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CELL-BASED DRUG DEVELOPMENT, SCREENING, AND TOXICOLOGY

Human Mesenchymal Stem Cells Impact Th17 and Th1 Responses Through a Prostaglandin E2 and Myeloid-Dependent Mechanism

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ABSTRACT

Human mesenchymal stem cells (hMSCs) are being increasingly pursued as potential therapies for immune-mediated conditions, including multiple sclerosis. Although they can suppress human Th1 responses, they reportedly can reciprocally enhance human Th17 responses. Here, we investigated the mechanisms underlying the capacity of hMSCs to modulate human Th1 and Th17 responses. Human adult bone marrow-derived MSCs were isolated, and their purity and differentiation capacity were confirmed. Human venous peripheral blood mononuclear cells (PBMC) were activated, alone, together with hMSC, or in the presence of hMSC-derived supernatants (sups). Cytokine expression by CD4+ T-cell subsets (intracellular staining by fluorescence-activated cell sorting) and secreted cytokines (enzyme-linked immunosorbent assay) were then quantified. The contribution of prostaglandin E2 (PGE2) as well as of myeloid cells to the hMSC-mediated regulation of T-cell responses was investigated by selective depletion of PGE2 from the hMSC sups (anti-PGE2 beads) and by the selective removal of CD14+ cells from the PBMC (magnetic-activated cell sorting separation). Human MSC-secreted products could reciprocally induce interleukin-17 expression while decreasing interferon-γ expression by human CD4+ T cells, both in coculture and through soluble products. Pre-exposure of hMSCs to IL-1β accentuated their capacity to reciprocally regulate Th1 and Th17 responses. Human MSCs secreted high levels of PGE2, which correlated with their capacity to regulate the T-cell responses. Selective removal of PGE2 from the hMSC supernatants abrogated the impact of hMSC on the T cells. Selective removal of CD14+ cells from the PBMCs also limited the capacity of hMSC-secreted PGE2 to affect T-cell responses. Our discovery of a novel PGE2-dependent and myeloid cell-mediated mechanism by which human MSCs can reciprocally induce human Th17 while suppressing Th1 responses has implications for the use of, as well as monitoring of, MSCs as a potential therapeutic for patients with multiple sclerosis and other immune-mediated diseases. STEM CELLS TRANSLATIONAL MEDICINE *2016;5:1506–1514*

SIGNIFICANCE

Although animal studies have generated a growing interest in the anti-inflammatory potential of mesenchymal stem cells (MSCs) for the treatment of autoimmune diseases, MSCs possess the capacity to both limit and promote immune responses. Yet relatively little is known about human-MSC modulation of human disease-implicated T-cell responses, or the mechanisms underlying such modulation. The current study reveals a novel prostaglandin E2-dependent and myeloid cell-mediated mechanism by which human MSCs can reciprocally regulate human Th17 and Th1 responses, with implications for the use of MSCs as a potential therapeutic for patients with multiple sclerosis and other immune-mediated diseases.

INTRODUCTION

Bone marrow-derived mesenchymal stem cells (MSCs), also referred to as multipotent mesenchymal stromal cells, are being actively pursued as a new potential therapeutic agent for a range of human conditions, including autoimmune diseases such as multiple sclerosis (MS) [1–4]. In MS, relapsing disease activity is thought to involve aberrant responses of proinflammatory effector Th1 and Th17 cells, faulty regulation by regulatory T cells (Treg), or both [5–7]. Involvement of other cells, including B cells and myeloid cells, is also likely [7, 8]. The potential for MSCs to limit central nervous system inflammatory disease has been studied extensively in the commonly used animal

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model of MS, experimental autoimmune encephalomyelitis (EAE). Both murine MSCs and human-derived MSCs (hMSCs) can ameliorate EAE disease severity [9, 10]. The benefit of MSCs in EAE is attributed to the general properties of MSCs, including their capacity to downregulate both Th1 and Th17 proinflammatory responses [11–15], as well as to enhance regulatory responses through multiple mechanisms [16–21]. The latter include licensing of regulatory T cells with involvement of interleukin (IL)-10, inducible nitric oxide synthase, the indoleamine 2,3-dioxygenase-mediated tryptophan pathway, and PD-1 [22–25] and expansion of regulatory T cells, both through de novo generation from naïve T cells and during Th1 and Th17 differentiation [16–18, 20]. The immune regulatory capacity of MSCs is also thought to reflect their ability to impair T-cell priming by myeloid cells including dendritic cells and macrophages [13, 14, 26].

This substantial body of work highlights anti-inflammatory properties of MSC; however, there have also been reports that MSCs can enhance proinflammatory responses [27–33]. Of particular interest have been seemingly contrasting reports on the effects of MSCs on responses of distinct subsets of effector T cells. Although MSCs have been shown to suppress both Th1 and Th17 responses in EAE [2, 4, 12], work with human peripheral blood mononuclear cells (PBMCs) revealed that soluble products of hMSCs could suppress Th1 responses while inducing Th17 responses of human T cells [31].

Here, we confirm the capacity of hMSCs to reciprocally regulate Th1 and Th17 effector T-cell subsets, and we show that this occurs through a prostaglandin E2 (PGE2)-dependent and myeloid cell-mediated mechanism. We first show that this reciprocal regulation of effector T-cell subsets by MSC soluble products persists also when the hMSCs can directly interact with the PBMCs in coculture. We report that the degree of Th17 induction by the hMSCs correlates with the levels of PGE2 secreted by the hMSC and that selective removal of PGE2 from the hMSC soluble products abrogates the ability of the hMSC to induce Th17 responses. We further demonstrate that the ability of hMSC secreted products to reciprocally affect responses of the distinct effector T-cell subsets within PBMCs is mediated through myeloid cells, and that this effect of myeloid cells is indeed PGE2 dependent. Our findings reinforce the potential for human MSCs to exert both anti-inflammatory and proinflammatory effects and define a novel PGE2-dependent and myeloid cell-mediated mechanism for reciprocal Th1/Th17 modulation.

