Human placental mesenchymal stem cells (pMSC) play a role as immune suppressive cells by shifting macrophage differentiation from inflammatory M1 to anti-inflammatory M2 macrophages.

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Running Title: Placental mesenchymal stem cells shift macrophage differentiation from inflammatory M1 to anti-inflammatory M2 type.

The authors declare no potential conflicts of interest

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Abstract

Background

Mesenchymal stem cells (MSCs) have a therapeutic potential in tissue repair because of capacity for multipotent differentiation and their ability to modulate the immune response. In this study, we examined the ability of human placental MSCs (pMSCs) to modify the differentiation of human monocytes into macrophages and assessed the influence of pMSCs on important macrophage functions.

Methods

We used GM-CSF to stimulate the differentiation of monocytes into the M1 macrophage pathway and then co-cultured these cells with pMSCs in the early stages of macrophage differentiation. We then evaluated the effect on differentiation by microscopic examination and by quantification of molecules important in the differentiation and immune functions of macrophages using flow cytometry and ELISA. The mechanism by which pMSCs could mediate their effects on macrophage differentiation was also studied.

Results

The co-culture of pMSCs with monocytes stimulated to follow the inflammatory M1 macrophage differentiation pathway resulted in a shift to anti-inflammatory M2-like macrophage differentiation. This transition was characterized by morphological of changes typical of M2 macrophages, and by changes in cell surface marker expression including CD14, CD36, CD163, CD204, CD206, B7-H4 and CD11b, which are distinctive of M2 macrophages. Co-culture with pMSCs reduced the expression of the costimulatory molecules (CD40, CD80 and CD86) and increased the expression of co-inhibitory molecules (CD273, CD274 and B7-H4) as well as the

surface expression of major histocompatibility complex (MHC-II) molecules. Furthermore, the secretion of IL-10 was increased while the secretion of IL-1β, IL-12 (p70) and MIP-1α was decreased; a profile typical of M2 macrophages. Finally, pMSCs induced the phagocytic activity and the phagocytosis of apoptotic cells associated with M2- like macrophages; again a profile typical of M2 macrophages. We found that the immunoregulatory effect of pMSCs on macrophage differentiation was mediated by soluble molecules acting partially via glucocorticoid and progesterone receptors.

Conclusions

We have shown that pMSCs can transition macrophages from an inflammatory M1 into an anti-inflammatory M2 phenotype. Our findings suggest a new immunosuppressive property of pMSCs that may be employed in the resolution of inflammation associated with inflammatory diseases and in tissue repair.

Keywords

Placenta mesenchymal stem cells; immune suppression; macrophages; inflammation

Introduction

Mesenchymal stem cells (MSCs) are the main subset of adult stem cells that can be isolated from different human tissues [1,2]. MSCs are described as multipotent cells that can differentiate into cells of multiple organs and systems such as bone, fat, cartilage, muscle, neurons and hepatocytes [1].

We have successfully isolated MSCs from the human term placenta, a tissue which is accessible, abundant and commonly discarded after normal delivery, capable of self-renewal, capable of differentiation into lineages of mesenchymal tissues and with immunoregulation properties [2,3]. These properties of placental MSCs (pMSCs) make them an attractive alternative source of MSCs for cell-based therapy. The mechanisms underlying the immunoregulatory functions of MSCs are complex and not fully understood.

MSCs have immunomodulatory effects, particularly on T and B lymphocytes [3,4]. However, relatively little attention has been paid to the potential interaction of MSCs with macrophages. A few studies reported that MSCs can induce anti-inflammatory phenotypes in macrophages *in vitro* and *in vivo* [5-11]. However, studies of the immunomodulatory effects of pMSCs on the functions of macrophages have not been undertaken.

Macrophages comprise a heterogeneous population of cells that are widely distributed in many different tissues and are potent immune regulators [12]. Macrophages play a crucial role in the resolution of tissue injury and promotion of tissue repair in various human diseases [12]. In response to chemotactic signals, monocytes migrate into tissues and subsequently differentiate into macrophages to replace aged ones or to participate in the initial phases of tissue defense in

response to harmful insults [12]. Macrophages also play an essential role in later phases of tissue homeostasis and repair, such as phagocytosis of apoptotic cells [12]. Functionally, macrophages can be polarized into distinct subpopulations of cells; each with specific functions related to the initiation and recovery phases of tissue injury [12]. The origin and activation state of macrophages and their microenvironment are critical determinants of their response to injury. Typically, macrophages are classified into two main groups: classically-activated macrophages (M1) and alternatively-activated macrophages (M2) [12].

M1 macrophages are pro-inflammatory cells with potent anti-microbial activities and promote T helper 1 (Th1) cell responses and have also been implicated in many inflammatory diseases [12]. Conversely, M2 macrophages are immunosuppressive cells that can support helper 2 (Th2)-associated effector functions. M2 macrophages are characterized based on their low production of proinflammatory cytokines, such as IL-1ß and IL-12, high production of anti-inflammatory cytokines such as IL-10 and have high phagocytic activities [12]. M2 macrophages play a major role in the resolution of inflammation, tissue remodeling and in wound repair by releasing IL-10, secreting trophic factors and enhancing the clearance of apoptotic cells [12].

Macrophage heterogeneity is likely to reflect the plasticity and versatility of these cells in response to microenvironmental signals. The differentiation of macrophages and dendritic cells from monocytes is reversible and this is determined by the microenvironments and the differentiation signals [13]. Various signals induce the differentiation of M1 or M2 macrophages. The differentiation pathway of M1 macrophage is activated by different molecules, such as interferon gamma (IFN-γ) or granulocyte macrophage colony stimulating factor (GM-CSF)

while the differentiation of M2 macrophages is promoted by different molecules, such as IL1 receptor antagonist, IL-4, IL-6, IL-10, IL-13, glucocorticoids, macrophage colony stimulating factors (M-CSF), vascular endothelial growth factor (VEGF), transforming growth factor beta- 1 (TGF-β1), leukemia inhibitory factor (LIF) and prostaglandin E2 (PGE2) [14-18].

Several studies have demonstrated the protective role of macrophages in cell therapy. The beneficial therapeutical effects of macrophages were shown in various tissue injury, such as central nervous system (CNS), heart and lungs [8,10,11,19,20]. For example, the immune system is activated following injury to the CNS. This affects the ability of neurons to survive and to regenerate damaged axons. However, the role of the immune system in the CNS is determined by the type and duration of the immune response and the balance between destructive and protective factors that ultimately define the net result of the neuro-immune interaction [21]. Injury in the CNS rapidly induces the response of M1 macrophages and then this response is shifted into M2 macrophages [21]. The response of M1 macrophages induces neuronal damage, whereas M2 macrophage response promotes neuronal regeneration [21]. Therefore, switching macrophages from M1 into M2 phenotype can promote the repair of CNS. Accordingly, modulating immune response seems to be a promising strategy for successful tissue repair. This reversibility in macrophage functions will be one of the useful therapeutic targets for the design of an efficient cellular therapy utilizing macrophages to remodel and repair tissue injury. Several recent studies showed that macrophages can be switched from inflammatory into anti-inflammatory phenotypes following their interaction with MSCs; this interaction resulted in the resolution of inflammation and tissue repair of heart, lung and skin [5-11]. These studies used MSCs from bone marrow, adipose tissues, gingiva and cord blood, but the interaction between pMSCs and macrophages has not been examined. pMSCs used in regenerative or reparative therapies are likely to interact

with macrophages and their immunoregulatory abilities could potentially influence macrophage differentiation and/or function. Therefore, we examined whether human pMSCs can induce anti-inflammatory phenotypes in human macrophages and also studied the mechanism by which pMSCs can shift macrophage differentiation.

Previous studies demonstrated that molecules with anti-inflammatory properties can mediate MSC induction of M2 macrophage differentiation [15-18,22], and therefore we examined the role of glucocorticoid receptor (GR) and progesterone receptor (PR) in mediating the anti-inflammatory effects of pMSCs on macrophages. These two receptors are involved in the production of anti-inflammatory proteins, promotion of phagocytosis of apoptotic cells and the differentiation of anti-inflammatory M2 macrophages [18,23]. We found that human pMSCs can induce an anti-inflammatory phenotype in macrophages by shifting the differentiation of macrophages from M1 into M2 macrophages and also by down-regulating the expression of costimulatory molecules (CD40, CD80 and CD86) with induced expression on co-inhibitory proteins (CD273, CD274 and B7H4). In addition, we found the immunoregulatory effects of pMSCs on human macrophages are mediated partially via GR and PR.

Materials and Methods

Ethics of Experimentation

This study was approved by the institutional research board at King Abdulla International Medical Research Centre/ King Abdulaziz Medical City, Riyadh, Saudi Arabia. All placentae were obtained with informed patient consent.

Placentae

Human placentae were obtained from uncomplicated pregnancies following normal vaginal delivery (38- 40 weeks of gestation). The gestational age and fetal viability of all pregnancies were confirmed by early ultrasound examination before 20 weeks gestation. The placentae were used within 2 h of delivery.

Isolation of mesenchymal stem cells from placental explants in vitro

pMSCs were isolated from chorionic villi of human term placenta as described previously [2]. Briefly, placental tissues were dissected and then washed thoroughly with sterile phosphate buffered saline (PBS), pH 7.4. After removing the maternal decidua, the chorionic villi from the fetal portion were cut into pieces of approximately 40 mg wet weight and washed with PBS and then incubated with 2.5% trypsin (Invitrogen, Saudi Arabia) diluted in DMEM-F12 medium (Invitrogen, Saudi Arabia) containing (271 unit/ml) DNase (Invitrogen, Saudi Arabia), 100 μg/ml streptomycin and 100 U/l penicillin with gentle rotation overnight at 4°C. Tissues were then washed thoroughly with PBS and allowed to adhere to the plastic in 6 well plates for 1 h at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air (a cell culture incubator). Then, DMEM-F12 medium containing 10% Mesenchymal Stem Cell Certified fetal bovine

serum (MSCFBS) (Invitrogen, Saudi Arabia), 100 μg/ml of L-glutamate, 100 μg/ml streptomycin and 100 U/l penicillin was gently added to the tissues and cultured at 37°C in a cell culture incubator. Every two days, the medium was removed and replaced with fresh medium. On day 14, the tissues were removed and cells migrated out from the cut ends of the tissues were then harvested with TrypLE™ Express detachment solution (Invitrogen, Saudi Arabia) and characterized by flow cytometry using MSC positive markers (CD44, CD90, CD146, CD166 and CD105) and hematopoietic negative markers (CD19, CD45, HLA-DR, CD80, CD86, and CD40) as described previously [2]. Cells at a density of 1 x 10^5 cells in 75 cm² flask (BD, Saudi Arabia) were re-cultured until they reached 75% confluency and then used in subsequent experiments.

Before any further experimental uses, pMSCs at passage 2 were assessed for differentiation into adipocytes, chondrocytes and osteocytes as described previously [2]. Adipogenic, osteogenic and chondrogenic differentiations were performed by incubating pMSCs in adipogenic (#390415), osteogenic (#390416) and chondrogenic (#390417) media, respectively. All differentiation media were purchased from R & D Systems (Saudi Arabia). Each differentiation medium was supplemented with 10% MSCFBS, 100 μg/ml of L-glutamate, 100 μg/ml streptomycin and 100 U/l penicillin. Adipocytes, osteoblasts and chondrocytes were identified using LipidTOX™ Green, Alizarin Red S and Alcian Blue dyes, respectively as described previously[2]. All antibodies were from Beckman Coulter (Saudi Arabia). Thirty placentae were used in this study.

Isolation of human monocytes

Peripheral blood mononuclear cells (PBMCs) were obtained from venous blood drawn from 30 normal healthy volunteers. PBMCs were isolated by centrifugation on a Ficoll-Paque Leucosep™ density gradient **(**Greiner Bio-One, Saudi Arabia) and suspended in RPMI-1640 medium. Monocytes were obtained using a magnetic cell separation system as described

previously [24]. PBMCs were incubated with anti-CD14 monoclonal antibody-coated microbeads, and monocytes were then isolated by passing the PBMCs through a column placed in a magnetic cell separator according to the manufacturer's instructions. The purity of monocytes was assessed by flow cytometry using an anti-CD14 monoclonal antibody. Samples with purity greater than 98% were used for experiments.

Culture of monocyte derived macrophages with different treatments of pMSCs (direct contact, supernatant and conditioned medium)

Monocytes were differentiated into M1 macrophages as described previously [25]. Monocytes at a density of 1 x 10^6 were seeded in 6-well culture plates in M1 differentiation medium consisting of RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 ng/mL GM-CSF (R and D Systems, Saudi Arabia), 50 U/mL penicillin and 50 µg/mL streptomycin and then incubated for 6 days at 37°C in a cell culture incubator. For intercellular direct contact experiments (ICpMSC), the 0.4µm pore size transwell chamber membrane culture system (Greiner Bio-One, Saudi Arabia) was used as described previously [26]. pMSCs were seeded on the reverse side of the membrane of the chamber until the cells were fully adhered and monocytes were seeded on the upper side of the membrane. For soluble factor experiments (SFpMSC), pMSCs were physically separated from monocytes by culturing them on the upper compartments while monocytes were cultured in the lower compartment. Both culture systems prevent the contamination of monocyte-derived macrophages with pMSCs and facilitate harvesting macrophages without pMSC contamination. In both systems, cells were cultured in M1 macrophage differentiation medium for six days as described previously [25]. For conditioned medium experiments (CMpMSC), supernatant from unstimulated pMSCs were added to monocytes cultured in M1 differentiation medium. To produce conditioned medium, 1x10^5

pMSCs were cultured in 75 cm² flasks containing DMEM-F12 medium with 10% MSCFBS, 100 µg/ml of L-glutamate, 100 µg/ml streptomycin and 100 U/l penicillin. Every two days, the medium was removed and replaced with fresh medium. When cells reached 75% confluency, cells were cultured in fresh medium for two days and conditioned medium was then harvested, centrifuged at 500 x g for 10 min and stored at -80ºC until use. On day 7, macrophages and pMSCs were harvested with TrypLE™ Express detachment solution and characterized by flow cytometry technique. Macrophages were characterized for the expression of M2 macrophage markers (CD14, CD11b, CD36, CD163, CD204, CD206 and B7H4), costimulatory molecules (CD40, CD80, CD86, CD273 and CD274) and MHC class II molecule, HLA-DR while pMSCs were characterized for the expression of the intracellular proteins including IL1-ß, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor- alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), indoleamine 2,3-dioxygenase (IDO) and B7H4). In some experiments ICpMSC, SFpMSC, CMpMSC were added to M1 macrophage culture on either day 3 or day 7 and incubated for further three days and then characterized as described above. Before the addition of pMSCs to the coculture experiments (ICpMSC and SFpMSC), pMSCs were treated with 25µg/ml Mitomycin C for 1 h at 37°C to inhibit their proliferation and then followed by five extensive washes with RPMI-1640 medium containing FBS as described previously [27]. To assess whether pMSCs effects on macrophage differentiation were reversible, ICpMSC or SFpMSC or CMpMSC were removed after three days of co-culturing with monocytes and macrophages were washed thoroughly and then cultured again in M1 macrophage differentiation medium containing GM-CSF for another three days. Each experiment was performed in duplicate and repeated with 30 independent preparations of monocyte-derived macrophages and pMSCs. Monocyte-derived macrophages cultured in a GM-CSF medium without pMSCs were included

as a negative control for all monocyte-derived macrophages cultured with pMSCs (ICpMSC, SFpMSC and CMpMSC).

pMSC induction of M2 macrophage differentiation

CMpMSC experiments were performed in which 500nM/mL Mifepristone (Tocris Bioscience, Saudi Arabia) in M1 macrophage differentiation medium containing 20% CMpMSC was added to monocyte culture on day zero. The dose of 500nM/mL Mifepristone was used because this dose was shown by others to result in a complete blockage of binding receptors [28]. Positive control was macrophages cultured in M1 macrophage differentiation medium containing 100µM/ mL dexamethasone in the presence and absence of Mifepristone. After six days in culture, macrophages were then harvested as described above. The mean number of differentiated cells was quantified by counting the number of cells in 10 random high-power fields (40×) on the culture plates. Monocytes cultured with CMpMSC without Mifepristone were included as a negative control. Each experiment was performed in duplicate and repeated with 10 independent preparations of monocytes- derived macrophages and pMSCs.

