ T cells in the presence or absence of hMSC and cytokine secretion was determined in the conditioned media. While unactivated monocytes secreted low levels of IL-12, TNF-α and IL-1β secretion, these levels were substantially increased upon SEB stimulation. In contrast, stimulation in the presence of hMSC resulted in significantly lower levels of SEB-induced IL-12 and TNF-α; however, there was no effect on IL-1β secretion (Fig 3A). Similar results were obtained when DC were used instead of monocytes (Fig. 3B), except that low levels of IL-1β were detected exclusively when hMSC were present (Fig. 3B, lower panel).

We further looked for the expression of the surface molecule on CD14+ (monocytes; Fig. 3C) and CD11c+ (DC; Fig. 3D) cells following co-culture with hMSC. Both, monocytes and DC expressed relatively low levels of CD86 and HLA-DR, which were increased upon SEB stimulation. HLA-DR upregulation in monocytes was significantly reduced by hMSC (Fig 3C, upper panel, and to a lesser degree in DC (Fig 3D, upper panel). On the other hand, the level of CD86 expression on monocytes and DC was not significantly affected by the presence of hMSC (Fig 3C and D, lower panels). These findings seem to represent a unique developmental stage for APC appearing to be similar to that of immature APC, yet differing from immature APC in their IL-1β and CD86 expression. Thus, this data suggest that hMSC partially block APC maturation leading to an aberrant APC phenotype, with dissociation of otherwise clustered molecular markers. These APC lack T cell activating capacity and possibly produce some inhibitory molecule(s) that can trigger T cell inhibitory events.
It is possible that these semi-mature, suppressive APC act indirectly through the induction or activation of regulatory T cells. Of the several populations of T cells exhibiting suppressive mechanisms, CD4^+CD25^+ are of special interest in our system since they arise spontaneously, and are present in the periphery. To determine whether CD4^+CD25^+ regulatory T cells play a role in hMSC-mediated inhibition, we depleted CD25^+ cells from the purified CD4^+ T cells, before adding them to co-cultures with hMSC, along with graded numbers of monocytes. In accordance with previous report 10, removing the regulatory CD4^+CD25^+ T cells did not prevent the hMSC-mediated inhibition (data not shown).

IL-10 and TGF-β are two anti-inflammatory cytokine candidates that may play a role in this system. First, IL-10 and TGF-β are potent suppressants of pro-inflammatory cytokine secretion by APC (such as IL-12 and TNF-α). Second, IL-10 has been shown to play a role in elicitation of the regulatory DC phenotype and both IL-10 and TGF-β mediate the activity of T regulatory 1 cells that are generated via the regulatory DC 16. We therefore tested whether these cytokines play a role in hMSC-mediated inhibition. First, the levels of TGF-β were determined in the conditioned media of either PBMCs or monocytes that were co-cultured with CD4^+ T cells, both in the presence or absence of hMSC or hMSC cultured alone (Fig. 4A). TGF-β was detected in conditioned media of both hMSC and immune cells. The level of TGF-β in the co-cultures was the expected additive amount of the two cell types, indicating that the interaction of immune cells:hMSC does not result in increased TGF-β secretion (Fig. 4A). Furthermore, the addition of neutralizing Abs (specific for human TGF-β1,2) had no effect on the inhibitory activity of hMSC as determined by IFN-γ (Fig. 4B). These data support the notion that TGF-β does not play a major role in hMSC-mediated inhibitory activity. Next, the possible role of IL-10 in mediating hMSC-induced immunomodulation was examined. Interestingly, while PBMC, monocytes, or both CD4^+ T cells and monocytes (but not DC; data not shown) secreted relatively low levels of IL-10, we detected up to a 10-fold increase in the concentrations of IL-10 in conditioned media of these cells when co-cultured with hMSC, regardless of the presence of SEB. Given that no IL-10 was detected in conditioned media of hMSC cultures (Fig. 4C, right panel), these data suggest that co-culturing hMSC with these immune cells induced the secretion of relatively high levels of IL-10 (Fig. 4C). To test whether IL-10
contributes to hMSC-derived T cell inhibition once APC are added to the system, we determined if addition of neutralizing anti-IL-10 receptor Abs abrogate inhibition and restore T cell responses. Although addition of anti-IL-10 receptor Abs increased IFN-γ and TNF-α secretion as well as the proliferative response, these responses were still lower as compared to control cultures. Thus, the inhibitory effect of hMSC was only partially reverted by blocking IL-10 activity (Fig. 4D).

