The handle http://hdl.handle.net/1887/24378 holds various files of this Leiden University dissertation.

Author: Melief, Sara Marie
Title: Immunomodulatory properties of human multipotent stromal cells
Issue Date: 2014-03-06
Multipotent stromal cells skew monocytes towards an anti-inflammatory IL-10 producing phenotype by production of IL-6

Sara M. Melief
Sacha B. Geutskens
Willem E. Fibbe
Helene Roelofs

Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

Haematologica, June 2013 98: 888-895
Abstract

Background
Multipotent stromal cells exhibit immunomodulatory capacities and have been applied in transplantation and autoimmune diseases. One of the effects of MSC involves the inhibition of dendritic cell differentiation. Since IL-6 and IL-10 are known to play a role in inhibiting immature dendritic cell differentiation, we hypothesized that these cytokines also may mediate the inhibitory effect of human MSC in immature dendritic cell differentiation.

Design and Methods
Monocytes were cultured with IL-4 and GM-CSF in the presence or absence of culture-expanded bone marrow-derived multipotent stromal cells. Neutralization and cytokine-depletion strategies were applied to reveal the cellular source and effect of IL-6 and IL-10.

Results
Addition of multipotent stromal cells to monocyte cultures significantly reduced the generation of immature dendritic cells (CD14-CD1a+) and resulted in the generation of CD14+CD1a- cells that displayed a significantly reduced immunostimulatory effect. We found that culture supernatants of co-cultures of multipotent stromal cells and monocytes contained higher concentrations of IL-6 and IL-10. multipotent stromal cells produce IL-6 and neutralizing this IL-6 reversed the inhibitory effect of multipotent stromal cells. IL-10 was not produced by multipotent stromal cells, but exclusively by monocytes after exposure to multipotent stromal cells-produced IL-6.

Conclusions
By the constitutive production of IL-6, multipotent stromal cells prevent the differentiation of monocytes towards antigen-presenting immunogenic cells and skew differentiation towards an anti-inflammatory IL-10 producing cell type.
Introduction

Multipotent stromal cells (MSC) are non-hematopoietic progenitor cells that have been identified in different tissues and are capable of differentiation towards adipogenic, osteogenic and chondrogenic lineages. Importantly, MSC display immunoregulatory properties. These properties have been the subject of many studies over the years and make MSC interesting candidates for further clinical application.

In several experimental disease models, MSC have been shown to modulate immune responses in vivo. Administration of MSC has been shown to prevent Graft-versus-host disease (GvHD)\(^1\), the development of experimental autoimmune encephalomyelitis in mice\(^2\) and results in prolonged skin graft survival in baboons\(^3\). The use of MSC as a cellular therapy is currently explored in clinical trials, including treatment of GvHD\(^4\), promotion of engraftment following hematopoietic stem cell transplantation\(^5\) and treatment of Crohn's disease\(^6\).

The mechanisms underlying the immune suppression by MSC are still unclear. It has been widely shown that MSC suppress the proliferation of T cells upon allogeneic or mitogenic stimulation\(^7,8\). Soluble factors proposed to be involved in this effect include indoleamine 2,3-dioxygenase, prostaglandin E2, TGF-β1, IL-6 and heme oxygenase\(^1\)\(^9\)-\(^13\). MSC also inhibit the proliferation of B cells and IL-2 stimulated NK cells\(^14,15\) and have been described to promote the formation of regulatory T cells (Tregs)\(^16\)-\(^18\).

MSC can also modulate immune responses through inhibition of monocyte-derived immature dendritic cell (iDC) differentiation\(^19,20\). Monocyte differentiation towards iDC can be inhibited by IL-6 and IL-10\(^21,22\) and several studies suggested that IL-6 may play a role in the inhibitory effect of MSC on the differentiation of monocyte to dendritic cells\(^19,20\). These data are however derived from in vitro co-culture experiments of MSC and monocytes, where the cellular source of cytokine production has not been identified. Therefore, the specific role of these cytokines for this inhibiting MSC functionality is still unclear.