Phagocytic function of monocyte derived macrophages

To study the phagocytic function of macrophages, the CytoSelect™ Phagocytosis kit was used (# CBA-224**,** Cell Biolabs, Saudi Arabia) according to the manufacturer's instructions. Briefly, monocyte-derived macrophages were cultured for 6 days with ICpMSC or SFpMSC or CMpMSC in 24-well flat-bottom culture plates containing M1 differentiation medium, consisting of RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 ng/mL GM-CSF, 50 U/mL penicillin and 50 µg/mL streptomycin. On day 7, pre-labelled- Zymosan

suspension was added to each well and then incubated for 2 h at 37°C in a cell culture incubator. Negative controls of macrophages without ICpMSC or SFpMSC or CMpMSC and cells without Zymosan particles were also included. Following removal of the culture medium, cells were washed, fixed and incubated with a blocking solution for 1 h at room temperature. Cells were then washed, permeabilized and incubated with a detection reagent for 1 h at room temperature. Following washing, cells were incubated with a detection buffer for 10 min at room temperature and then incubated with a substrate to initiate the reaction for 20 min at 37ºC. Finally, the reaction was stopped and the optical density was read at 405 nm. Each experiment was performed in duplicate. 10 individual preparations of monocyte-derived macrophages and 10 pMSCs were used in this study.

Phagocytosis of apoptotic cells by macrophages

Following six days of coculture of monocyte- derived macrophages with CMpMSC, macrophages were labelled with 5 μM green fluorescent cell tracker stain (5-chloromethylfluorescin diacetate; CMFDA; Molecular Probes, Invitrogen, Saudi Arabia) in DMEM/F12 Medium for 4 h. Apoptotic pMSCs were generated by freezing and thawing method as described previously [29] and labelled with 5 μM red fluorescent cell tracker stain 5-(and-6)-carboxy SNARF®-1, acetoxymethyl ester, acetate (SNARF; Molecular Probes, Invitrogen, Saudi Arabia) for 4 h and then added to macrophage culture at a ratio of 1 (Apoptotic pMSCs): 10 (macrophages) for 24 h in the presence or absence of 10 μg/mL cytochalasin B (Invitrogen, Saudi Arabia). Apoptotic pMSCs from each placenta were added to macrophages isolated from an individual (independent) donor. Remaining apoptotic cells were removed by extensive washing with PBS, and macrophages were fixed with 4% (w/v) paraformaldehyde and visualized

on an inverted fluorescent microscope (Nikon ECLIPSE Ti U) and photomicrographs recorded using a Nikon DS-Qi1 camera and software. The percentage of macrophages containing apoptotic cells was reported as the phagocytic index using the following formula:

Phagocytic index (PI) = Apoptotic cells associated with macrophages/Total number of macrophages X 100

Comparison between the effects of pMSCs and bone marrow derived MSCs on the differentiation of macrophages

To compare between the effects of pMSCs and BMMSCs on macrophage differentiation, we used BMMSCs (Catalogue # C-12974, Promocell, Saudi Arabia). BMMSCs (1 x 10^5 cells) were cultured in in 75 cm² flask containing αMEM supplemented with 10% FBS, 100 µg/ml of L-glutamate, 100 µg/ml streptomycin and 100 U/l penicillin. Cells were then harvested and characterized by flow cytometry and used for co-culture studies. BMMSCs were positive for MSC markers (CD44, CD90 and CD105) and negative for hematopoietic markers (CD45) (data not shown). Intracellular contact (IC) and soluble factor (SF) experiments were performed as described above. Macrophage-like cells were characterized for the expression of M2 macrophage markers (CD163 and CD206) by flow cytometry. Soluble factors [IL-10 and IL12 (p70)], which are secreted by macrophages, were measured by ELISA. Each experiment was performed in duplicate. Three independent experiments were performed in this study.

Quantification of human cytokines

ELISA Kits (R & D Systems or MyBiosource, Saudi Arabia) were used according to the manufacturer's instructions to quantify IL-1β, IL10, IL-12 (p70), MIP-1α and MCP-1 in the

supernatants obtained from macrophage culture, whilst M-CSF, TGFß-1, VEGF, LIF, B7-H4, and PEGE2 were quantified in the CM of unstimulated pMSCs or pMSC stimulated with macrophages. Both unstimulated and stimulated pMSCs were cultured at confluency for two days. To produce CM of stimulated pMSCs, soluble factor experiments (SFpMSC) were performed as described above. On day 7, the transwell coated pMSCs were washed thoroughly with PBS and then cultured in DMEM-F12 medium containing 10% MSCFBS, 100 µg/ml of L-glutamate, 100 µg/ml streptomycin and 100 U/l penicillin for two days. Complete RPM-1640 and DMEM-F12 were included as a negative control.

Flow Cytometry

Cells were harvested using TrypLE™ Express detachment solution. For analysis, 1 x 10^5 of cells were stained with antibodies (Table 1) for 30 min and then washed twice with cold PBS by centrifugation at 150 x g for 5 min at 8°C. To analyse intracellular expression of IL1-ß, IL-6, IL-8, IL-10, IL-12, TNF-α, MCP-1, IDO and B7H4, cells were fixed with 4% paraformaldehyde in sterile PBS, pH 7.4 for 10 min at room temperature and then permeabilized using a sterile PBS, pH 7.4 containing 0.1% saponin for 5 min at room temperature. The expression of the corresponding intracellular and cell surface proteins was assayed by an FC500 (Beckman Coulter, Saudi Arabia) flow cytometer. As a negative control for analysis, cells in a separate tube were treated with FITC or PE-labeled mouse IgG or IgM antibody

Statistical analysis

Data were analyzed using the Mann-Witney U and Kruskal- Wallis tests for non-parametric data. These analyses were performed using GraphPad Prism 5. Results were considered to be statistically significant if $P < 0.05$.

Results

Isolation and characterization of pMSCs from human placental chorionic villi

All pMSCs derived from normal healthy placenta at passage 2 were more than 95% positive for MSC markers (CD44, CD90, CD146, CD166 and CD105) and negative for hematopoietic markers (CD19, CD45, HLA-DR, CD80, CD86, and CD40), this was consistent with our published study [2]. pMSCs derived from the placenta in this study were successfully differentiated into bone, fat and cartilage *in vitro* using appropriate growth factors and methods we described recently (data not shown) [2]. Based on the above criteria, we used pMSCs at passage 2 in the subsequent experiments.

Human pMSCs secrete molecules inducing immunoinhibitory M2 macrophage differentiation

Molecules, such as M-CSF, VEGF, TGFß-1, LIF, B7-H4 and PGE2, induce the differentiation of M2 macrophages or are present in the M2 macrophage environment [17]. Therefore, we screened conditioned medium obtained from pMSCs stimulated with macrophages (as described above) or unstimulated pMSCs, for the secretion of these molecules. ELISA assays detected M-CSF (1217 pg/ml ±80), VEGF (100 pg/ml ±15), TGFß-1 (40 pg/ml ±5), LIF (300 pg/ml ±10), B7-H4 (100ng/ml ±7) and PGE2 (200 pg/ml ±21) in the CM of unstimulated pMSC while we detected M-CSF (2640 pg/ml ±60), VEGF (180 pg/ml ±20), TGFß-1 (120 pg/ml ±15), LIF (450 pg/ml ±25), B7-H4 (210ng/ml ±14) and PGE2 (350 pg/ml ±30) in the CM of pMSC stimulated with macrophages for six days. There were 2.16, 1.8, 3, 1.5, 2.1 and 1.75 fold increases in the concentration of M-CSF, VEGF, TGFß-1, LIF, B7-H4 and PGE2, respectively in the conditioned

medium of stimulated pMSCs suggesting that the pre-stimulation of pMSCs with macrophages induced the secretion of these molecules by pMSCs.

Monocytes cultured in direct or indirect contact with human pMSCs or with pMSC conditioned medium, shifted the initial differentiation of human monocytes from M1 inflammatory into M2-like anti-inflammatory macrophages.

$CD14^+$ monocytes isolated from normal human peripheral blood were induced to differentiate into M1 macrophages through culture with GM-CSF for six days. On day 7, monocytes gave rise to "fried egg-shaped" morphology; a typical feature of M1-like macrophages [30] (Fig. 1A). These M1-like macrophages expressed the monocytic marker CD14 in the absence of the typical dendritic cell marker CD1a (data not shown). To examine the ability of pMSCs in inducing anti-inflammatory phenotype in macrophages, we examined the effects of pMSCs on the initial differentiation of macrophages. Monocytes were differentiated with GM-CSF in the presence or absence of pMSCs (ICpMSC, SFpMSC and CMpMSC). For ICpMSC and SFpMSC experiments, dose-response experiments were conducted in triplicate, using ratios of 1:1, 1:5 or 1:10 pMSCs: macrophages. The data indicated that with increasing amounts of pMSCs, there was increasing responsiveness of the monocyte-derived macrophages (Table 2), but morphologically, monocytes did not change into M1 or M2 like macrophages and remained loosely adhered to the surface of the culture plate at ratios 1: 1 and 1: 5. Therefore, we used a ratio of 1:10 pMSCs: macrophages for all ICpMSC and SFpMSC experiments conducted in this study. In addition, dose-response experiments using different concentrations of conditioned medium, 1%, 5%, 10%, 20%, 40%, 60%, were performed. We found that the concentration which gives a maximum response of macrophages was 20% and therefore we used a

concentration of 20% in this study (Table 3). Compared to untreated monocytes, pMSCs (ICpMSC or SFpMSC or CMpMSC) strongly inhibited the differentiation of monocytes into M1-like macrophages as cells displayed an elongated, spindle-like morphology, a typical feature of M2 macrophages [30] (Fig. 1B) and continued to express CD14 in the absence of CD1a (data not shown). In addition, we evaluated whether monocytes, differentiated in the presence of ICpMSC or SFpMSC or CMpMSC, expressed molecules characteristic of M2 macrophages including CD14 [30], CD36 [31], CD163 [16,30], CD204 [32], CD206 [5], B7-H4 [33] and CD11b [30]. These functional markers were studied by flow cytometry and expression of CD14, CD36, CD163, CD204, CD206 and CD11b was recorded as median fluorescence intensity (MFI) while the expression of B7-H4 was recorded as median percentage of positive cells. After six days culture with pMSCs (ICpMSC or SFpMSC or CMpMSC), the expression by M2-like macrophages of CD14, CD36, CD163, CD204 and CD206 and B7-H4, was significantly increased ($P<0.0001$ with ICpMSC or SFpMSC but $P<0.01$ with CMpMSC) compared to untreated M1-like macrophages (Fig. 1C- H). In contrast, the expression of CD11b was significantly decreased ($p<0.0001$) on M2-like macrophages following culturing with pMSCs (ICpMSC or SFpMSC or CMpMSC) (Fig. 1I).

The data of SFpMSC experiments provided evidence that soluble factors secreted from pMSCs were capable of shifting monocyte differentiation from M1 into M2 macrophages while the data with CMpMSC experiments suggested that the production of inhibitory soluble factors produced by pMSCs does not require paracrine signals from monocyte/macrophage cells. Compared with ICpMSC, SFpMSC showed less profound suppression effects on macrophage differentiation (Fig. 1), but when the results of CMpMSC were compared with ICpMSC or SFpMSC, the CMpMSC showed less profound suppression effects on macrophage differentiation (Fig. 1).