Notwithstanding that this hMSC-induced IL-10 is not inhibitory on its own (given that hMSC on their own activate T cells, despite the presence of IL-10 in the conditioned media of this co-culture (data not shown) and the moderate effect of the neutralizing anti-IL10 receptor Ab), there remains the possibility that IL-10 works in concert with other immunoinhibitory factor(s) derived from hMSC-conditioned APC.
Discussion

Previous studies have suggested that MSC inhibit T cell activation and can induce immune tolerance. However, the precise mechanism underlying this phenomenon is still unclear. While one study suggested that hMSC interfere with T cell:APC contact, a second study suggested that the hMSC themselves act as veto cells. The present data offer a different view, namely, an indirect inhibitory effect of hMSC mediated by the APC. Accordingly, the primary effect of hMSC is to convert APC into what is effectively a “deletional APC” with active immunoregulatory properties, as opposed to simply being activation incompetent. The critical observation here is that hMSC can efficiently activate highly purified T cells (possibly due to expression of HLA-DR and CD80 by the hMSC) and surprisingly, addition of APC attenuates this activation event. Obviously, our findings differ from those described by Krampera et.al. Using mouse MSC, Krampera et al., have demonstrated contact-dependent inhibition of T cell activation. However, the authors of that study have concluded that MSC hinder T cell from the contact with APC in a noncognate fashion, and that the presence of APC is not required for MSC to inhibit. At this stage, it is not clear why our data departs from the previous report (e.g. perhaps a species human versus mouse difference), but in any case, our results, using human cells, support the hypothesis proposed here, namely that in the presence of hMSC, APC are transformed into regulatory APC.

There is a large and growing literature bearing upon the regulatory APC concept (reviewed in 14,18). Over the past several years, the limited conception of APC, and DC in particular, as positive regulators (i.e., as natural “adjuvants” that promote immune responses to foreign antigens) has been expanded to encompass the notion of APC as negative regulators, with the recognition that APC involved in immune induction are likely to also be involved in the induction of tolerance to self-antigens. Two general mechanisms have been proposed by which DC might maintain peripheral tolerance. The first is that a subtype of specialized regulatory DC maintains peripheral tolerance. The second is that all DC have the ability to induce tolerance, with the capacity to induce tolerance or immunity dependent on the maturation or activation state of the DC. According to this mechanism the functional activities of DC are mainly dependent on their
state of activation and maturation; that is, terminally-differentiated, mature DC can efficiently induce the development of T effector cells, whereas “immature” or “semi-mature” DC maintain peripheral tolerance. Full APC maturation can be triggered by Toll-like receptors (TLRs; e.g. with LPS, CpG oligodexynucleotides) or by ligation of CD40 by CD40L. Given our observation that LPS or anti-CD40 mAb can partially override hMSC-driven T cell inhibition (Fig. 1D), we have tested the possibility that hMSC somehow disrupt APC maturation. The data presented establish that via cell:cell contact, hMSC convert APC into T cell inhibitors (as opposed to APC that are lacking T cell activating capacity) essentially by locking them in a semi-mature or in an aberrant maturation phenotype. This unique developmental stage includes not only low levels of expression of several proinflammatory molecules (such as IL-12, TNF-α and MHC II expression), despite high expression of IL-1β and the costimulatory molecule, CD86, but also the increased expression of the anti-inflammatory cytokine IL-10. Further work will be required to clarify how these phenotypes are associated with each other, and how these intriguing developmental alternations in APC maturation contribute to their capacity to regulate T cell responses. Interestingly, while the regulatory APC literature deals almost exclusively with DC, in this study we demonstrate that like DC, under specific conditions, monocytes can also develop into functionally regulatory APC that actively inhibit T cell activation.

As an aside, the model suggested here whereby hMSC’s immuno-regulatory activity is mediated by the induction of regulatory APC links two previous seemingly unrelated findings. On the one hand, regulatory DC have been shown to protect mice from acute GVHD and leukemia relapse, and on the other hand, hMSC have been successfully used in leukemic patient as a treatment of acute GVHD resulting from bone marrow transplantation. Thus, this model provides a possible mechanism through which hMSC can alleviate GVHD.