In this study, we further investigated the mechanism of inhibition by MSC during the differentiation of monocyte towards iDC and focussed on the exact source of the cytokines involved. To this end we performed co-culture, transwell and supernatant-supplemented experiments of monocytes and MSC and applied neutralization and depletion strategies to reveal the cellular interactions that are crucial for the immunomodulating effect of MSC on monocyte differentiation. We found that MSC inhibit monocyte-derived iDC differentiation and skew monocytes towards a cell type with a low allostimulatory capacity. We show that this process is dependent on the IL-6 produced by MSC, that subsequently induces monocyte-derived cells to produce IL-10, a cytokine involved in immune suppression and that is not produced by MSC.

In conclusion, our results reveal a potential powerful role for MSC that, through their production of IL-6, program monocytes to become anti-inflammatory cells that prevent or reduce tissue damage and modulate the immune response.
**Design and Methods**

**Generation of human MSC**

After obtaining informed consent, adult bone marrow was harvested from healthy donors or from orthopedic patients. Mononuclear cells were isolated using a Ficoll-Paque density gradient (1.077 g/cm$^3$) and were plated at 1.3 x 10$^5$/cm$^2$ in DMEM-low glucose (Invitrogen Corp., Paisley, UK) supplemented with 10% fetal calf serum (FCS; Greiner Bio-one) and Penicillin/Streptomycin (P/S; Invitrogen Corp., Paisley, UK). After 3-4 days the non-adherent cells were removed and medium was refreshed every 3-4 days until the cells reached confluency. The MSC monolayer was detached using trypsin/EDTA (Invitrogen Corp., Paisley, UK) and reseeded at 4,000 cells/cm$^2$ for expansion. The MSC were characterized by FACS analysis and used in the co-culture experiments at passage 2-5.

**Isolation of monocytes**

Human PBMC from healthy donors were isolated from buffy coats obtained from Sanquin Blood Supply using a Ficoll-Paque density gradient (1.077 g/cm$^3$). From the freshly prepared mononuclear cell fraction, CD14 positive monocytes were purified by MACS using CD14 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cells were separated with a MACS LS column according to the manufacturer’s recommendations.

**Differentiation of monocytes**

Freshly isolated monocytes (CD14$^+$) were cultured at a concentration of 1.0 x 10$^6$ cells/well in 6-wells plates (Corning BV Life Sciences, Amsterdam, The Netherlands) in RPMI (Invitrogen Corp., Paisley, UK) containing P/S, L-glutamin (Invitrogen Corp., Paisley, UK) and 10% FCS supplemented with the growth factors IL-4 (10 ng/ml) and GM-CSF (5 ng/ml) (both from Invitrogen Corp., Paisley, UK) for 6 days, resulting in the generation of iDC (CD14$^-$/CD1a$^+$). To examine the effect of MSC on monocyte differentiation, irradiated MSC (60Gy) were added to the culture at a MSC:monocyte ratio of 1:10 as was described before (23, 24). The co-culture experiments were performed in direct cell-cell contact and in a transwell coculture system (pore size 0.4 μM; Corning Inc., Lowell, MA, USA). In the transwell experiments, MSC were plated in the lower well and monocytes were added to the transwell insert. All experiments were performed in duplicate. In some experiments IL-10 (20 ng/ml), IL-6 (100 ng/ml), anti-IL-10 (2–20 μg/ml) or anti-IL-6 (2.5 μg/ml) (all from R&D systems Europe Ltd., Abingdon, UK) was added. The involvement of secreted factors was further assessed by addition of MSC conditioned medium (MSC-CM) to the monocyte differentiation culture. Conditioned medium was generated by collecting cell-free culture supernatant from MSC alone (MSC-CM), MSC cultured with growth factors (MSC+GF CM) and from the monocyte-MSC cocultures (MSC+mono CM). In some experiments, IL-6
was depleted from the MSC-CM using the μMACS Streptavidin kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cell free MSC supernatant was incubated with biotinylated anti-IL-6 (2 μg/ml, BD Biosciences, San Diego, CA, USA) and μMACS Streptavidin microbeads. IL-6 was depleted from the medium using a μMACS column according to the manufacturer’s recommendations. MSC-CM was used in the monocyte differentiation in a 2:1 ratio with fresh medium.

