Human mesenchymal stem cells modulate allogeneic immune cell responses

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Mesenchymal stem cells (MSCs) are multipotent cells found in several adult tissues. Transplanted allogeneic MSCs can be detected in recipients at extended time points, indicating a lack of immune recognition and clearance. As well, a role for bone marrow–derived MSCs in reducing the incidence and severity of graft-versus-host disease (GVHD) during allogeneic transplantation has recently been reported; however, the mechanisms remain to be investigated. We examined the immunomodulatory functions of human MSCs (hMSCs) by coculturing them with purified subpopulations of immune cells and report here that hMSCs altered the cytokine secretion profile of dendritic cells (DCs), naive and effector T cells (T helper 1 [T_H1] and T_H2), and natural killer (NK) cells to induce a more anti-inflammatory or tolerant phenotype. Specifically, the hMSCs caused mature DCs type 1 (DC1) to decrease tumor necrosis factor α (TNF-α) secretion and mature DC2 to increase interleukin-10 (IL-10) secretion; hMSCs caused T_H1 cells to decrease interferon γ (IFN-γ) and caused the T_H2 cells to increase secretion of IL-4; hMSCs caused an increase in the proportion of regulatory T cells (T_{Regs}) present; and hMSCs decreased secretion of IFN-γ from the NK cells. Mechanistically, the hMSCs produced elevated prostaglandin E2 (PGE_2) in co-cultures, and inhibitors of PGE_2 production mitigated hMSC-mediated immune modulation. These data offer insight into the interactions between allogeneic MSCs and immune cells and provide mechanisms likely involved with the in vivo MSC-mediated induction of tolerance that could be therapeutic for reduction of GVHD, rejection, and modulation of inflammation. (Blood. 2005;105:1815-1822)

Introduction

The isolation of stem cell populations has burgeoned in the last 10 years, opening many new opportunities to evaluate the stem cells and their use in tissue regeneration. Hematopoietic stem cells (HSCs) were identified after a long search for cells that would allow survival following radiation exposure. During this period, several studies demonstrated that bone marrow would form new bone when transplanted to an ectopic site.[1] The isolation and culture of cells from bone marrow that could form this ectopic bone were first demonstrated by Friedenstein et al[2] using the guinea pig as a model. Later, several groups published similar data for rat and rabbit cells harvested from bone marrow and other tissues.[2-4] Similar to the hematopoietic stem cell and its lineages, the concept emerged that there may be a mesenchymal stem cell (MSC), a single cell capable of forming bone, cartilage, and other mesenchymal tissues.[3,4] Haynesworth et al[5] developed a reliable in vivo bone-forming assay and were able to isolate and culture human MSCs in therapeutic quantities.

Subsequently, in vitro experiments demonstrated that clonal human MSCs are able to differentiate into various lineages including osteoblasts, chondrocytes, and adipocytes.[6,7] In vitro and in vivo studies have also indicated the capability of MSCs to differentiate into muscle,[8] neural precursors,[9,10] cardiomyocytes,[11-13] and possibly other cell types.[14,15] In addition, MSCs have been shown to provide cytokine and growth factor support for expansion of hematopoietic and embryonic stem cells.[16-19] Numerous studies with a variety of animal models have shown that MSCs may be useful in the repair or regeneration of myocardial tissues,[13,20,21] damaged bone,[22-25] tendon,[26] cartilage,[27] and meniscus.[28] Perhaps one of the most remarkable and least understood findings is the ability of MSCs to migrate to sites of tissue injury.[13,29-31] Several clinical studies using autologous whole bone marrow, presumably containing MSCs, HSCs, and/or endothelial progenitor cells have also been reported for patients with myocardial infarcts.[32-34] Importantly, encouraging results have been reported for ex vivo–cultured MSCs in early clinical use including engraftment of autologous[35,36] or allogeneic[37] (also Lazarus et al, manuscript submitted, August 2004) bone marrow transplants, allogeneic MSCs for the collagen I genetic disease osteogenesis imperfecta,[38] and recently for the treatment of graft-versus-host disease (GVHD).[39]

Human MSCs (hMSCs) can be isolated from several, perhaps most, tissues, although bone marrow is most often used. Human MSCs express intermediate levels of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I, and they can be induced to express MHC class II antigen[40-43] and Fas ligand by interferon γ (IFN-γ) treatment. MSCs do not express costimulatory molecules B7-1, B7-2, CD40, and CD40 ligand and probably, therefore, do not activate alloreactive T cells.[41,43,44] In addition, MSCs differentiated into various mesenchymal lineages do not appear to alter their interaction with T cells.[45,46] MSCs isolated from humans and other mammalian species including baboon, canine, caprine, and rodents do not elicit a proliferative response

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S.A. and M.F.P. are currently employed at Osiris Therapeutics, Inc, which is developing cellular therapeutics based on human mesenchymal stem cells.

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from allogeneic lymphocytes.[24,47-50] In fact, MSCs suppress proliferation of allogenic T cells in an MHC-independent manner.[40,41,44] Few studies have investigated the interaction of MSCs with specific immune cell subtypes.[51-53]

In order to understand the mechanisms involved in the observed MSC-mediated immunosuppressive action, we used a step-wise approach in which we isolated various immune cell populations, cocultured them with hMSCs, and then examined the alterations in cytokine secretion profile by these cells. We observed that each interaction resulted in altered phenotypic expression of factors by the immune cells. Furthermore, as most of the observed MSC-mediated T-cell suppressive activity in vitro has been attributed to soluble mediators, we evaluated several factors and identified the role of one of the factors, prostaglandin E2 (PGE_2), produced by MSCs in their immunomodulatory activity.