METHODS

Subjects and hMSC and Peripheral Blood Mononuclear Cell Isolation

All donors of hMSCs were recruited from the Cleveland Clinic Mellen Center, following institutional review board approval of the study protocol and informed consents. Bone marrow aspirates were obtained from posterior superior iliac crest in the Dahms Clinical Research Unit at University Hospitals Case Medical Center. The hMSCs were then isolated from the bone marrow aspirates and expanded in culture according to standard procedures used in the Cellular Therapy Laboratory at Case Western Reserve University [27]. After 1–3 passages in optimized medium (Dulbecco's modified Eagle's medium [DMEM]-low glucose + 10% fetal bovine serum [FBS] + 1% antibiotic/antimycotic + 1% Glutamax [Thermo Fisher Scientific Life Sciences, Waltham, MA, https://www.thermofisher.com] + 10 ng/ml fibroblast growth factor [FGF-2]), 4×10^6 cells per millliliter were frozen in cryovials using Plasma-Lyte A, containing 10% dimethyl sulfoxide and 5% human serum albumin as the freezing medium. Venous blood PBMCs were obtained following standard Ficoll gradient centrifugation of whole blood from antecubital venipuncture of healthy volunteers, using our established protocols [8, 31] and following informed consent as approved by the institutional review board. Aliquots of cryopreserved hMSCs and PBMCs were subsequently transferred to the Montreal Neurological Institute at McGill University.

Characterization of hMSCs and Generation of hMSC-Conditioned Media

Frozen hMSC aliquots were thawed, washed twice, and then cultured at a density of 7.8×10^3 cells per cm^2 in a 75-cm^2 flask until reaching confluence of 80%, followed by trypsinization and reseeding at the same density for the next passages. Culture medium was DMEM-low glucose (Thermo Fisher Scientific) with 10% FBS (Thermo Fisher; prescreened for optimal MSC growth), 5 ml pen/strep, 5 ml L-glutamine, and 10 ng/ml human FGF-2 (PeproTech, Rocky Hill, NJ, https://www.peprotech.com). All MSCs were characterized phenotypically and functionally by flow cytometry following 2–3 passages. Phenotyping was done using directly conjugated antibodies to the known MSC markers CD73, CD90, CD105, and CD44, as well as for the lineage-negative markers CD31, CD34, and CD45, with appropriate isotype controls (all BD Biosciences, San Diego, CA, http://www.bdbiosciences.com). Functional capacity to differentiate was confirmed using distinct differentiation media, added in parallel wells, and changed twice a week until cells differentiated: For osteocytes, STEMPRO osteogenesis differentiation kit (Thermo Fisher Scientific) was used per standard protocol with gentamycin (5 μg/ml) added. Cells were subsequently washed; alizarin red S staining solution was added for 5 minutes at room temperature; solution was then removed, and cells were washed twice with ddH_2O prior to microscopy and digital photography. For adipocytes, STEMPRO adipogenesis differentiation kit (Thermo Fisher Scientific) was used per standard protocol with gentamycin (5 μg/ml) added. Cells were subsequently fixed with paraformaldehyde (PFA) 4% for 1hr at room temperature. PFA was then removed, and 2 ml of diluted oil red staining solution (3 parts oil red solution in 2 parts distilled water) was added for 10 minutes at room temperature. Staining solution was then removed, and cells were washed twice with ddH_2O, followed by microscopy and photography. For all experiments, human-MSC conditioned media (hMSC supernatants, or sups) were obtained by culturing hMSCs as above until reaching confluence of 80%. After removal of medium and a series of washes, the cells were cultured (RPMI 1640 medium, 10% fetal calf serum, Pen-Strep, and L-glutamine) for another 24 hours, with or without exposure to IL-1β (10 ng/ml; R&D, Minneapolis, MN, https://www.rndsystems.com), then washed carefully with fresh media added. The MSC-conditioned medium was then collected 24 hours later and stored at -80°C until use.

Human PBMC Activation in Presence of hMSC Supernatants

PBMC aliquots were thawed, washed twice, and activated with soluble anti-CD3 (0.3 μg/ml; eBioscience, San Diego, CA, http://www.ebioscience.com), anti-CD28 (1 μg/ml; eBioscience), recombinant IL-23 (10 ng/ml; R&D Systems), anti-IL-4 (5.0 μg/ml; R&D Systems, Minneapolis, MN, https://www.rndsystems.com), and anti-interferon (IFN)-γ (5.0 μg/ml; R&D Systems) for 3 days, as previously described [31]. When MSC-conditioned media or control media

were added, they constituted 75% of the final volume. Cytokine secretion from the activated PBMCs was quantified at the time points indicated by using standard enzyme-linked immunosorbent assay (ELISA) for IL-17A (eBioscience) as the prototypical Th17 marker and for IFNγ (BD Biosciences) as the prototypical Th1 marker, according to the manufacturers' instructions. Intracellular cytokine staining was performed with anti-CD4 peridinin chlorophyll, anti-IFNγ adenomatous polyposis coli, and anti-IL-17A phycoerythrin (PE) antibodies (all BD Biosciences) to examine cytokine expression by $CD4^+$ T cells, as previously described [31].

hMSC-PBMC Coculture Experiments

hMSCs were cultured as above with or without 24-hour pre-exposure to IL-1β (10 ng/ml). The hMSCs were then removed from the flasks, washed carefully, and added to wells of a 96-well plate, into which PBMCs (2×10^5 cells per well) were subsequently added at the indicated ratios. PBMC activation was achieved, as described above. After 3 days, coculture sups were collected for cytokine quantification by ELISA, and cells were analyzed by flow cytometry to assess intracellular cytokine staining within CD4+ T cells. Similarly activated PBMCs that were cultured alone (without hMSCs) were also used as a control.