In some experiments, the effects of pMSCs (ICpMSC, SFpMSC and CMpMSC) on macrophage differentiation was examined after adding pMSCs to monocyte culture in the middle or after the end of the culture (on day 3 or day 7) and then cultured for another three days. Similar to the results described above, pMSCs (ICpMSC, SFpMSC and CMpMSC) induced M2-like macrophage differentiation (data not shown). These data suggest that pMSCs can affect macrophage differentiation at various times during culture. Similarly, pMSCs (ICpMSC, SFpMSC and CMpMSC) induced M2-like macrophage differentiation after coculture with monocytes in the absence of GM-CSF for seven days (data not shown). Thus, these data suggest that pMSCs can induce M2 macrophage differentiation from monocytes whether monocytes cultured in M1 macrophage differentiation medium (Inflammatory environment) or cultured in RPM1640 culture medium without GMCSF (Normal environment). Thus, these experimental conditions demonstrate that pMSCs can induce M2 macrophage differentiation from monocytes regardless to the environment of monocytes.

pMSC conversion of M1 macrophage to M2-like macrophages is reversible

Next, we investigated whether the inhibitory effects of pMSCs (ICpMSC, SFpMSC and CMpMSC) on the initial differentiation of macrophages was persistent or reversible. The aim of this study is to investigate whether pMSCs can shift the initial differentiation of monocytes from M1 macrophages into M2 macrophages. Therefore, we examined the effects of pMSCs on macrophage differentiation using the three- day co-cultures experiment of pMSCs and monocytes rather than a six-day co-culture experiment. pMSCs were harvested from monocyte culture on day 3 and then monocyte- derived macrophages were washed and cultured alone in fresh M1-like macrophage differentiation medium for another 3 days. The differentiation of M1-

like macrophages under these conditions occurred since macrophages displayed fried egg-shaped morphology (M1-like macrophages) on day seven (data not shown). In addition, these M1-like macrophages expressed reduced levels of CD14, CD163, CD204, B7-H4 and CD206 (Fig. 1A- D and F) and displayed increased levels of CD11b (Fig.1E) as compared to M2-like macrophages cocultured with pMSCs. These data indicated that the inhibitory effect of pMSCs on monocyte differentiation was reversible.

pMSCs significantly modulated the expression of M2-like macrophage markers CD40, CD80, CD86, CD273, CD274 and MHC class II complex

To evaluate whether the coculture of pMSCs (ICpMSC, SFpMSC and CMpMSC) with monocyte-derived macrophages would alter the functions of M2-like macrophages, a variety of immune functional markers were studied by flow cytometry and expression recorded as median fluorescence intensity. After six days culture with pMSCs, M2-like macrophage expression of CD40, CD80 and CD86 was significantly reduced ($P<0.01$) compared to untreated M1-like macrophages (Fig. 3A- C). In contrast, the expression of CD273, CD274 and HLA-DR was significantly increased ($p<0.01$) on M2-like macrophages after incubation with the indicated treatments (Fig. 3D- F).

Modulation of IL-10, IL1β, IL-12 (p70), MIP-1α and MCP-1 secretion by M2-like macrophages following culturing macrophages with pMSCs

To further confirm that pMSCs induced anti-inflammatory phenotypes in M2-like macrophages, the secretion of cytokines by M2-like macrophages was determined following culturing monocyte- derived macrophages with SFpMSC for six days. The level of IL-10 secreted by M2-like macrophages was significantly increased ($P<0.0001$) following culturing with SFpMSC (Fig. 4A). In contrast, SFpMSC significantly decreased the secretions of IL-1β, IL-12 (p70) and MIP-1α ($P<0.0001$) (Fig. 4B-D), while the secretion of MCP-1 by M2-like macrophages was not significant ($P= 0.06$) as compared to untreated M1-like macrophages (Fig. 4E).

M2-like macrophages cocultured with pMSCs showed increased phagocytic activity

As described above, pMSCs can shift the initial differentiation of monocytes from M1 inflammatory into M2 anti-inflammatory macrophages. In addition, pMSCs significantly modified the functions of M2-like macrophages by altering the expression of various immunological markers including CD40, CD80, CD86, CD273, CD274 and HLA-DR. Therefore, we determined whether the immunoinhibitory effects of pMSCs on M2-like macrophage function would affect the phagocytic function of M2-like macrophages. For this purpose, the difference in the phagocytic function between M2-like macrophages initially cultured with different pMSC treatments (ICpMSC, SFpMSC and CMpMSC) for 6 days and untreated M1-like macrophages was measured using the CytoSelect™ phagocytosis functional assay. Untreated and treated macrophages were incubated with or without Zymosan particles, and a colorimetric reaction was then performed to detect the engulfed particles by reading the optical density at 405nm. Assays performed at 37°C to allow phagocytosis showed higher

activity in M2-like macrophages cultured with different pMSC treatments compared to untreated M1-like macrophages (Fig. 5A and B). Fold increases of phagocytic functions of M2-like macrophages treated with ICpMSC, SFpMSC, CMpMSC over untreated M1-like macrophages, as determined by optical density, were 3.5±0.53 (P = 0.0002), 2.75±0.49 (P = 0.002) and 2.0 (P = 0.0002), respectively. pMSCs in direct contact with monocytes (ICpMSC) showed more profound effects on the phagocytic activity of M2-like macrophages than soluble factors (SFpMSC) and CMpMSC (Fig. 5A and B). The phagocytic functions of untreated and treated macrophages were achieved by the incubation with 2µM Cytochalasin D (phagocytosis inhibitor; Invitrogen, Saudi Arabia) for 1 h at 37ºC before addition of Zymosan particles (data not shown).

pMSCs stimulate phagocytosis of apoptotic cells by M2-like macrophages

Similar to other immune cells such as dendritic cells and T cells, macrophages can differentiate into different profiles when suitable microenvironments and differentiation signals are provided [34,35]. As discussed above, the expression of functional molecules, and the profile of cytokines secreted by M2-like macrophages cultured in the presence of pMSCs, suggest that macrophages differentiated into anti-inflammatory M2-like macrophages with the ability to resolve inflammation by secreting anti-inflammatory cytokines and also by stimulating the phagocytosis of apoptotic cells [35,36]. Therefore, we evaluated whether pMSCs stimulated the ability of M2-like macrophages to phagocytose apoptotic cells. Experiments were performed by generating M2-like macrophages in the presence of pMSCs in direct contact with pMSCs (ICMSC) for six days, as discussed above. Then, apoptotic cells (apoptotic pMSCs) were added to M2-like macrophage culture (M: apoptotic cells ratio = 10:1) for 24 h at 37°C. Phagocytosis was evaluated by fluorescence microscopy using apoptotic cells labeled with the red fluorescent dye CMFDA and

M2-like macrophages were labelled with the green fluorescent dye SNARF. Results in Figure 5D showed that pMSC stimulated phagocytosis of apoptotic cells by M2-like macrophages and the phagocytic index was calculated to be 87% (±3.50 S.E) (Fig. 5C). Phagocytosis of apoptotic cells was reduced (phagocytic index 13% ±2.85 S.E) by the inhibitor of phagocytosis, cytochalasin B confirming that the apoptotic cells had been phagocytosed and was not just adherent to the surface of the M2-like macrophages (Fig. 5C). Control M2-like macrophages not exposed to apoptotic cells did not exhibit any red fluorescence. These results further confirm the phagocytic activity of M2-like macrophages generated in the presence of pMSCs as discussed above. The phagocytosis results confirm our results discussed in Figure 1 which demonstrated the increased expression of receptors involved in the clearance of apoptotic cells [37] including the scavenger receptor CD36 and the glycosylphosphatidylinositol-anchored LPS receptor CD14 [37,38]. Interestingly, a similar enhancing effect was found using conditioned medium collected from unstimulated pMSC (CMpMSC) (data not shown), supporting the notion that stimulation of phagocytosis of apoptotic cells is mediated by the release of factors constitutively secreted by pMSC.

Mechanism of pMSC shifting macrophage differentiation from M1 into M2 macrophages

To gain further insight into the mechanism underlying the immunoinhibitory effects of pMSCs on macrophage differentiation, we tested the hypothesis that conditioned medium from unstimulated pMSCs contains soluble factors that can mediate the immunoinhibitory effects of pMSCs on macrophages via the glucocorticoid receptor (GR) and progesterone receptor (PR). For this purpose, we cultured monocytes in medium containing GM-CSF and conditioned medium of unstimulated pMSCs in the presence or absence of Mifepristone, a potent GR and PR antagonist for 6 days. Positive control was monocytes cultured in medium containing GM-CSF

and 100µM/ml dexamethasone in the presence or absence of Mifepristone. Compared to untreated M2-like macrophages, Mifepristone partially blocked the immunoinhibitory effects of pMSC conditioned medium on macrophage differentiation since the culture contained a heterogonous population of cells including M1-like macrophages (6%) and M2-like macrophages (40%) as well as non-adherent cells (54%) (Fig. 6C). In addition, the expression of CD163, CD204, CD206 and B7-H4 by monocytes-derived cells (M1-like, M2-like macrophages and non-adherent cells,) was also examined. After six days incubation with Mifepristone, the expression by monocytes-derived cells of CD163 and CD204 was significantly decreased ($P = 0.0002$) compared to M2-like macrophage-treated with pMSC conditioned medium, but was not changed significantly ($P>0.05$) as compared to untreated M1-like macrophages (Fig. 6E and G). In addition, the expression of B7-H4 was significantly decreased ($P= 0.0002$) on monocytes-derived cells after incubation with Mifepristone as compared with M2-like macrophage treated with pMSC conditioned medium, but was significantly increased ($P= 0.0002$) as compared to untreated M1- like macrophages (Fig. 6D). In contrast, the expression of CD206 was increased but not significantly ($P> 0.05$) on monocyte-derived cells after incubation with Mifepristone compared to M2-like macrophages treated with pMSC conditioned medium (CMpMSC) but significantly increased ($P= 0.0002$) as compared to untreated M1-like macrophages (Fig. 6F).

We also measured the concentrations of cytokines in the supernatant of monocytes-derived cells -treated with pMSC conditioned medium in the presence and absence of Mifepristone. The levels of IL-10 secreted by monocytes- derived cells was significantly decreased ($P<0.0001$) following culturing with Mifepristone as compared to M2- like macrophages treated with conditioned medium of pMSCs (Fig. 6H). In contrast, Mifepristone significantly increased the secretion of IL-1β, IL-12 (p70) and MIP-1α ($P<0.0001$) by monocyte-derived cells as compared to M2-like

macrophages treated with conditioned medium of pMSC (CMpMSC) (Fig. 6I- L). The levels of IL-10, IL12 (p70), MIP-1α and IL-1ß were not changed in the presence of Mifepristone when compared to untreated M1-like macrophages (Fig. 6H- L).

Macrophages up-regulated the expression of inflammatory and anti-inflammatory proteins in pMSCs

Macrophages and MSCs are highly plastic cells that undergo changes in their phenotypes according to the types of signals received from the neighbouring cells in the local environments [39]. These two cell types contribute significantly to the components of inflammation. Most importantly, they may interact during the course of inflammation. Therefore, we determined the effects of macrophages on pMSC phenotypes by identifying changes in the expression of intracellular proteins in pMSCs following their interaction with macrophages in the ICpMSC and SFpMSC. pMSCs showed increased protein expression indicative of a inflammatory phenotype (IL-8, IL-12 and MCP-1) and of an anti-inflammatory phenotype (IL-6, IL-10, IDO and B7H4) as shown in Fig 7.

pMSCs influence macrophage differentiation to a greater degree than BMMSCs

We compared the effects of pMSCs on macrophage differentiation, with a well-characterized type of MSCs; the BMMSC. We compared the effect of these MSC types on macrophage differentiation in the intercellular contact (IC) assay and the soluble factor (SF) assay as described above. Monocytes were cultured with pMSCs (ICpMSCs and SFpMSCs) or BMMSCs (ICBMMSCs and SFBMMSCs). Compared with BMMSCs, pMSCs induced significantly more

expression of CD163 and CD206 (Fig. 8 A and B respectively) in monocytes-derived cells (i.e. M2-like macrophages) after six days in culture. We also measured the concentrations of cytokines in the supernatant of monocytes-derived cells -treated with pMSCs and BMMSCs. The level of IL-10 secreted by monocytes- derived cells was significantly increased (P=0.02) while the level of IL-12 (p70) was not significantly changed (p>0.05) following culture of monocytes with pMSCs as compared to culturing with BMMSCs (Fig. 8C and D).

Discussion

In this study, we evaluated the effects of human placental MSCs on the initial differentiation of macrophages. This was accomplished by co-culturing pMSCs with macrophages, which were derived from normal human peripheral blood monocytes grown in medium containing GM-CSF; an inducer of M1 macrophage differentiation [25]. After 6 days culture, monocytes in GM-CSF medium differentiated into "fried egg-shaped" cells typical of M1-like macrophages (Fig. 1A). These M1-like macrophages expressed the monocytic marker CD14 but lacked the expression of the characteristic dendritic cell marker CD1a [40]. After adding pMSCs to the initial culture of monocytes in GM-CSF supplemented medium, monocytes differentiated into elongated, spindle-like cells; a distinctive feature of M2-like macrophages (Fig. 1B). We also observed that the expression of cell surface markers CD14, CD36, CD163, CD204, CD206 and B7-H4 by M2-like macrophages was significantly increased (Fig. 1C-H). These markers are usually upregulated in M2-like macrophages [16,30,32,33]. In addition, the expression of CD11b by M2-like macrophages was significantly decreased (Fig. 1I). This further characterizes M2 macrophages; thus further supporting the notion that the differentiation of monocytes into M2-like macrophages occurs in the presence of MSCs [30]. These results demonstrated that pMSCs can shift the initial differentiation of monocytes from M1 into M2 macrophages. Kinetic experiments with monocyte-derived macrophages revealed that the addition of pMSCs to monocytes cultured in GM-CSF medium on day 3, shifted macrophage differentiation from M1 into M2-like macrophages and this was similar to the effects of pMSCs when added to the culture on day zero. Importantly, experiments with monocytes in GM-CSF supplemented medium treated with pMSCs from day zero, with subsequent removal of pMSCs on day three, revealed that pMSC effects on monocyte differentiation were reversible as the fried egg-shaped morphology (M1-like

macrophages) appeared at the end of the culture. In addition, these M1-like macrophages expressed reduced levels of CD14, CD163, CD204, CD206 and B7-H4 and displayed increased levels of CD11b (Fig. 2A- F). This further confirms that the effects of pMSCs on monocyte differentiation were reversible.

Further evidence that pMSCs can induce an anti-inflammatory/suppressive phenotype in M2-like macrophages was our finding that pMSCs significantly down-regulated the expression of CD40, CD80 and CD86 by M2-like macrophages and that this was associated with increased expression of B7-H4 (Fig. 1H and 3A- C). The reduction in expression of CD40, CD80 and CD86 is important because *in vivo,* the blockade of CD28–B7 and CD40–CD40 ligand interactions prolongs graft survival and suppresses autoimmunity [41]. The increased expression of B7-H4 is an important immunosuppressive feature of M2 macrophages and it reflects the immunosuppressive properties of pMSCs. B7-H4 is an inhibitory co-regulatory molecule which belongs to the B7 family of proteins. It is expressed on antigen-presenting cells and non-immune cells, and interacts with program death receptor-1 (PD-1) on T cells leading to the inhibition of T-cell proliferation [42]. Several studies have shown the significance of the immunosuppressive properties of B7-H4. Blocking B7-H4 action in an experimental autoimmune encephalomyelitis (EAE) mouse model promoted T-cell responses and exacerbated disease [43]. Conversely, adenoviral-mediated transduction of islets with B7-H4 protected them from rejection when transplanted into allogeneic mice [44].