Materials and methods

Materials

Recombinant cytokines and antibodies against cytokines or CD antigens were from BD Biosciences, San Diego, CA, unless noted otherwise. The enzyme-linked immunosorbent assay (ELISA) kits to detect the cytokines interleukin-6 (IL-6), IL-8, vascular endothelial growth factor (VEGF), or PGE_2 were purchased from R&D Systems, Minneapolis, MN.

Methods

Culture of the human MSCs. Bone marrow aspirates drawn from the iliac crest of donors were obtained from Poietics Technologies (Gaithersburg, MD) following informed consent. Human MSCs were isolated using previously described methods.[6,54] Briefly, the bone marrow aspirate (10 mL) was combined with Dulbecco phosphate-buffered saline (DPBS; 40 mL) and centrifuged at 900*g* for 10 minutes at 20°C. The cells were then resuspended and gently layered onto a Percoll cushion (density, 1.073 g/mL; Invitrogen, Carlsbad, CA) at 1 to 3×10^8 nucleated cells/25 mL. The low-density hMSC-enriched mononuclear fraction was collected, washed with 25 mL DPBS, and centrifuged to collect the cells. Cells were resuspended in hMSC complete culture medium (Dulbecco modified Eagle medium with low glucose [Invitrogen], 10% fetal bovine serum [FBS] from preselected lots [JRH BioSciences, Lenexa, KS], antibiotic/antimycotic [Invitrogen], and glutamax [Invitrogen]) and plated at 3×10^7 cells/185 cm^2. The cultures were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 and subcultured prior to confluency. The hMSCs expanded in culture showed positive surface staining for CD73 (SH-3), CD105 (SH-2), and CD44, but lacked CD14, CD34, and CD45 surface expression. The hMSCs were routinely frozen in medium containing 10% dimethyl sulfoxide (DMSO) in 90% FBS. The hMSCs retained the capacity to differentiate into adipogenic, osteogenic, and chondrogenic lineages (data not shown).

For in vitro experiments, frozen aliquots of hMSCs were thawed and cultured in complete medium containing DMEM, 10% selected FBS, and 1% antibiotics (Invitrogen). Human MSCs grew as adherent cells and were detached by incubation with trypsin (0.05% trypsin at 37°C for 3 minutes). The donor population used in these experiments consisted of 9 donors designated hMSC nos. 57, 59, 68, 71, 75, 101, 124, 151, and 218.

Isolation of the immune cell populations. Human peripheral blood mononuclear cells (hPBMCs) were prepared from leucopheresis packs (Cambrex, East Rutherford, NJ) by centrifugation on a Ficoll Hypaque density gradient. Aliquots of the isolated hPBMCs were frozen and stored at −140°C until further use. For in vitro experiments, frozen aliquots of the hPBMCs were randomly chosen from the 5 unrelated donors, thawed, and used.

Dendritic cells type 1 (DC1)

Precursors of DC1 belong to the myeloid lineage of leukocytes and are $CD1c^+$. These cells were selected from hPBMCs using a 2-step magnetic isolation method according to Dzionek et al.[55] First, the CD1c-expressing B cells were depleted using CD19-labeled magnetic beads. Secondly, the B-cell–depleted flow-through fraction was labeled with biotin-labeled CD1c antibody (BDCA-1^+; Miltenyi Biotech, Auburn, CA) and selected by the antibiotin antibody-labeled micromagnetic beads. These labeled cells were separated from the unlabeled cell fraction using magnetic columns according to the manufacturer's instructions (Miltenyi Biotech). The isolated cell population was stained with the phycoerythrin (PE)–antibiotin BDCA-1^+ antibody and was always more than 95% positive. For maturation of the DC1 population, recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF, 1×10^3 IU/mL) and recombinant human IL-4 (rhIL-4, 1×10^3 IU/mL) were added at the initiation of a 2-day culture.[56]

Dendritic cells type 2 (DC2)

Precursors of DC2 belong to the plasmacytoid lineage of leukocytes and express BDCA-4 antigen on their cell surface. These cells were isolated directly from hPBMCs by immunomagnetic selection of BDCA-4^+ cells (anti–BDCA-4; Miltenyi Biotech). The isolated cell population was stained with PE–anti–BDCA-2 (another antigen coexpressed on BDCA-4^+ cells; Miltenyi Biotech) and was always more than 80% positive. The DC2 population was matured by adding recombinant IL-3 (10 ng/mL) at the initiation of a 2-day culture.

Naive T cells

For the isolation of naive T cells, the hPBMCs were magnetically labeled with CD45RA microbeads (Miltenyi Biotech) and then loaded onto the column in a magnetic field according to the manufacturer's instructions. The magnetically labeled CD45RA$^+$ cells were retained on the column and were eluted as the positively selected cell fraction. An aliquot of the isolated cell population was labeled with the fluorescein isothiocyanate (FITC)–CD45RA antibody and was always more than 95% positive.

Natural killer (NK) cells

Natural killer cells were isolated by immunodepletion of the non-NK cells. First, hPBMCs were magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies (anti-CD3, -CD4, -CD14, -CD15, -CD19, -CD36, -CD123, and 235a [glycophorin A]) and magnetic antibiotin microbeads. Next, the labeled non-NK cells were retained in the magnetic field, while the NK cells passed through. A small aliquot of the lineage-negative flow-through population was stained with PE-conjugated CD56 antibody and was always more than 95% $CD56^+$.

MSC–immune cell coculture

MSCs–dendritic cells. Human MSCs and the DC1 population isolated from unrelated donors were cocultured (hMSC to DC1 ratio, 1:10) in the presence of GM-CSF + IL-4. After 2 days, inflammatory bacterial lipopolysaccharide (LPS, 1 ng/mL) was added and the culture supernatant was then analyzed for tumor necrosis factor α (TNF-α) levels after 16 hours. The experiment was performed with 5 MSC and 5 unrelated DC1 donor pairs.