PGE2 Measurements and Effects of Selective PGE2 Removal From hMSC Supernatants

To assess hMSC PGE2 secretion, hMSC sups were obtained under basal culture conditions or following 24-hour pre-exposure to IL-1β (10 ng/ml), as above. PGE2 levels were then quantified by the PGE2 ELISA kit (Cayman Chemical, Ann Arbor, MI, https://www.caymanchem.com). The same hMSC sups were used to assess the ability of hMSC-soluble products to affect T-cell responses within PBMCs. This enabled correlation between levels of PGE2 measured in the hMSC-soluble products, with the degree of change in the T-cell cytokine responses. Depletion of PGE2 from the hMSC sups was performed by adding anti-PGE2 antibody (2b5) to hMSC sups, spinning at 4°C, and then running the sups through a well-washed protein A/G column with Agarose beads (Santa Cruz Biotechnology, Dallas, TX, https://www3.scbt.com). Complete removal of PGE2 from the flow through was confirmed by ELISA. In subsequent experiments, PBMCs were activated (as above) in culture media containing 75% hMSC sups that were either nondepleted or PGE2 depleted, and T-cell cytokine responses were subsequently assessed. hMSC supernatants treated with the appropriate mIgG1 isotype, and protein A/G column used with or without Agarose beads, were used as negative controls.

Isolating CD14+ Myeloid Cells and Assessing Their Contribution to hMSC-Mediated Effects on T-Cell Responses

CD14+ myeloid cells were isolated from PBMCs using magnetic-activated cell sorting (MACS) CD14-positive selection kit (Miltenyi Biotec, San Diego, CA, http://www.miltenyibiotec.com). Purity of the isolated CD14+ cells (>97%), as well as the complete removal of CD14+ cells from the CD14-depleted PBMCs, was routinely confirmed by flow cytometry. Purified CD14+ cells (4×10^4 cells) were pre-exposed to either hMSC supernatants (hMSC sup) or control media for 24 hours in a well of a 96-well plate, then washed twice, and 1.6×10^5 CD14-depleted PBMCs were added to the pre-exposed CD14+ cells. As a comparison, the CD14-depleted PBMC were pre-exposed to the same hMSC sups or to control media and, subsequently, added to the purified CD14+ cells (pre-exposed only to control media). As a negative control, both CD14+ and CD14-depleted PBMCs were pre-exposed to control media only, prior to combining them. All cultures were activated (as described above) for 72 hours, and secretion of IFNγ and IL-17 was then quantified in culture supernatants by ELISA. To examine whether PGE2 in the hMSC sups was responsible for the myeloid-mediated effects of hMSC sups on T-cell responses, we carried out similar experiments with hMSC sups that were either selectively depleted of PGE2 or sham-depleted in the control conditions, as detailed above.

Statistical Approach

Percentage change was calculated by subtracting the respective control media value from treatment value, dividing by the control value ($\times 100$). Repeated-measures analysis of variance followed by Bonferroni test or unpaired Student's *t* test were used where appropriate. A cutoff of $p \leq .05$ was used to indicate statistical significance. Statistical computations were performed using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, http://www.graphpad.com)

RESULTS

Confirmation of Adult Human (h)MSC Phenotypic and Functional Capacities

As is shown in Figure 1, hMSC cultures were routinely highly pure, stained positively for the established MSC markers CD73, CD90, CD105, and CD44; were appropriately negative for markers of other lineages (CD31, CD34, and CD45) (Fig. 1A); and retained the expected capacity to differentiate into osteocytes and adipocytes under the appropriate lineage differentiation conditions (Fig. 1B). In keeping with prior reports, the hMSCs were also able to limit proliferation of T cells within activated PBMCs (supplemental online Fig. 1).

hMSCs Inhibit Th1 Responses yet Induce Th17 Responses, Both in Coculture and Through Soluble Products

We previously reported that soluble products of hMSCs could downregulate IFNγ expression while surprisingly inducing IL-17 expression within activated PBMCs [31]. In the context of in vivo therapy, however, one must consider the potential for hMSCs to directly interact with immune cells through cell-cell contact. Such contact could include molecular interactions that might deliver inhibitory signals to the immune cells, which could conceivably abrogate the apparent IL-17-inducing capacity of hMSC-secreted products. We therefore first assessed whether hMSCs preserve their ability to induce IL-17 responses of PBMCs in direct coculture (Fig. 2). We found that presence of hMSCs in coculture with activated PBMCs indeed resulted in increased secretion of IL-17 (Fig. 2A, 2C; $n = 6$, $p = .031$), while decreasing IFNγ (Fig. 2B, 2C; $p = .0083$), similar to the effects observed when adding only the hMSC supernatants to the activated PBMCs (Fig. 2D–2F). Using intracellular cytokine staining together with surface staining to define T-cell subsets, we could confirm that activated CD4+ T cells within the PBMCs exhibited the reciprocal regulation of IL-17 and IFNγ expression when exposed to the hMSC products (Fig. 2G). To address whether different sources of hMSC may have different effects on Th1 and Th17 responses, we carried out a series of experiments assessing the impact of MSC obtained from multiple different healthy donors on cytokine responses of a

