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Immunomodulatory properties of human multipotent stromal cells

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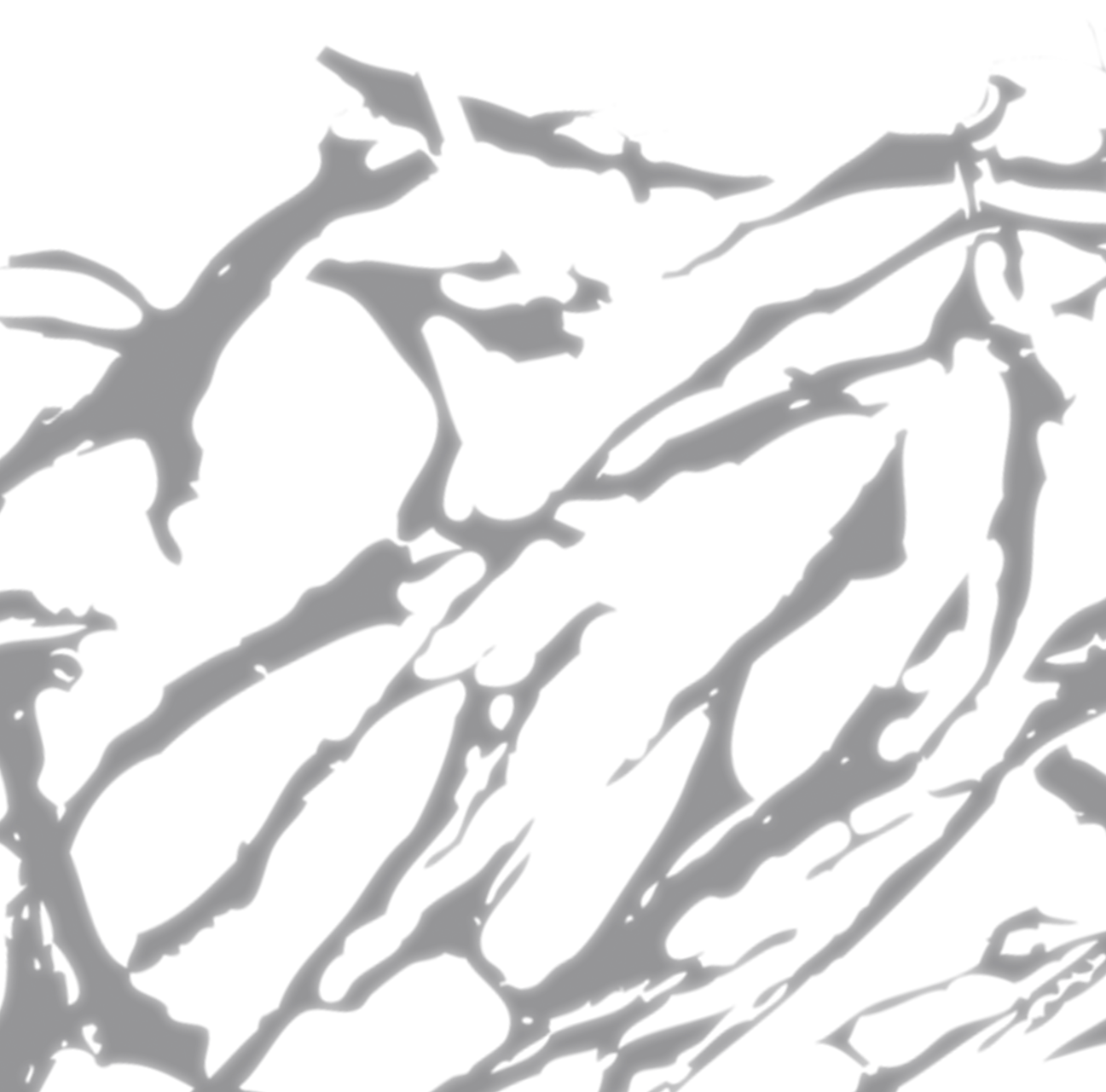
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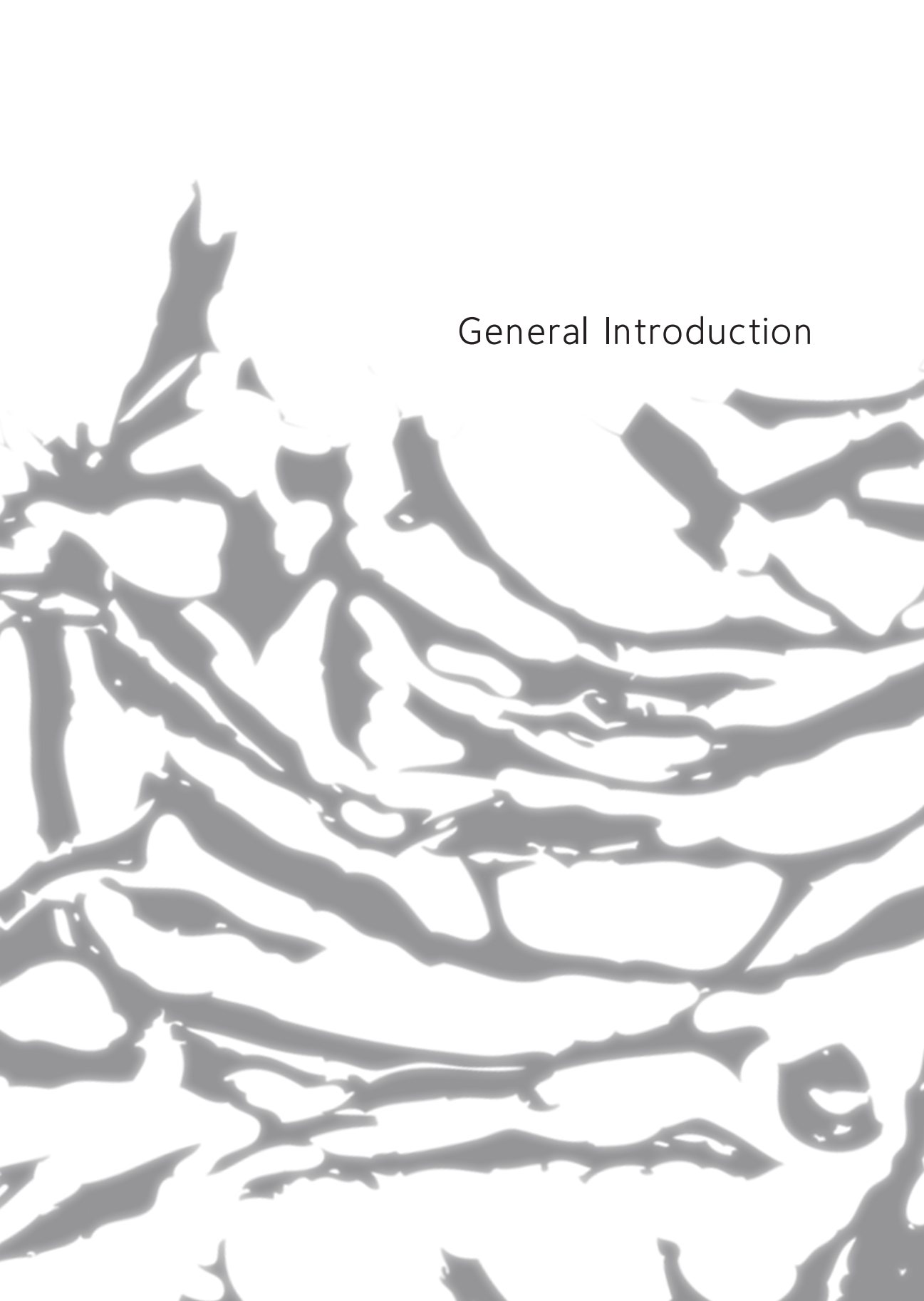
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Chapter 1





General Introduction

Introduction

The bone marrow (BM) contains a pool of multipotent stromal cells (MSC), that was first described by Friedenstein and colleagues¹. MSC are non-hematopoietic cells and are defined by the ability of plastic adherence and by their capacity to differentiate towards mesenchymal lineages¹⁻³. Friedenstein proposed the concept of 'stromal stem cells', showing the potential of MSC to give rise to bone and the hematopoietic environment. Later, Caplan proposed the term 'mesenchymal stem cells', but the debate on the nature, identity, function, ways of isolation and therefore also nomenclature of MSC in the research field continued. The International Society for Cellular Therapy (ISCT) made an effort to clarify the controversy in the field by introducing the term 'multipotent mesenchymal stromal cells'. A hallmark of MSC is their capacity to differentiate *in vitro* towards the osteogenic, adipogenic and chondrogenic lineage. This multilineage differentiation capacity *in vitro* and *in vivo* has raised interest for their use in regenerative medicine.

The regenerative effects of MSC may partly be a result of the secretion of factors that stimulate angiogenesis and suppress inflammation in a local or systemic manner. MSC have been shown to migrate to sites of inflammation *in vivo*, which might be relevant for their anti-inflammatory properties. The ability to suppress immune responses is an important feature of MSC that is currently under investigation aiming at the treatment of immune-related diseases, including Graft-versus-host disease, auto-immune diseases and inflammatory diseases like Crohn's disease.

Characterization of MSC

Source and expansion of MSC

After their first identification in BM, MSC have been isolated from a variety of different tissues, including adipose tissue (AT) ⁴, umbilical cord blood (UCB) ⁵, umbilical cord ^{6,7}, placenta ⁸, synovium ⁹, skin ¹⁰, kidney and lung ¹¹ and different fetal tissues ^{12,13}. Kern et al. ¹⁴ compared BM-MSC, AT-MSC and UCB-MSC and found that MSC could be isolated from all BM and AT samples, but only from 63% of the UCB samples. The proliferative capacity of UCB-MSC, was higher than that of AT-MSC and BM-MSC. Regarding clinical application, adipose tissue has become attractive as an alternative source for MSC. It contains a high frequency of MSC ¹⁵ and in addition, adipose tissue from liposuctions is at present discarded as waste ¹⁶ and is therefore readily available.

MSC are currently expanded in fetal calfs serum (FCS) and the use of bovine-derived FCS has raised concerns since this might be a potential source of prions. Therefore, alternatives are under investigation, including human serum and human platelet lysate (HPL). HPL has been proven to be superior over FCS in promoting proliferation of MSC in culture, while the morphology, immunophenotype and functionality of MSC cultured in both HPL and FCS was similar ¹⁷⁻¹⁹.

Phenotype of MSC

As proposed by the ISCT, that set the minimum criteria to define MSC ³, culture expanded MSC should express the markers CD105, CD90 and CD73 and lack expression of the markers CD45, CD34, CD11b, CD19 and HLA-DR. Small differences in immunophenotype were described for MSC isolated from different sources, e.g. early passage AT-MSC contain a population of CD34 expressing cells, that is not detected in MSC derived from other sources ^{20,21}. Although expression levels may vary, CD34 expression is maintained upon prolonged culture. The proposed surface markers are not related to specific functional characteristics used to define MSC. In search for specific subpopulations within the heterogeneous culture expanded MSC, the endothelial adhesion molecule CD146 (MCAM) has been identified as a marker to isolate MSC with a higher bone-forming capacity ²².

The markers that are used to characterize culture expanded MSC are not necessarily useful for isolating MSC prospectively. Surface markers can be up- or downregulated in culture and little is known about the physiological features of the cells that give rise to the plastic adherent MSC that are expanded in culture.

Enrichment strategies were applied to prospectively isolate primary MSC from the resident tissue. CD271 (low affinity nerve growth factor receptor), CD105 (endoglin) and MSCA-1 were reported to enrich for MSC in the human bone marrow ²³⁻²⁵. Only the CD271^{bright} population within the BM-derived mononuclear cells (MNC) contains colony-forming-unit-fibroblasts (CFU-F) ^{23,26} and CFU-F were even more enriched by selecting for MSCA-1⁺ cells ²⁴. Further isolation on basis of CD56 expression could differentiate between subpopulations with chondrogenic (CD56⁺) or adipogenic (CD56⁻) differentiation potential ²⁴.

Several studies showed that isolation of human perivascular cells, derived from a

variety of tissues using CD146 or STRO-1 expression, resulted in long-term cultures that exhibited a phenotype that was very similar to bone marrow-derived MSC and had the capacity of tri-lineage differentiation ²⁷⁻²⁹. Moreover, using confocal microscopy, Crisan et al. demonstrated co-localization of the perivascular markers CD146⁺, NG2⁺, PDGF- β ⁺ with the MSC markers CD44, CD90, CD73 and CD105 on pericytes at their native location ²⁹. These data suggest that cells with MSC-like characteristics actually reside in all organs of the adult organism.

Hierarchy within the MSC population

Ex vivo expanded MSC populations exist of a heterogeneous mix of multipotent, bipotent and unipotent cells ^{30,31}. For the hematopoietic system, the hierarchical model describing the relations between stem cells and the various progenitor and mature cells is well established ³². Far less is known about potential hierarchical relationships between MSC subpopulations. Single cells residing in the bone marrow of mice and humans were found that can give rise to an ectopic hematopoietic microenvironment ^{33,34}. This indicates that bone marrow contains multipotent mesenchymal precursor cells for the different cell types that compose bone i.e. bone, cartilage, adipose tissue, fibroblasts and hematopoiesis-supporting stromal cells. Additionally, the self-renewal capacity was indicated by colony assays that showed that the CFU-F frequencies of secondary MSC clones were similar to the CFU-F frequencies of primary MSC clones ^{35,36}. Aiming at defining the true stem cells within the MSC compartment of the bone marrow, Sacchetti showed evidence of self-renewal and multipotency of the CD45⁻CD146⁺ fraction of the human BM-MNC ³⁴. Together, these data suggest that MSC populations contain stem cells. In the murine bone marrow, SSEA-1⁺ cells were defined as primitive stem cells that are able to contribute to different mesenchymal cell types, but also endodermal and hematopoietic mesodermal lineages and can give rise to SSEA-1⁻ mesenchymal cells ³⁷. SSEA-1 was also found on primary human BM-MSC ³⁸, but it remains unclear whether these cells were primitive stem cells similar to their murine counterparts.

The existence of a common multipotent mesenchymal stem cell that can generate progenitors with different multilineage differentiation potentials ³⁹, indicates a hierarchical model of lineage commitment. The initial reports on MSC hierarchy described a consecutive loss of lineages, where the adipogenic lineages diverges first and the osteogenic lineage is default ^{30,31,36,40}. Subsequent studies, however, indicated a greater variety in bi- and unipotent clones and suggested a more complex model of hierarchical relations ^{41,42}. Since no specific markers have been identified for the prospective isolation of multipotent stem or progenitor cells, the study of the hierarchy of MSC population has proven to be challenging.

Physiological role of MSC

While MSC seem to be present in all tissues of the body, the physiological function of MSC *in vivo* is largely unknown. The role of MSC in the hematopoietic stem cell niche has been investigated in mice. These studies revealed that MSC are key players for the support of hematopoiesis ⁴³ and as osteoprogenitors (reviewed by Bianco et al. ⁴⁴). Mendez-Ferrer et al. showed that MSC in the murine bone marrow

express nestin and that HSC were reduced in the bone marrow after depletion of nestin⁺ cells ⁴³. In a later study, Chow et al. showed that depletion of macrophages resulted in the reduction in expression of HSC retention genes by nestin⁺ MSC. This indicated that the crosstalk of nestin positive stromal cells and macrophages in the murine hematopoietic niche seems essential for the maintenance of the hematopoietic niche ^{45,46}.