We also found that pMSCs significantly increased the expression of CD273 and CD274 by M2-like macrophages (Fig. 3D and E). The CD273 and CD274 signaling pathways play an important role in maintaining immune tolerance to allografted tissue and blockade of the CD273 and

CD274 signaling pathway can accelerate allograft rejection [45]. Our results suggest that the changes induced in the expression of macrophage signaling molecules following culture with pMSCs suggest that pMSCs may play an important role in the resolution of inflammation in patients affected by inflammatory diseases by changing the environment from inflammatory into anti-inflammatory as a result of shifting macrophage differentiation from M1 into M2 macrophages.

We further confirmed the immunosuppressive properties of pMSCs on M2-like macrophages by determining the levels of cytokines in the supernatant of M2-like macrophages culture. The results confirmed that pMSCs can modulate the differentiation of monocytes with increased secretion of anti-inflammatory cytokine IL-10 and reduced secretion of pro-inflammatory cytokines IL-1β, IL-12p70 and MIP-1α by M2-like macrophages (Fig. 4A- D). Importantly, the ability of pMSCs to inhibit the secretion of IL-1β, IL-12p70 and MIP-1α suggests that pMSCs may be able to reduce inflammation not only by inhibiting the secretion of pro-inflammatory cytokines during the early step of the inflammatory process, but also by inhibiting the activation of Th1 functions, which depend on Il-1β and IL-12 (p70) [46,47]. Our results also suggest that pMSCs induce an anti-inflammatory profile in M2-like macrophages not only by inhibiting the secretion of pro-inflammatory cytokines and increasing the secretion of anti-inflammatory cytokines, but also by inducing both the expression of cell surface MHC/HLA class II molecules (Fig. 3F) and the phagocytic function of M2-like macrophages (Fig. 5). Placental MSCs were able to increase the uptake of Zymosan particles by M2-like macrophages. In addition, pMSCs induced the phagocytosis of apoptotic cells by professional phagocytes as demonstrated in this study (Fig. 5C- E). The clearance of apoptotic cells plays a critical role in the resolution of

inflammation [37,38]. Generally, inflammation induces the recruitment of a huge number of leukocytes, which undergo apoptosis at the inflammatory site. If apoptotic cells are not efficiently removed, they will release intracellular components that are able to further increase the development of inflammation [37,38].

Interestingly, all these effects of pMSCs on M2-like macrophages were induced by pMSCs in direct or indirect contact with monocytes and by CM from unstimulated pMSCs. Many studies support our findings that soluble factors secreted from MSCs mediate the immunomodulatory effects on immune cells since the separation of MSCs and immune cells by a semi-permeable membrane (Transwell), does not prevent the immunomodulatory effects that MSCs exert on immune cells [48-50]. Importantly, our results also demonstrate that the immunosuppressive effects of pMSCs on macrophages are mediated by soluble factors secreted by unstimulated pMSCs suggesting that a crosstalk between pMSCs and immune cells is unnecessary. This is in contrast with previous studies which reported the necessity of activating MSCs for their immunomodulatory effects on immune cells [10,51,52]. However, our results showed that pre-stimulation of pMSCs with monocytes/ macrophages can induce the secretion of soluble factors by pMSCs. Interestingly, monocyte- derived macrophages induced the expression of intracellular inflammatory proteins IL8, IL-12 MCP-1 and anti-inflammatory proteins IL-10, IDO and B7H4 in pMSC cocultured with monocyte- derived macrophages for six days. These data suggest that macrophages can induce phenotype changes in pMSCs. The increased expression of IL-8 and MCP-1 (Fig. 7 B and E) in pMSCs by macrophages indicates that the migration of pMSCs towards macrophages is possible in inflammatory environment. Interestingly, macrophages induced a significant expression of IL-12 in pMSCs (Fig 7 C). This is an important finding which may indicate that pMSCs can deliver an anti-tumour activity via the expression of IL-12

upon interaction with macrophages in local tumour environment [53]. In addition, these pMSCs expressed high levels of immunosuppressive proteins IL-6, IL-10, IDO and B7H4 (Fig. 7F-I). These proteins can modulate the inflammatory effects of T cells. Thus, these data provide a framework for understanding the impact of macrophages on MSC phenotypes. However, other types of immune cells, such as dendritic and T cells may have differential effects on the biology of pMSCs. Therefore, further investigation of the interaction between immune cells and pMSCs in different microenvironments may lead to a more complete understanding of the mechanisms of MSC response during processes, such as tissue repair. Such study may provide the framework for tissue repair.

As we discussed above, our results show that pMSCs can induce many alterations in M2-like macrophage functions. Remarkably, each of these alterations may involve different mechanisms that all can resolve inflammation. One of the mechanisms that may be implicated in the resolution of inflammation is the phagocytosis of apoptotic cells. Our pMSCs upregulated the expression of receptors involved in the clearance of apoptotic cells including scavenger receptor A (CD204), scavenger receptor B (CD36) and CD14 [54-56] in M2-like macrophages. pMSCs also stimulated the ability of M2-like macrophages to phagocytose apoptotic cells. In addition, our study showed that pMSCs could upregulate the expression of hemoglobin (Hb) scavenger molecule (CD163), a receptor that resolves inflammation by limiting free Hb associated damage [57] and induce the secretion of anti-inflammatory cytokines [58,59], in M2-like macrophages. Moreover, our pMSCs upregulated the expression of the macrophage mannose receptor (CD206), which is involved in the resolution of inflammation during the course of an innate immune response [60], in M2-like macrophages. In this study, we attempted to elucidate the mechanism underlying the immunomodulatory effects of pMSCs on the differentiation of

macrophages. As is the case with MSCs from a variety of sources, placental MSCs produce several molecules, such as IL1R antagonist, IL-6, IL-10, M-CSF, LIF, VEGF, TGFß-1, PEGE2 and B7-H4. These molecules can induce the differentiation of M2 macrophages [14-18,61] or can provide immunosuppressive environment as in the case of B7-H4 [33]. However, the role of B7-H4 in M2-like macrophage differentiation is unknown, but based on its anti-inflammatory properties, we propose that B7-H4 may induce M2-like macrophage differentiation; further study is required to confirm this. In addition, pMSCs express a broad spectrum of molecules [2] that can induce an immunosuppressive environment as demonstrated in this study. Thus, pMSCs share similarity with glucocorticoids and progesterone with respect to the resolution of inflammation. This assumption was supported by the experiments performed with Mifepristone, an antagonist to PR and GR. Interestingly, the addition of Mifepristone to monocytes cultured in GM-CSF and CM of unstimulated pMSCs on day zero of culture partially inhibited the effects of pMSCs on macrophage (Fig. 5). However, we were surprised since neither pMSCs, nor other sources of MSCs secrete glucocorticoids/progesterone. Thus, suggesting that pMSCs secrete molecules that can modulate the functions of GR or PR as was previously reported that the functions of GR in human cells can be modulated by cytokines such as IL-1 and TGF beta [62,63]. Therefore, we propose that pMSCs secrete molecules with a therapeutical value that can induce anti-inflammatory environment via a mechanism that partially involves GR or PR. However, these factors and the mechanisms of their action need to be determined for the development of an efficient treatment that benefits patients with inflammatory diseases. Interestingly, we also found that placental pMSCs have more significant immunomodulatory effects through shifting the differentiation of monocytes from M1 into M2-like macrophages than bone marrow derived MSCS (Fig. 8). These results suggest that the use of placental pMSCs would be advantageous

where the resolution of inflammation is important and this may further assist in promoting tissue remodeling and wound repair. However, more extensive comparisons, involving additional markers, are required with other MSC sources, such as umbilical cord MSCs and adipose tissue MSCs, to validate these findings.

Conclusion

This is the first study to show that human pMSCs can induce the differentiation of human M2 macrophages by direct or indirect contact mechanisms or soluble factors secreted from unstimulated pMSCs (Fig. 9). We showed for the first time that pMSC soluble factors can partially act on GR or PR to induce an immunosuppressive phenotype in macrophages. We also showed that this effect of pMSCs on macrophage differentiation is reversible. In view of this study, human pMSCs are good candidates for the development of a cell-based therapy that can target macrophages in the resolution of inflammation. However, further studies are required to fully elucidate the mechanisms by which pMSCs induce this effect on macrophages and also to determine the molecules that mediate pMSC effects on macrophages.

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Fig. 1: Morphological effects of human pMSCs on the GM-CSF–stimulated human monocyte to macrophage differentiation analysed by microscopic examination (Panels A and B) and flow cytometric analysis of cell surface molecules (CD14, CD36, CD163, CD204, CD206, B7-H4 and CD11b). Representative phase contrast microscopic images revealing (A) M1-like macrophages (fried egg-shaped morphology, scale bar 10µm) differentiated from monocytes cultured for six days in medium containing GM-CSF and (B) elongated, spindle-like morphology (M2-like anti-inflammatory macrophages, scale bar 100µm) differentiated from monocytes cultured for six days in medium containing GM-CSF and pMSCs. The expression profiles of thirty individual experiments demonstrating that pMSCs [ICpMSC (Intracellular Direct Contact), SFpMSC (Soluble Factor), CMpMSC (Conditioned Medium)] as compared to untreated (GM-CSF treated monocytes without pMSCs) induced changes in membrane expression of (C- I) CD14, CD36, CD163, CD204, CD206, B7-H4 and CD11b on macrophages at day 7 of the culture of monocytes with GM-CSF. ICpMSC experiments were conducted using 0.4µm pore size transwell chamber membrane culture system in which pMSCs were seeded on the reverse side of the membrane of the chamber and monocytes were seeded on the upper side of the membrane. For SFpMSC experiments, pMSCs were cultured on the upper compartments while monocytes were cultured in the lower compartment. For CMpMSC experiments, supernatant from pMSCs were added to monocytes cultured in M1 differentiation medium. pMSCs were added to the monocyte cultures on day 1. The levels of expression are presented as median fluorescent intensity (MFI) or median percentage of B7-H4 positive cells as determined by flow cytometry. Experiments were conducted in triplicate using the indicated pMSC treatments. Thirty independent placentae were used to prepare pMSCs and monocytes were from the peripheral

blood of thirty individual healthy donors. *P, **P and ***P<0.0.0001. Bars represent 5-95 percentiles.

Fig. 2: Flow cytometric analysis of cell surface molecules (CD14, CD163, CD204, CD206, CD11b and B7-H4). The expression profiles of 10 individual experiments demonstrating that pMSCs [ICpMSC (Intracellular Direct Contact), SFpMSC (Soluble Factor), CMpMSC (Conditioned Medium)] have reversible effects on the membrane expression of (A- F) CD14, CD163, CD204, CD206, CD11b and B7-H4 on macrophages. pMSCs were removed after three days of co-culturing with monocytes and macrophages, washed thoroughly and then cultured again in M1 macrophage differentiation medium containing GM-CSF for another three days. On day seven, M1-like macrophages expressed reduced levels of (A- D and F) CD14, CD163, CD204, CD206 and B7-H4 and displayed increased levels of (E) CD11b cocultured without pMSCs (-ICpMSC, -SFpMSC and –CMpMSC) as compared to M2-like macrophages cocultured with pMSCs. The levels of expression are presented as median fluorescent intensity (MFI) or median percentage of B7-H4 positive cells as determined by flow cytometry. Experiments were conducted in triplicate using the indicated pMSC treatments. 10 independent placentae were used to prepare pMSCs and monocytes were from the peripheral blood of 10 individual healthy donors. *$P<0.01$. Bars represent standard errors.

Fig. 3: Phenotypic analysis of GM-CSF–driven human monocyte to macrophage differentiation following six days culture in medium containing GM-CSF alone (Untreated) and with different treatment of pMSCs (ICpMSC, SFpMSC and CMpMSC) analysed by flow cytometry. The expression profiles of thirty individual experiments demonstrating that GM-CSF alone (Untreated) gave an M1 phenotype. Addition of pMSCs (ICpMSC, SFpMSC) or pMSC conditioned medium (CMpMSC) significantly decreased the expression of (A- C) CD40, CD80 and CD86 whilst increasing the expression of (D- F) CD273, CD274 and HLA-DR to give an M2-like macrophage phenotype. The levels of expression are presented as median fluorescent intensity (MFI) as determined by flow cytometry. Experiments were conducted in triplicate using indicated pMSC treatments. Thirty independent placentae were used to prepare pMSCs and monocytes were from the peripheral blood of thirty individual healthy donors. *P, **P and ***P<0.0.0001. Bars represent 5-95 percentiles.

Fig. 4: Effects of pMSCs on the GM-CSF–stimulated human monocyte to macrophage differentiation analysed by the secretion of IL-10, IL-1ß, IL-12 (p70), MIP-1α and MCP-1 by sandwich ELISA. The secretion profiles of cytokines showed soluble factors secreted from pMSCs (SFpMSC) significantly increased the secretion of (A) IL-10 (*P< 0.0001) and significantly decreased the secretions of (B- D) IL-1ß, IL-12 (p70) and MIP-1α (*P< 0.0001). Secretion of (E) MCP-1 was not significantly increased (P= 0.06) by macrophages on day 7 of culture of GM-CSF treated monocytes with pMSC soluble factors (SFpMSC) as compared to GM-CSF alone (Untreated) macrophages. Experiments were conducted in triplicate using pMSCs prepared from 30 independent placentae and monocytes were from the peripheral blood of 30 individual healthy donors. Bars represent 5-95 percentiles.

Fig. 5: Functional assay for the effects of human pMSCs on the phagocytic activity of macrophages differentiated from GM-CSF–stimulated human monocytes. The assay measured phagocytosed Zymosan particles by reading the optical density at 405nm. The samples were also examined microscopically for evidence of phagocytosing apoptotic cells. The optical density demonstrating (A and B) the phagocytosis of Zymosan particles by macrophages was significantly increased by pMSC treatments compared to GM-CSF alone (Untreated) macrophages. (C) The phagocytic index of phagocytosis of apoptotic cells by M2- like macrophages was 87% while it was reduced to 13% by the inhibitor of phagocytosis, cytochalasin B. Representative fluorescence photomicrographs demonstrating (D) phagocytosis of red fluorescent apoptotic cells by green fluorescent-labelled macrophages, and (E) the inhibition of phagocytosis of apoptotic cells by cytochalasin B. Experiments were conducted in triplicate and 10 independent experiments were carried out. Bar = 20 μm.*P, **P and ***P = 0.0002. Bars represent standard errors.