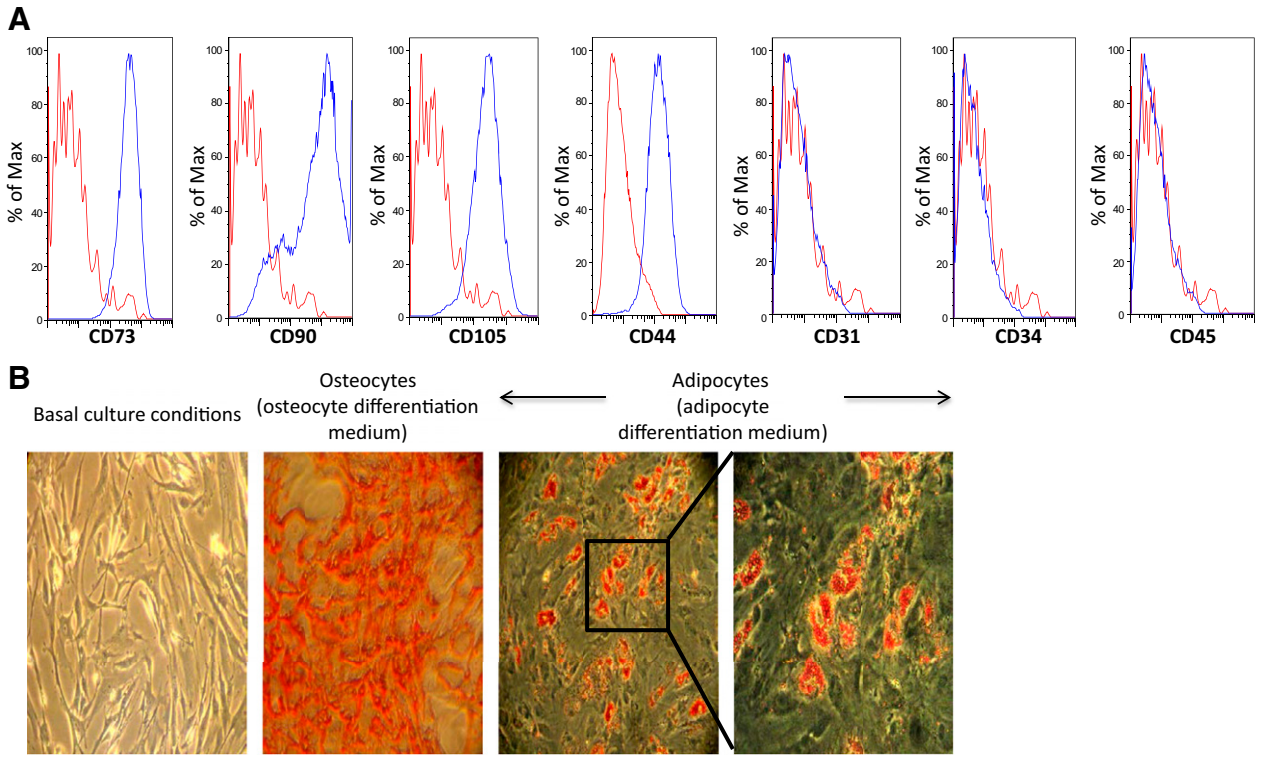

Figure 1. Purity, phenotype, and differentiation capacity of bone marrow-derived human mesenchymal stem cells (hMSCs). **(A):** Purity and phenotype of bone marrow hMSCs used in experiments were routinely confirmed by flow cytometry using antibodies to lineage-positive (CD73, CD90, CD105, CD44) and lineage-negative (CD31, CD34, CD45) markers (red lines denote staining with appropriate isotype controls). **(B):** Confirming capacity of the hMSCs to differentiate into osteocytes (using STEMPRO osteogenesis differentiation kit by Thermo Fisher Scientific/Gibco, followed by alizarin red S staining) and adipocytes (STEMPRO adipogenesis differentiation kit by Thermo Fisher Scientific/Gibco, followed by paraformaldehyde 4% fixation, and subsequent oil red staining). Images obtained at ×10 magnification (insert at ×20).

single source of PBMCs. We found that the magnitude of the effect of different MSC supernatants on both Th1 and Th17 responses of the same PBMCs differed across MSC preparations, although the immune modulatory effects were qualitatively similar, meaning the MSCs in our assays consistently inhibited Th1 responses and consistently enhanced Th17 responses, although to differing extents (data not shown). In subsequent experiments examining the mechanism involved in hMSC-mediated induction of human T-cell IL-17 secretion, we focused on the effects of soluble products within the hMSC-conditioned media (hMSC supernatants, or sups).

Induction of Human Th17 Responses by hMSC Is Mediated by PGE2

We considered whether our PBMCs exposed to hMSC supernatants exhibited changes in regulatory T-cell subsets as a possible explanation for the observed changes in effector T-cell responses. Neither the frequencies of phenotypically defined $CD25^{hi}CD127^{low}$ Treg, or of Treg- expressing glucocorticoid-induced tumor necrosis factor receptor or CD39 (implicated as particularly involved in the regulation of Th17 cells), were appreciably altered by the hMSC supernatants in our short-term cultures, nor did we appreciate changes in levels of Foxp3 expressed by the T cells (data not shown). We next considered whether a single MSC-soluble factor could be responsible for enhancing IL-17 expression, while inhibiting IFNγ expression, by activated T cells. An attractive candidate, on the basis of the literature, was PGE2, which has been reported to increase Th17 responses while decreasing Th1 responses [34, 35] and is also known to be produced by hMSCs [36, 37]. We first confirmed that our hMSCs could secrete PGE2 and also that pre-exposure of the hMSC with IL-1β (which we previously demonstrated could enhance the ability of hMSCs to induce IL-17 production by human T cells [31]) significantly increased the hMSC secretion of PGE2 (Fig. 3A; $p < .0001$). Using the soluble products of these unexposed or IL-1β pre-exposed hMSCs, we confirmed that the IL-1β pre-exposed hMSCs could induce significantly greater levels of IL-17 from activated PBMCs (Fig. 3B; $p = .0068$) and that a strong correlation existed between the levels of IL-17 induced by the PBMCs and the levels of PGE2 secreted by the hMSCs, whether the hMSCs were grown under basal conditions (Fig. 3C; $r^2 = .3919$; $p = .0221$) or pre-exposed to IL-1β (Fig. 3D; $r^2 = .6662$; $p = .0004$). To determine whether hMSC-secreted PGE2 was indeed responsible for the induction of IL-17 in T cells, we selectively removed PGE2 from the hMSC supernatants using anti-PGE2 beads or appropriate controls (confirmed by ELISA; supplemental online Fig. 2). We could then show that removal of PGE2 from the hMSC supernatants reversed the ability of the hMSCs to induce T-cell IL-17 responses (Fig. 3E; $n = 6$; ***, $p \leq .001$). As an additional confirmation, PGE2 was added directly to activated PBMCs and found to enhance IL-17 while inhibiting IFNγ responses (supplemental online Fig. 3).

hMSC-Derived PGE2 Effects on Th1 and Th17 Responses Can Be Mediated via CD14+ Cells

We next considered whether the demonstrated effects of hMSC-derived PGE2 on PBMC responses reflect direct effects of PGE2 on the T cells or indirect effects through other cells present within