Several studies compared MSC from patients with those from healthy controls to gain more insight in the physiological role of MSC. In patients suffering from severe aplastic anemia ⁴⁷, B cell chronic lymphocytic leukemia ⁴⁸ and systemic lupus erythematosus ⁴⁹ aberrations were found in the immunoregulatory properties of MSC. From these comparative studies, it is however difficult to determine whether these aberrations in MSC are involved in the development of disease or a result of the progressing disease. Most studies on the immunomodulatory effects of MSC *in vivo* were performed with large numbers of cells that were expanded *ex vivo* before infusion. It is as yet unclear whether the observed immunomodulatory effects of MSC are biologically relevant in normal tissue homeostasis.

MSC have been isolated from placenta ^{8,50,51} and fetal membranes and a strong inhibitory effect on activated T cells was found to be exerted by fetal MSC, derived from amnion and decidua, as well as by maternal MSC derived from these tissues ⁵². These data are compatible with the hypothesis that MSC in the placenta may play a role in maintaining fetal-maternal tolerance.

MSC and the immune system

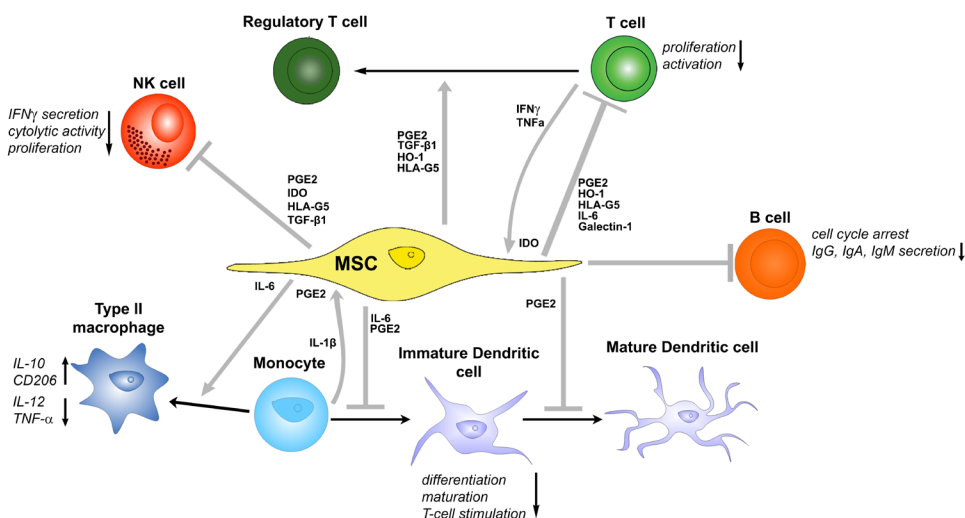
Immunogenicity

MSC are considered as low immunogenic, in accordance with their immunophenotype with a low expression of HLA class II and co-stimulatory molecules^{3;53;54}, which suggests that they are not readily recognized by the immune system. However, murine studies showed that MSC are immunogenic in an allogeneic host and that they can be rejected following injection into MHC class I and class II mismatched mice⁵⁵⁻⁵⁷. Moreover, several reports showed that MSC can elicit allogeneic T cell responses^{58,59} and that they can be lysed by activated NK cells⁶⁰.

Immunomodulation

It has been widely shown that MSC are able to suppress the proliferation of T cells stimulated with allo-antigens and mitogens^{53;61-66}. They effectively inhibit dendritic cell differentiation and maturation⁶⁷⁻⁶⁹ and the proliferation of B cells⁷⁰⁻⁷² and IL-2 stimulated NK cells⁷³⁻⁷⁷. Also the formation of regulatory T cells (Tregs) is promoted by MSC^{74;78-80}. Factors involved in these processes include indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), interleukin-6 (IL-6), galectin-1, heme oxygenase 1 (HO-1) and HLA-G5^{62;64-66;69;75;81-84}. Besides soluble factors, direct cell-cell contact is involved in the immunomodulation by MSC⁸⁴. The interactions of human MSC through cell-cell contact with the different cells of the immune system and the involved soluble factors is highlighted below.

Figure 1. MSC interact with different cells of the immune system and a range of soluble factors is involved.



T cells

The suppression of T cell proliferation is one of the most studied immunomodulatory effects of MSC^{53;85}. Activated T cells secrete IFN- γ and TNF- α , which induce expression of IDO in MSC^{64;86}. This results in a negative feedback loop, since IDO catalyzes the degradation of tryptophan, which is required for T cell proliferation⁸⁷. The level of induced IDO expression has been correlated to the immunosuppressive potential^{74;81;88}. Addition of the IDO-inhibitor 1-MT or neutralizing antibodies against IFN- γ inhibited the suppressive effect of MSC on T cell proliferation and IFN- γ production^{64;76;88}. In mice, MSC primarily produce inducible nitric oxide synthase (iNOS) rather than IDO after stimulation with IFN- γ ^{81;82}, indicating primary effector molecules are different between mice and humans.

MSC constitutively express PGE2 that suppresses the proliferation of T cells by several mechanisms, including the inhibition of IL-2 production and inhibition of intracellular calcium release (reviewed by Harris et al.⁸⁹). PGE2 expression in MSC was upregulated upon stimulation with IFN- γ and TNF- α , secreted by activated T cells⁷⁴ and upon stimulation with IL-1b that was secreted by monocytes⁹⁰. This resulted in increased concentrations of PGE2 in co-cultures of activated PBMC with MSC^{65;74}. Addition of a PGE2 inhibitor to these co-cultures resulted in restoration of PBMC proliferation^{65;74;90}, but did not reverse the MSC-mediated inhibition of pro-inflammatory cytokine secretion by PBMC⁶⁵.

Galectin-1 belongs to the family of animal lectins and has immunosuppressive and anti-inflammatory effects⁹¹. MSC constitutively express and secrete galectin-1⁸³ and in co-cultures of MSC with activated T cells, galectin-1 concentrations were increased⁸⁴. Recombinant galectin-1 showed a similar inhibitory effect as MSC on T cell proliferation⁸⁴ and a stable knockdown of galectin-1 in MSC resulted in restoration of the T cell proliferation and IFN- γ secretion, which was also observed after blocking galectin-1 activity in these co-cultures^{83;84}.

The potent immunosuppressive enzyme HO-1^{92;93} is also constitutively expressed by MSC, and the suppressive effect of MSC on T cell proliferation was abolished following inhibition of HO-1 activity^{62;94}. HO-1 expression was decreased in MSC that were co-cultured with lymphocytes in a mixed lymphocyte reaction, while the suppressive activity of these MSC was not diminished⁹⁴. This suggests that, besides HO-1, other factors are involved in MSC-mediated immunosuppression.

HLA-G, belonging to the non-classical HLA class I molecules, has also been implicated in the immunomodulatory properties of MSC. The secretion of the HLA class I molecule HLA-G5 by MSC is induced by IL-10 and cell-cell contact with allostimulated T cells. Addition of neutralizing antibodies against HLA-G resulted in a reduced inhibition of allogeneic T cell proliferation by MSC, demonstrating the contribution of HLA-G5 to the immunomodulating effect of MSC⁷⁵.

Studies that compared MSC derived from bone marrow, adipose tissue, umbilical cord blood or Wharton's jelly showed that they all share the capacity to suppress the proliferation of stimulated T cells^{7;66;95;96}. Functional differences between MSC from different sources were also demonstrated. In a comparison of fetal and maternal MSC from the amnion and decidua, fetal MSC exhibited a more profound inhibitory effect on T cell proliferation than maternal MSC⁵².

Regulatory T cells

The effect of MSC on the generation of Tregs has mainly been studied *in vitro* and the cytokines involved are not yet completely clear. In different experimental settings, MSC were able to induce an increase in the frequency of functionally suppressive CD4⁺CD25^{hi}FoxP3⁺ cells when co-cultured with PBMC or T cells^{79;80;97;98} or in an allogeneic MLR^{75;78;94}. Other cells besides CD4 T cells seem to play a role, since co-cultures of MSC with whole PBMC populations resulted in Treg induction, while this was not observed in co-cultures with CD4⁺T alone^{79;80}.

Several factors have been suggested to play a role in MSC-induced formation of Tregs. TGF- β 1 was described to promote induction of Tregs in mice⁹⁹ and is constitutively produced by MSC⁷. In co-cultures of MSC with unstimulated PBMC, English et al. showed that TGF- β 1 and PGE2 are involved in MSC-mediated promotion of Treg formation⁷⁹, while others showed that blocking these two factors in MSC-PBMC co-cultures did not affect the MSC-mediated promotion of Treg formation⁹⁸. Other studies, performing co-cultures of allo-activated PBMC with MSC, report a role for HO-1⁹⁴ and HLA-G5⁷⁵ in the MSC-mediated induction of Treg formation. Despite these results, no consensus has been reached about the factors that are involved in the MSC-induced Treg formation. Moreover, it is as yet unknown whether the Tregs generated under influence of MSC are newly induced from CD4 effector T cells (iTregs) or that they are expanded natural occurring Tregs (nTregs).

In preclinical models of allergic airway inflammation and heart transplantation, MSC induced the generation of Tregs *in vivo*^{100;101}. A recent study also showed an increased percentage of Tregs in kidney transplantation recipients after MSC infusion¹⁰².

Natural killer cells

Natural killer (NK) cells are cells of the innate immune system that are activated depending on the balance of stimulatory and inhibitory signals that they receive through their receptors. NK cells play an important role in the innate immune response to tumours and viruses and target cells that lack the expression of MHC class I^{103;104}. MSC escaped lysis by naïve, non-activated NK cells, but were susceptible to lysis by allogeneic and autologous activated NK cells^{60;105-108}. NK-mediated lysis of MSC was shown to be inversely correlated to the level of HLA class I expression on MSC⁶⁰.

Inversely, MSC also affect natural killer (NK) cells by inhibition of proliferation and of cytokine production⁷⁴⁻⁷⁸. MSC inhibited the IL-2 induced NK proliferation, which was mediated by PGE2 and TGF- β 1^{73;76;77}. Moreover, the cytolytic activity of NK cells was impaired by MSC, dependent on MSC-derived PGE2 and HLA-G5^{73;75}.

B cells

The research on the immunosuppression by MSC has focussed predominantly on T cells and data on the effect of MSC on B cells are scarce¹⁰⁹. Studies with murine cells showed that MSC affect the proliferation, activation and differentiation of B cells stimulated with anti-CD40 and IL-4¹¹⁰ or mitogens^{111;112}. B cell proliferation is inhibited by MSC through arrest in the G0/G1 phase of the cell cycle^{70;72}. MSC

also inhibit B cell differentiation by hampering the production of IgM, IgA and IgG^{70;71;111} and AT-MSC show a stronger suppression of immunoglobulin production than BM-MSC¹¹³. These inhibitory effects of MSC on B cells are mediated by, as yet unknown, soluble factors¹¹². In contrast to the inhibition of activation or proliferation of B cells, MSC promote the survival of naïve B cells¹¹⁴, a process that might be dependent on IL-6, that stimulated B cell proliferation¹¹⁵.

Monocytes, macrophages and dendritic cells

Monocytes are important regulators of inflammation as part of the innate immune response. *In vitro*, monocytes promote or are even required for the inhibitory effect of MSC on T cell activation and proliferation^{59;90;116}. It was reported that MSC promote the activation of purified T cells and only suppress T cell activation in the presence of monocytes⁵⁹. The interaction between MSC and monocytes results in skewing of monocytes towards a type of macrophage with anti-inflammatory properties^{88;117}. Monocyte-derived macrophages that were cultured with MSC showed characteristics of type 2 macrophages, including increased expression of CD206 and secretion of high levels of IL-10 and low levels of IL-12 and TNF- α ^{59;116;117}. Moreover, MSC impair the allostimulatory function of monocytes¹¹⁶.

Interaction with MSC also affects the differentiation and function of dendritic cells (DC). *In vitro*, MSC inhibited CD34-derived and monocyte-derived immature dendritic cell (iDC) differentiation^{68;69;118;119}. Besides prevention of differentiation, MSC also impaired the antigen-presenting ability of iDC¹²⁰, the ability of mature DC to induce allogeneic T cell proliferation¹¹⁸ and the migratory properties of DC¹²¹. Studies on the molecular mechanisms behind these effects of MSC revealed a role for PGE2 and IL-6. Inhibition of PGE2 expression reversed the MSC-mediated inhibition of iDC differentiation¹²². Several studies proposed IL-6 as important factor, since IL-6 has a similar inhibitory effect on dendritic cell differentiation as MSC⁶⁷ and neutralising IL-6 abolished the MSC-induced inhibition of iDC differentiation *in vitro*^{68;69;119}.

The involvement of monocytes/macrophages and DC in the therapeutic effect of MSC was studied in several animal models. Infusion of culture-expanded MSC impairs capacity of DC to migrate and to prime T cells *in vivo*¹²³. Also infusion of *ex vivo* modulated BM-derived macrophages, by co-culture with AT-MSC or AT-MSC conditioned medium, ameliorated colitis and decreased mortality in experimental IBD and sepsis in mice¹²⁴. In another model of sepsis, where transfer of MSC decreased mortality, depletion of macrophages eliminated the beneficial effect of MSC¹²⁵. Furthermore, infusion of MSC in mice with an experimental acute myocardial infarction resulted in an increased fraction of anti-inflammatory type 2 macrophages in the blood and the heart¹²⁶.

Overall, these studies suggest that monocytes play a decisive role in enabling MSC to exert their immunomodulatory activity. MSC induce the generation of regulatory macrophages that exert anti-inflammatory activity and this may also play a role in tissue repair and wound healing.

Immunomodulation by MSC

Animal models

The effect of both human and murine MSC administration in mice was investigated in several models of autoimmune diseases (reviewed by Uccelli et al. ¹²⁷). MSC were administered in mice with experimental autoimmune encephalomyelitis (EAE) ^{128;129}, collagen-induced arthritis ¹³⁰ and experimental diabetes ¹³¹. In these models, infusion of MSC decreased the severity and incidence of disease, measured by clinical scores and inflammatory responses in the affected organs, were reduced. EAE-mice that were treated with MSC showed decreased demyelination and their T cells showed a reduced response against myelin oligodendrocyte glycoprotein ^{129;132}. In mice with collagen-induced arthritis, MSC treatment resulted in a reduction of Th1/Th17 expansion and of the production of inflammatory cytokines and in an increase of IL-10 production in the joints ¹³⁰. Also in inflammatory diseases like experimental colitis, MSC alleviated the clinical symptoms. In mice with TNBS-induced colitis, MSC treatment resulted in reduction of pro-inflammatory cytokines in the colon ^{133;134}.

In these models, the transfusion of xenogenic, allogeneic and syngeneic MSC produced similar effects and the beneficial effect of MSC in these animal models supported the use of MSC as therapy for autoimmune and inflammatory diseases. Another area of potential therapeutic application of MSC is the transplantation setting. Syngeneic ⁵⁵ and xenogenic ¹³⁵ MSC were shown to enhance long-term engraftment of allogeneic bone marrow transplants in mice, which was associated with tolerance to host and donor antigens ⁵⁵. In solid organ transplantation, MSC induced Treg mediated tolerance and a synergy of MSC with immunosuppressive treatment was reported (reviewed by Casiraghi et al. ¹³⁶).

Co-transplantation of both allogeneic and syngeneic mouse MSC has been investigated for the prevention and treatment of GvHD after hematopoietic stem cell transplantation ¹³⁷⁻¹³⁹. Prevention or delay of GvHD development was observed in models where MHC-mismatched as well as MHC-haploidentical or xenogeneic MSC were used ¹⁴⁰. The suppression of GvHD development by MSC was shown to be dependent on IFN- γ activation of MSC ¹³⁹, which confirms the molecular data found in *in vitro* experiments on the immunomodulatory mechanisms of MSC. MSC treatment failed to prevent GvHD after disease onset, which makes it more challenging to use MSC to manage established GvHD.