Fig. 6: The effects of mifepristone on the GM-CSF–stimulated human monocyte to macrophage differentiation in the presence of pMSC conditioned medium (CMpMSC). Outcomes were analysed by 1) morphological examination, 2) the expression of cell surface molecules (CD163, CD204, CD206 and B7-H4) assessed by flow cytometry and 3) the secretion of IL-10, IL-1ß, IL-12 (p70) and MIP-1α measured by sandwich ELISA. Representative phase contrast microscopic images revealing (A) M1-like macrophages (fried egg-shaped morphology, scale bar 10µm) differentiated from monocytes cultured for six days in medium containing GM-CSF, (B) elongated, spindle-like morphology (M2-like anti-inflammatory macrophages, scale bar 100µm) differentiated from monocytes cultured for six days in medium containing GM-CSF and pMSCs and (C) non-adherent cells (arrow head), fried egg-shaped cells (dashed arrow) and elongated, spindle-like cells (arrow), scale bar 10µm, differentiated from monocytes cultured for six days in medium containing GM-CSF, CMpMSC and mifepristone. The expression profiles of cell surface molecules demonstrated mifepristone significantly inhibited the effects of CMpMSC on the expression of (D, E and G) CD163, CD204 and B7-H4 (*P= 0.0002) while not significantly stimulated the effects of CMpMSC on the expression of (F) CD206 on macrophages on day 7 compared to monocyte cultured with GM-CSF and CMpMSC. Mifepristone significantly inhibited the effects of dexamethasone (Dex; positive control) on the expression of (D- G) CD163, CD204, CD206 and B7-H4 (*P<= 0.0002) on macrophages on day 7 compared to monocyte cultured with GM-CSF and dexamethasone. The secretion profiles of cytokines demonstrating mifepristone inhibiting the effects of dexamethasone and CMpMSC on the secretion of (H- L) IL-10, IL-1ß, IL-12 (p70) and MIP-1α by macrophages on day 7 compared to monocyte cultured with GM-CSF and CMpMSC , *P< 0.0001. The levels of expression are presented as median fluorescent intensity (MFI) or median percentage of positive cells as

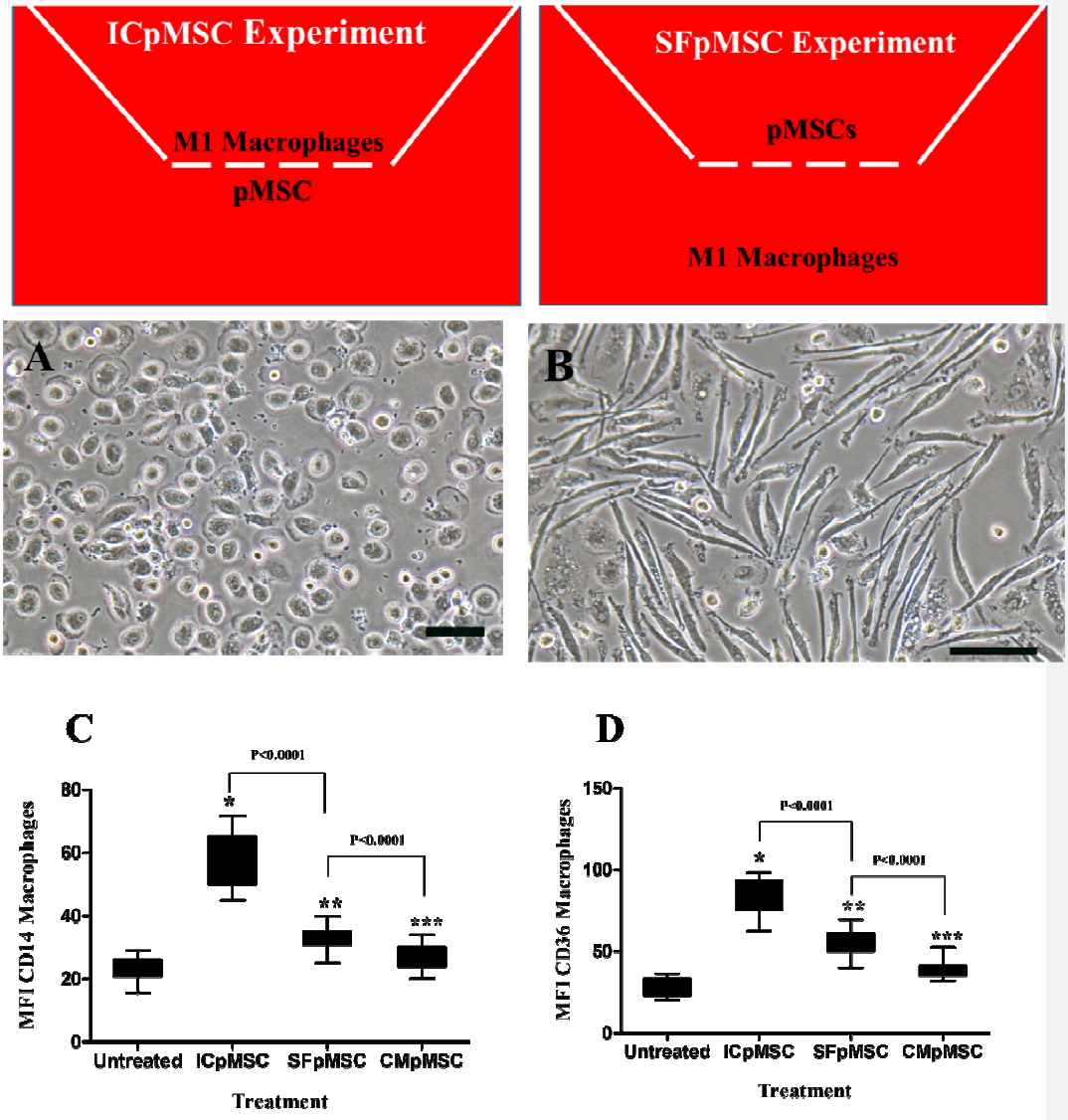
determined by flow cytometry. Experiments were conducted in triplicate using CM prepared from 10 independent placentae and monocytes were from the peripheral blood of 10 individual healthy donors. Bars represent 5-95 percentiles.

Fig. 7: Flow cytometric analysis of intracellular molecules (IL-1ß, IL-8, IL-12, TNF-α, MCP-1, IL-6, IL-10, IDO and B7-H4). The expression profiles of 10 individual experiments demonstrating that macrophages significantly increased the expression of IL-8, MCP-1, IL-12, IL-6, IL-10, IDO and B7H4 in pMSCs while the expressions of IL-1ß and TNF-α were not changed significantly in pMSCs as compared to untreated pMSCs. The levels of expression are presented as median fluorescent intensity (MFI) as determined by flow cytometry. Experiments were conducted in triplicate using the indicated pMSC treatments. 10 independent placentae were used to prepare pMSCs and monocytes were from the peripheral blood of 10 individual healthy donors. *P<0.01 and **P< 0.05. Bars represent standard errors.

Fig. 8: The comparison between the effects of pMSCs and BMMSCs on the GM-CSF–stimulated human monocyte to macrophage differentiation. Outcomes were analysed by the expression of cell surface molecules (CD163 and CD206, A and B respectively) as assessed by flow cytometry and for the secretion of IL-10 and IL-12 (p70) (C and D respectively) as measured by sandwich ELISA. pMSCs (ICpMSC and SFpMSC) had a greater effect than BMMSCs (ICBMMSC and SFBMMSC) on the expression of CD163 and CD206 (*P<0.05 and **P=0.005) on macrophages. The secretion profiles of cytokines showed that pMSCs (ICpMSC and SFpMSC) had a greater effect than BMMSCs (ICBMMSC and SFBMMSC) on the secretion of IL-10 by macrophages, *P< 0.05. The levels of expression in A and B are presented as median fluorescent intensity (MFI). Experiments were conducted in triplicate and three independent experiments were carried out. Bars represent standard errors.

Fig. 9: Illustration showing the effects of placental mesenchymal stem cells (pMSCs) on modulating the differentiation and functions of macrophages. pMSCs induce a shift in the differentiation of human monocyte-derived macrophages from inflammatory M1 into anti-inflammatory macrophages as demonstrated by the changed expression of M2 like- macrophages markers (CD14, CD36, CD163, CD204, CD206, B7-H4 and CD11b) by physical contact (cell–cell contact) or by soluble factors via a mechanism that partially involving glucocorticoid receptor (GR) and progesterone receptor (PR). pMSCs also upregulated the expression of co-inhibitory proteins (CD273, CD274 and B7-H4) and down-regulated the expression of CD40, CD80 and CD86 by M2 like-macrophages. In addition, the secretion of IL-10 was increased while the secretion of IL1β, IL12 (p70) and MIP-1α was decreased by M2 like-macrophages under the influence of pMSCs.

Figure 1


ICpMSC Experiment
M1 Macrophages
pMSC
SFpMSC Experiment
pMSCs
M1 Macrophages
A
B
C
MFI CD14 Macrophages
P<0.0001
P<0.0001
Untreated
ICpMSC
SFpMSC
CMpMSC
Treatment
D
MFI CD36 Macrophages
P<0.0001
P<0.0001
Untreated
ICpMSC
SFpMSC
CMpMSC
Treatment

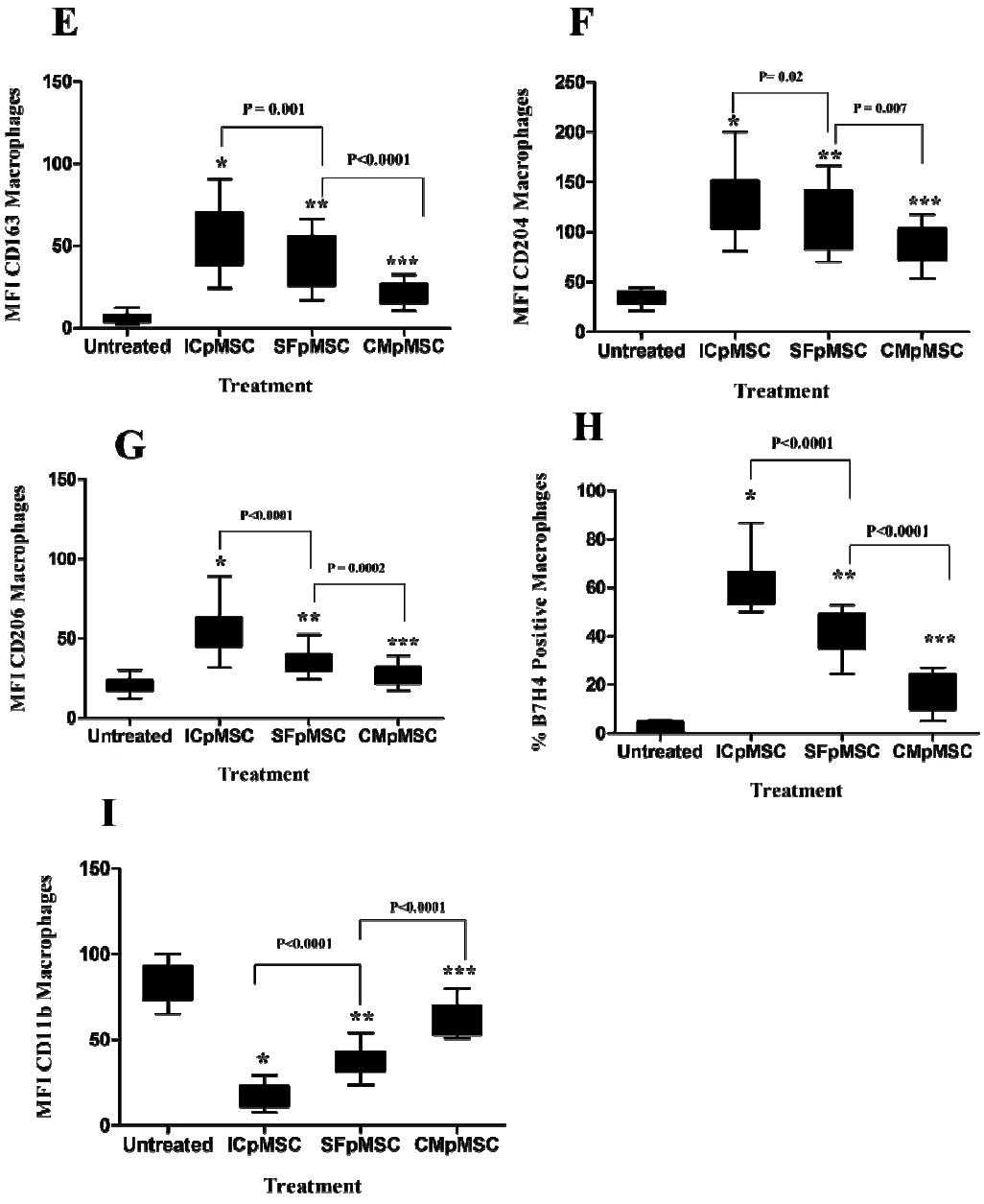

E
MFI CD163 Macrophages
150
100
50
0
P = 0.001
P<0.0001
*
**
***
Untreated
ICpMSC
SFpMSC
CMpMSC
Treatment
F
MFI CD204 Macrophages
250
200
150
100
50
0
P= 0.02
P = 0.007
*
**
***
Untreated
ICpMSC
SFpMSC
CMpMSC
Treatment
G
MFI CD206 Macrophages
150
100
50
0
P<0.0001
P = 0.0002
*
**
***
Untreated
ICpMSC
SFpMSC
CMpMSC
Treatment
H
% B7H4 Positive Macrophages
100
80
60
40
20
0
P<0.0001
P<0.0001
*
**
***
Untreated
ICpMSC
SFpMSC
CMpMSC
Treatment
I
MFI CD11b Macrophages
150
100
50
0
P<0.0001
P<0.0001
*
**
***
Untreated
ICpMSC
SFpMSC
CMpMSC
Treatment