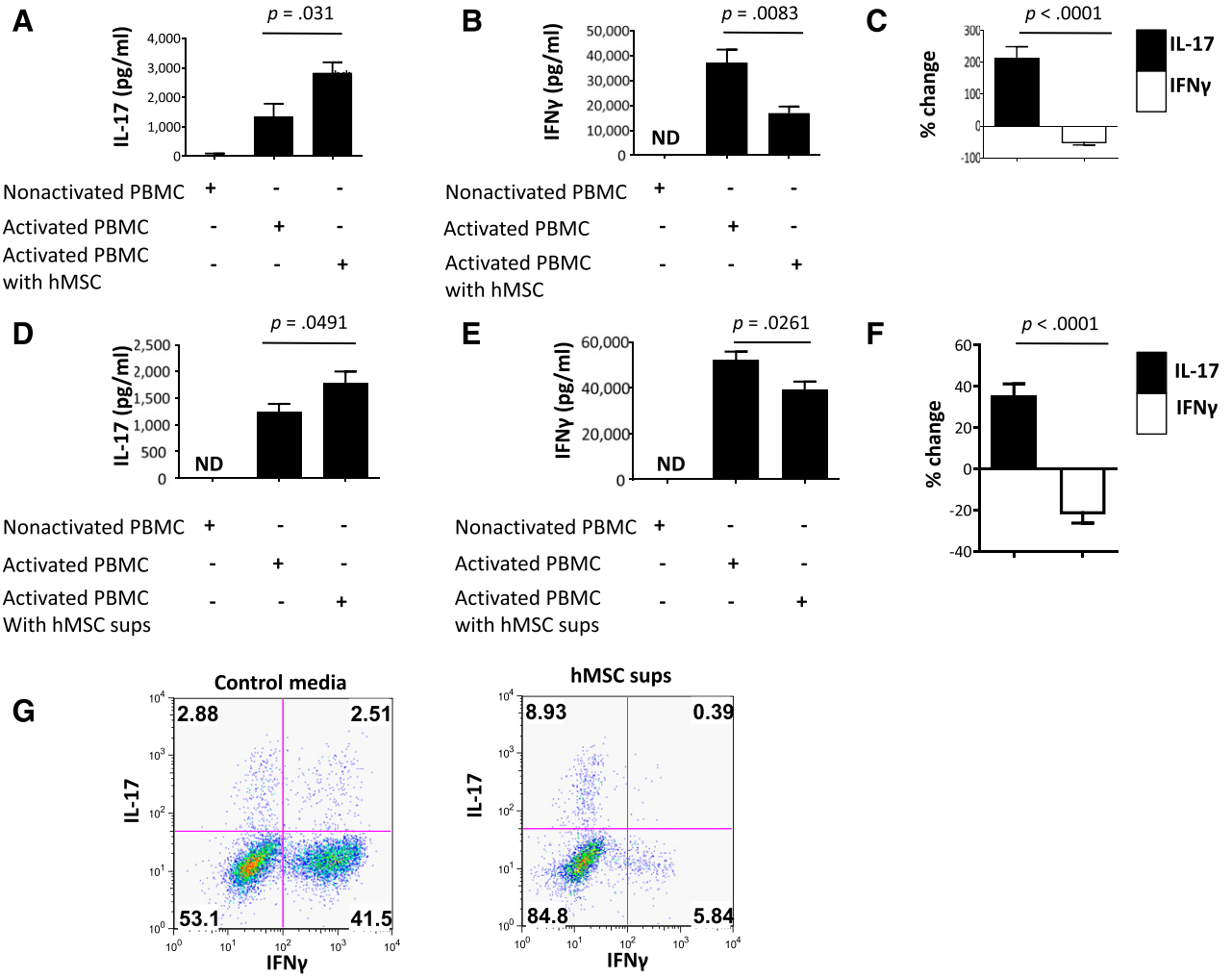

Figure 2. hMSCs inhibit Th1 responses yet induce Th17 responses both in coculture and through soluble products. **(A–C):** hMSCs were cocultured with PBMCs (ratio of hMSC : PBMC 1:10), followed by 3-day PBMC activation using anti-CD3 (0.3 μg/ml; eBioscience), anti-CD28 (1 μg/ml; eBioscience), recombinant interleukin (IL)-23 (10 ng/ml; R&D Systems), anti-IL-4 (0.5 μg/ml; R&D Systems), and anti-IFNγ (0.5 μg/ml; R&D Systems). Presence of hMSCs in coculture resulted in, increased PBMC IL-17 secretion (n = 6; p = .031) **(A)** and decreased IFNγ secretion (p = .0083) **(B)**, as assessed by enzyme-linked immunosorbent assay. This represented reciprocal regulation of IL-17 and IFNγ responses in the cocultures in the presence of hMSCs ($p < .0001$) **(C)**. **(D–F):** Addition of soluble products of the hMSC (hMSC sups) to PBMCs had similar effects on cytokine responses of activated PBMCs, including increased IL-17 responses (p = .0491; n = 11) **(D)** and decreased IFNγ responses (p = .0261; n = 7) **(E)**; again, representing reciprocal regulation of IL-17 and IFNγ responses ($p < .0001$) **(F)**. **(G):** PBMCs were activated as above for 3 days with control media (left panel) or with hMSC sups (right panel) followed by flow cytometry analysis of surface staining (for CD3 and CD4) and intracellular cytokine staining. Representative dot plots are gated on CD4+ CD3+ T cells and indicate that hMSC sups induce the frequency of CD4 T cells expressing IL-17 while decreasing the frequency of IFNγ expressing CD4 T cells (representative of 11 independent experiments). Abbreviations: hMSC, human mesenchymal stem cells; IFNγ, interferon-γ; IL, interleukin; ND, nondetectable; PBMC, peripheral blood mononuclear cells; sups, supernatants.