Safety studies

Before starting the first studies in humans, the safety of application of xenogeneic, allogeneic and syngeneic MSC has been investigated in animal models. It was shown that murine and rhesus monkey MSC develop aneuploidies in culture and are prone to malignant transformation after numerous passages. Murine MSC showed chromosomal aberrations when expanded in culture and injection of murine MSC in mice resulted in sarcoma in the lungs and other organs ¹⁴¹⁻¹⁴³. This has not been observed for human MSC, that could be culture expanded *in vitro* for up to 25 passages without signs of malignant transformation ^{142;144}. Aneuploidies, however, were reported in cultivated human MSC populations. This phenomenon

was probably donor-dependent since multiple preparations of MSC samples from the same donor showed the same genetic alterations. Moreover, these cells showed normal senescence and no selective growth advantage or signs of malignant transformation were observed ¹⁴⁵. An analysis of adverse events in clinical trials with MSC revealed no severe adverse events related to MSC infusion, but showed a significant association between MSC administration and transient fever, probably due to acute inflammatory reactions to preparations of MSC ¹⁴⁶.

Clinical studies

The use of MSC as a cellular therapy is currently explored in phase I, II and III clinical trials, aiming to promote of engraftment following solid organ transplantation ¹³⁶ and hematopoietic stem cell transplantation¹⁴⁷ and to improve treatment of GvHD ¹⁴⁸⁻¹⁵⁰, neurological diseases ¹⁵¹, ischemia reperfusion injury ¹⁵² and inflammatory diseases like Crohn's disease ¹⁵³.

For the promotion of engraftment, MSC have been co-transplanted with hematopoietic stem cells or stem cell grafts. In a study by Ball et al., 14 children undergoing transplantation of HLA-disparate CD34⁺ cells were co-transplanted with MSC. All patients showed sustained hematopoietic engraftment, compared to 15% graft failure in historical controls ¹⁴⁷. Infusion of MSC was demonstrated to be safe and feasible and seemed to promote the engraftment of HSC patients receiving an autologous HSCT ^{154;155}.

Also for the treatment of GvHD, promising results have been reported. The first report was a case study of a 9-year old boy that was successfully treated with MSC for severe steroid resistant GvHD that occurred after transplantation of HSC from a haploidentical donor ¹⁵⁰. MSC treatment resulted in a rapid healing of the gut epithelium. Further studies on larger patient groups with steroid refractory GvHD, showed that infusion of MSC resulted in resolution of GvHD in the gut, liver and skin and enhanced survival compared to non-responding patients ^{148;149}. For these studies, MSC were derived from HLA-identical siblings, haploidentical donors or HLA-mismatched third party donors and no relation was found between the response rate and the HLA match.

In the field of inflammatory diseases, the potential treatment of inflammatory bowel disease by MSC has been explored. Systemic infusion of autologous MSC in patients with refractory Crohn's disease proved to be safe ¹⁵⁶. Furthermore, local intrafistular injections of MSC resulted in healing of fistulising lesions in patients with fistulising Crohn's disease ¹⁵³.

Biodistribution of MSC

The fate of MSC after infusion has not fully been elucidated. Intravenous administration of MSC resulted in entrapment of a large proportion of the infused cells in the lungs ^{157;158}. In experimental colitis, only after pretreatment of MSC with IFN- γ , more MSC were present in the inflamed tissue compared to the surrounding tissues after intraperitoneal injection ¹³³. Studies on long-term engraftment in human and mouse suggested that MSC do not robustly distribute in the donor tissues beyond the lungs and no clear evidence of sustained engraftment has been demonstrated ^{159;160}. These data suggest that delivery of MSC to the site of

injury might not be required and that the therapeutic effect of MSC is the result of paracrine signalling induced by secreted factors from the MSC. With regards to the safety of MSC infusion, clearance of MSC from the system might limit the risks of ectopic tissue formation and malignant transformation.

Scope of this thesis

This thesis focuses on elucidating mechanisms underlying the immunomodulatory properties of human MSC. In addition, studies on the developmental hierarchy of expanded MSC are included. All studies were performed *in vitro*, using human MSC derived from bone marrow or adipose tissue. The cells were expanded according to the protocol that is used for the application of MSC in the clinical trials that are ongoing in the Leiden University Medical Center.

Chapter 2 describes a study aiming at unravelling the clonal composition and hierarchical relationships within the MSC population. By studying the differentiation and proliferative capacity of primary and secondary MSC clones derived from bone marrow aspirates, we aimed to find correlates to the proliferative potential of MSC populations.

Chapter 3, 4 and 5 focus on the immunomodulatory properties of MSC. These studies aim at selecting the most optimal source of MSC for the purpose of immunomodulation and at investigating the molecular mechanisms and cellular interactions that are involved.

Adipose tissue has been described as an promising alternative for bone marrow MSC. We performed a systematic comparison of the immunomodulatory capacities of bone marrow-derived MSC (BM-MSC) and adipose tissue-derived MSC (AT-MSC) derived from age-matched donors. The results of this study are described in chapter 3.

In chapter 4 and 5, the immunomodulatory properties of MSC are studied and experiments were performed to investigate the cellular interactions and molecular mechanisms involved in immunomodulation by MSC.

MSC may inhibit monocyte-derived immature dendritic cell (iDC) differentiation. The cytokines IL-6 and IL-10 have been determined as important factors in this process. In chapter 4 we describe studies on the cellular source of IL-6 and IL-10 and their role in the MSC-induced inhibition of monocytes-derived iDC differentiation.

Another important immunomodulatory effect of MSC is related to their capacity to induce the formation of Tregs. The mechanisms by which MSC modulate Treg formation are largely unclear and conflicting reports about the factors involved are published. In chapter 5, a novel indirect pathway, involving skewing of monocytes towards anti-inflammatory macrophages, is presented.

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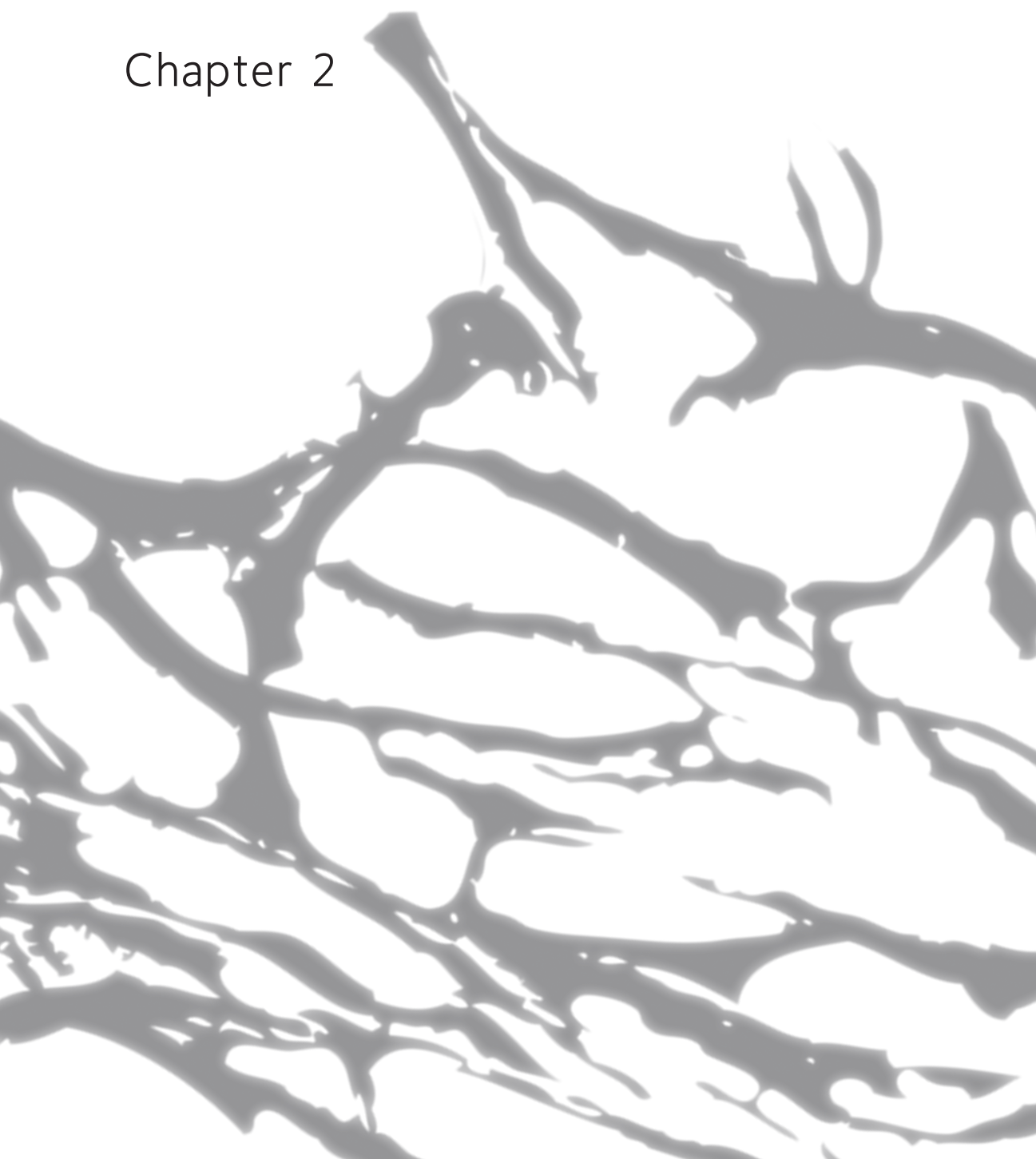
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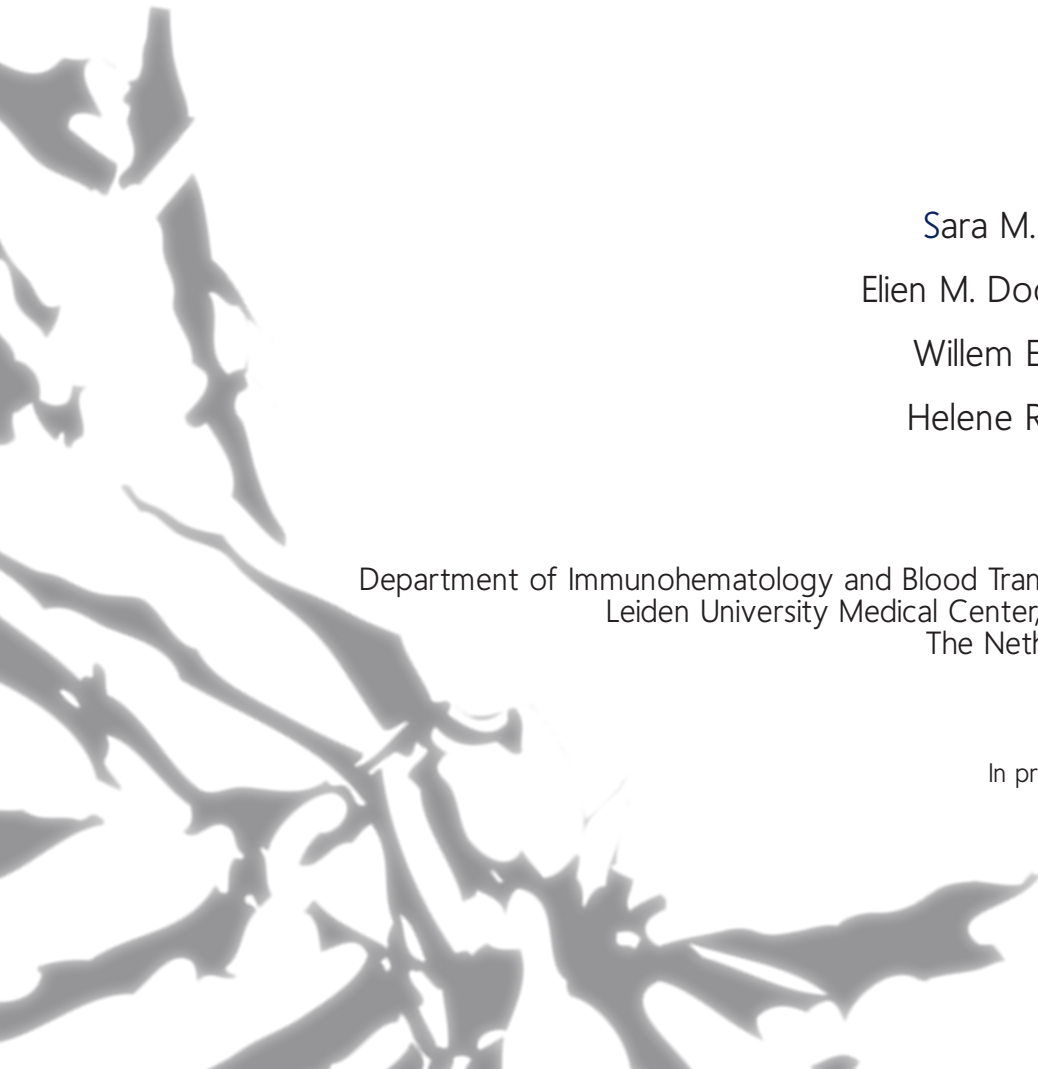
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Chapter 2





The osteogenic differentiation potential of bone marrow-derived multipotent stromal cells correlates with their proliferative capacity: a clonal analysis

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In preparation

Abstract

Background

Multipotent stromal cells (MSC) are expanded *ex vivo* for various clinical applications. Between MSC cultures from different donors a large variation is observed in proliferative potential and in the capacity to differentiate into mesenchymal lineages.

Methods

We generated primary and secondary MSC colonies (CFU-F) from human bone marrow and investigated their frequency, differentiation potential and proliferation rate in order to find correlates to the proliferative potential of MSC cultured in bulk and to unravel the hierarchical relations

Results

We observed a correlation between the proliferative potential and the osteogenic differentiation potential, i.e. colonies with osteogenic differentiation capacity showed a high proliferative potential. Vice versa, fast proliferating colonies had a higher osteogenic differentiation capacity than slowly proliferating colonies.

Discussion

We propose a hierarchical model in which osteogenic colonies are more primitive than unipotent adipogenic colonies and in which cells gradually lose their differentiation potential, concurrently with a loss in proliferative capacity.

Introduction

The bone marrow contains a pool of multipotent stromal cells (MSC), which are non-hematopoietic progenitor cells that possess the capacity to differentiate into various mesenchymal lineages^{1,2} and have been shown to possess immunomodulatory properties³. For their clinical application, MSC have to be expanded *ex vivo* to millions of cells. A large variation between donors exists with respect to the expandability of bone marrow (BM)-derived MSC populations. This variation cannot be explained by differences in donor age or gender, since no correlation was found with either proliferation capacity or CFU-F frequencies of MSC⁴. Due to the variation in expansion potential, the required cell numbers for clinical use are not always reached, thus limiting their therapeutic use.

Ex vivo expanded MSC populations are composed of cells that vary in their differentiation capacity^{5,6}. The lack of markers that uniquely identify MSC in the resident tissue has precluded the analysis of prospectively isolated single MSC and instead single-cell derived MSC colonies have been studied to unravel the hierarchical relationships between MSC with different capacities to differentiate. MSC clones with multilineage differentiation potential have been identified in different tissues⁷⁻⁹ and several hierarchical models have been proposed in which common multipotent stromal cells generate progeny with more restricted differentiation potentials^{5,8-14}. Studies on BM-derived MSC^{5,10} showed no evidence for the presence of purely adipogenic colonies, while in skin-derived MSC these colonies were found⁹. Based on these previous studies, deterministic hierarchy models in which the adipogenic lineage forfeits first and the chondrogenic and osteogenic lineage proceed together, diverging later^{5,8}, or models with a more stochastic consecutive loss of lineage potential¹¹ were proposed.

In this study we classified primary human BM-derived MSC colonies from expanded MSC populations with respect to their proliferative potential and searched for correlates that are associated with a high expansion potential. Furthermore, we aimed to further dissect the developmental hierarchy within expanded MSC at the earliest possible timepoint, i.e. by assessing the primary colonies and their progeny. By studying primary MSC colonies we formulate a model that describes the heterogeneity in differentiation potential that is observed in the early *ex vivo* MSC progeny.