**FACS analysis**
At day 6 the monocyte-derived cell population was harvested from cultures and analyzed for surface marker expression using CD1a-FITC and CD14-PE (BD Biosciences, San Diego, CA, USA) by flow cytometry. MSC were trypsinized and analyzed for expression of surface markers using CD90-FITC, CD73-PE, CD45-FITC, CD34-PE, HLA-DR-PE, HLA-ABC-FITC, CD80-PE (BD Biosciences, San Diego, USA) and CD105-FITC (Ancell Corp., Bayport, MN, USA). For surface staining, cells were collected and primary antibodies were added and incubated for 30 minutes at 4°C in the dark. Cells were washed with PBS/1%GPO and analyzed using a FACSCanto II (BD Biosciences, San Diego, CA, USA). The analysis of the acquired data was done with FlowJo software version 7.6.1 (Tree Star Inc. Ashland, OR, USA). Statistical analysis was performed using the Student t test.

**Analysis of cytokines**
Cytokine concentrations were measured in cell-free supernatants collected at day 6 from cultures of monocytes with or without MSC. To distinguish between cytokines produced by MSC or monocytes, the cells from the transwell co-cultures were separated at day 6 and cultured further in fresh medium for an additional 2 days. IL-6 protein concentrations were determined in supernatants of these cultures.

We also measured the of cytokine concentrations in supernatants of unstimulated MSC and MSC cultures containing IL-4 and GM-CSF. Culture supernatants were stored at -20°C until use. Cytokine concentrations were measured using the Bio-Plex Pro Human Cytokine assay (Bio-Rad laboratories, Inc, Hercules, CA, USA) or by sandwich ELISA (BD Biosciences, San Diego, CA, USA). Statistical analysis was performed using a Student t-test and a \( p<0.05 \) was considered statistically significant.

**Gene expression**
Total RNA was extracted from MSC and monocyte populations using the RNeasy micro kit (Qiagen GmbH, Hilden, Germany). cDNA was synthesized with Superscript III RT (Invitrogen Corp., Carlsbad, CA, USA). Q-PCR analyses were performed on a StepOnePlus real-time PCR system (Applied Biosystems, Foster city, CA, USA) using Sybr Green. The following primer sets were used:
Gene | Forward | Reverse
--- | --- | ---
IL-6 | 5’-TTCAATGAGGAGACTTGCCTG-3’ | 5’-ACAACAACAATCTGAGGTGCC-3’
IL-10 | 5’-CCGAGATGCTTCAGCAGAG-3’ | 5’-GGTCTTGGTTCTCAGCTTGG-3’
β-actin | 5’-AGGCATCCTCACCTGAGTA-3’ | 5’-CACACGCAGTCATTGTAGA-3’

All data were normalized using β-actin as a reference gene.

**Allogeneic mixed lymphocyte reaction assay**

Monocytes cultured for 6 days in the presence or absence of MSC in a transwell co-culture, were tested for their ability to stimulate the proliferation of allogeneic T cells. Allogeneic CD4\(^+\)CD25\(^-\) T cells were isolated from PBMC by MACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) using the CD4\(^+\)CD25\(^+\) regulatory T cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). CD4 T cells were isolated from PBMC using a cocktail of biotin-labeled antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and CD235a and anti-biotin microbeads followed by separation of CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^+\) T cells with CD25 microbeads. The CD4\(^+\)CD25\(^-\) T cells were stimulated with iDC or the monocyte-derived cells from the monocyte-MSC co-culture (MDC) at various ratios. After four days incubation, cells were pulsed overnight with 0.5μCi of \([\text{H}]\)-thymidine to determine T cell proliferation. Thymidine incorporation was expressed as mean corrected counts per minute (CCPM).

**Statistical analysis**

All data represent the average and standard deviation of multiple MSC donors. Unless otherwise specified, statistical analysis was tested by the Student’s t test for two groups and by a two-way ANOVA with a Bonferroni posttest for comparison of 3 groups using Prism5 software (GraphPad Software, Inc., CA, USA).
Results

**MSC skew the differentiation of monocytes to the formation of CD14<sup>+</sup> cell with low allostimulatory capacity**

Monocytes (purity after MACS isolation was more than 90% CD14<sup>+</sup> cells) cultured in the presence of IL-4 and GM-CSF differentiated into CD1a<sup>-</sup>CD14<sup>+</sup> immature dendritic cells (iDC). Following addition of MSC to these cultures, monocyte differentiation towards iDC was inhibited (Figure 1A-B). In the presence of MSC, a significantly lower fraction of monocytes acquired CD1a (15.1 ± 7.4% versus 70.6 ± 8.8%, p = 0.002, n = 7) and a significantly lower fraction lost CD14 expression (52.2 ± 8.1 % versus 99.5 ± 0.1 %, p = 0.001, n = 7) (Figure 1B).