Human MSCs and the DC2 populations isolated from unrelated donors were cocultured (hMSC to DC2 ratio, 1:1) in the presence of IL-3 for 2 days, following which LPS (1 ng/mL) was added and the levels of IL-10 were then examined after 16 hours. The hMSC to DC2 ratio used was 1:1 due to an inability to obtain sufficient DC2 from hPBMCs. The experiment was performed with 5 unrelated hMSC-DC2 donor pairs.

MSCs–T cells. Human MSCs were plated into 12-well plates containing T cells (CD45RA$^+$; 2×10^5 cells/mL) from an unrelated donor (hMSC to T-cell ratio, 1:10) in the presence of T helper 1 (T_H1)–inducing conditions (anti-CD3 [5 μg/mL], anti-CD28 [1 μg/mL], recombinant human IL-2 [rhIL-2, 4 ng/mL], rhIL-12 [1 μg/mL], and anti–IL-4 [1 μg/mL]) or T_H2-inducing conditions (anti-CD3 [5 μg/mL], anti-CD28 [1 μg/mL], rhIL-2 [4 ng/mL], rhIL-4 [1 μg/mL], and anti–IFN-γ [1 μg/mL]). After 48 hours, the nonadherent cells were harvested, washed extensively, and stimulated with phytohemagglutinin (PHA, 2.5 μg/mL) for another 16 hours, and the levels of IFN-γ for T_H1 and IL-4 for T_H2 cocultures were determined. The experiments were performed with 3 hMSC and 3 unrelated

hPBMC donor pairs. Data were expressed as percent change (mean ± SD) in IFN-γ or IL-4 in cultures incubated with or without hMSCs.

For the regulatory T cells (T_{Regs}), frozen aliquots of hMSCs were thawed and expanded using standard culture conditions and used within passage 6. The hMSCs were harvested, counted, and added into wells (2×10^4 cells/mL) containing hPBMCs from an unrelated donor (hMSC to PBMC ratio, 1:10) in the presence of rhIL-2 (300 U/mL). After 5 days of coculture, nonadherent T cells were harvested and evaluated for the proportion of T_{Regs} present by flow cytometry using FITC-CD4 and PE-CD25 antibodies.[57,58]

MSCs–NK cells. Human MSCs were incubated with isolated NK cells (hMSC to NK cell ratio, 1:1) from an unrelated hPBMC donor in the presence of rhIL-2 (300 U/mL). Levels of IFN-γ were measured in coculture supernatants collected at 24 hours. The experiment was performed with 3 unrelated hMSC and hPBMC donor combinations.

Proliferation assay

Human MSCs were plated in triplicates onto 96-well plates at 2×10^4 cells/mL in 100 μL complete media and were allowed to adhere to the plate for 1 to 2 hours. Human PBMCs, resuspended at 2×10^5 cells/mL were added to wells (in 100 μL volume) containing or lacking hMSCs in the presence of the mitogen PHA (2.5 μg/mL). The hMSC to hPBMC ratio was 1:10. Cocultures without PHA were used as controls. The culture was continued and ^{3}H-thymidine (1 μCi [0.037 MBq]) was added 4 hours before the end of the 72-hour culture. The cells were harvested and counted using a 1450 Microbeta TriLux apparatus (Perkin Elmer, Boston, MA). The T-cell proliferation was represented as the incorporated radioactivity in counts per minute (cpm). In selected experiments, the data were expressed as percent change due to the variability in the baseline proliferative response to PHA by hPBMCs from different donors. Percent change is defined as follows: % change = [{(mean cpm of triplicate wells of hPBMC + PHA) − (mean cpm of triplicate wells of hPBMC + hMSC + PHA)}/(mean cpm of triplicate wells of hPBMC + PHA)] × 100.

PGE_2 determination

The levels for PGE_2 in the cell-culture supernatant were determined using ELISA. For the PGE_2 inhibition experiments, the human MSCs were resuspended in complete media in the presence or absence of PGE_2 inhibitors NS-398 (5 μM; Cayman Chemicals, Ann Arbor, MI) or indomethacin (5 μM; ICN Chemicals, Irvine, CA). These concentrations of PGE_2 inhibitors resulted in complete inhibition of PGE_2 secretion (< 1-2 pg/mL) and were chosen after performing a dose analysis (data not shown), and all cells remained healthy at these concentrations. In order to examine if PGE_2 inhibitors had any effect on T-cell proliferation, the hMSCs were allowed to adhere to the plate surface of a 96-well flat-bottom plate for 1 to 2 hours, and hPBMCs from an unrelated donor (hMSC to PBMC ratio, 1:10), also resuspended in PGE_2 inhibitor–containing culture media, were added in the presence of the mitogen PHA (2.5 μg/mL). Following 3 days of coculture, ^{3}H-thymidine was added during the last 4 hours, and the PBMCs were harvested and ^{3}H-thymidine was counted using a 1450 Microbeta TriLux apparatus. The experiment was performed with 3 unrelated hMSC-hPBMC donor pairs.

In parallel experiments, the hMSCs were resuspended in complete media containing or lacking the PGE_2 synthesis inhibitor NS-398 and were plated in a 12-well plate (2×10^4 cells/mL). Isolated DC1 or $CD45RA^+$ T cells from an unrelated donor were resuspended in media containing a PGE_2 inhibitor and added to the hMSC cultures under TNF-α–producing (LPS; 1 ng/mL) or IFN-γ–producing (PHA; 5 μg/mL) conditions. Following a 24-hour incubation, the levels of TNF-α from DC/hMSC cocultures and levels of IFN-γ from T-cell/hMSC cocultures were measured and calculated as the percent change in cytokine levels between cultures with or without PGE_2 inhibitor NS-398. The DCs or T cells treated with NS-398, without the added hMSCs, were used as controls.