the PBMCs. Because MSCs have been shown to directly affect myeloid cell responses [13, 14], we wished to assess whether myeloid cells within the PBMCs were involved in mediating the effects of hMSC-derived PGE2 on T-cell responses. To determine this, we first removed CD14+ myeloid cells from a fraction of PBMCs by using CD14+ MACS isolation and confirmed both the purity of the isolated CD14+ cells and the complete removal of CD14+ cells from the CD14-depleted PBMC fraction (supplemental online Fig. 4). We then exposed isolated CD14+ cells to either hMSC supernatants or to corresponding control media for 24 hours and, following two washes, added back the CD14+ cells to the CD14-depleted PBMCs. As a comparison, the CD14-depleted PBMCs were pre-exposed to the hMSC supernatants, then washed prior to adding back unexposed CD14+ cells. All culture conditions were then activated, as above, to assess T-cell cytokine (IFNγ and IL-17) secretion following 72 hours. We found that the ability of hMSC supernatants to induce Th17 (Fig. 4A) and to inhibit Th1 (Fig. 4B) responses within PBMCs was dependent on initial exposure of CD14+ cells (not T cells within the PBMCs) to the hMSC supernatants. To confirm that PGE2 within the hMSC supernatants was responsible for the myeloid cell-mediated effect on T-cell responses, we repeated the above experiments but first used the same approach to selectively remove PGE2 from the hMSC supernatants. Our results indicate that PGE2 within the hMSC supernatants mediates the CD14+ cell-dependent reciprocal regulation of T-cell IL-17 (Fig. 5A; **, $p < .01$) and IFNγ (Fig. 5B; ***, $p < .001$). This capacity of myeloid cells to mediate the

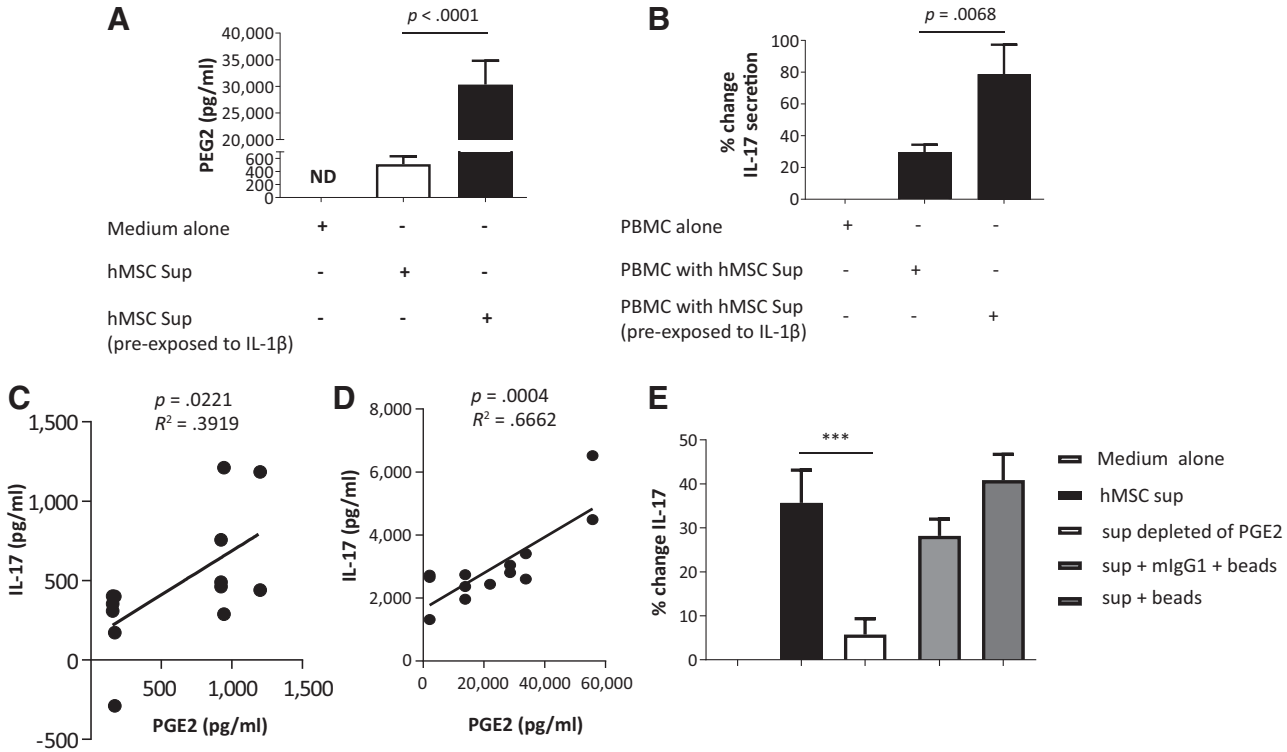

Figure 3. Induction of human Th17 responses by human mesenchymal stem cells (hMSCs) is mediated by PGE2. **(A):** hMSCs secrete PGE2 under basal culture conditions, and this secretion is induced when the hMSCs are pre-exposed to IL-1β ($p < .001$; $n = 12$). **(B):** hMSC sups obtained after the hMSCs were pre-exposed to IL-1β for 24 hours (then washed twice and cultured for another 24 hours in fresh media), which further enhanced the ability of hMSC sups to induce IL-17 responses from PBMCs. Shown is the percentage change in PBMC IL-17 secretion measured by enzyme-linked immunosorbent assay (ELISA) ($p = .0068$). **(C, D):** Correlations (linear regression test) are noted between the amount of IL-17 induced in the PBMCs by the hMSC supernatants and the amount of PGE2 present in the hMSC supernatants, both for unexposed hMSCs ($n = 12$; $r^2 = .3919$; $p = .0221$) **(C)** and IL-1β pre-exposed hMSCs ($n = 12$; $r^2 = .6662$; $p = .0004$) **(D)**. The IL-17 values shown reflect the difference between IL-17 secretion from activated PBMCs with hMSC exposure versus the IL-17 secretion from activated PBMCs that were exposed to control media. **(E):** PGE2 removal from hMSC supernatants (using the 2B5 anti-PGE2 antibody + agarose beads, then run on protein A/G column) abrogates the ability of the hMSCs to induce Th17 responses within PBMCs ($n = 6$; $p \leq .001$). Controls included hMSC sups following sham removal of PGE2 using an appropriate mouse (m)IgG1 antibody together with the agarose beads or the beads alone. ELISA was routinely used to confirm successful depletion or lack of depletion of PGE2 in the hMSC sups (supplemental online Fig. 1). ***, $p \leq .001$. Abbreviations: hMSC, human mesenchymal stem cells; IL, interleukin; ND, nondetectable; PBMC, peripheral blood mononuclear cells; PGE2, prostaglandin E2; sup, supernatant.

enhanced Th17 responses following exposure to MSC supernatants occurred in spite of the ability of the MSC supernatants to enhance myeloid-cell secretion of IL-10 and limit myeloid secretion of TNFα (supplemental online Fig. 5).