Figure 2

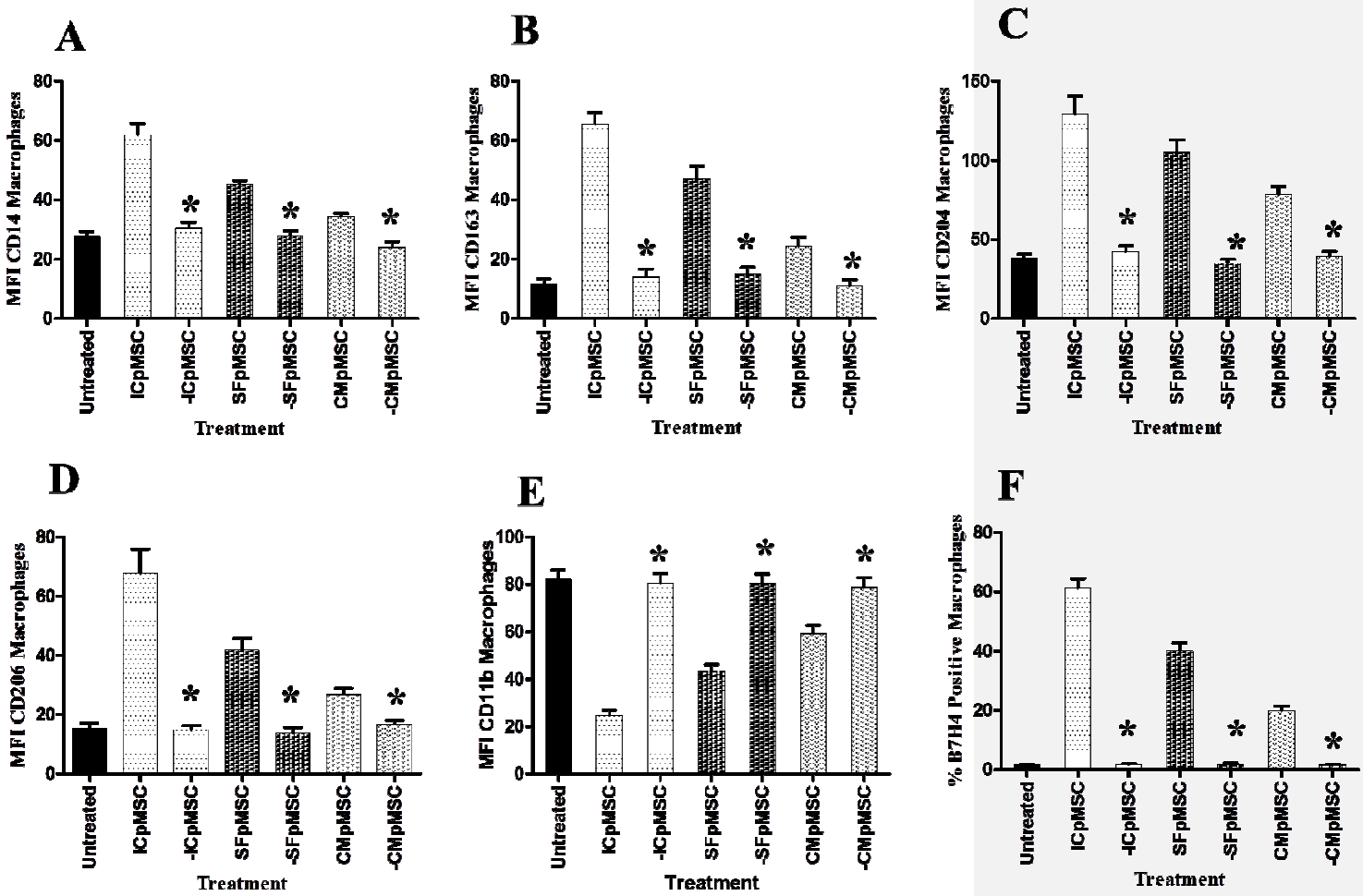

A
MFI CD14 Macrophages
0
20
40
60
80
Untreated
ICpMSC
-ICpMSC
SFpMSC
-SFpMSC
CMpMSC
-CMpMSC
Treatment
B
MFI CD163 Macrophages
0
20
40
60
80
Untreated
ICpMSC
-ICpMSC
SFpMSC
-SFpMSC
CMpMSC
-CMpMSC
Treatment
C
MFI CD204 Macrophages
0
50
100
150
Untreated
ICpMSC
-ICpMSC
SFpMSC
-SFpMSC
CMpMSC
-CMpMSC
Treatment
D
MFI CD206 Macrophages
0
20
40
60
80
Untreated
ICpMSC
-ICpMSC
SFpMSC
-SFpMSC
CMpMSC
-CMpMSC
Treatment
E
MFI CD11b Macrophages
0
20
40
60
80
100
Untreated
ICpMSC
-ICpMSC
SFpMSC
-SFpMSC
CMpMSC
-CMpMSC
Treatment
F
% B7H4 Positive Macrophages
0
20
40
60
80
Untreated
ICpMSC
-ICpMSC
SFpMSC
-SFpMSC
CMpMSC
-CMpMSC
Treatment

Figure 3

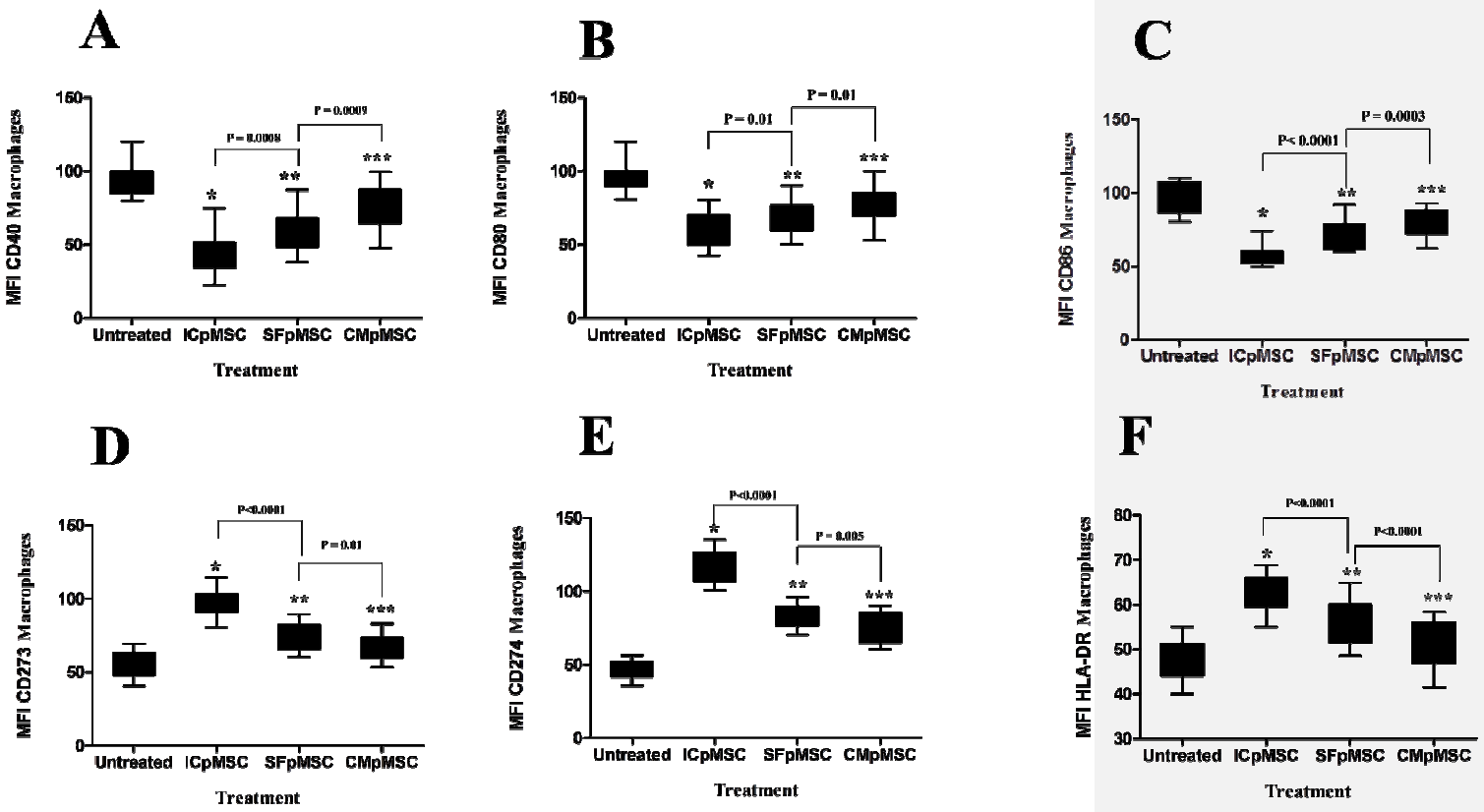

A
MFI CD40 Macrophages
P = 0.0008
P = 0.0009
Untreated
ICpMSC
SFpMSC
CMpMSC
Treatment
B
MFI CD80 Macrophages
P = 0.01
P = 0.01
Treatment
C
MFI CD86 Macrophages
P< 0.0001
P = 0.0003
Treatment
D
MFI CD273 Macrophages
P<0.0001
P = 0.01
Treatment
E
MFI CD274 Macrophages
P<0.0001
P = 0.005
Treatment
F
MFI HLA-DR Macrophages
P<0.0001
P<0.0001
Treatment

Figure 4

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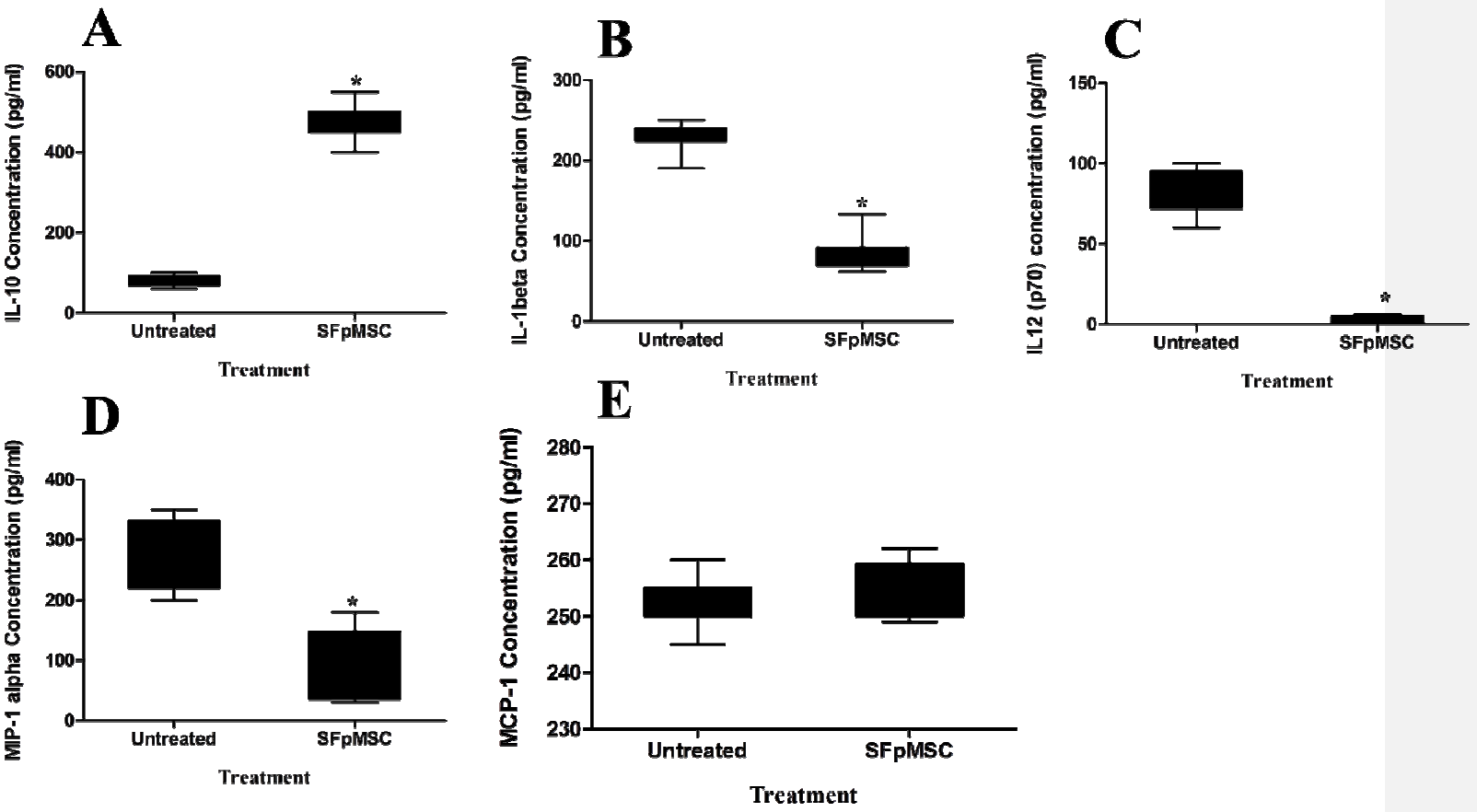
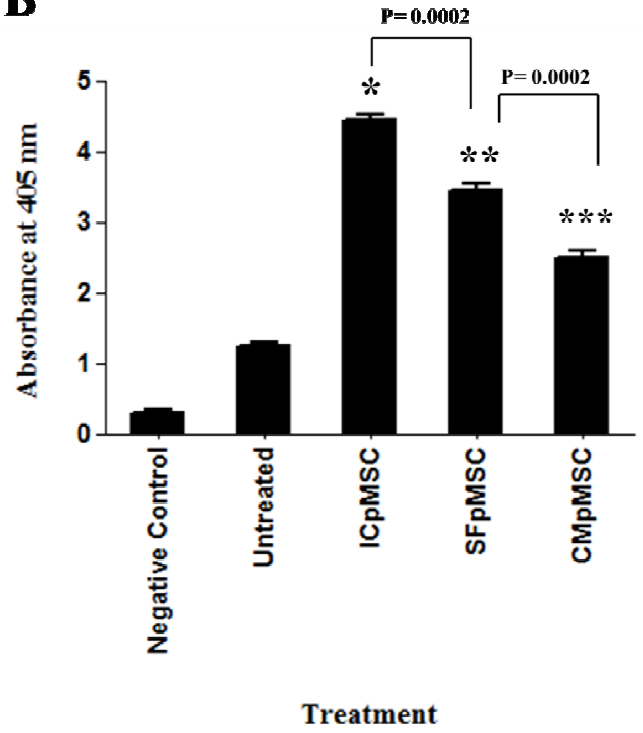


Figure 5

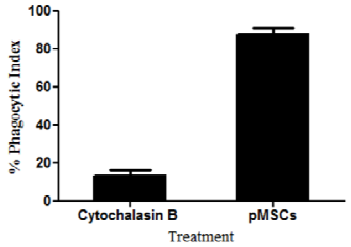
A

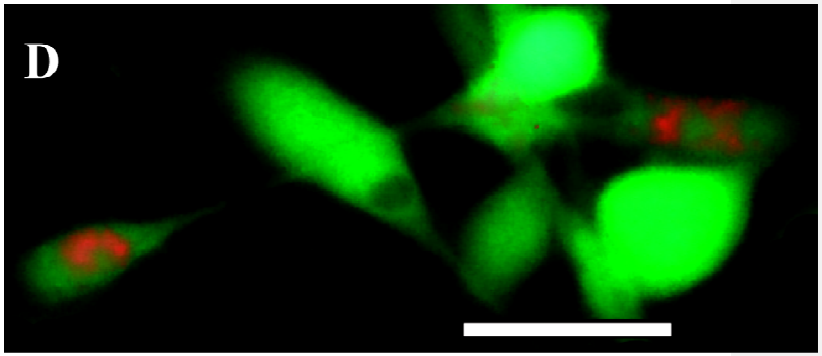
	ICpMSC	SFpMSC	CMpMSC
Fold increase in phagocytic activity	3.5±0.53 (P = 0.0002)	2.75±0.49 (P = 0.002)	2.0 (P = 0.0002)