Statistical analysis

Data were presented as the mean ± the standard deviation from 3 or more experiments. The statistical significance was assessed by 2-tailed Student *t* test.

Results

MSC–immune cell interaction

Previous reports have demonstrated that MSCs are able to suppress an ongoing immune response by inhibiting the T-cell proliferation stimulated in a mixed-lymphocyte culture or by incubation with mitogens.[40-44] Therefore, we first examined the hMSC-mediated immunosuppressive effect of the hMSCs. All of the studies were performed using HLA-unmatched donor populations of hMSCs and hPBMCs. Human MSCs from 5 donors were cocultured with hPBMCs isolated from 3 unrelated donors. Results from these experiments are summarized in Table 1. In all the experiments, the presence of hMSCs with hPBMCs resulted in a statistically significant ($P < .0001$) decrease in PHA-induced proliferation. Although the PHA-induced proliferation rate was different for each donor hPBMC, the inhibition in the presence of hMSCs was 50% to 60%.

MSC-DC interaction. Purified populations of the DC1 were cocultured with hMSCs as described in "Materials and methods." For DC1/hMSC cocultures, there was a significant ($P < .0001$) decrease in TNF-α levels secreted. The absolute levels of LPS-induced TNF-α differed for each donor DC1, however, the average decrease in the cytokine levels in the presence of hMSCs from 5 independent experiments was 50 ± 5% (Figure 1A). Treatment of the hMSCs alone with GM-CSF, IL-4, or LPS did not result in any detectable TNF-α production. As DC1 were matured using rIL-4, which can interfere with IL-10 determination, levels of IL-10 secreted were not measured following DC1 activation. A similar degree of hMSC-mediated TNF-α inhibition was seen within 16 hours when the hMSCs were cocultured with LPS-activated monocytes (data not shown).

Table 1. Inhibition of PHA-stimulated PBMC proliferation by human MSCs

No. MSCs	PBMC no. 1001		PBMC no. 1002		PBMC no. 11-30338	
	PBMCs + PHA	PBMCs + PHA + MSCs	PBMCs + PHA	PBMCs + PHA + MSCs	PBMCs + PHA	PBMCs + PHA + MSCs
51	25 044 ± 2849	9 717 ± 1379	12 188 ± 2105	5564 ± 594	34 799 ± 3312	10 474 ± 3954
57	25 044 ± 2849	12 727 ± 958	12 188 ± 2105	5434 ± 4039	34 799 ± 3312	15 159 ± 3359
78	25 044 ± 2849	11 236 ± 1250	12 188 ± 2105	7458 ± 973	34 799 ± 3312	11 649 ± 2319
101	25 044 ± 2849	12 070 ± 2207	12 188 ± 2105	6841 ± 3334	34 799 ± 3312	10 556 ± 4461
151	25 044 ± 2849	8 519 ± 1359	12 188 ± 2105	4172 ± 582	34 799 ± 3312	9521 ± 597

Human MSCs from 5 different donors were plated onto a 96-well plate (2×10^4 cells/mL) in a 100-μL volume. Following adherence of MSCs, PBMCs from an unrelated donor were added in the presence of PHA (hMSC to PBMC ratio, 1:10). ^{3}H-thymidine incorporation was measured at 72 hours of coculture (set in triplicates) and data were expressed as mean cpm ± SD. The experiment was performed with 2 additional hPBMC donors. The cpm for MSC alone or hPBMC alone was less than 1500 in all experiments.

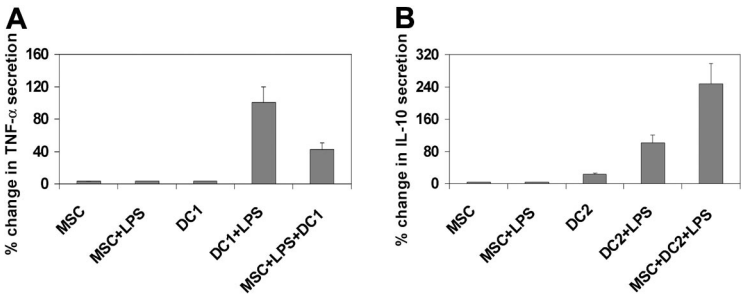

Figure 1. MSCs alter cytokine secretion from DC1 and DC2. Human MSCs were cocultured with (A) mature monocytic dendritic cells (DC1) or (B) mature plasmacytoid dendritic cells (DC2) in the presence or absence of inflammatory stimulus LPS (1 ng/mL). When hMSCs were present, there was a more than 50% decrease in the secretion of TNF-α by activated DC1 (TNF-α levels secreted by LPS-treated DC1 = 100%). The coculture of hMSCs with DC2 consistently increased the secretion of IL-10 by more than 100% (IL-10 levels secreted by LPS-treated DC1 = 100%). The graphs represent the cytokine levels (mean percent change ± SD) from 5 independent experiments in the presence or absence of hMSCs.

The activated DC2 secreted moderate levels of IL-10 in culture upon LPS stimulation. Figure 1B shows the percent increase in IL-10 levels from 5 experiments when hMSCs were cocultured with the activated DC2. The average increase in the IL-10 levels (mean ± SD) in the presence of hMSCs was 140 ± 15% and was statistically significant ($P < .001$). The treatment of the hMSCs alone with IL-3 or LPS did not result in a significant increase in IL-10 production. Changes in the TNF-α levels were not measured in the activated DC2 because the preparation may contain approximately 10% $DC1^+$, and therefore, may result in false positives upon LPS stimulation.