Discussion

A substantial body of work investigating MSCs (both murine and human derived) has underscored their anti-inflammatory effects and hence their therapeutic potential in MS and other immune-mediated diseases. Our study, however, points toward the potential for hMSCs to induce certain proinflammatory responses, in keeping with several prior reports [27–33]. In particular, we note that hMSCs can induce Th17 responses while limiting Th1 responses. Such differential impact of MSCs on distinct subsets of effector T cells is supported by a recent clinical trial of autologous MSCs in patients with MS, in which the authors documented decreased Th1 but not Th17 responses in vivo [38]. As reviewed by Uccelli and colleagues [33], the inflammatory context may importantly affect the consequence of MSC : immune interactions. Relevant contextual factors that may affect the T-cell immune modulatory effects of MSCs in addition to other soluble factors in the local environment may include the presence of other immune cells and their products (indirect vs. direct effects on the T cells), pathogen-associated molecular signals, and the timing of introduction of the MSCs to the inflammatory milieu. The latter is supported by the recent report that early administration of MSCs into animals developing EAE ameliorated disease, whereas later administration was not helpful and indeed resulted in development of atypical EAE that is more commonly seen with Th17-mediated rather than classical Th1 EAE [39]. Although considerable work has been done to elucidate mechanisms underlying anti-inflammatory properties of MSCs, relatively less is known about mechanisms by which MSCs may promote inflammatory responses. Our study implicates both PGE2 and myeloid cells as mediators of the capacity of hMSCs to induce Th17 responses, supporting the concept that both soluble factors and indirect effects mediated through third-party cells may modulate the impact of MSCs on immune responses. The role of PGE2 in mediating MSC effects, however, appears complex. Our results, indicating that PGE2 can enhance human Th17 responses, are consistent with a number of prior reports of PGE2 enhancing proinflammatory T-cell

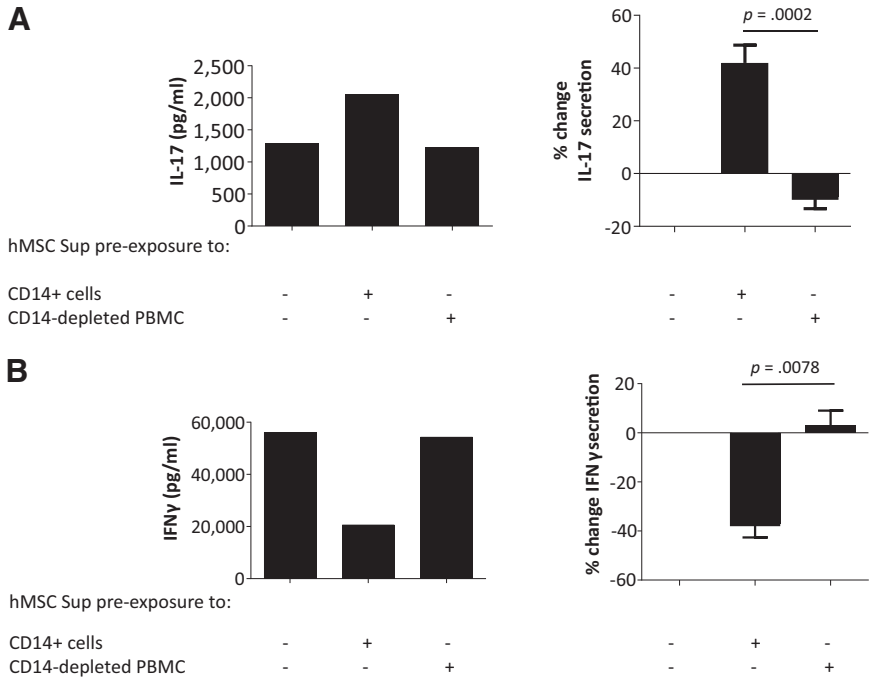

Figure 4. The capacity of hMSC sups to reciprocally regulate PBMC IL-17 and IFNγ responses is mediated by CD14+ myeloid cells. CD14+ myeloid cells were isolated from PBMCs by using CD14+ magnetic-activated cell sorting isolation, and the purity of the isolated CD14+ cells, as well as the complete removal of CD14+ cells from the CD14-depleted PBMCs, were confirmed by flow cytometry (supplemental online Fig. 4). Either the isolated CD14+ cells, or the CD14-depleted PBMCs, were pre-exposed to hMSC supernatants (hMSC sup) or control media for 24 hours, then washed twice. The CD14-depleted PBMCs were then added to the CD14+ cells with subsequent activation. At 72 hours following activation, IFNγ and IL-17 secretion were quantified in all cultures by enzyme-linked immunosorbent assay. The ability of hMSC supernatants to induce IL-17 **(A)**, representative of five independent experiments ($p = .0002$) and to inhibit IFNγ **(B)** ($p = .0078$) responses within PBMCs was dependent on initial exposure of CD14+ cells (but not on the CD14-depleted PBMC fraction) to hMSC sups. Abbreviations: hMSC, human mesenchymal stem cells; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cells; sup, supernatant.

response [34, 35, 40, 41]. However, PGE2 has also been implicated as part of the mechanisms mediating anti-inflammatory effects of MSCs, including their capacity to limit T-cell proliferation [42], suppress Th17 responses through cell-contact-mediated inhibition [43], induce regulatory T-cell responses [16], and inhibit $\gamma\delta$ T cells and invariant natural killer T-cell expansion [44].