The MSC were analyzed for expression of surface markers before and after co-culture with monocytes. MSC expressed HLA-class I, CD73, CD105 and CD90 and did not show expression of HLA-class II, CD31, CD45, CD80 and CD34. No changes were observed after co-culture with monocytes.

The monocyte-derived populations, formed in the absence or presence of MSC in transwell co-cultures, were functionally tested for their ability to induce proliferation of allogeneic T cells (Figure 1C). iDC effectively induced proliferation of allogeneic CD4<sup>+</sup>CD25<sup>-</sup> T cells. The monocyte-derived population that was generated in the presence of MSC, showed a significantly reduced allostimulatory capacity compared to the iDC (corrected counts per minute (CCPM), *, p<0.05; **, p< 0.01; ***, p<0.001, n = 3).

Figure 1. MSC prevent the differentiation of iDC.

(A) Representative FACS plots of the differentiation of CD14<sup>+</sup> monocytes towards iDC with or without MSC in a direct co-culture. (B) Cumulative data of 7 independent experiments with 6 different MSC donors and 7 different monocyte donors (data are mean ± SEM significance is relative to −MSC *p<0.05). (C) Corrected counts per minute (CCPM) of iDC and day 6 MDC from transwell co-cultures that were incubated with CD4<sup>+</sup>CD25<sup>-</sup> T cells to assess their allostimulatory capacity. Data are from 2 different MSC donors and the experiment was performed in triplo; significance is relative to iDC (*p<0.05, ***p<0.001).
Both IL-6 and IL-10 concentrations are increased in co-cultures of monocytes and MSC

Monocytes were cultured in the presence of IL-4 and GM-CSF and in the presence or absence of MSC and cytokine concentrations were measured in cell-free culture supernatants of the direct co-cultures. Supernatants from monocytes that were cultured in the presence of MSC contained significantly higher concentrations of IL-6 and IL-10 than control supernatants, derived from cultures without MSC (IL-6: 984.6 ± 122.6 pg/ml versus 333.4 ± 15.1 pg/ml, n = 3, p<0.0001; IL-10: 221.6 ± 30.0 pg/ml versus 11.9 ± 4.4 pg/ml, n = 3, p<0.05; Figure 2A-B).

Figure 2. Cytokine expression in MSC and monocytes in the co-cultures.

Cytokine protein levels in the supernatant of monocyte cultures at day 6 for IL-10 (A) and IL-6 (B) in the presence or absence of MSC (IL-10: mean ± SEM of 3 independent experiments with 3 different monocyte donors and 6 different MSC donors; IL-6: mean of 6 independent experiments with 6 different monocyte donors and 7 different MSC donors; *p<0.05, **p<0.01, ***p<0.001). (C) IL-6 and IL-10 mRNA expression in MSC after 6 days of culture with and without monocytes and growth factors in a transwell co-culture system. Fold change is relative to MSC alone. (D) IL-6 and IL-10 mRNA expression in the monocyte-derived cell population from the transwell co-culture at day 6 of differentiation in the absence or presence of MSC. Fold change is relative to MDC differentiated in the absence of MSC. (E) IL-6 secretion of MSC after 2 days of culture in fresh medium following 6 days culture with or without growth factors and monocytes. (F) IL-6 secretion of monocyte-derived-cells (MDC) after 2 days of culture in fresh medium following differentiation in the absence or presence of MSC. (C-F: data are mean ± SEM of 3 different MSC donors, *p<0.05, **p<0.01, ***p<0.001).
Cytokine expression by MSC and monocytes
The MSC-induced changes in surface marker phenotype and cytokine concentrations in the monocyte cultures were also observed when the experiments were performed in a transwell system (data not shown). To assess which population was responsible for the observed increase in cytokine concentrations, the MSC and the monocyte-derived cell populations from such transwell cultures were assessed at day 6 for expression of IL-6 and IL-10 mRNA. Neither unstimulated MSC, nor MSC cultured in the presence of IL-4 and GM-CSF and in the presence or absence of monocytes, were found to express IL-10 mRNA (Figure 2C). In agreement with this observation, IL-10 protein was not detected in culture supernatants from unstimulated MSC or from MSC cultured in the presence of IL-4 and GM-CSF (data not shown). Monocytes-derived cells, however, expressed detectable levels of IL-10 mRNA (fold change of $3.47 \times 10^{-4} \pm 8.5 \times 10^{-5}$ relative to β-actin) and these expression levels were considerably increased in the presence of MSC ($p<0.05$; Figure 2D). These data indicate that the IL-10 protein detected in the supernatants of monocyte-MSC co-cultures was produced by the monocyte-derived cell population.