Materials and methods

Generation of MSC from bone marrow

To isolate human MSC, bone marrow aspirates were harvested from the iliac crest of healthy individuals (age 14-69) after written informed consent, according to procedures that were approved by the medical ethical committee. Mononuclear cells (MNC) were isolated with a Ficoll-Paque density gradient (1.077 g/cm³). To expand MSC, the MNC were plated at $1.3 \times 10^5/\text{cm}^2$ in culture medium (DMEM-low glucose with glutamax (Invitrogen Corp., Paisley, UK) supplemented with 10% fetal calfs serum (FCS; Greiner Bio-one B.V. Alphen a/d Rijn, The Netherlands) and Penicillin/Streptomycin (P/S; Invitrogen Corp., Paisley, UK)). The cells were incubated at 37°C and 5% humidified CO₂ and refreshed with culture medium every 3-4 days until confluency was reached. The MSC monolayer was detached using trypsin/EDTA (Invitrogen Corp., Paisley, UK) and the MSC were reseeded at 4,000/cm² for further expansion.

Generation of primary MSC colonies

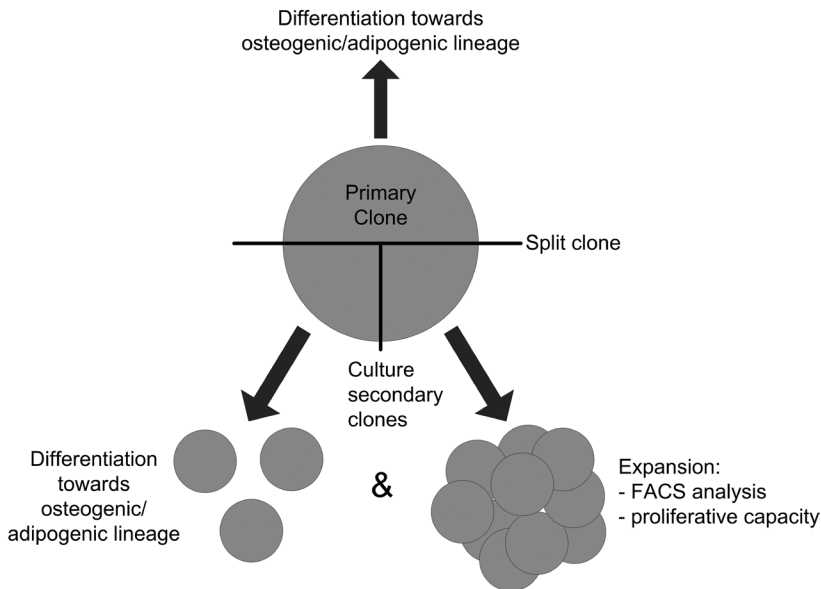
To generate primary separate MSC colonies (CFU-F), mononuclear cells were plated at lower density than was used for expansion (1.0×10^4 - 1.0×10^5 cells/cm² in 12-wells plates (Corning BV Life Sciences, Amsterdam, The Netherlands). Primary, single cell-derived MSC colonies were observed after 2-3 weeks of culture and only wells that contained separated colonies were used for further analysis. To study the difference between fast and slowly proliferating colonies, primary colonies of two different donors were divided into 4 groups based on their size. Colonies were analyzed at 100x magnification and categorized by size ('--' = < 0.25; '-' = 0.25 - 0.5, '+/-' = 0.5 - 1.0, '+' = > 1.0 microscopic field). The selected colonies were assayed for their osteogenic differentiation potential.

Generation of secondary colonies and expansion of clones

Primary colonies from three different MSC donors were selected for further analysis of the hierarchical relationships within the MSC population. Solitary primary colonies were identified and half of each colony was scraped off with a pipette tip. The scraped cells were collected in culture medium and divided in two equal volumes to be transferred to two separate wells of a 12-wells plate (figure 1). One well was used for further expansion of the cells derived from the primary colony to perform surface marker analysis and analysis of expansion potential as described below. The other well was used for the analysis of the secondary colonies that were generated after replating of $\frac{1}{4}$ of the cells from the primary colony. These secondary colonies and the remaining half of the primary colonies were assayed for their osteogenic and adipogenic differentiation potential.

Analysis of expansion potential

MSC were expanded in culture until 80% confluency was reached. Cells were then harvested with 0.25% trypsin and 1mM EDTA, counted and replated at a density of 4000 cells/cm². This was repeated until the cells stopped proliferating. The number of population doublings was calculated with the following formula; PD =

Figure 1. Experimental setup for analysis of primary and secondary colonies.

$\log N / \log 2$, where N is the cell number of the harvested MSC divided by the initial number of cells seeded. The expansion potential was defined as the total number of cells that could be obtained by expansion.

Osteogenic and adipogenic differentiation

The cells were cultured with osteogenic, adipogenic or osteogenic/adipogenic differentiation medium.

For osteogenic differentiation, MSC were cultured in α -MEM (Invitrogen Corp., Paisley, UK) with L-glutamin (200nM, Invitrogen Corp., Paisley, UK), P/S and 10% FCS supplemented with 10^{-7} M dexamethason and 50 μ g/ml Vitamin C (both from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). After the first week 5 mM β -glycerophosphate (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was added to the differentiation medium.

For combined osteogenic/adipogenic differentiation, MSC were cultured in differentiation medium that consisted of α -MEM with L-glutamin, P/S and 10% FCS supplemented with 10^{-7} M dexamethasone, 50 μ g/ml Vitamin C, insulin (10 μ g/ml), indomethacin (5 μ M) and IBMX (5 μ M) (all from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). After the first week 5 mM β -glycerophosphate was added to the differentiation medium.

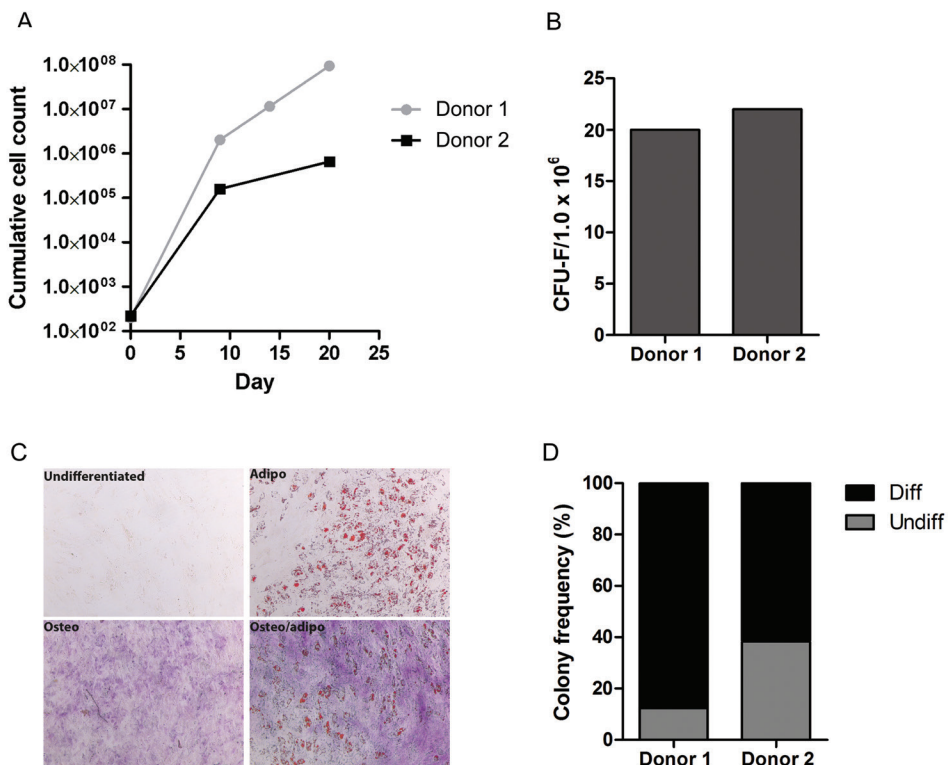
Cultures were refreshed once a week. After 21 days of differentiation, the cultures were stained for alkaline phosphatase activity with Fast Blue (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and the formation of lipid droplets was

visualized with Oil-red O staining (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). All colonies were photographed and assessed for the presence of ALP positive cells and/or lipid droplets.

Flow cytometry

For analysis of surface marker expression, MSC from each expanded colony were trypsinized and resuspended in staining buffer (PBS/1% GPO). Primary antibodies were added and incubated for 30 minutes at 4°C in the dark. Antibodies used were CD90-FITC, CD73-PE, CD45-FITC, CD34-PE, HLA-DR-PE, HLA-ABC-FITC, CD80-PE (all from BD Biosciences, San Diego, USA) and CD105-FITC (Ansell Corp., Bayport, MN, USA). Cells were washed with staining buffer and analyzed using a FACSCalibur (BD Biosciences, San Diego, CA, US). The analysis of the acquired data was done with FlowJo software version 7.6.1 (Tree Star Inc. Ashland, OR, USA).

Figure 2. Heterogeneity between BM-MSc populations.



(A) Expansion curves of BM-MSc populations. (B) CFU-F frequency of per 10 x 10⁶ MNC. (C) Representative images of clones with no differentiation capacity, only adipogenic differentiation capacity, only osteogenic differentiation capacity and combined adipogenic/osteogenic differentiation capacity. (D) Frequency of differentiated/undifferentiated primary clones after culture in combined osteogenic/adipogenic differentiation medium. Diff = differentiated clones; Undiff = undifferentiated clones.

Results

Heterogeneity in expansion potential and clonal composition

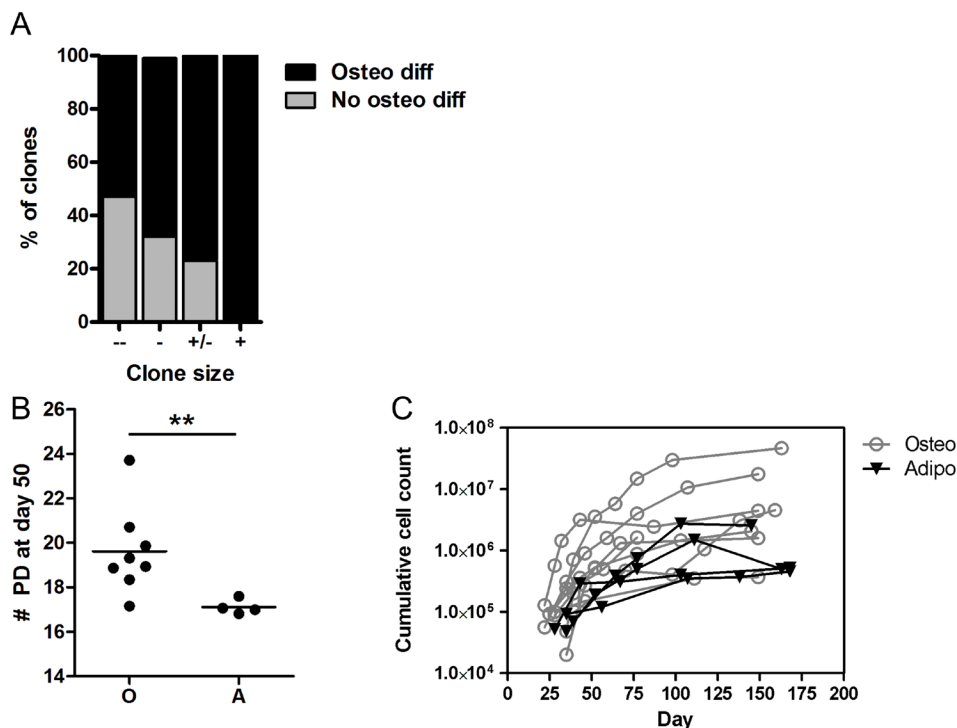
We analyzed CFU-F frequencies and expansion potential of MSC populations from two different donors and tested their primary colonies for differentiation capacity. Donor 1 showed a markedly higher expansion capacity than donor 2 in cultures that were started with 10×10^6 MNC (figure 2A). The theoretical cumulative cell count at day 20 of expansion was 9.4×10^7 cells for donor 1 versus 6.6×10^5 cells for donor 2. The CFU-F frequencies for the BM aspirates, however, were similar. Where donor 1 contained 20 CFU-F per 1.0×10^6 MNC, donor 2 contained 22 CFU-F per 1.0×10^6 MSC (figure 2B). 359 primary colonies of donor 1 and 91 primary colonies of donor 2 were cultured in osteogenic/adipogenic differentiation medium. The cultures derived from donor 1 showed a higher frequency of clones with differentiation potential (osteogenic, adipogenic or both; figure 2C) compared to the cultures derived from donor 2 (figure 2D). These data show heterogeneity in the clonal composition of the primary MSC population between these two donors and we hypothesize that the differentiation capacity of the primary clones is correlated to the expansion potential of the MSC population.

Expansion potential is positively related to osteogenic differentiation capacity

To study the possible correlation between expansion potential and differentiation capacity, we discriminated between large and small (see M&M) colonies, assuming that the large colonies represented rapidly expanding clones and the small colonies represented slowly expanding clones. New MSC cultures were initiated from fresh BM aspirates from 2 different donors and 85 primary colonies (46 for donor 1 and 39 for donor 2) were first categorized by size and subsequently cultured in osteogenic differentiation medium. Almost half (47%) of the very small colonies lacked osteogenic differentiation capacity, while all large ('+') colonies showed osteogenic differentiation (figure 3A). This difference in the phenotype distribution between small and large colonies was statistically significant ($p < 0.0001$). The progenitor cells forming small colonies exhibited a limited proliferative capacity and had lost the ability to differentiate into the osteogenic lineage. The progenitor cells that formed the large colonies exhibited a relatively high proliferative capacity and had retained the ability of osteogenic differentiation. These data indicate that MSC with a relatively high proliferative capacity maintain a high capacity for differentiation.

Additionally, we investigated the relationship between expansion potential and differentiation potential, starting from the other end of the equation, i.e. by assessing the expansion capacity of colonies selected on basis of their differentiation potential. To this end new cultures were initiated and primary colonies from 3 different donors were split in half and the cells in the remaining half were differentiated. The other half was divided in two equal parts and one part was used for further expansion (figure 1). The expanded cells, derived from primary colonies displaying osteogenic differentiation, showed a higher expansion potential compared to the expanded cells derived from primary colonies that were

Figure 3. Primary colonies with osteogenic differentiation capacity show a higher expansion potential than colonies with adipogenic differentiation capacity.



(A) The frequency of osteogenic colonies correlated with the size of the colonies (chi-square test, $P < 0.0001$). (B) Number of population doublings at day 50 of expanded cells derived from primary colonies that showed either osteogenic or adipogenic differentiation capacity (unpaired t-test with Welch's correction; *, $P < .05$; **, $P < 0.01$). (C) Expansion curves of clones with osteogenic or adipogenic differentiation potential.