Our findings also indicate that the ability of PGE2 to enhance Th17 responses, and indeed contribute to reciprocal regulation of the balance between Th1 and Th17 responses, may be mediated at least in part by presence of myeloid cells. Interactions between myeloid cells and MSCs are well established and have previously been highlighted in the context of anti-inflammatory effects of MSCs. It has been shown that MSCs can limit dendritic cell maturation, activation, and trafficking, and their subsequent capacity to prime T cells [14, 45, 46]. The abilities of MSCs to indirectly inhibit B cell differentiation (via MSC release of interleukin-1 receptor antagonist resulting in anti-inflammatory macrophage [26]) and to downregulate CD8 T-cell responses (via MSC soluble factors affecting CD14+ monocytes [47]) have also been highlighted. In the latter study, similar to our work, removal of CD14+ cells from PBMCs abrogated the effect of MSC-soluble products on the T-cell responses [47]. The potential for MSCs to mediate either pro- or anti-inflammatory immune responses appears not to be restricted to effects on T cells or myeloid cells, because MSC have been reported to either up- or downregulate B cell responses, including their proliferation and differentiation [48–50]. These effects were attributed to soluble factors and three-way interactions among MSC, B cells, and T cells, again highlighting the complexities involved in MSC immune modulation.

The accumulating evidence therefore underscores the diverse ways in which MSCs can shape responses of innate and adaptive immune cells, both directly and indirectly [33]. The importance of the "inflammatory context" in shaping how MSCs affect immune responses also highlights the challenge of translating findings from animal studies to humans who, in addition to substantially greater genetic diversity, experience a much broader range of environmental exposures that can influence the internal inflammatory milieu.

Although our discovery of a PGE2-dependent, myeloid cell-mediated mechanism by which MSCs may enhance Th17 responses benefits from use of human-derived MSCs and immune cells, it is limited to experiments carried out in vitro. It remains possible that, in vivo, the multiple reported mechanisms by which MSCs can induce anti-inflammatory responses [16–25] are sufficient to downregulate any proinflammatory T-cell responses that they may also induce. Nonetheless, it is important to appreciate any proinflammatory potential of hMSCs, because for example, patients who may have some deficiency in their capacity to induce regulatory T cells (as part of their own illness; history of immune suppressive treatment, etc.) may not be able to mount as effective a regulatory response, in which case, unopposed induction of Th17 responses by MSC therapy could have deleterious consequences.

Further preclinical work is warranted to rigorously examine the effects of hMSC on disease-relevant immune responses. Ultimately, however, ascertaining the in vivo consequences of hMSC

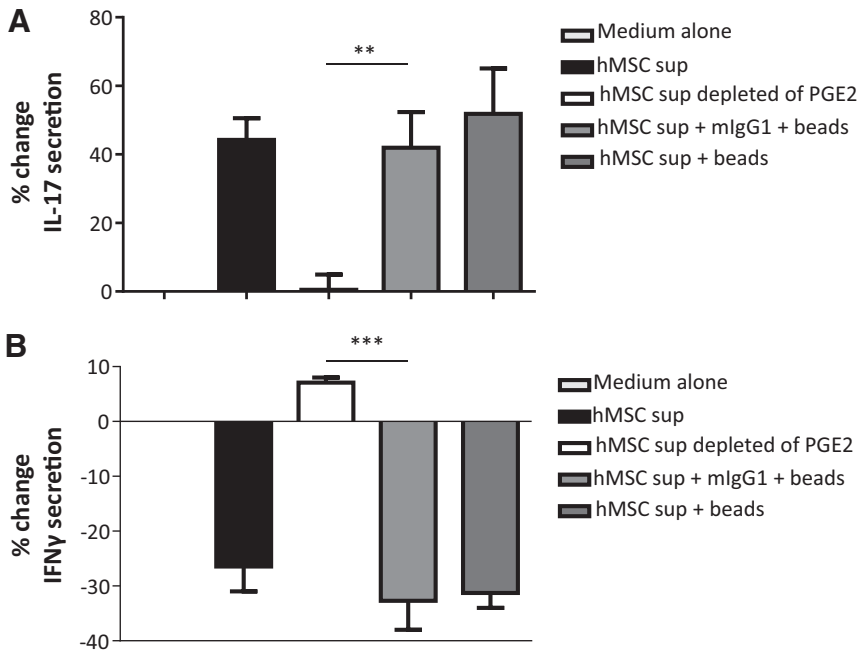

Figure 5. The capacity of myeloid cells to mediate the effects of hMSCs on peripheral blood mononuclear cell cytokine responses is PGE2-dependent. The ability of CD14+ cells that were pre-exposed to hMSC sups to enhance T-cell expression of IL-17 (n = 5, **, $p \leq .01$) **(A)** and to suppress T-cell expression of IFNγ (***, $p \leq .001$) **(B)** was abrogated if the hMSC sups were depleted of PGE2 prior to being added to the CD14+ cells. Abrogation of these effects was not seen under the control conditions. Abbreviations: hMSC, human mesenchymal stem cells; IFNγ, interferon-γ; IL, interleukin; PGE2, prostaglandin E2; sup, supernatant.

therapy in humans will only be possible in the context of carefully designed clinical trials. We advocate for close monitoring of disease activity as well as thoughtful incorporation of immune-monitoring strategies that could assess immune response profiles of individual patients, because MSCs are being introduced into patients with MS and other autoimmune diseases.

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Author Contributions

A. Rozenberg: performance of the experiments, data analysis and interpretation, manuscript writing, final approval of manuscript; A. Rezk, M.-N.B., P.J.D., M.N., R.L., and F.J.: collection and/or assembly of data, technical advice; R.W. and E.A.A.: figure revisions; A.U.: conception and design, data analysis and interpretation, and manuscript writing; J.S.R.: coordination and quality control of hMSC isolation process from donors; S.M.P. and J.A.C.: provision of study material or patients, study coordination, and manuscript writing; A.B.-O.: study oversight, supervision of the experiments, data analysis, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

J.A.C. is a compensated consultant for Genentech, Genzyme, and Novartis; has compensated honoraria from Teva; and has research funding from Genzyme, Novartis, Receptos, Synthon, and Teva. The other authors indicated no potential conflicts of interest.

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