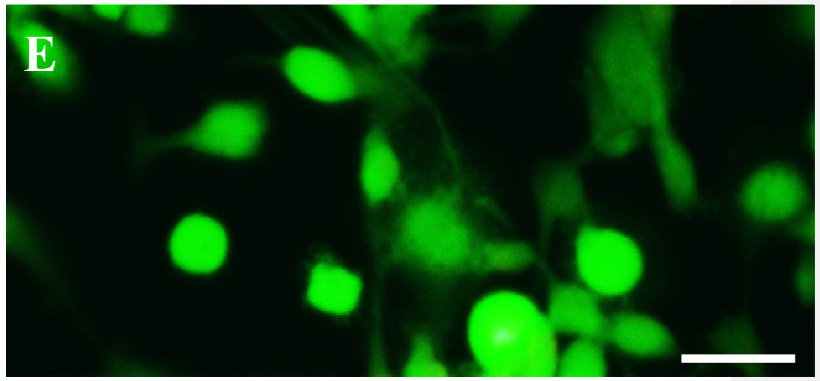
B



C







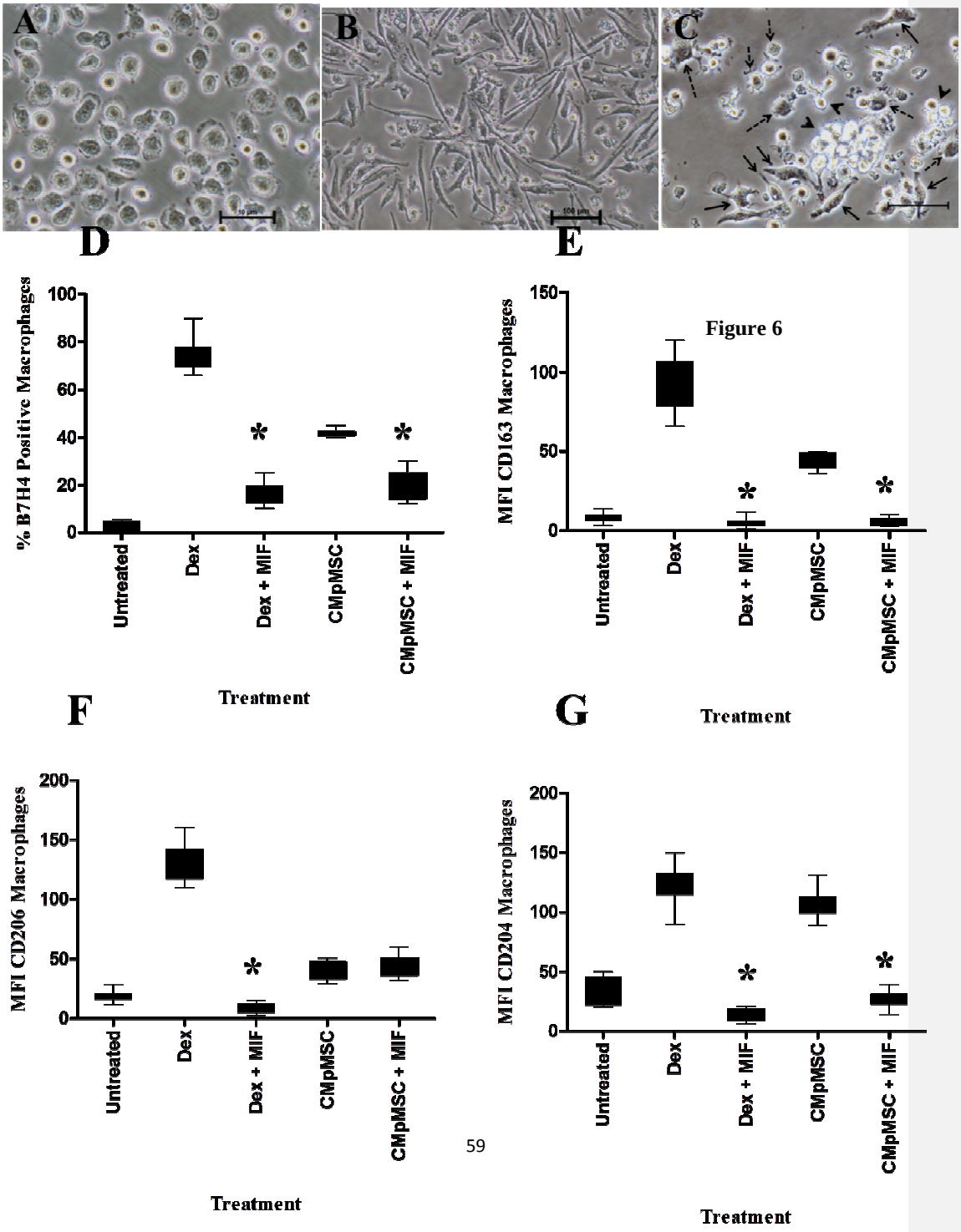

A
B
C
D
% B7H4 Positive Macrophages
100
80
60
40
20
0
Untreated
Dex
Dex + MIF
CMpMSC
CMpMSC + MIF
Treatment
E
MFI CD163 Macrophages
150
100
50
0
Untreated
Dex
Dex + MIF
CMpMSC
CMpMSC + MIF
Treatment
F
MFI CD206 Macrophages
200
150
100
50
0
Untreated
Dex
Dex + MIF
CMpMSC
CMpMSC + MIF
Treatment
G
MFI CD204 Macrophages
200
150
100
50
0
Untreated
Dex
Dex + MIF
CMpMSC
CMpMSC + MIF
Treatment

Figure 6

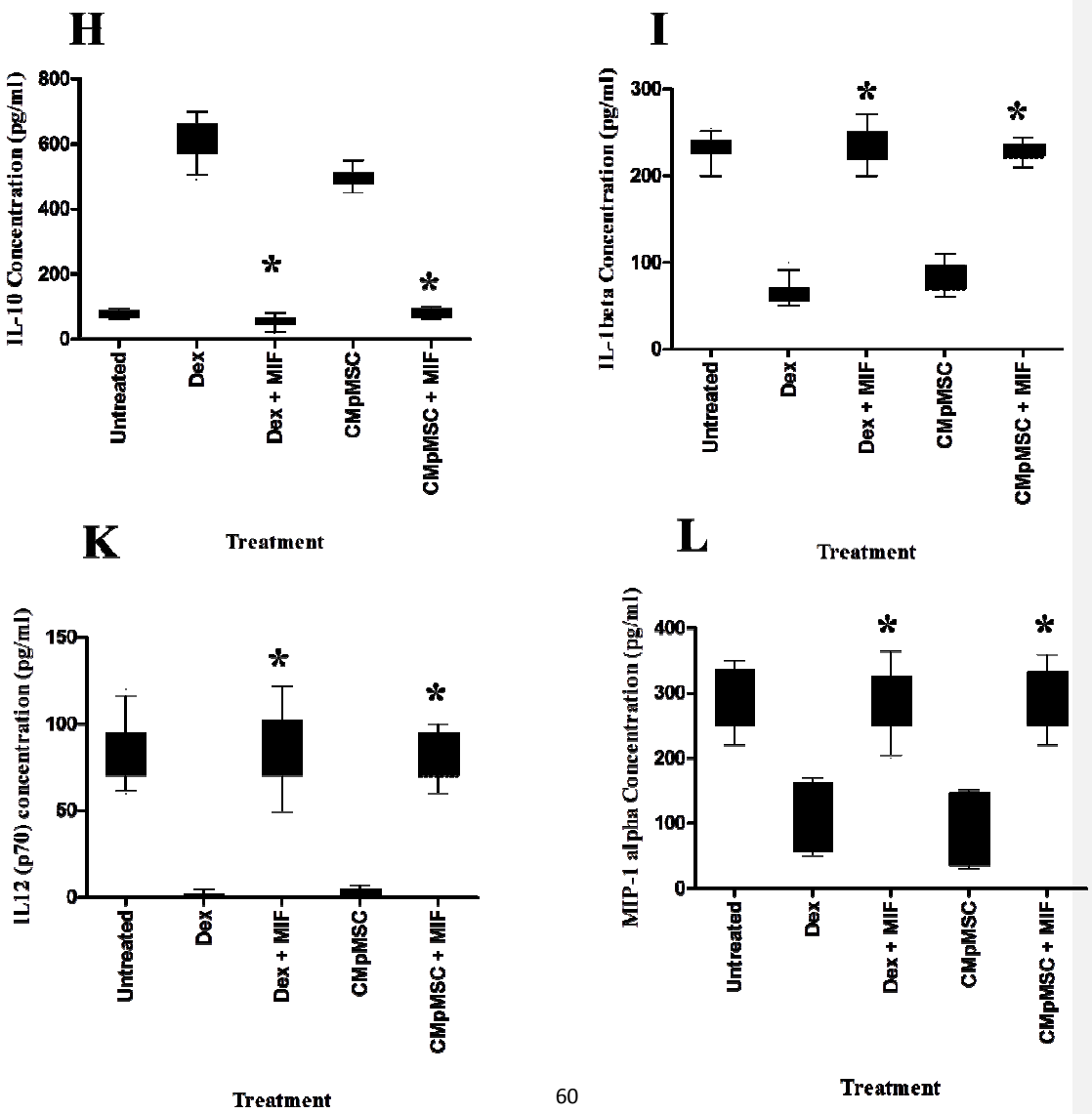

H
IL-10 Concentration (pg/ml)
800
600
400
200
0
Untreated
Dex
Dex + MIF
CMpMSC
CMpMSC + MIF
*
*
I
IL-1beta Concentration (pg/ml)
300
200
100
0
Untreated
Dex
Dex + MIF
CMpMSC
CMpMSC + MIF
*
*
Treatment
K
IL12 (p70) concentration (pg/ml)
150
100
50
0
Untreated
Dex
Dex + MIF
CMpMSC
CMpMSC + MIF
*
*
Treatment
L
MIP-1 alpha Concentration (pg/ml)
400
300
200
100
0
Untreated
Dex
Dex + MIF
CMpMSC
CMpMSC + MIF
*
*
Treatment

Fig. 7

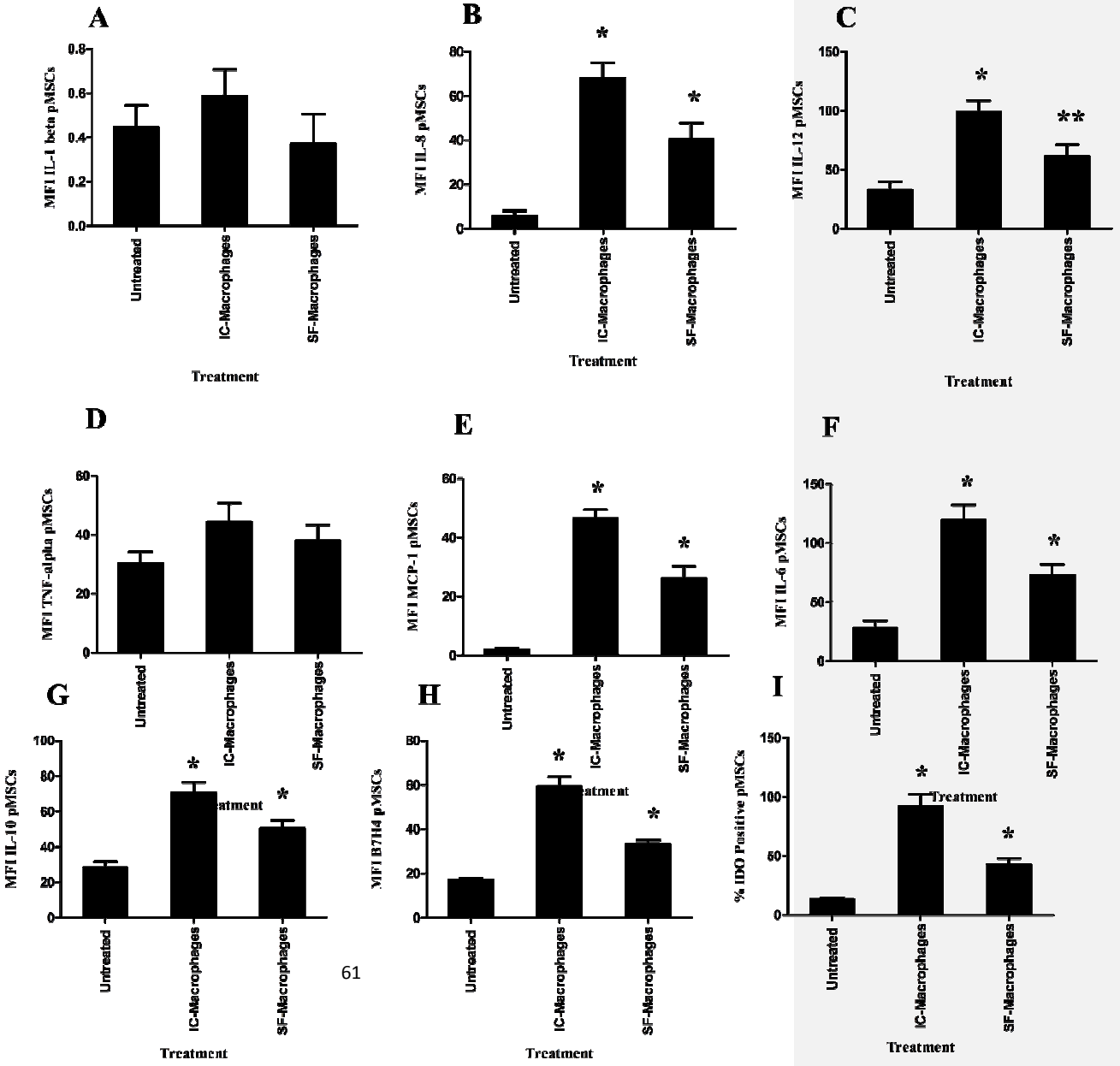

A
MFI IL-1 beta pMSCs
0.0
0.2
0.4
0.6
0.8
Untreated
IC-Macrophages
SF-Macrophages
Treatment
B
MFI IL-8 pMSCs
0
20
40
60
80
*
*
Untreated
IC-Macrophages
SF-Macrophages
Treatment
C
MFI IL-12 pMSCs
0
50
100
150
*
**
Untreated
IC-Macrophages
SF-Macrophages
Treatment
D
MFI TNF-alpha pMSCs
0
20
40
60
Untreated
IC-Macrophages
SF-Macrophages
E
MFI MCP-1 pMSCs
0
20
40
60
*
*
Untreated
IC-Macrophages
SF-Macrophages
F
MFI IL-6 pMSCs
0
50
100
150
*
*
Untreated
IC-Macrophages
SF-Macrophages
G
MFI IL-10 pMSCs
0
20
40
60
80
100
*
*
Untreated
IC-Macrophages
SF-Macrophages
Treatment
H
MFI B7H4 pMSCs
0
20
40
60
80
*
*
Untreated
IC-Macrophages
SF-Macrophages
Treatment
I
% IDO Positive pMSCs
0
50
100
150
*
*
Treatment
Untreated
IC-Macrophages
SF-Macrophages
Treatment