MSC–T-cell interaction. Naive T cells were activated to differentiate into T_H1 effector cells in the presence or absence of hMSCs. The effector T cells undergoing T_H1 differentiation secreted moderate levels of IFN-γ. However, when the hMSCs were present during the T-cell differentiation, there was a significant ($P < .0001$) decrease in the amount of IFN-γ produced. As the baseline levels of IFN-γ produced by each donor's cells differed, the data were expressed as the percent change (mean ± SD) in IFN-γ in cultures with or without hMSCs. The results in Figure 2A show that the decrease in IFN-γ levels in the presence of hMSCs from 3 independent experiments was 60 ± 5%.

Naive T cells were also activated to differentiate into T_H2 effector cells in the presence or absence of hMSCs. The effector T cells undergoing T_H2 differentiation secreted moderate levels of IL-4. However, when hMSCs were present during the differentiation, there was a significant ($P < .0001$) increase in the amount of IL-4 produced. As the baseline levels of IL-4 produced by each donor differed, the data were expressed as the percent change (mean ± SD) in secreted IL-4 in cultures with or without hMSCs. Results (Figure 2B) showed that the average increase in IL-4 levels in the presence of hMSCs from 3 independent experiments was 500 ± 45%.

Figure 2C shows the paired comparison of the percentage of $CD4^+CD25^+$ regulatory T cells detected from 5 different experiments in the presence or absence of hMSCs. There was a significant ($P < .0001$) increase in the T_{Reg} population when the PBMCs were cocultured with hMSCs (also see Supplemental Figures, available on the *Blood* website; click on the Supplemental Figures link at the top of the online article). Figure 2D shows a representative flow cytometry scatter plot from 1 experiment.

MSC–NK cell interaction. Purified NK cells in culture will secrete IFN-γ when stimulated with IL-2. The results in Figure 3 show that when hMSCs were cocultured with IL-2–stimulated NK cells, there was a statistically significant ($P < .0001$) decrease in IFN-γ levels produced. The amount of IFN-γ produced by NK cells upon IL-2 stimulation differed for each donor, however, the average decrease in IFN-γ secretion in the presence of hMSCs was 80 ± 10%. The presence of IL-2 did not result in any detectable IFN-γ secretion from hMSCs alone.

PGE_2 as MSC-derived immune modulator

Previous reports from our colleagues have indicated that MSCs can modify T-cell functions by soluble factor(s).[41,44,73] Therefore, we attempted to identify molecules involved in the hMSC modulation of immune cell activities. We observed that hMSCs in culture secrete several factors including IL-6, IL-8, PGE_2, and vascular endothelial growth factor (VEGF). When PBMCs from unrelated donors were added to hMSCs (hMSC to PBMC ratio, 1:10), the levels of each factor significantly ($P < .001$) increased several fold (Figure 4A). The experiments were repeated with 2 or more independent hMSC/PBMC donor pairs and the data were presented as the measured mean cytokine levels (pg/mL ± SD). This enhancement of PGE_2 production was reproducible also when the hMSCs were incubated with the proinflammatory recombinant cytokines TNF-α or IFN-γ as shown in Figure 4B. The contributions of each cell type to the increase in secreted PGE_2 and VEGF are under investigation.

As PGE_2 has been shown to modulate a wide variety of immune functions in vitro,[59] we examined whether inhibiting production of PGE_2 leads to reversal of any of the hMSC-mediated immunomodulatory effects. The data depicted in Figure 5 demonstrate that, in the presence of PGE_2 synthesis inhibitors, there was a more than 70%

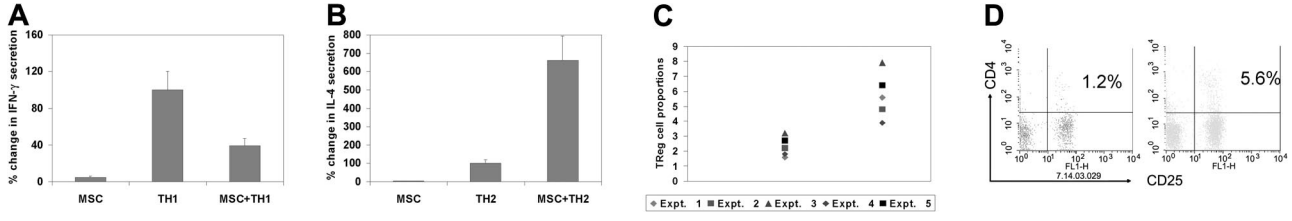

Figure 2. MSCs interact with T cells and induce a T_H1 to T_H2 shift. Human MSCs were cocultured with hPBMCs from unrelated donors under (A) T_H1-, (B) T_H2-, or (C) T_{Reg}-inducing conditions as described. In the presence of hMSCs, there was a more than 50% decrease in IFN-γ secreted from T_H1 cells compared with controls without hMSCs present (IFN-γ levels secreted by T_H1 cells alone = 100%). For T_H2 cultures, there was a more than 500% increase in IL-4 produced when hMSCs were present (control T_H2 cells = 100%). Bars indicate the change in cytokine levels (mean % change ± SD) in cultures incubated with or without hMSCs from 3 independent experiments. For T_{Reg} cultures, the proportion of $CD4^+CD25^+$ cells from 5 experiments is plotted. Results indicated that when hMSCs were present, there was a consistent increase in the proportion of $CD4^+CD25^+$ T_{Regs}. (D) A representative flow diagram from one experiment in which T_{Regs} were generated with or without hMSCs is shown.