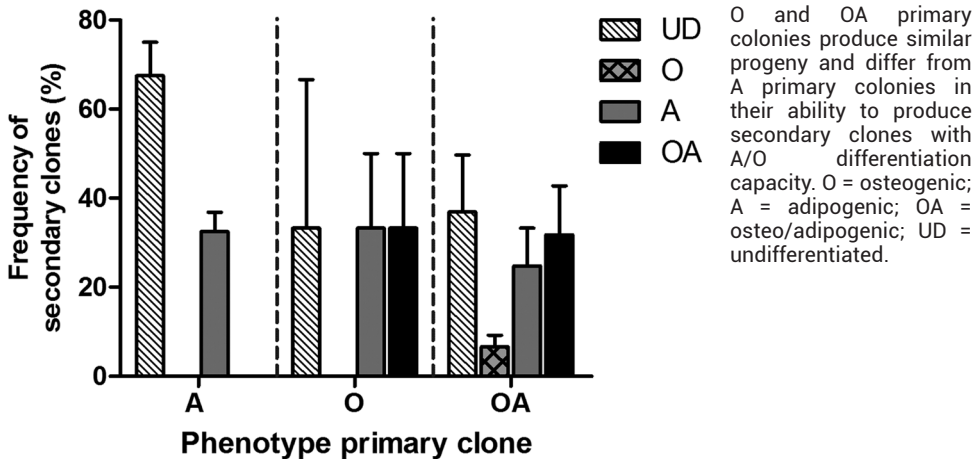
only capable to differentiate along the adipogenic lineage. This resulted in a higher number of population doublings at day 50 of the culture (figure 3B) and a higher total number of cells that could be expanded from the $\frac{1}{4}$ part of the primary colony (figure 3C). The combined data show that colonies that possess the capacity to differentiate towards the osteogenic lineage have a higher expansion potential and suggest that these colonies contain more primitive cells than the colonies that are committed to the adipogenic lineage. Interestingly, some of the colonies that did not show either osteogenic or adipogenic differentiation displayed a proliferative potential comparable to the osteogenic colonies.

All expanded MSC clones, irrespective of their differentiation potential or proliferative capacity, showed a similar immunophenotype in accordance with the phenotypical definition of MSC by the International Society for Cellular Therapy¹⁵. The cells were positive for the expression of CD90, CD73, HLA-ABC, CD105 and negative for the expression of CD45, CD34, HLA-DR and CD80. This shows that there is considerable functional heterogeneity within the same immunophenotype.

Differentiation potential is sequentially lost in culture

From the remaining $\frac{1}{4}$ of the primary colony, secondary colonies were derived by plating at low cell density. To study the hierarchical relationships within the MSC population, primary colonies and their secondary 'daughter' colonies were tested for osteogenic and adipogenic differentiation potential by culturing the colonies in osteogenic/adipogenic differentiation medium. All possible differentiation phenotypes were observed among the primary colonies: colonies that showed only osteogenic (O) differentiation, only adipogenic (A) differentiation, both osteogenic and adipogenic (OA) differentiation or no differentiation potential (UD). All 4 different differentiation lineages (O, A, OA and UD) were represented in the pool of secondary colonies, but they had a skewed differentiation potential, i.e. secondary colonies, derived from OA or O primary colonies often showed differentiation towards only one lineage or no differentiation at all (figure 4). Furthermore, the differentiation of the secondary colonies did not necessarily resemble that of the primary colony. Primary colonies that showed only osteogenic differentiation could give rise to secondary colonies showing only adipogenic differentiation, indicating that osteogenic differentiation capacity could be disguised. However the opposite, a primary adipogenic colony giving rise to a osteogenic secondary colony, was never observed (figure 4), indicating a permanent loss of osteogenic differentiation capacity.

Figure 4. Differentiation capacity profile of secondary colonies is dependent on differentiation capacity of primary MSC colony.



Discussion

In this study we investigated the clonal composition of BM-MSC populations aiming to dissect the hierarchical relationships between MSC colonies in expanded MSC populations. In addition, we searched for parameters that can be used to identify populations with high expansion potential to better meet the requirements with respect to cell numbers that are needed for clinical application of MSC. We confirm previous data showing that primary MSC populations consist of a mixture of colonies with multilineage, unilineage or no osteogenic/adipogenic differentiation potential and show that these colonies give rise to secondary colonies with a skewed differentiation potential. We showed that the differences in MSC expansion capacity between donors cannot be predicted by the frequency of CFU-F, but correlate with the frequency of colonies with multilineage or osteogenic differentiation capacity. The colonies with osteogenic differentiation capacity exhibited a higher proliferation rate, formed larger colonies and upon replating the frequency of secondary colonies with differentiation capacity was higher compared to colonies with only adipogenic differentiation capacity. We hypothesize that colonies with osteogenic differentiation capacity are more immature than colonies with adipogenic differentiation capacity, suggesting a developmental hierarchy.

The MSC populations that can be expanded from different donor BM aspirates vary largely in cell number. These variations are influenced by the amount of blood that is co-aspirated, but also qualitative differences might be important. Heterogeneity between donors with respect to CFU-F frequency, differentiation potential of the colonies and proliferative potential of MSC has been described earlier^{11,16}. In our experiments we found heterogeneity in the proliferative potential that was not reflected in the CFU-F frequencies. Our combined data showing that colonies that were selected for their osteogenic capacity have a higher expansion capacity and, vice versa, that colonies selected for their fast proliferation have osteogenic differentiation capacity, indicate a relation between proliferative and differentiation parameters. This correlation between proliferation and differentiation confirms and extends previous studies pointing at this relationship^{10,17-19}. The positive correlation between differentiation capacity and expansion capacity implies that the number of MSC that can be expanded from a BM-MNC population is not only directly proportional to the plating efficiency of the cells but also highly dependent on qualitative parameters, such as differentiation capacity. To translate these findings to the clinic, a fast and effective way of identifying osteogenic, fast expanding colonies would increase the efficiency of the preparation of MSC products for therapeutic use. Identifying unique markers to prospectively isolate these MSC from the bone marrow would greatly progress this research field.

As opposed to the studies by Muraglia et al.⁵ and Banfi et al.¹⁰, we detected purely adipogenic BM-MSC colonies. In our study, we also investigated the hierarchical relations in BM-MSC populations focussing on the differentiation capacities, but, in contrast to previous reports, we investigated the earliest primary MSC colonies as well as their progeny. Based on our results we also propose a model for BM-MSC hierarchy with sequential loss of differentiation potential. However, in our model, the osteogenic differentiation is default and the adipogenic differentiation

capacity only becomes apparent upon loss of the osteogenic differentiation capacity. This allows for the occurrence of secondary colonies with only adipogenic differentiation capacity that are derived from primary clones that show only osteogenic differentiation. Recently, Russell et al. also showed the emergence of unipotent adipogenic MSC clones from bone marrow and proposed a more complex hierarchical relationship, where tripotent osteogenic/adipogenic/chondrogenic (OAC) MSC commit via bipotent OC, OA and AC states towards unipotent O, A and C progenitors ¹¹. The differences between our model for BM-MSc hierarchy and earlier described models are most likely due to the fact that we have analyzed the clonal differentiation capacity at the earliest stage i.e. without further propagation and subcloning of the primary MSC colonies. By using a combined osteogenic/adipogenic differentiation, we were able to study bilineage differentiation potential in the very early colonies.

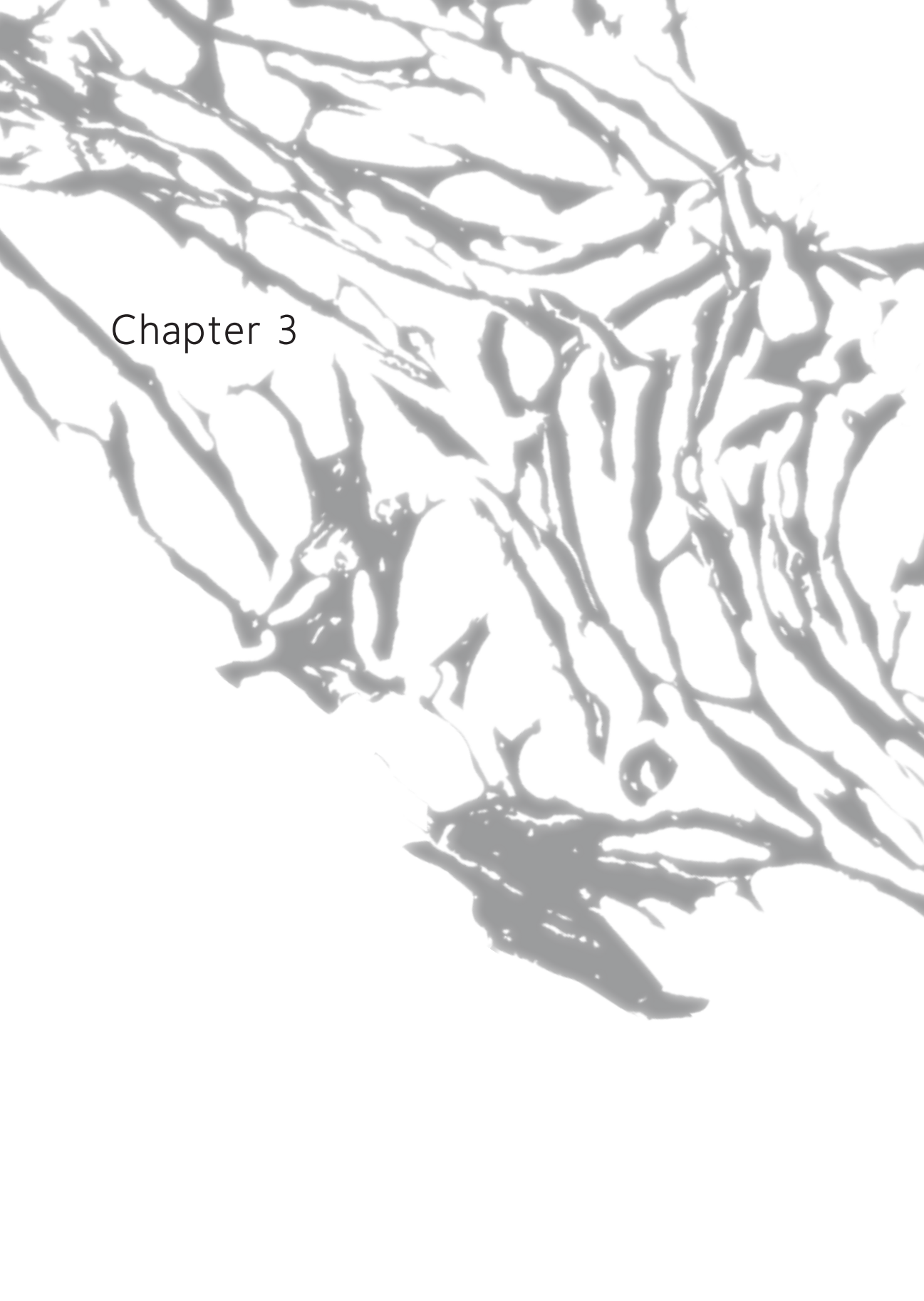
Our interpretation that osteogenic, fast proliferating MSC are more primitive than MSC that have lost their osteogenic differentiation capacity, fits with previously published gene expression data revealing that MSC populations with a high proliferation rate show lower expression of cell senescence genes and higher expression of self-renewal genes compared to clones with a low proliferation rate ¹⁹. The positive correlation between proliferation rate and differentiation capacity fits in a hierarchical model where cells gradually lose their differentiation potential, with a concurrent loss in proliferation rate. According to this model it would be expected that the colonies that do not show either osteogenic or adipogenic differentiation expand slowly. In our data set, however, we have observed fast expanding clones without differentiation capacity. We currently interpret this cell population that is not capable of differentiation to be a mixture of MSCs that have lost their capacity to differentiate and other cells (e.g. fibroblasts) unequipped with differentiation capacity.

In conclusion, we show that a high variation in differentiation phenotypes is present within the primary bone marrow-derived MSC population and that the osteogenic differentiation potential is correlated to proliferative capacity. We propose a model in which colonies with osteogenic potential are more immature than colonies with unilineage adipogenic potential. Our results contribute to the understanding of the composition of the MSC pool, which might add to the optimization of MSC expansion for clinical application.

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A close-up photograph of a tree branch with green leaves and a small, dark, textured object (possibly a bird's nest or a small animal) visible among the foliage.

Chapter 3



Adipose tissue-derived multipotent stromal cells have a higher immunomodulatory capacity than their bone marrow-derived counterparts

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Abstract

AT-MSC are studied as an alternative for BM-MSC for immunomodulatory treatment. In this study we systematically compared the immunomodulatory capacities of BM-MSC and AT-MSC, derived from age-matched donors. We found that BM-MSC and AT-MSC share a similar immunophenotype and capacity for in vitro multilineage differentiation. BM-MSC and AT-MSC showed comparable immunomodulatory effects as they were both capable to suppress proliferation of stimulated PBMC and to inhibit differentiation of monocyte-derived immature dendritic cells (iDC). However, at equal cell numbers, the AT-MSC showed more potent immunomodulatory effects in both assays as compared to BM-MSC. Moreover, AT-MSC showed a higher level of secretion of cytokines that have been implicated in the immunomodulatory modes of action of MSC, such as IL-6 and TGF- β 1. This is correlated with higher metabolic activity of AT-MSC compared to BM-MSC. We conclude that the immunomodulatory capacities of BM-MSC and AT-MSC are similar, but that differences in cytokine secretion cause AT-MSC to have more potent immunomodulatory effects than BM-MSC. Therefore, lower numbers of AT-MSC evoke the same level of immunomodulation. These data indicate that AT-MSC can be considered as a good alternative to BM-MSC for immunomodulatory therapy.

Introduction

Multipotent stromal cells (MSC), originally detected in bone marrow, are non-hematopoietic progenitor cells that are defined by their ability to adhere to plastic surfaces and their capacity to differentiate towards different mesodermic lineages, including adipocytes, osteocytes and chondrocytes¹. An important characteristic of MSC is their immunomodulatory capacity. MSC interfere with differentiation and functions of multiple immunomodulatory cells. *In vitro*, MSC suppress the proliferation of T cells upon allogeneic and mitogenic stimulation^{2,3}, they inhibit monocyte-derived dendritic cell differentiation and maturation^{4,5} and the proliferation of B cells and NK-cells^{6,7} and they promote the generation of regulatory T cells⁸⁻¹⁰. Multiple factors have been implicated in the immunomodulatory effects of MSC, including indoleamine 2,3-dioxygenase (IDO), IL-6, prostaglandin E2 (PGE2) and TGF- β 1^{9,11-14}. The potential therapeutic application of MSC for immunomodulation has been the subject of many studies over the years. In experimental models, administration of MSC resulted in prolonged skin graft survival in baboons¹⁵ and in prevention of both Graft-versus-host disease (GvHD)¹⁶ and the development of experimental autoimmune encephalomyelitis in mice¹⁷. The use of MSC as a cellular therapy is currently explored in clinical trials, aiming at the promotion of engraftment following hematopoietic stem cell transplantation¹⁸ and treatment of GvHD¹⁹ and Crohn's disease²⁰.

Expanded bone marrow-derived MSC have been most widely used for clinical application, but alternative sources or subpopulations are currently under investigation^{21,22}. The frequency of MSC in bone marrow is low (0.001% - 0.01% of the total mononuclear cell fraction²³) and aspirating bone marrow is an invasive procedure. Adipose tissue is an interesting alternative to bone marrow, since it contains approximately a 500-fold higher frequency of MSC^{24,25} and tissue collection is simple. Moreover, 400,000 liposuctions a year are performed in the US alone, where the aspirated adipose tissue is regarded as waste²⁶, and could be collected without any additional burden or risk for the donor. Adipose tissue-derived MSC (AT-MSC) are already applied in clinical trials^{21,22}, but a systematic comparative study of bone marrow-derived MSC (BM-MSC) and AT-MSC has not been performed. Studies that compared MSC derived from different sources focused on phenotype, transcription profiling and differentiation potential²⁷⁻³³. A few studies compared the immunomodulatory properties^{30,32,34-36} and showed qualitative similarities between AT-MSC and BM-MSC. Quantitative analysis could not be performed in these studies, since different patient groups and different age groups were analyzed. Age is an important variable, since the composition of the MSC population and the functional properties of MSC change with aging^{37,38}.

In this study we systematically compared the *in vitro* immunomodulatory capacities of BM-MSC and AT-MSC, derived from age-matched donors, to further define the advantages and disadvantages related to the use of the different tissue sources for MSC therapy. We show that both AT-MSC and BM-MSC are capable to suppress proliferation of stimulated PBMC and to inhibit differentiation of monocyte-derived immature dendritic cells (iDC). However, AT-MSC show more potent immunomodulatory effects, which is related to higher levels of cytokine secretion.