Figure 8

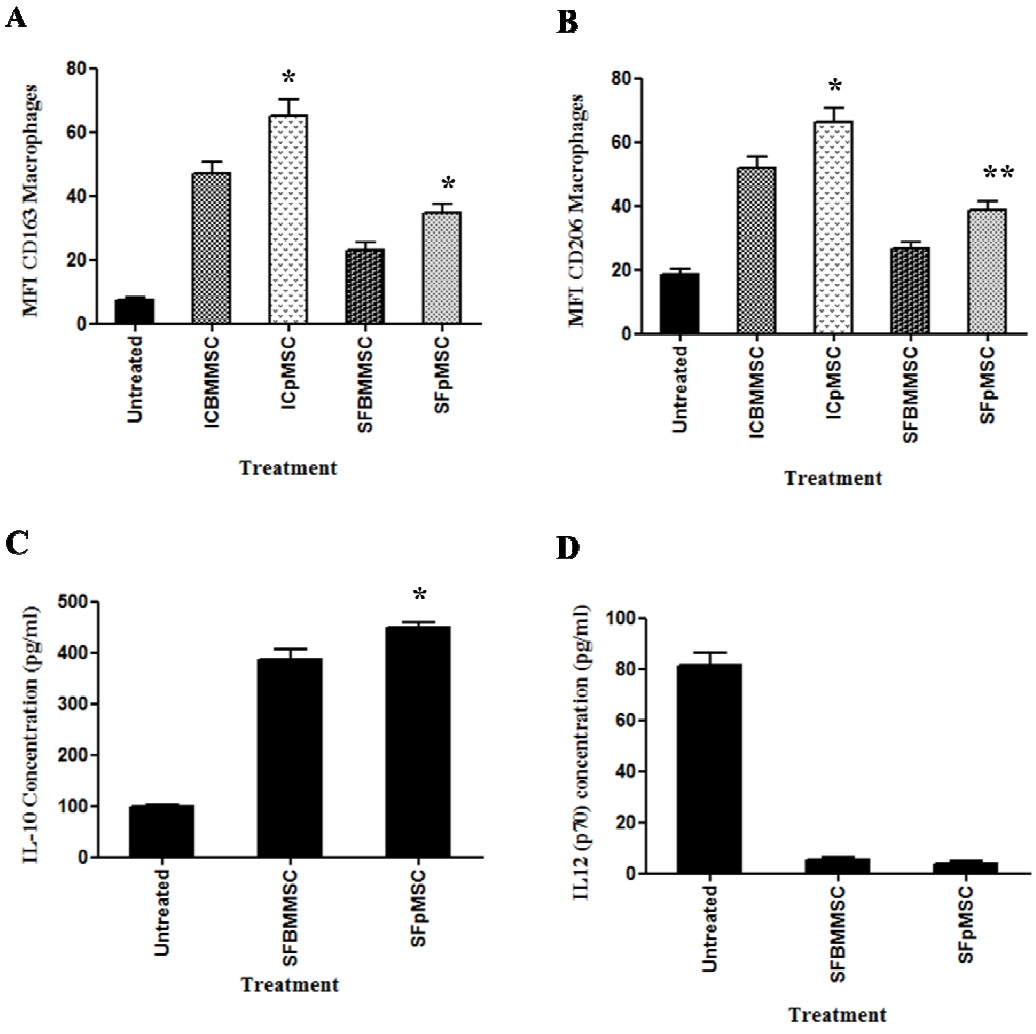

A
MFI CD163 Macrophages
80
60
40
20
0
*
*
Untreated
ICBMMSC
ICpMSC
SFBMMSC
SFpMSC
Treatment
B
MFI CD206 Macrophages
80
60
40
20
0
*
**
Untreated
ICBMMSC
ICpMSC
SFBMMSC
SFpMSC
Treatment
C
IL-10 Concentration (pg/ml)
500
400
300
200
100
0
*
Untreated
SFBMMSC
SFpMSC
Treatment
D
IL12 (p70) concentration (pg/ml)
100
80
60
40
20
0
Untreated
SFBMMSC
SFpMSC
Treatment

Figure 9

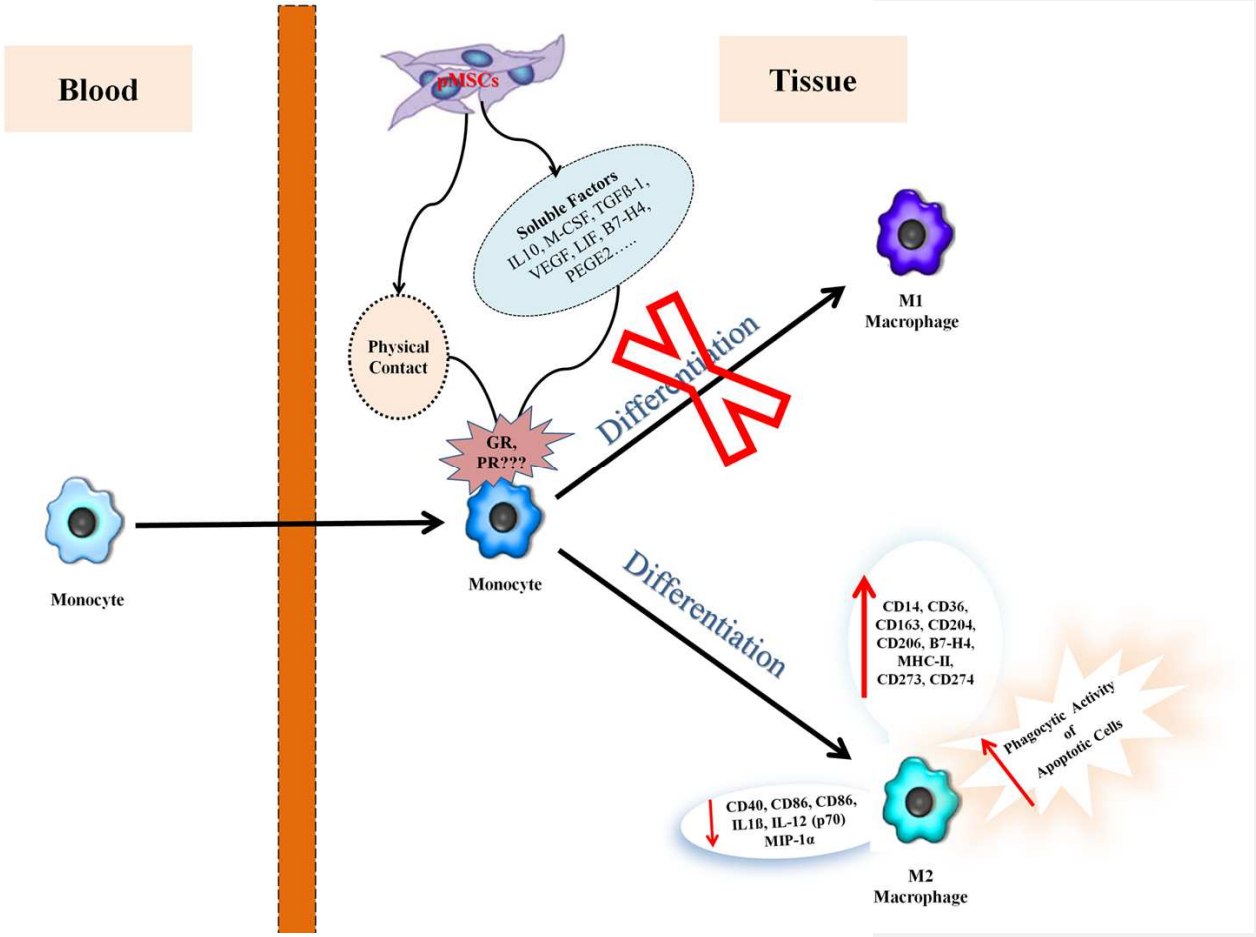

Blood
Tissue
pMSCs
Soluble Factors
IL10, M-CSF, TGFβ-1,
VEGF, LIF, B7-H4,
PEGE2.....
Physical
Contact
GR,
PR???
Differentiation
M1
Macrophage
Monocyte
Monocyte
Differentiation
CD14, CD36,
CD163, CD204,
CD206, B7-H4,
MHC-II,
CD273, CD274
Phagocytic Activity
of
Apoptotic Cells
CD40, CD86, CD86,
IL1ß, IL-12 (p70)
MIP-1α
M2
Macrophage

Table 1 Antibodies used in this study to characterize the isolated placental mesenchymal stem cells (pMSCs) and monocyte derived macrophages.

Antibody	Conjugate	Dilution	Clone	Manufacturer	Antibody	Conjugate	Dilution	Clone	Manufacturer
CD45	FITC	1:10	J33	Beckman Coulter	**CD273**	PC7	1:10	PD-L2	Beckman Coulter
CD19	FITC	1:10	J3-119	Beckman Coulter	**CD274**	PC7	1:10	PDL1.3.1	Beckman Coulter
CD90	FITC	1:10	F15-42-1-5	Beckman Coulter	**IL1ß**	PE	1:10	8516	R & D Systems
CD44	FITC	1:10	J.173	Beckman Coulter	**IL-6**	PE	1:10	1936	R & D Systems
HLA-ABC	FITC	1:10	B9.12.1	Beckman Coulter	**IL-8**	PE	1:10	6217	R & D Systems
CD105	PE	1:10	1G2	Beckman Coulter	**IL-12**	PE	1:10	27537	R & D Systems
CD146	PE	1:10	TEA 1/34	Beckman Coulter	**IL-10**	PE	1:10	127107	R & D Systems
CD166	PE	1:10	3A6	Beckman Coulter	**MCP-1**	PE	1:10	2H5	Abacm
CD14	PE	1:10	RMO52	Beckman Coulter	**TNF-α**	PE	1:10	6402	R & D Systems
CD11B	PE	1:10	M1/70	R & D Systems	**IDO**	PE	1:10	700838	R & D Systems
CD163	PE	1:10	215927	R & D Systems	**IgG/IgM**	FITC/ PE	1:10		Beckman Coulter

CD204	PE	1:10	351615	R & D Systems					
CD206	PE	1:10	685641	R& D Systems					
B7H4	PE	1:10	MIH43	Abcam					
CD80	PE	1:10	MAB104	Beckman Coulter					
CD86	PE	1:10	HA5.2B7	Beckman Coulter					
CD40	PE	1:10	MAB89	Beckman Coulter					
HLA-DR	FITC	1:10	B8.12.2	Beckman Coulter					

Table 2 The expression profiles of dose- response experiments using ratios of 1:1, 1:5 or 1:10 pMSCs ((ICpMSC (Intracellular Direct Contact) and SFpMSC (Soluble Factor)): macrophages demonstrating that pMSCs as compared to untreated (GM-CSF treated monocytes without pMSCs) induced changes in membrane expression of CD14, CD163, CD206, and CD11b on macrophages on day 7 of the culture of monocytes with GM-CSF. The mean of expression are presented as median fluorescent intensity (MFI) as determined by flow cytometry 10 independent placentae were used to prepare pMSCs and monocytes were from the peripheral blood of 10 individual healthy donors.

Treatment / Markers	**Untreated**	**ICpMSC 1:1**	**SFpMSC 1: 5**	**ICpMSC 1: 10**	**SFpMSC 1: 1**	**SFpMSC 1: 5**	**SFpMSC 1: 10**
CD14	20.74± 1.236	93.20± 5.876	75.40± 4.031	54.40± 3.550	77.80± 2.715	62.50± 2.197	42.90± 4.473
CD163	8.320± 1.694	100.8± 7.222	78.04± 5.598	53.87± 7.449	87.80± 4.657	62.31± 4.811	45.56± 7.133
CD206	17.63± 5.170	117.0± 6.333	93.00± 6.333	50.90± 6.627	102.0± 7.272	69.71± 7.090	33.84± 4.188
CD11b	90.37± 4.540	82.00± 5.011	63.50± 5.167	22.83± 2.611	68.50± 4.658	51.00± 4.876	35.79± 3.757

Table 3 The expression profiles of dose- response experiments using different concentrations (1%, 5%, 10%, 20%, 40% and 60) of conditioned medium of pMSCs (CMpMSC) demonstrating that pMSCs as compared to untreated (GM-CSF treated monocytes without pMSCs) induced changes in membrane expression of CD14, CD163, CD206, and CD11b on macrophages on day 7 of the culture of monocytes with GM-CSF. The mean of expression are presented as median fluorescent intensity (MFI) as determined by flow cytometry 10 independent placentae were used to prepare pMSCs and monocytes were from the peripheral blood of 10 individual healthy donors.

Treatment / Markers	**Untreated**	**CMpMSC 1%**	**CMpMSC 5%**	**CMpMSC 10%**	**CMpMSC 20%**	**CMpMSC 40%**	**CMpMSC 60%**
CD14	20.74± 1.236	19.60± 1.614	22.70± 1.106	24.60± 1.118	29.70± 0.9894	23.90± 0.6904	20.70± 0.8172
CD163	8.320± 1.694	9.800± 1.731	14.20± 1.632	16.20± 1.459	22.06± 1.832	17.70± 1.044	10.50± 1.003
CD206	17.63± 5.170	19.50± 4.965	22.00± 4.814	24.50± 4.902	29.80± 1.867	19.00± 1.350	9.540± 1.214
CD11b	90.37± 4.540	83.40± 4.230	78.30± 3.742	73.00± 3.350	55.70± 1.915	60.80± 2.118	67.20± 2.507

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