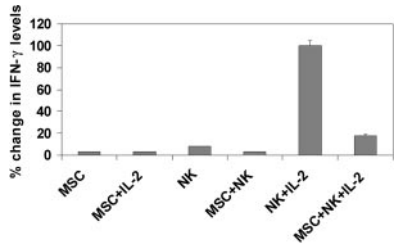

Figure 3. MSCs inhibit IFN-γ secretion from purified NK cells. Natural killer cells were purified and cocultured with hMSCs for different times in the presence of rhIL-2. The levels of IFN-γ in the supernatant were quantified in the presence or absence of hMSCs after 24 hours of coculture. The bars show the percent change in IFN-γ secreted compared with IL-2–stimulated NK cells (= 100%). When hMSCs were present, there was a more than 80% decrease in levels of IFN-γ secreted by IL-2–stimulated NK cells. Results from 3 independent hMSC/NK experiments are plotted. Error bars represent mean ± SD.

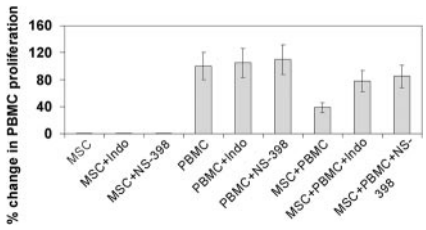

Figure 5. MSCs' immunomodulatory effects are mediated in vitro via secretion of PGE_2. Human MSCs and PBMCs from unrelated donors were cocultured in media containing PGE_2 inhibitors indomethacin (Indo) or NS-398 for 3 days in the presence of PHA (2.5 μg/mL). ^{3}H-thymidine incorporation was measured as an indication of PBMC proliferation and data were presented as percent change in incorporated ^{3}H-thymidine in presence or absence of inhibitors (hPBMCs stimulated with PHA in absence of MSCs = 100%). Bars represent percent change in ^{3}H-thymidine (mean ± SD) from 3 independent experiments.

increase in ^{3}H-thymidine incorporation and the difference was statistically significant ($P < .001$). These stimulated levels were similar to the levels achieved when hMSCs were not present, suggesting that PGE_2 synthesis inhibitors negated the hMSC-mediated immunosuppressive effects on T cells. Furthermore, in the presence of a PGE_2 inhibitor, there was a statistically significant increase in TNF-α and IFN-γ from the activated DCs and T cells, respectively ($P < .001$ and $P < .005$, respectively), indicating that hMSC-secreted PGE_2 plays an important role in hMSC-mediated immunomodulatory effects. The results from 3 independent experiments are summarized in Table 2.

Discussion

In this paper, we have examined interactions between culture-expanded hMSCs and different immune cells in order to better understand the mechanisms of MSC-mediated immune modulation. This is the first report showing that hMSCs interact with each of the isolated cells of the immune system and that hMSCs are capable of altering the outcome of the immune cell response by inhibiting 2 of the most important proinflammatory cytokines (ie TNF-α and IFN-γ) and by increasing expression of suppressive cytokines, including IL-10. Mechanistically, we have shown that inhibitors of PGE_2 synthesis mitigated the overall hMSC suppressive effects, suggesting that PGE_2 may be responsible for much of the hMSC-mediated immunomodulatory effects in vitro.

The inhibition of TNF-α secretion by DCs has been shown to inhibit their maturation, migration to lymph nodes, and ability to stimulate allo-T cells by altering the expression of several receptors and coreceptors necessary for antigen capture and processing.[60-62] We have not examined the effects of hMSCs on DC surface expression, although recently, the effects of hMSCs on differentiation, maturation, and function of monocyte-derived DCs were reported by Zhang et al.[53] Using a similar in vitro MSC/DC coculture system, these authors have shown that hMSCs inhibit the up-regulation of several of the maturation markers on DCs, resulting in their decreased capacity to activate allo-reactive T cells. Our results are in agreement with this report, and further strengthen the hypothesis that MSCs, due to their ability to inhibit TNF-α secretion by DCs, may lead to an immunologic tolerance state.

The ability of MSCs to interact with HLA-unrelated immune cells and modulate their response has important implications in transplantation biology. Recipients of allogeneic transplants often experience acute GVHD due to alloreactive T cells present in the allograft.[63-66] Graft-versus-host disease involves a pathophysiology that includes host tissue damage, increased secretion of proinflammatory cytokines (TNF-α, IFN-γ, IL-1, IL-2, IL-12), and the activation of DCs and macrophages, NK cells, and cytotoxic T cells.[67] All of these events are very important components of GVHD management, wherein inhibition of proinflammatory cytokines has been shown to be beneficial in resolution of the severity and incidence of GVHD.[68,69] Our in vitro data suggest that hMSCs, in their ability to inhibit IFN-γ and increase IL-4 secretion, may orchestrate a shift from the prominence of proinflammatory T_H1 cells toward an increase in anti-inflammatory T_H2 cells, beneficial for GVHD management. In fact, recently, Le Blanc et al[39] reported that by using haploidentical hMSCs, they were able to treat a patient suffering with acute GVHD. Our results may offer one explanation for the clinical outcome observed by the authors.