Materials and methods

Patients

Adipose tissue was dissected as medical waste from 9 cadaveric pancreata that were donated for islet transplantation in our center, according to the institutional guidelines regarding the use of waste material. Bone marrow from a group of 9 age-matched donors was obtained from the iliac crest of healthy donors and from orthopedic patients after written informed consent, according to procedures that were approved by the medical ethical committee. The mean age of bone marrow donors was 49.2 ± 13.2 years and the mean age of adipose tissue donors was 54.2 ± 16.0 years (table 1).

Table 1. Ages and origins of the MSC donors.

Donor	Age	Type of donor/cause of death	Fig 1B	Fig 2	Fig 3A	Fig 3B	Fig 3C	Fig 4A	Fig 4B	Fig 4C	Fig 4D	Fig 5A	Fig 5B	Fig 5C
BM-1	44	healthy donor	x	x	x	x			x	x	x	x	x	
BM-2	40	healthy donor	x	x	x		x		x			x		x
BM-3	40	healthy donor	x	x			x				x	x	x	x
BM-4	39	healthy donor	x	x	x		x				x	x	x	x
BM-5	41	orthopedic patient		x	x	x		x	x		x			
BM-6	40	orthopedic patient		x	x						x			
BM-7	59	orthopedic patient			x									
BM-8	70	orthopedic patient			x									
BM-9	70	orthopedic patient			x	x					x			
AT-1	52	myocardial infarction	x	x	x		x					x		x
AT-2	21	car accident	x	x	x	x	x		x	x	x	x	x	x
AT-3	65	myocardial infarction	x	x	x									
AT-4	49	CVA	x	x	x									
AT-5	73	CVA			x									
AT-6	67	CVA	x	x	x	x			x		x	x	x	
AT-7	45	pneumococcal meningitis		x	x			x						
AT-8	67	CVA		x	x							x		
AT-9	49	unknown		x	x	x	x		x		x		x	x

Table indicates the analyses for which each donor's cells were used.

Generation of MSC from bone marrow and adipose tissue

Bone marrow-derived mononuclear cells were isolated using a Ficoll-Paque density gradient (1.077 g/cm^3) and plated at $1.3 \times 10^5/\text{cm}^2$ in proliferation medium (DMEM-low glucose (DMEM-LG; Invitrogen Corp., Paisley, UK) supplemented with 10% fetal calf serum (FCS; Greiner Bio-one) and Penicillin/Streptomycin (P/S; Invitrogen Corp., Paisley, UK)).

The adipose tissue was mechanically disrupted and subsequently digested using collagenase (2 mg/ml; Sigma) in proliferation medium. After digestion, the cell suspension was centrifuged to separate the adipocytes from the stromal vascular fraction (SVF). The SVF was filtered and the cells were plated in culture flasks in culture medium in a density of $1.4 \times 10^4/\text{cm}^2$.

Cultures were incubated at 37°C and 5% CO_2 , after 3-4 days non-adherent cells were removed and medium was refreshed every 3-4 days until confluency was reached. The MSC monolayer was detached using trypsin/EDTA (Invitrogen Corp., Paisley, UK) and cells were reseeded at $4,000/\text{cm}^2$ for further expansion. The MSC were characterized by immune phenotyping and used in further experiments at passage 2-5.

Isolation of human peripheral blood mononuclear cells and monocytes

Human PBMC were isolated from buffy coats obtained from healthy donors from Sanquin Blood Supply (Leiden, The Netherlands) using a Ficoll-Paque density gradient. Monocytes were purified from the freshly prepared mononuclear cell fraction 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 experiments

BM-MSc and AT-MSc were tested for their capacity to differentiate towards the osteogenic and adipogenic lineage. 100,00 MSC were plated in 24-wells plates (Corning BV Life Sciences, Amsterdam, The Netherlands) and incubated with the appropriate medium for induction of differentiation as stated below. All cultures were refreshed weekly.

For osteogenic differentiation, MSC were cultured in α -MEM (Invitrogen Corp., Paisley, UK) supplemented with L-glutamin (200nM, Invitrogen Corp., Paisley, UK), P/S and 10% FCS, 10^{-7} M dexamethason and 50 μ g/ml Vitamin C (both from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). After the first week, 5 mM β -glycerophosphate (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was added.

For adipogenic differentiation, MSC were incubated with medium consisting of α -MEM supplemented with L-glutamin, P/S and 10% FCS, 10^{-7} M dexamethason, insulin (10 μ g/ml), indomethacin (5 μ M) and IBMX (5 μ M) (all from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands).

After 3 weeks of culture, cells from all differentiation cultures were stained for alkaline phosphatase activity with Fast Blue (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and calcium deposition was determined with Alizarine Red (MP Biomedicals LLC, Illkirch Cedex, France) staining. Formation of lipid droplets was visualized using Oil-red O staining (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands).

Suppression of PBMC proliferation

The immunosuppressive capacity of MSC was tested in a co-culture of MSC and PBMC. MSC were plated in graded doses in a 96-well flat-bottom plate in DMEM-LG (Invitrogen Corp., Paisley, UK) supplemented with 10% FCS (Greiner Bio-one) and P/S (Invitrogen Corp., Paisley, UK). Following adherence overnight, PBMC (1.0×10^5 /well) were added and stimulated with human T-activator CD3/CD28 dynabeads (Invitrogen Corp., Paisley, UK) in a bead:PBMC ratio [1:5]. After 5 days of culture, cells were pulsed with [3 H]-thymidine (0.5 μ Ci/well) and incubated for 16 h at 37°C. The cultures were harvested on a glass fiber filter and thymidine incorporation was measured with a liquid scintillation counter (Wallac, Turku, Finland). Data were expressed as mean corrected counts per minute (CCPM) of triplicate co-cultures. Control experiments were performed, replacing MSC with K562 cells.

Inhibition of monocyte-derived immature dendritic cell differentiation

Immature dendritic cells (iDC) were generated from freshly isolated CD14⁺ monocytes by culturing 1.0×10^6 cells in 6-wells plates in RPMI (Invitrogen Corp., Paisley, UK) containing P/S, L-glutamin and 10% FCS supplemented with the growth factors IL-4 (10 ng/ml; Invitrogen Corp., Paisley, UK) and GM-CSF (5 ng/ml; Novartis) for 6 days. To examine the effect of MSC on monocyte differentiation, irradiated (60 G) BM-MSC and AT-MSC were added to the culture at a MSC:monocyte ratio of [1:5]-[1:50]. All experiments were performed in duplicate. The experiments were performed in direct co-culture and in a transwell co-culture system (pore size 0.4 μ M; Corning Inc., Lowell, MA, USA). In the transwell experiments, MSC were plated in the lower well and monocytes were added in the transwell insert. At day 6 the iDC were harvested from the co-culture and analyzed for expression of CD1a and CD14 by flow cytometry.

Flow cytometry

After trypsinization, MSC cultures were 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 (Ansell Corp., Bayport, MN, USA). The monocytes from the co-culture experiments were collected at day 6 and analyzed for surface marker expression using CD1a-FITC and CD14-PE (BD Biosciences, San Diego, CA, USA). Cells were incubated with fluorescent-labeled antibodies 30 minutes at 4°C in the dark. After washing with PBS/1% albumin they were analyzed using a FACSCanto II (BD Biosciences, San Diego, CA, US). The analysis of the acquired data was done with FlowJo software version 7.6.1 (Tree Star Inc. Ashland, OR, USA).

Cytokine assay

To determine cytokine concentrations in the supernatants of PBMC proliferation cultures and the monocyte cultures, cell-free supernatant was collected at day 5 and 6 respectively and stored at -20°C until use. Cytokine concentrations were measured using the Bio-Plex Pro Human Cytokine Th1/Th2 panel (Bio-Rad laboratories, Inc, Hercules, CA, USA). To analyze constitutive cytokine secretion, BM-MSC and AT-MSC cells were plated at confluent cell concentrations (2.0×10^5 cells in a 12-wells plate) and cultured without medium replacement. At day 7, cell-free culture supernatant was collected and stored at -20°C and MSC were harvested and counted. Cytokine concentrations were measured using the Bio-Plex Pro Human Cytokine 27-plex panel (Bio-Rad laboratories, Inc, Hercules, CA, USA) or sandwich ELISA (BD Biosciences, San Diego, CA, USA).

MTT assay

In a 96-wells plate, 2.0×10^4 MSC were plated in DMEM-LG (Invitrogen Corp., Paisley, UK) supplemented with 10% FCS (Greiner Bio-one) and P/S (Invitrogen Corp., Paisley, UK). After overnight adherence, the medium was replaced with 100 μ l colorless IMDM medium (Invitrogen Corp., Paisley, UK) supplemented with P/S and 10% FCS. For measurement of the metabolic activity 10 μ l of 12mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) stock solution

(Life Technologies, Grand Island, NY, USA) was added to each well and incubated for 4 hours at 37°C. Then 75 µl of the medium was removed and 100 µl DMSO was added and incubated for 10 minutes at 37°C. Absorbance was read at 540 nm.

IFN-γ stimulation of MSC

To analyse IDO upregulation induced by IFN-γ, MSC were plated at a concentration of 2.0×10^5 cells per well in a 12-wells plate and IFN-γ was added to the culture medium (500 U/ml; Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). At timepoints 0, 4, 8 and 24 hours, RNA was extracted and cell-free supernatant was collected and stored for further analysis at -20°C.

Gene expression analysis

RNA was extracted from MSC using the RNeasy micro kit from Qiagen. cDNA was synthesized with Superscript III RT (Invitrogen, GmbH, Hilden Germany). RT-qPCR analyses were performed on a StepOnePlus real-time PCR system (Applied Biosystems, Foster city, CA, USA) using SYBR Green reagent (Roche Diagnostics, Almere, The Netherlands). The following primer sets were used:

Gene	Forward	Reverse
IDO	5'-CCTGAGGAGCTACCATCTGC-3'	5'-TCAGTGCCTCCAGTTCCTTT-3'
IL-6	5'-TTCAATGAGGAGACTTGCCTG-3'	5'-ACAACAACAATCTGAGGTGCC-3'
β-actin	5'-AGGCATCCTCACCCTGAAGTA-3'	5'-CACACGCAGCTCATTGTAGA-3'

All RT-qPCR Data were normalized to β-actin expression and the data were analyzed using the delta-Ct method.

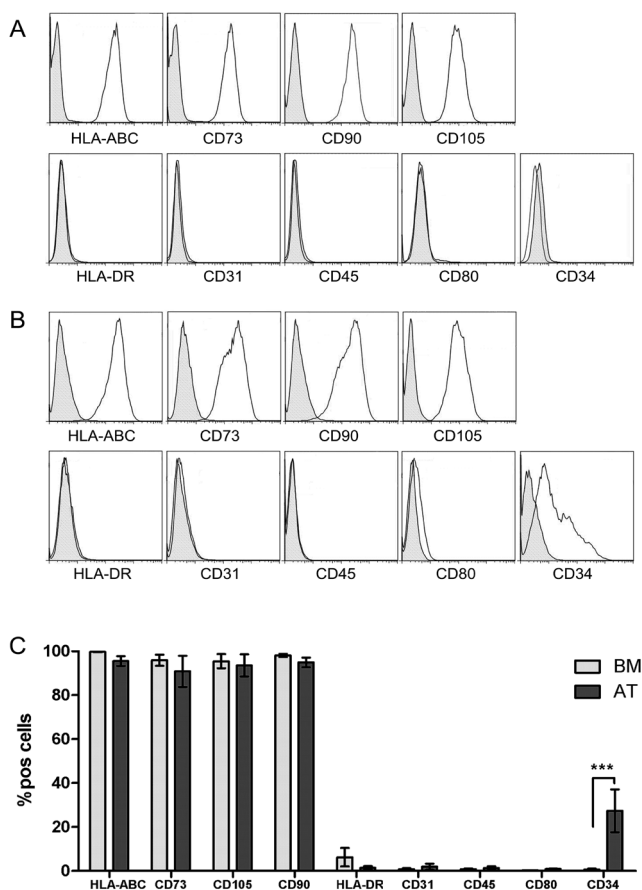
Results

BM-MSC and AT-MSC have similar phenotype and differentiation capacity

Immunophenotypic analysis of BM-MSC and AT-MSC showed a similar surface marker expression profile (figure 1A-C). This profile is in accordance with the phenotypical definition of MSC by the International Society for Cellular Therapy¹, with the exception of CD34, that was expressed only on a fraction of AT-MSC (27.4% $\pm$ 9.8%, n = 4) (figure 1C).

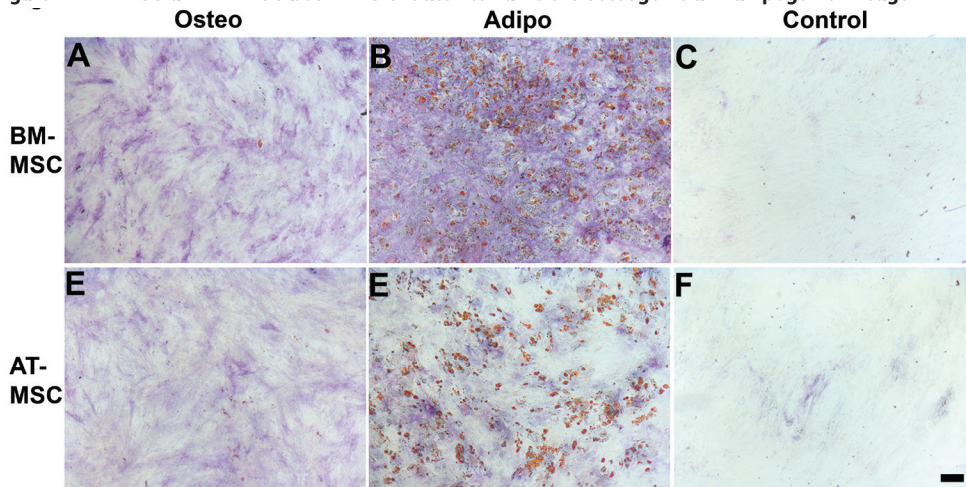
Both BM-MSC and AT-MSC were able to differentiate towards the osteogenic and the adipogenic lineage (figure 2). No differentiation was observed for MSC that were cultured in medium only.

Figure 1. BM-MSC and AT-MSC show the same immunophenotype, except for CD34 expression.



Representative histograms of BM-MSC (A) and AT-MSC (B) FACS analysis. All AT-MSC donors show a CD34⁺ population. Grey histograms are isotype control. (C) Cumulative data of FACS analysis of BM-MSC and AT-MSC for several surface markers. The expression of CD34 is significantly higher on AT-MSC compared to BM-MSC (data are means from 4 BM-MSC and 5 AT-MSC donors; statistical analysis was performed using a 2-way ANOVA; *, p < 0.05; **, p < 0.01; ***, p < 0.001).

Figure 2. BM-MSC and AT-MSC both differentiated towards the osteogenic and adipogenic lineage.



Confluent cultures of BM-MSC (A,B,C) and AT-MSC (D,E,F) were maintained in osteogenic differentiation medium (A,D), adipogenic differentiation medium (B,E) or control medium (C,F). After three weeks of culture in differentiation medium, both BM-MSC and AT-MSC were positive for alkaline phosphatase activity (A,D) and lipid droplets were formed (B,E). Control cultures in proliferation medium did not show alkaline phosphatase activity or the formation of lipid droplets. MSC populations from 6 BM-MSC donors and from 8 AT-MSC donors were tested for their differentiation capacity, representative pictures are shown for BM-MSC and AT-MSC. Bar = 50µm.