This description of the outcome of stem cells interface with immune cells resembles the effect of apoptotic cells on APC activation. Whereas necrotic cells induce an immune response by activating APC, exposure of APC to apoptotic cells results in an APC maturation blockade. In this way, apoptotic material may play a role in the induction of peripheral tolerance to self-antigens derived from apoptotic cells. Specifically, uptake of apoptotic material by APC (macrophages, monocytes and DC) decreases the secretion of
pro-inflammatory cytokine (TNF-α, IL-1β, and IL-12) while increasing the secretion of the anti-inflammatory cytokine, IL-10. Similar to our findings, antigen presentation and consequently the secretion of IFN-γ, which is induced by IL-12 and inhibited by IL-10, was also reduced in the presence of apoptotic cells, and could be partially restored by the addition of neutralizing anti-IL10 antibodies.

What locks APC in a distinct differentiation and functional stage *in situ* has not as yet been determined. The data presented in this study suggest that in at least a certain cellular microenvironment, cell:cell interactions may uncouple APC activation events and drives the differentiation of tolerogenic APC with an intermediate phenotype. Thus, the plasticity of the APC maturation program allows them to modulate their function according to the nature and condition of the tissue, which in turn shapes T cell response, giving rise to distinct functional outputs such as effector, memory T cell generation or T cell tolerance. As suggested, we favor the hypothesis that APC induce and maintain tolerance in the periphery by mechanisms that uncouple APC activation events and involve cell:cell interaction within the tissue micro-environment. This may provide an explanation for other examples whereby in certain *in vivo* milieus, APC maturation is altered acquiring a tolerogenic function. For example, Suter T. et al, have demonstrated that DC isolated from mice with experimental autoimmune encephalomyelitis (EAE) exhibit a maturational phenotype similar to that of immature bone DC. In particular, these central nervous system (CNS)-DC are unable to prime naïve T cells and inhibit T cell priming by bone marrow-derived DC. TGF-β, IL-10 and TRAIL were found to significantly contribute to the CNS-DC-mediated inhibition of T cell proliferation. Thus, under the conditions of the privileged site of the brain, a special DC subset exists with an immature phenotype that secretes IL-10 and TGF-β. Interestingly, reminiscent of our LPS data, infectious diseases of the CNS, overrule the standard immunosuppressive program of CNS-DC, and under this conditions CNS-DC function as potent APC. Other examples include tumors that have been shown to promote altered maturation of DC, possibly representing a mechanism for tumors to evade immune detection, and pathogens shown to evade the immune responses by inducing IL-10 production by APC (DC and macrophages). Our experimental system may offer a unique setting to study how the development of a distinct APC population is regulated and to look for hMSC surface ligand(s) and their corresponding receptor(s) on...
the APC that are linked to induction of the regulatory APC phenotype.
References


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Figure Legends

Figure 1. Characterization of hMSC-induced inhibition of IFN-γ secretion.
A; hMSC inhibit the response of SEB stimulated T cells in a dose dependent fashion. PBMC were stimulated with SEB (1ng/ml) in the absence or presence of increasing numbers of hMSC. The levels of IFN-γ in the culture media were determined by ELISA (left panel). Proliferation was determined by [³H]-thymidine incorporation (right panel). The data represent the mean values of triplicate samples and standard deviations.
B; The inhibitory activity of hMSC do not correlate with SEB concentration. PBMC were stimulated with various concentrations of SEB in the absence or presence of hMSC (5x10⁴ cells). IFN-γ secretion was determined in the conditioned medium. C; Inhibition of IFN-γ secretion by hMSC requires cell contact. hMSC and PBMC were co-cultured together or on opposite sides of a transwell in the presence of SEB (1 ng/ml) for 72h. IFN-γ secretion was measured in the conditioned media. D; Proliferation and IFN-γ inhibition by hMSC is partially restored by adding LPS or anti CD40 monoclonal antibodies. PBMC were co-cultured with hMSC (5x10⁴ cells) and SEB (1ng/ml). Either LPS (1µg/ml; left and right panels) or anti-CD40 mAb (1µg/ml; middle panel) were added and either IFN-γ secretion or incorporation of [³H]-thymidine was determined, after 72h. The data in B-D were calculated as the percent inhibition of IFN-γ secretion in the presence of hMSC as compared to PBMC stimulated in the absence of hMSC. The results shown are representative of three separate experiments.

Figure 2. hMSC convert activating APC into T cell inhibitors
Either CD4⁺ T cells (A and C) or CD8⁺ T cells (B) were stimulated with SEB (1ng/ml) in the absence (open symbol) or presence (closed symbol) of hMSC (5x10⁴ cells) and increasing numbers of either monocytes (A-B) or DC (C). T cell activation was followed by either IFN-γ secretion, incorporation of [³H]-thymidine or IL-5 secretion to the media. Each panel shows one representative experiment out of three.