Several studies have now shown that hMSCs in culture can mediate suppression of T-cell proliferation by a secreted factor. Among the favored explanations is the secretion of immunosuppressive cytokines TGF-β and/or IL-10 by MSCs.[41,44] However, blocking these factors with antibodies does not completely reverse the MSC-mediated immunosuppression.[41,44] Another potential mechanism to explain the MSC-mediated immunosuppression is a veto cell–like activity.[42] However, veto cells suppress cytotoxic T lymphocyte (CTL) precursor functions against antigens present on their own cell surface, but not against third-party allogeneic cells. This suppression mechanism contrasts with the extensive data with

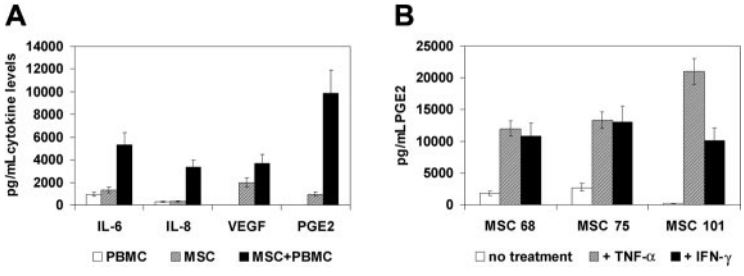

Figure 4. MSCs secrete important factors, the levels of which increase upon PBMC coculture. Human MSCs and human PBMCs isolated from unrelated donors were cocultured (MSC to PBMC ratio, 1:10) for 24 hours, and cell supernatants were collected and analyzed for various secreted factors by ELISA. (A) Human MSCs secreted factors IL-6, IL-8, VEGF, and PGE_2, and the levels of each of these factors increased more than 3-fold upon hPBMC coculture (IL-6: 6-fold; IL-8: 6-fold; VEGF: 3-fold; and PGE_2: 10-fold). Bars represent cytokine levels (mean pg/mL ± SD) from 2 independent hMSC and 1 hPBMC donors. (B) Human MSCs from 3 independent donors were cultured in the presence of TNF-α and IFN-γ for 24 hours, following which PGE_2 levels in the supernatant were determined using ELISA. The results shown are from duplicate cultures performed in parallel (mean ± SD).

Table 2. Cytokine secretion and effect of PGE_2 inhibitor NS-398 on immune cells

	Cytokine secretion							
	DCs, TNF-α, pg/mL*				T cells, IFN-γ, pg/mL†			
	Without MSCs		With MSCs		Without MSCs		With MSCs	
Inhibitor	−	+	−	+	−	+	−	+
Experiment no. 1	789 ± 42	880 ± 46	460 ± 44	624 ± 92	1750 ± 32	1600 ± 58	695 ± 100	1390 ± 121
Experiment no. 2	1630 ± 55	1600 ± 57	289 ± 12	1289 ± 57	2287 ± 60	2800 ± 75	1235 ± 80	2764 ± 85
Experiment no. 3	458 ± 30	524 ± 38	144 ± 64	369 ± 48	3196 ± 101	ND	1657 ± 34	2677 ± 74

ND indicates not done; −, no inhibitor; +, 5 μM NS-398.

*Human MSCs and mature DCs were cocultured with or without PGE_2 inhibitor NS-398 in the presence of activating stimulus LPS. The TNF-α levels in the supernatant were determined by ELISA. Inhibition of PGE_2 always resulted in an increase in TNF-α levels.

†Human MSCs and T cells were cocultured with or without PGE_2 inhibitor NS-398 in the presence of mitogen PHA and levels of IFN-γ were determined by ELISA. Results show that inhibition of PGE_2 always resulted in an increase in IFN-γ levels.

MSCs wherein T-cell proliferation is inhibited across strain (allo-) and across species (xeno-).[40,41,44,49] Therefore, the veto cell–like activity may play an important role in the low immunogenecity of MSCs, but certainly does not explain the suppression of T-cell proliferation by the MSCs. More recently, the role of tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO)–mediated tryptophan degradation has been suggested to play a role in the MSC-mediated immunosuppression.[70] In that report, Meisel et al have shown that MSCs, in the presence of IFN-γ, express IDO activity that in turn degrades essential tryptophan in the media, and hence, may lead to inhibition of T-cell proliferation.[70] Although tryptophan degradation may contribute to the inhibition of T-cell proliferation at later time points (the authors show appearance of IDO expression at 5 days of MSC + IFN-γ culture), this is in disagreement with the inhibition kinetics of T cells by MSCs (within 2 days in the case of PHA stimulation). Furthermore, a previous report[44] and our own work demonstrate that MSC/PBMC coculture does not lead to T-cell apoptosis, normally a definite outcome of tryptophan depletion from the media.

We have shown that hMSCs can secrete PGE_2 in quantities that may be sufficient to be involved in MSC-mediated immunomodulation. Spontaneous and continuous production of cyclooxygenase-2 (COX-2)–dependent PGE_2 has been observed in murine small intestine lamina propria cells[71] and cells lining the airway mucus membranes,[72] probably as a mechanism to down-regulate an inappropriate inflammatory response to nonpathogenic antigens residing in the environmental flora of these organs. Tse et al[45] also reported low levels of PGE_2 secretion by MSCs, but they did not see significant reversal in T-cell proliferation when using PGE_2 synthesis inhibitors. This disagreement with the results reported