AT-MSC are more potent in their suppression of PBMC proliferation than BM-MSC

Functional suppression

Both BM-MSC and AT-MSC were able to suppress the proliferation of PBMC in a dose dependent fashion (figure 3A). However, addition of equal numbers of AT-MSC resulted in significantly higher levels of suppression of PBMC proliferation compared to BM-MSC (figure 3A). Approximately 3 times more BM-MSC were needed to obtain the suppressive effect that was observed with AT-MSC. At a ratio MSC:PBMC of [1:3], both BM-MSC and AT-MSC showed almost complete inhibition of PBMC proliferation. Control experiments, replacing MSC with K562 cells, showed that the effect on proliferation was not due to cell crowding or exhaustion of the culture medium (data not shown).

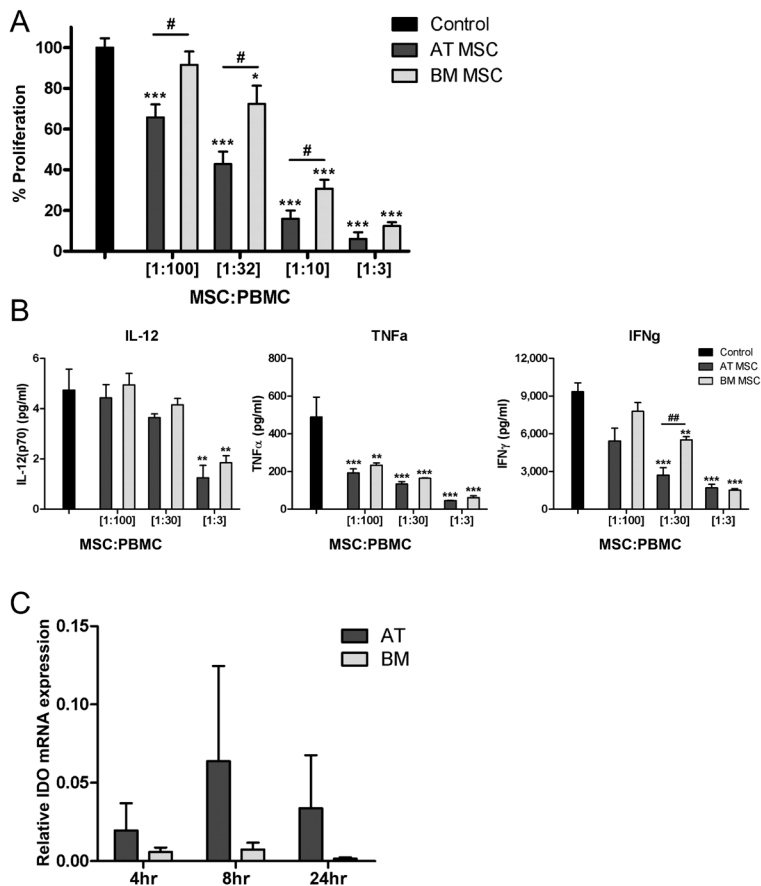
Cytokine production

In the presence of increasing amounts of both BM-MSC and AT-MSC, the concentrations of the pro-inflammatory cytokines IL-12, TNF- α and IFN- γ , in the culture supernatants of PBMC-MSC co-culture, decreased in a dose-dependent fashion (figure 3B). At a ratio MSC:PBMC of [1:30], the IFN- γ concentrations in the culture supernatant were significantly lower ($p < 0.01$) in the presence of AT-MSC compared to BM-MSC (figure 3B).

The upregulation of IDO expression in MSC following IFN- γ stimulation is

regarded as an important mechanism for the MSC-induced suppression of PBMC proliferation. Therefore, we investigated the level and speed of induction of IDO expression in response to IFN- γ stimulation in BM-MSC and AT-MSC. Following IFN- γ stimulation, expression of IDO mRNA was induced in both BM-MSC and AT-MSC populations. Between 4 and 24 hr after IFN- γ stimulation, the average IDO expression in AT-MSC was higher than in BM-MSC. However, due to the high variation, the difference did not reach statistical significance. At 8 hours, the

Figure 3. AT-MSC are more potent in suppressing PBMC proliferation compared to BM-MSC.



(A) MSC suppress PBMC proliferation in a dose dependent fashion. AT-MSC show a significant stronger suppression of proliferation at MSC:PBMC ratios [1:100], [1:32], and [1:10] (2 separate experiments, $n = 9$ for AT-MSC, $n = 8$ for BM-MSC). (B) From one experiment, culture supernatants of PBMC proliferation in the presence and absence of MSC were assayed for cytokine concentrations at day 5 of co-culture ($n = 3$ for both groups). Statistical analysis was performed using the student's t-test (data are mean $\pm$ SEM; * = compared to control; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; # = AT-MSC compared to BM-MSC; #, $p < 0.05$; ##, $p < 0.01$). (C) After IFN- γ stimulation of MSC, both BM-MSC and AT-MSC show IDO mRNA upregulation, with an optimum at 8 hours. IDO mRNA expression is relative to β -actin mRNA expression. Statistical analysis was performed using a student's t-test ($n = 3$ for both groups).

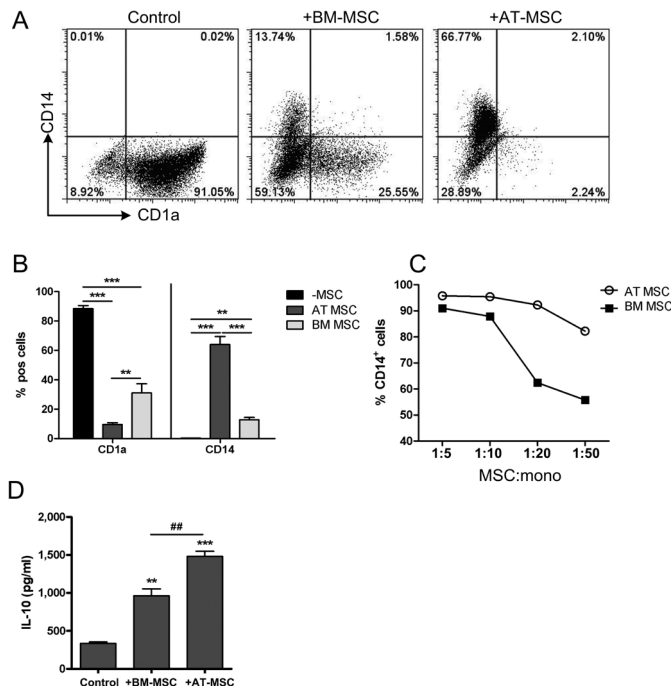
highest expression levels of IDO were observed for both AT-MSC and BM-MSC (figure 3C).

AT-MSC are more potent in inhibiting dendritic cell formation than BM-MSC

BM-MSC and AT-MSC were further tested for their capacity to inhibit the differentiation of CD1a⁺CD14⁺ monocytes towards CD1a⁺CD14⁺ immature dendritic cells (iDC). Both AT-MSC and BM-MSC exhibited this inhibitory effect at a MSC:mono ratio of [1:10] (figure 4A,B). Also in this immunomodulation assay, a clear difference in the dose-response relation was observed, in favor of AT-MSC (figure 4C). At a MSC:monocyte ratio of [1:20], AT-MSC showed the same level of inhibition as BM-MSC showed at a ratio of [1:5].

Addition of BM-MSC and AT-MSC increased the concentrations of the anti-inflammatory cytokine IL-10 in supernatants of cultures from monocytes that

Figure 4. AT-MSC are more potent inhibitors of monocyte differentiation than BM-MSC.



(A) Representative dotplots of monocyte differentiation towards iDC in the absence and presence of BM-MSC and AT-MSC (MSC:monocyte ratio of [1:10]). (B) Cumulative data of CD1a and CD14 expression on differentiated monocytes. Data are means from 3 different BM-MSC and 3 different AT-MSC (MSC:monocyte ratio of [1:10]), statistical analysis was performed using the student's t-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (C) Dose-response curves of the percentage of CD14⁺ cells in the presence of BM-MSC or AT-MSC. (D) IL-10 concentrations in culture supernatants from day 6 of the monocyte differentiation in the presence of BM-MSC and AT-MSC are increased compared to the differentiation without MSC. Data are means from 3 different experiments with 6 different BM-MSC and 3 different AT-MSC (MSC:monocyte ratio of [1:10]), statistical analysis was performed using a student's t-test (* = compared to -MSC; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; # = BM-MSC compared to AT-MSC; #, $p < 0.05$; ##, $p < 0.01$).

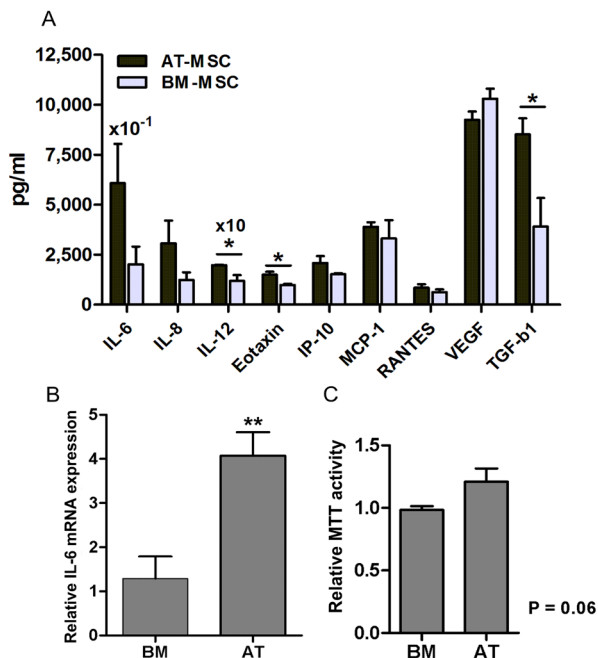
were differentiated towards iDC (figure 4D). Again, addition of AT-MSC resulted in a significantly higher concentrations of IL-10 than addition of BM-MSC (1481.0 ± 118.8 pg/ml versus 961.8 ± 159.5 pg/ml, $n = 3$, $p < 0.01$).

AT-MSC secrete higher concentrations of cytokines compared to BM-MSC

To investigate whether the difference in potency in the various immunomodulatory assays could be explained by a generally increased cytokine secretion by AT-MSC compared to BM-MSC, cytokine profiling was performed in culture supernatant of unstimulated MSC. After 7 days of culture, no differences were observed in the number of cells between AT-MSC and BM-MSC cultures. Culture supernatants from AT-MSC cultures generally contained higher concentrations of cytokines than culture supernatants from BM-MSC and the reverse phenomenon was never observed. The differences were significant for the cytokines IL-6, IL-8, IL-12, Eotaxin and TGF- β 1 (figure 5A). The largest differences were found for IL-6 and TGF- β 1. For IL-6, this difference in cytokine concentrations was confirmed at the transcriptional level (figure 5B).

We hypothesized that the generally enhanced levels of multiple cytokines in AT-MSC are the result of a higher metabolic activity in AT-MSC. To test this hypothesis, we directly analyzed the metabolic activity of BM-MSC and AT-MSC using an MTT assay. Indeed the AT-MSC showed a higher average metabolic activity compared to BM-MSC, however this observation reached only borderline significance ($P = 0.06$) (figure 5C).

Figure 5. AT-MSC secrete higher levels of cytokines compared to BM-MSC.



(A) Cytokine concentrations measured in culture supernatant were higher for AT-MSC compared to BM-MSC. A representative experiment is shown from 3 separate experiments. ($n = 4$ for both groups; IL-6 values represent 1.0×10^{-1} of the measured concentrations, represented IL-12 values are 10x the measured concentrations). (B) IL-6 mRNA expression was also increased in AT-MSC compared to BM-MSC ($n = 3$ for both groups). (C) AT-MSC show a slightly enhanced MTT activity; MTT activity AT-MSC is relative to BM-MSC ($n = 3$ for both groups). Data are means $\pm$ SEM from 3 different experiments, statistical analysis was performed using a student's t-test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Discussion

AT-MSC are considered as an alternative to BM-MSC for clinical application^{16,30,39}. In this study we performed an *in vitro* systematic comparison of BM-MSC and AT-MSC from age-matched donors. We found a similar phenotype and capacity for multilineage *in vitro* differentiation towards the osteogenic and adipogenic lineages. We observed that both BM-MSC and AT-MSC exhibit the capacity to inhibit PBMC proliferation as well as monocyte differentiation towards iDC. However, the dose-response curve for AT-MSC was clearly different than for BM-MSC. To acquire the same level of suppression, a lower number of AT-MSC were required than BM-MSC. Furthermore, AT-MSC secreted higher levels of cytokines than BM-MSC, which corresponded to a higher metabolic activity.

The expression of surface marker CD34 on a subpopulation of the AT-MSC has previously been described. This expression decreases during culture expansion⁴⁰⁻⁴², explaining the observed variation. The relevance of CD34 expression on AT-MSC is currently unclear. CD34⁺/CD31⁻ cells have been detected at perivascular locations in adipose tissue⁴³. These cells can differentiate *in vitro* towards endothelial cells⁴⁴, which suggests that they might play role in vasculogenesis. Such preferred role for these CD34⁺/CD31⁻ cells is not supported by the findings of Suga et al., who showed similar capabilities for capillary-network formation for CD34⁺ and CD34⁻ AT-MSC⁴⁵.

Several studies have compared BM-MSC and AT-MSC, but mostly small groups of donors and different patient groups and age groups were investigated. The reports described that AT-MSC and BM-MSC both exhibit the capacity to inhibit PBMC proliferation, but indicated no differences between the MSC populations^{30,32,34-36}. We also show that both populations are able to inhibit PBMC proliferation, but with a different dose-response curve. Others used MSC concentrations at the plateau phase of the dose-response curve and MSC from different patient groups and age. Therefore they did not find differences in potency between AT-MSC and BM-MSC. We show that differences are only found at concentrations in the linear phase of the dose-response curve.

The more potent suppression of PBMC proliferation by AT-MSC than by BM-MSC was also reflected in the cytokine concentrations measured in the co-culture supernatants of the PBMC-MSC co-cultures. Our data suggest that both BM-MSC and AT-MSC induce a shift from a Th1 towards a Th2 response, since the pro-inflammatory cytokines IL-12, TNF α and IFN- γ concentrations were significantly reduced in culture supernatants of PBMC cultured in the presence of either type of MSC. This effect was stronger in the presence of AT-MSC than in the presence of BM-MSC and for IFN- γ this difference between AT-MSC and BM-MSC became statistically significant.

IFN- γ has also been implicated in the initiation of the immunosuppressive function of MSC. Activation of MSC by IFN- γ is involved in the inhibitory effect of MSC on T cell proliferation through induction of expression of IDO, an enzyme that catabolizes tryptophan¹³. The level of induced IDO expression differs between MSC populations and has been correlated to their immunosuppressive potential⁴⁶. We found that, in response to IFN- γ , IDO expression is induced to higher levels

in AT-MSC than in BM-MSC. The more potent inhibitory effect of AT-MSC on PBMC proliferation can be explained by this stronger response to IFN- γ , i.e. the IFN- γ that is produced by the activated PBMC causes a stronger or more adequate response of the AT-MSC resulting in stronger inhibition of the PBMC proliferation, finally resulting in lower concentrations of IFN- γ and other pro-inflammatory cytokines. We have shown *in vitro* that MSC inhibit monocyte differentiation towards iDC and rather skew monocytes towards a tolerogenic cell type, with increased secretion of the immunomodulatory cytokine IL-10⁴⁷. In our monocyte-MSC co-cultures, the secretion of the immunomodulatory cytokine IL-10 was more pronounced in the presence of AT-MSC than in the presence of BM-MSC. This indicates that the monocytes have become more tolerogenic in the presence of AT-MSC. Since activated monocytes have been shown to increase their IL-10 expression upon IL-10 exposure, creating a positive feedback loop⁴⁸, this difference in effect of AT-versus BM-MSC might even be further enhanced.