Figure 3. hMSC-conditioned APC exhibit a particular pattern of activation markers.
Purified CD4⁺ T cells were stimulated with SEB (1ng/ml) in the presence of either monocytes (A and C) or DC (B and D) in the absence (-) or presence (+) of hMSC (5x10⁴ cells of each cell type were plated). After 72h, conditioned media were collected and the levels of the indicated cytokines in the media were determined using the appropriate ELISA kit (A-B). The data represent the mean values of triplicate samples and standard deviations. A representative of four separate experiments is shown. The cells were collected and the levels of CD86 and HLA-DR surface expression either on CD14⁺ cells (C) or CD11c⁺ cells (D) were determined by immunofluorescence and flow cytometry. Data are presented as mean fluorescent intensity of CD86 and HLA-DR staining. One of three experiments is shown. *= P<0.001, based on t test.

Figure 4. The inhibitory effect of hMSC is partially mediated by IL-10 but not by TGF-β.

A; CD4⁺ T cells, monocytes and hMSC were cultured alone (black bars) or in combination (grey bars), in the presence or absence of SEB, as indicated. The levels of TGF-β in the conditioned media were assayed by ELISA, after 72h. The data represent the mean of triplicate samples and standard deviations. B; PBMC were stimulated with SEB in the absence or presence of hMSC (5x10⁴ cells of each cell type). Neutralizing anti-TGF-β₁₋₂ Ab (1µg/ml) were added and IFN-γ secretion was measured in the conditioned media, after 72h. The data are presented as percentage of inhibition of IFN-γ secretion in the presence of hMSC as compared to cells stimulated in the absence of hMSC. The data in A and B are from one experiment; similar results were obtained in two other experiments.

C; Co-culturing hMSC with monocytes induce IL-10 secretion. PBMC were either left untreated or were stimulated with SEB (1ng/ml) in the absence (black bars) or presence (grey bars) of hMSC (left panel). Either monocytes or monocytes and CD4⁺ T cells that were either untreated or stimulated with SEB (1 ng/ml), were cultured alone (black bars) or in the presence (grey bars) of hMSC (5x10⁴ cells per well; right panel). After 72h, the level IL-10 in the various conditioned media was determined by ELISA. No IL-10 was detected in conditioned media of hMSC cultured alone (right panel). The data represent the mean values of triplicate samples and standard deviations. D; Purified CD4+ T cells were stimulated with SEB in the presence of monocytes and in the absence or presence of
hMSC (5x10^4 cells of each cell type were plated). Neutralizing anti-IL-10 receptor Ab (1µg/ml) were added and either proliferation (right panel) or IFN-γ (left panel) and TNF-α (middle panel) secretion was measured in the conditioned media, after 72h. Similar results were obtained when whole PBMC were used (data not shown). The data are presented as percentage of inhibition of proliferation and cytokines secretion in the presence of hMSC as compared to cells stimulated in the absence of hMSC. The results shown in C and D are representative of three separate experiments.
Figure 1

A

B

C

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Figure 2

A

IFN-γ secretion (ng/ml)

[3H] Thymidine incorporation (cpm)

IL-5 secretion (pg/ml)

monocyte number (x10^3)

B

IFN-γ secretion (ng/ml)

monocyte number (x10^3)

C

IFN-γ secretion (ng/ml)

[3H] Thymidine incorporation (cpm)

DC number (x10^3)
Figure 3

A

MSC

TNF-α

IL-12

IL-1β

B

MSC

TNF-α

IL-12

IL-1β

C

MSC

SEB

CD86

HLA-DR

D

MSC

SEB

CD86

HLA-DR

*
Figure 4

A

T cells
monocytes
hMSC
monocytes + T cells
monocytes + T cells + SEB

TGF-β secretion (pg/ml)

0 250 500 750 1000 1250

% inhibition of thymidine incorporation

None -IL10R

B

% inhibition of TNF-α secretion

None -IL-10R

C

PBMC
PBMC + SEB

IL-10 secretion (pg/ml)

0 500 1000 1500 2000 2500

% inhibition of IFN-γ secretion

hMSC

Monocytes
Monocytes + T cells
Monocytes + T cells + SEB

IL-10 secretion (pg/ml)

0 1000 2000 3000 4000

D

% inhibition of IFN-γ secretion

% inhibition of TNF-α secretion

% inhibition of thymidine incorporation

None -IL-10R
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