Next, we analyzed expression levels of IL-6. Figure 2C shows that unstimulated MSC express IL-6 mRNA. A 5-fold increase in the average level of IL-6 mRNA expression was observed in MSC from cultures containing IL-4 and GM-CSF, but this increase did not reach statistical significance ($p = 0.06$), which was probably due to the high variation that was observed between the different MSC samples. No further increase in IL-6 mRNA expression was observed in MSC that were co-cultured with monocytes. After the 6 days co-culture, MSC and monocytes were separated and cultured for another 2 days to measure the cytokine concentrations secreted by both populations after co-culture. Similar to the IL-6 mRNA expression in MSC, the concentrations of IL-6 protein in culture supernatants of MSC cultured in the presence of IL-4 and GM-CSF and in the presence or absence of monocytes were not significantly increased compared to unstimulated MSC (Figure 2E). IL-6 mRNA was also expressed in the monocyte-derived iDC. Significantly increased expression levels ($p<0.001$) were found in monocyte-derived populations that were generated in the presence of MSC (Figure 2D). In agreement, the concentrations of IL-6 in supernatant of monocytes cultured for an additional 2 days after the 6 day MSC-monocyte co-culture were significantly increased compared to the control culture of monocytes with IL-4 and GM-CSF ($p<0.05$; Figure 2F).

Secreted factors from unstimulated MSC are responsible for the inhibition of monocyte differentiation
The observation that MSC also displayed their inhibitory effect on the differentiation of monocytes when the co-cultures were performed in a transwell system, showed that cell-cell contact between monocytes and MSC was not required and that soluble factors were responsible for this effect. We investigated whether these soluble factors were secreted by culture-expanded MSC or only by MSC stimulated in the co-cultures. Therefore, three types of conditioned medium were collected and tested for their ability to inhibit monocyte differentiation: 1) conditioned medium from unstimulated MSC (MSC-CM), 2) conditioned medium
from MSC cultured with the growth factors IL-4 and GM-CSF (MSC+GF CM) and 3) conditioned medium from the monocyte-MSC direct co-cultures (MSC+mono CM). Figure 3 shows that the differentiation of CD1a CD14⁺ monocytes into CD1a⁺CD14⁻ iDC was completely inhibited in the presence of CM derived from stimulated, but also from unstimulated MSC. This shows that the MSC constitutively produce and secrete the essential factor(s) that cause their inhibitory effect on monocyte differentiation to iDC.

**Figure 3.** Secreted factors from unstimulated MSC are responsible for the inhibition of monocyte differentiation.

(A) Representative dotplots of monocytes differentiated for 6 days in the presence of various conditioned media. (B) Cumulative data of CD1a and CD14 expression on day 6 monocytes differentiated in the presence of various conditioned media (data are collected from two different MSC donors and 1 monocyte donor). MSC-CM, MSC conditioned medium; MSC+GF CM, conditioned medium from MSC cultures containing the growth factors IL-4 and GM-CSF; MSC+mono CM, conditioned medium from the monocytes differentiated with growth factors in the presence of MSC in a direct co-culture. Statistical significance is compared to control (*p<0.05,**p<0.01).
Figure 4. IL-6, but not IL-10, is directly involved in the inhibitory effect of MSC on monocyte differentiation.