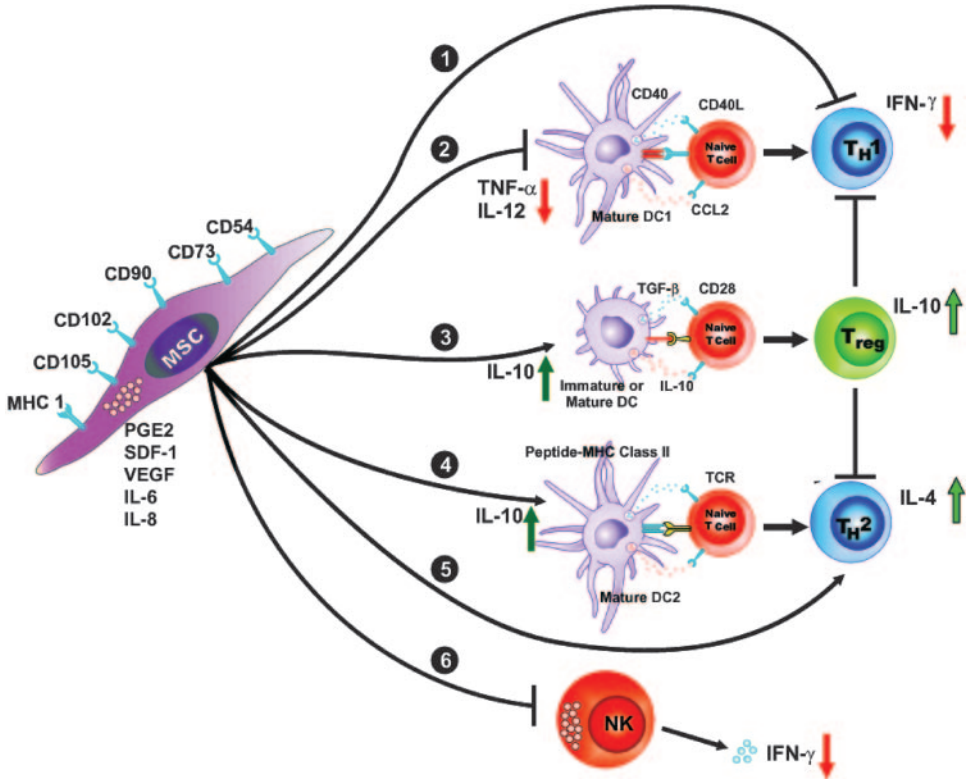

Figure 6. Proposed mechanisms of action of MSCs. We propose that MSCs mediate their immunomodulatory effects by interacting with cells from both the innate (DC, pathways 2-4; NK cell, pathway 6) and adaptive immunity systems (T cell, pathways 1 and 5). MSC inhibition of TNF-α secretion and promotion of IL-10 secretion may affect DC maturation state and their functional properties, resulting in skewing the immune response toward in an anti-inflammatory/tolerant phenotype. Alternatively, when MSCs are present an inflammatory microenvironment, they inhibit IFN-γ secretion from T_H1 and NK cells and increase IL-4 secretion from T_H2 cells, thereby promoting a $T_H1 \rightarrow T_H2$ shift. It is likely that MSCs also mediate their immunomodulatory actions by direct cell-cell contact as well as by secreted factors. Several MSC cell-surface molecules and secreted molecules are depicted. CCL indicates chemokine ligand; TCR, T-cell receptor.

herein may be due to differences in experimental design. We observed that hMSCs exhibit a bell-shaped time-dependent curve of PGE_2 secretion (ie, after an initial increase there is a decrease in levels of PGE_2 after 4 to 5 days in culture [data not shown]). Therefore, an inability to observe any significant effect of PGE_2 inhibitors by Tse et al[45] may be due to performing their evaluations at 5 days.

As shown here, hMSCs, when cocultured with immune cells resulted in increased PGE_2 and VEGF secretion. This can be explained only by a synergistic rather than an additive effect, suggesting MSC–immune cell cooperation at sites of inflammation. Although the exact contributions of each cell source to the increased levels of secreted factors are not known, our preliminary experiments in which human VEGF secretion was measured following rat MSCs (rMSCs)–human PBMCs coculture, and vice versa, showed that the hPBMCs, following coculture with rMSCs, increased secretion of human VEGF (data not shown). What other PGE_2-secreting cells are also capable of mediating this effect remains to be explored.

To the question of whether differentiated MSCs induce a T-cell proliferative response, we detected comparable amounts of PGE_2 in supernatants derived from cultures under control and adipocyte differentiation conditions (data not shown), and therefore, expect comparable levels of inhibition of T-cell proliferation. Le Blanc et al found that differentiated MSCs had immunologic properties similar to the undifferentiated MSCs.[46] We have detected allogeneic MSCs in vivo in several animal models at extended time periods (months) wherein the allo-MSCs expressed markers of differentiated cell types, suggesting differentiated MSCs are not rejected. However, some caution should be exercised in predicting the in vivo role of MSC-mediated PGE_2 secretion, as it could be an in vitro phenomenon. Development of an animal model is under way to investigate the in vivo effects further.

To summarize, through the interactions of hMSCs with the various immune cells, it appears that hMSCs inhibit or limit inflammatory responses and promote the mitigating and anti-inflammatory pathways. A proposed model of these interactions is shown in Figure 6, which illustrates that hMSCs alter the outcome of the immune response by inhibiting inflammatory DC1 signaling (pathway 2) and promoting anti-inflammatory DC2 signaling (pathway 4). Also, when immature effector T cells are present, hMSCs may interact with them directly and inhibit the development of proinflammatory T_H1 and NK signaling (pathway 1 and 6, respectively) and promote anti-inflammatory T_H2 (pathway 5) and/or suppressive T_{Reg} signaling (pathway 3). The results imply that when hMSCs are present in an inflammatory environment (such as that artificially created by activating DCs, macrophages, NK cells, or T cells using various stimuli), they may alter the outcome of the on-going immune response by altering the cytokine secretion profile of DC subsets (DC1 and DC2) and T-cell subsets (T_H1, T_H2, or T_{Regs}), thereby resulting in a shift from a proinflammatory environment toward an anti-inflammatory or tolerant cell environment. Such a model of the hMSC–immune cell interaction is consistent with the experiments reported here.

In conclusion, while the complete mechanism of immune modulation by MSCs will require further investigations, the studies presented here provide a working model to understand MSC-mediated immunomodulation that may be an active component in inflammation modulation, tolerance induction, and reduction of transplantation complications such as rejection and GVHD.

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