The role of IL-10 and IL-6 in the MSC-mediated inhibition of monocyte differentiation was investigated. (A) Adding a neutralizing antibody against IL-10 did not restore the iDC formation (data are representative dotplots from 2 independent experiments with 2 different MSC donors and 2 different monocyte donors). (B, C) The addition of a neutralizing antibody against IL-6 (2.5 µg/ml) to the transwell co-culture significantly reduces the inhibitory effect of MSC (data are of 3 independent experiments with 4 different MSC donors and 4 different monocyte donors; means ± SEM of; Statistical analysis was performed using the Wilcoxon signed rank test *p<0.05). (D, E) The increase of IL-6 and IL-10 mRNA expression on day 6 monocytes (fold change is relative to iDC)(D) and protein secretion in the co-culture at day 6 (E) is reversed when an IL-6 neutralizing antibody is added to the culture (data are means ± SD of two different MSC donors and 1 monocyte donor; *p<0.05).
Role of IL-6 and IL-10 in the MSC-induced inhibition of monocyte differentiation

To study the exact role of IL-6 and IL-10 in the inhibition of monocyte differentiation by MSC, neutralizing antibodies against IL-6 and IL-10 were added to the transwell co-cultures. Addition of neutralizing antibodies against IL-10 did not result in a restored iDC formation (Figure 4A). Addition of neutralizing antibodies against IL-6, however, caused a partly, but significant reduction of the inhibitory effect of MSC and resulted in an increased CD1a expression and a decrease in the expression of CD14 on monocytes (Figure 4B-C). This neutralization of IL-6 substantially reduced the upregulation of IL-6 mRNA and IL-10 mRNA expression in the monocyte-derived population (Figure 4D) and reduced IL-10 protein concentrations (Figure 4E) in the monocytes cultures with MSC or MSC-CM.

We confirmed the observation that addition of either IL-6 or IL-10 resulted in inhibition of the monocyte differentiation (Figure 5A-B). However, since MSC do not produce IL-10 (Figure 2), only IL-6 can be an MSC-derived factor that is relevant for the inhibitory effect of MSC. Indeed, we showed that addition of only IL-6 to the monocyte cultures induces IL-10 expression in monocytes, similar to the effect of MSC or MSC-CM (Figure 5A). To confirm that IL-6 is indeed the key factor produced by MSC, we depleted IL-6 from MSC-CM and studied the effect of this IL-6 depletion on monocyte differentiation. After depletion of IL-6, 84-96% of the IL-6 was depleted from the MSC-CM. In Figure 5C is shown that the inhibition of monocyte differentiation by MSC-CM was reversed by depletion of IL-6 from the CM. IL-6 depletion completely reversed the formation of a CD14+ monocyte-derived population, but did not reverse the MSC-CM-mediated inhibition of CD1a upregulation. Functional testing revealed that the MSC-CM-induced IL-10 secretion in the monocyte cultures was completely abolished following IL-6 depletion (Figure 5D). The allostimulatory capacity, however, was not restored by depleting IL-6 from the MSC-CM (figure 5E). Overall, our results show that MSC-derived IL-6 is an essential factor for the inhibition of monocyte differentiation by MSC and for inducing an IL-10 producing, monocyte-derived CD14+CD1a- cell population. The neutralizing and depletion experiments show that IL-6 is an important, but not the only factor, that is produced by MSC to exert the inhibitory effect on monocyte differentiation.
IL-6 (A) and IL-10 (B) were added to the monocyte differentiation cultures. Both IL-6 and IL-10 inhibit the differentiation to iDC similar to the effect of co-culture with MSC/MSC-CM and addition of IL-6 directly increases the IL-10 secretion by monocyte-derived cells (A) (data are means ± SD from two different MSC; *p<0.05). (C) Depletion of IL-6 from the MSC conditioned medium partly reverses the inhibiting effect of MSC on the monocyte-derived iDC differentiation (representative dotplot of 3 different MSC donors and 2 different monocyte donors. (D) MSC-derived IL-6 drives the IL-10 upregulation in the monocyte-derived cell population (C; data are means ± SD from two different MSC ; ***p<0.001). (E) Corrected counts per minute (CCPM) of iDC and day 6 MDC from cultures in MSC-CM and IL-6 depleted MSC-CM that were incubated with CD4+CD25 T cells to assess their allostimulatory capacity. Data are from 2 different MSC donors and the experiment was performed in triplo; significance is relative to iDC (*p<0.05, **p<0.01, ***p<0.001).
Discussion

It has been acknowledged that MSC inhibit the differentiation of monocytes towards iDC. The cytokines IL-6 and IL-10 have been described to play a role in the differentiation of monocytes towards immature dendritic cells (iDC) \(^{21,22}\). However, the role of these cytokines in the MSC-induced inhibition of this process is still unclear. In this study, we have used an MSC-monocyte co-culture system to identify the cytokines that are crucial in this process and reveal their exact cellular source of production. The addition of MSC to the monocyte cultures containing IL-4 and GM-CSF inhibited the monocyte-derived iDC differentiation and significantly increased the concentrations of the cytokines IL-6 and IL-10. Transwell experiments showed that cell-cell contact was not required for the inhibiting effect of MSC on monocyte differentiation, indicating that soluble factors were responsible for this effect. Subsequent experiments with MSC-CM showed that unstimulated MSC produced the soluble factors responsible for inhibition of monocyte differentiation. Previous studies suggested IL-6 to play a role in the inhibitory effect of MSC on the differentiation of monocytes to dendritic cells \(^{19,20}\). Whether this would be a direct or indirect effect remained unclear since both monocytes and MSC are capable of IL-6 production and secretion. We show that IL-6 production by MSC is constitutive and not significantly enhanced upon contact with monocytes. Since the effect of MSC could be replaced by MSC-CM, we considered MSC-derived IL-6 to be responsible for the MSC-induced inhibition of monocytes differentiation. Indeed, addition of neutralizing antibodies to IL-6 significantly reversed the inhibitory effect of MSC and depletion of IL-6 reversed the inhibitory effect of MSC-CM. These data clearly indicate that MSC-derived IL-6 regulates in vitro monocyte differentiation, thereby skewing monocytes towards an IL-10 producing cell type with a reduced allostimulatory capacity. Additional factors might however play a role in the complete inhibition of monocyte differentiation towards iDC by MSC, since the allostimulatory capacity of the monocyte-derived cells was not restored by depleting IL-6 from the MSC-CM.

We found that, in spite of a number of reports making this claim \(^{10,25-32}\), MSC do not produce IL-10, but that IL-10 is exclusively produced by monocytes in this system. Several reports claiming that the immunomodulatory properties of MSC are mediated by IL-10 have used antibodies to neutralize IL-10 in co-cultures of MSC and other cell types, including monocytes. These studies were therefore not designed to identify the cells that produce IL-10. Many reports indicate that MSC need to be activated in order to exert their immunomodulatory activities or to produce IL-10 \(^{26,33,34}\). Our data clearly show that activation of MSC is not required for the IL-10 induction in monocytes. Activated monocytes have been shown to increase their IL-10 expression upon IL-10 exposure \(^{35}\). Such increased IL-10 production could further enhance the immunomodulatory effect of MSC. Increased IL-10 secretion is a characteristic of type II activated macrophages, which have an immunomodulatory, anti-inflammatory function \(^{36,37}\). Type-II-activated macrophages have also been shown to be able to induce regulatory T cells \(^{38}\), another potential additional indirect immunomodulatory effect of MSC.
Taken together, we propose the hypothesis that MSC, by inducing IL-10 production in monocyte-derived cells, play a powerful regulatory role in multiple anti-inflammatory mechanisms (Figure 6), which could explain their clinical benefits in immunotherapy e.g. the treatment of GvHD. Thereby MSC may act as potent modulators of the (innate) immune response inducing an anti-inflammatory environment.

Figure 6. Hypothetical model of immunomodulating mechanisms exerted by MSC through production of IL-6.

By secreting IL-6, MSC skew monocytes from differentiation towards an antigen-presenting DC towards an anti-inflammatory cell that produces IL-10.
Reference List


17. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic


34. Groh ME, Maitra B, Szekely E, Koc ON. Human mesenchymal stem cells require monocyte-mediated activation to suppress


MSC skew monocytes through the production of IL-6