Compared to BM-MSC, AT-MSC constitutively secrete higher levels of multiple cytokines that have been implicated in MSC-mediated immunomodulation, such as IL-6 and TGF- β 1^{5,11,49,50}. We have shown that IL-6 is a key factor for the MSC-induced inhibition of monocyte differentiation towards iDC and the induction of IL-10 secretion by monocytes⁴⁷. The higher IL-6 secretion by AT-MSC can therefore explain the more pronounced inhibition of iDC generation and the increased IL-10 protein concentrations that were observed in the monocyte cultures in the presence of AT-MSC. TGF- β 1 has been implicated in the generation of regulatory T cells by MSC^{11,14}. Therefore, we hypothesize that the observed higher TGF- β 1 production by AT-MSC may also be associated with an increased ability to induce regulatory T cells.

The overall explanation for the observed functional superiority of AT-MSC may be the its higher metabolic activity, resulting in production of higher levels of cytokines that are involved in the immunosuppressive mechanisms of MSC. It is therefore conceivable that this also holds true for other factors that are involved in immunomodulation by MSC, like PGE2^{30,51}, galectin-1^{52,53} and HLA-G5⁵⁴.

Conclusion

Our data show that BM-MSC and AT-MSC share a similar immunophenotype and capacity for *in vitro* multilineage differentiation. Also functionally, BM-MSC and AT-MSC show similar immunomodulatory effects, but with a different dose-response curve, in favor of AT-MSC. We show that the differences in immunomodulatory properties between BM-MSC and AT-MSC are primarily due to the quantitative aspects of the suppression, which are likely related to their respective metabolic activity. These data support the notion that AT-MSC could be a promising alternative for BM-MSC for future clinical immunomodulatory applications.

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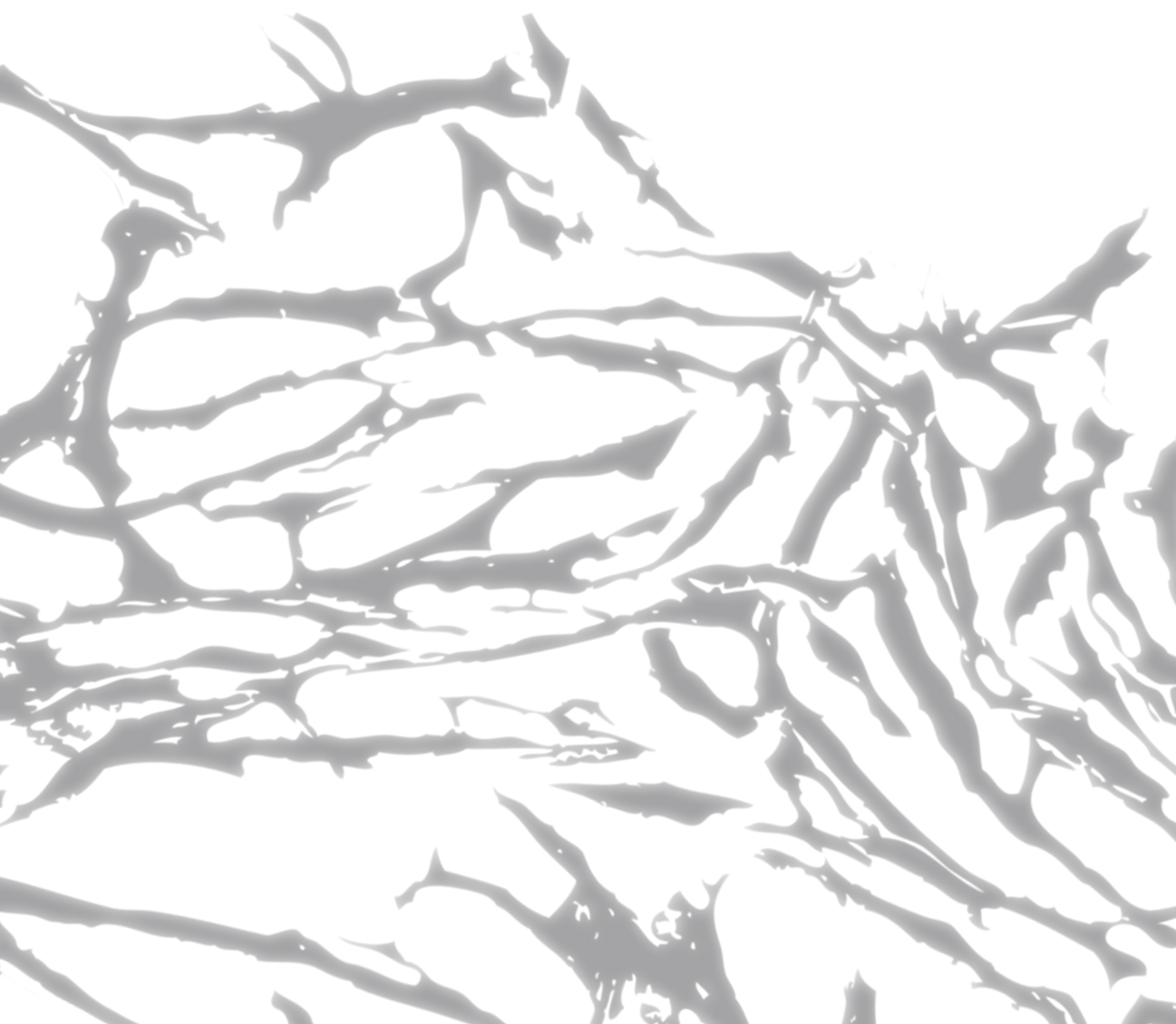
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Chapter 4



Multipotent stromal cells skew monocytes towards an anti-inflammatory IL-10 producing phenotype by production of IL-6

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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- β 1, IL-6 and heme oxygenase 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/cm³) and were plated at 1.3×10^5 /cm² 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/cm² 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/cm³). 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 1.0×10^6 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 co-culture system (pore size 0.4 μ M; 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 μ g/ml) or anti-IL-6 (2.5 μ g/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 co-cultures (MSC+mono CM). In some experiments, IL-6

was depleted from the MSC-CM using the μ MACS Streptavidin kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cell free MSC supernatant was incubated with biotinylated anti-IL-6 (2 μ g/ml, BD Biosciences, San Diego, CA, USA) and μ MACS Streptavidin microbeads. IL-6 was depleted from the medium using a μ MACS 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, US). 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'-CCGAGATGCCTTCAGCAGAG-3'	5'-GGTCTTGGTTCTCAGCTTGG-3'
β-actin	5'-AGGCATCCTCACCTGAAGTA-3'	5'-CACACGCAGCTCATTGTAGA-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, TCR γ/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 0.5 μ Ci of [³H]-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

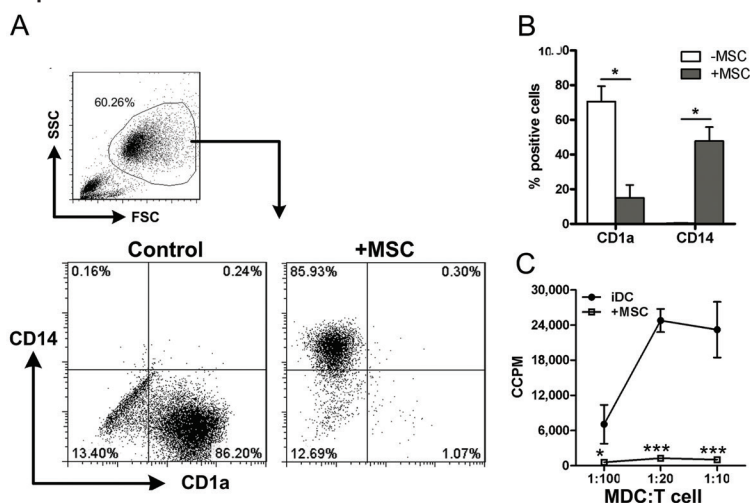
MSC skew the differentiation of monocytes to the formation of CD14⁺ cell with low allostimulatory capacity

Monocytes (purity after MACS isolation was more than 90% CD14⁺ cells) cultured in the presence of IL-4 and GM-CSF differentiated into CD1a⁺CD14⁺ 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 ($15.1 \pm 7.4\%$ versus $70.6 \pm 8.8\%$, $p = 0.002$, $n = 7$) and a significantly lower fraction lost CD14 expression ($52.2 \pm 8.1\%$ versus $99.5 \pm 0.1\%$, $p = 0.001$, $n = 7$) (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⁺CD25⁻ 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⁺ 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 $\pm$ 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⁺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 (* $p < 0.05$, *** $p < 0.001$).

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 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).

A

IL-10 (pg/ml)

MSC

-MSC +MSC

B

IL-6 (pg/ml)

-MSC +MSC

C

MSC

Fold change

IL6 IL10

MSC GF Mono

n.d. n.d. n.d.

D

MDC

Fold change

IL6 IL10

MSC GF Mono

E

MSC

IL-6 (pg/ml)

MSC GF Mono

F

MDC

IL-6 (pg/ml)

MSC GF Mono

78

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).

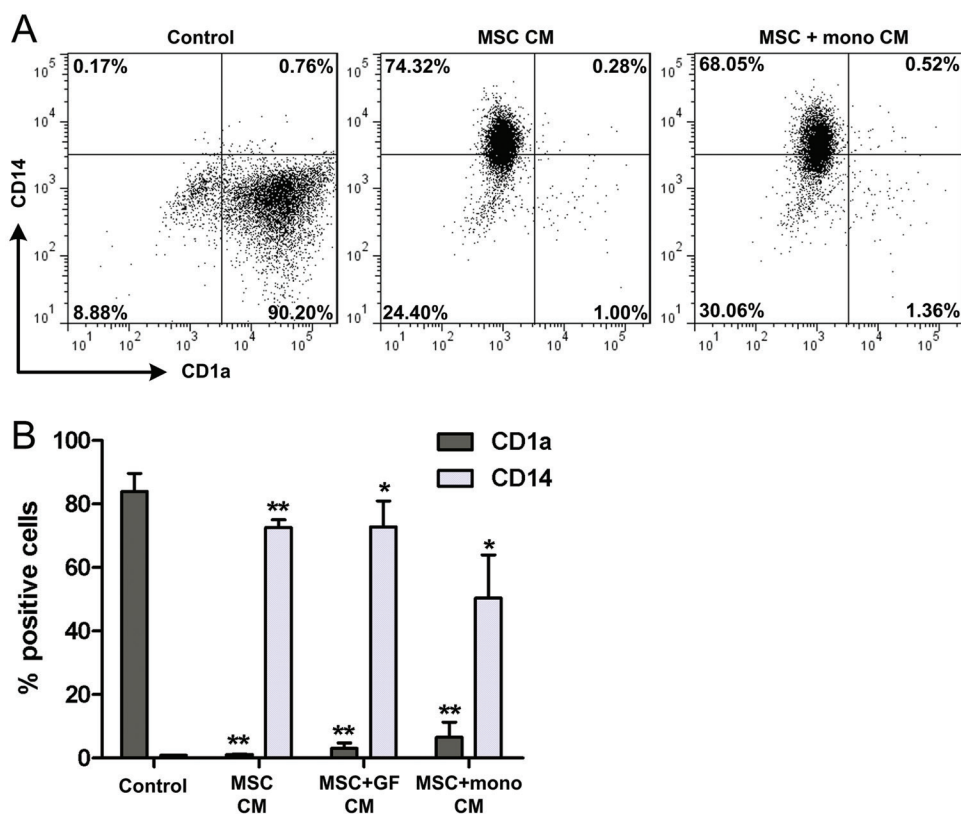
IL-6 mRNA was also expressed in the monocyte-derived iDC. Significantly increased expression levels ($p < 0.001$) were found in monocyte-derived populations that were generated in the presence of MSC (Figure 2D). In agreement, the concentrations of IL-6 in supernatant of monocytes cultured for an additional 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

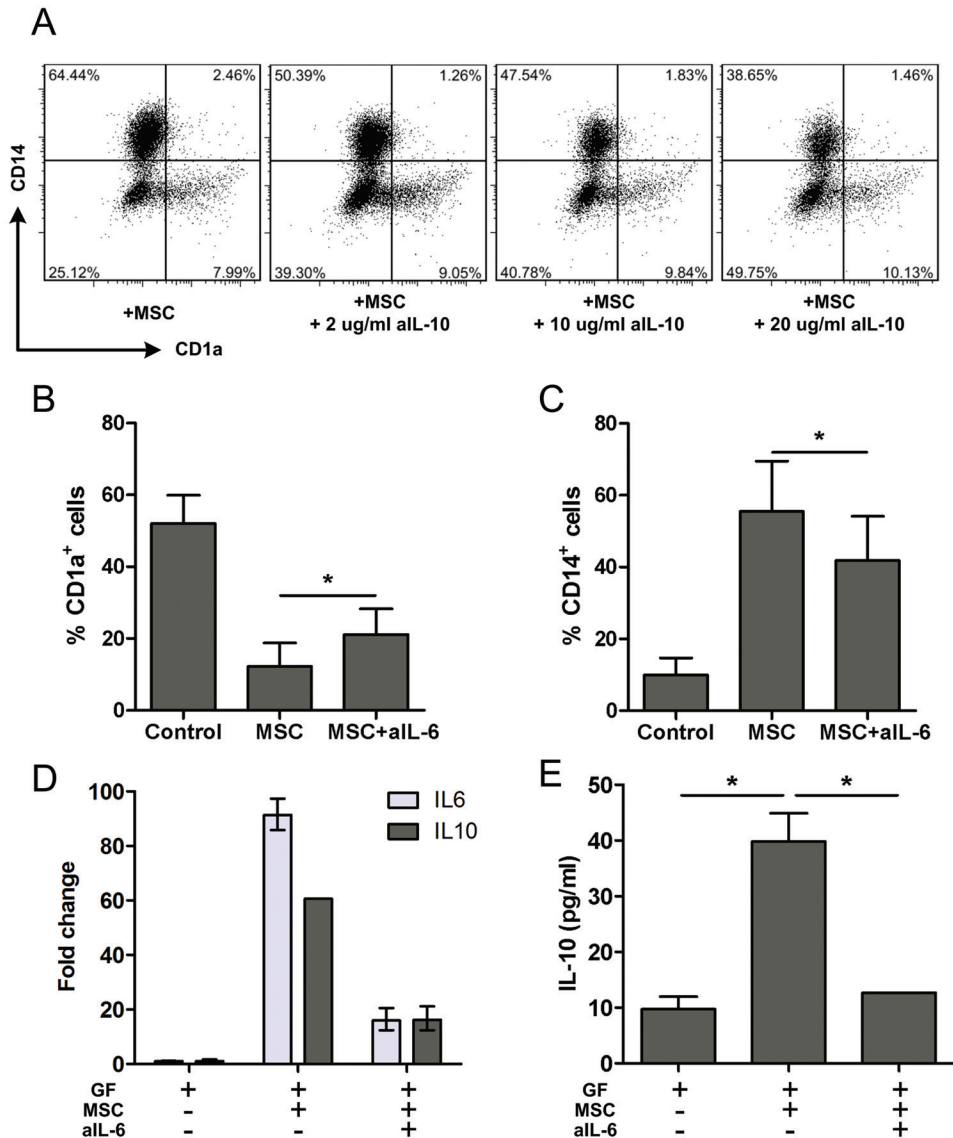
from MSC cultured with the growth factors IL-4 and GM-CSF (MSC+GF CM) and 3) conditioned medium from the monocyte-MSC direct co-cultures (MSC+mono CM). Figure 3 shows that the differentiation of CD1a⁺CD14⁺ monocytes into CD1a⁺CD14⁺ iDC was completely inhibited in the presence of CM derived from stimulated MSC, 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.



Monocytes were differentiated in the presence of different conditioned media. (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.

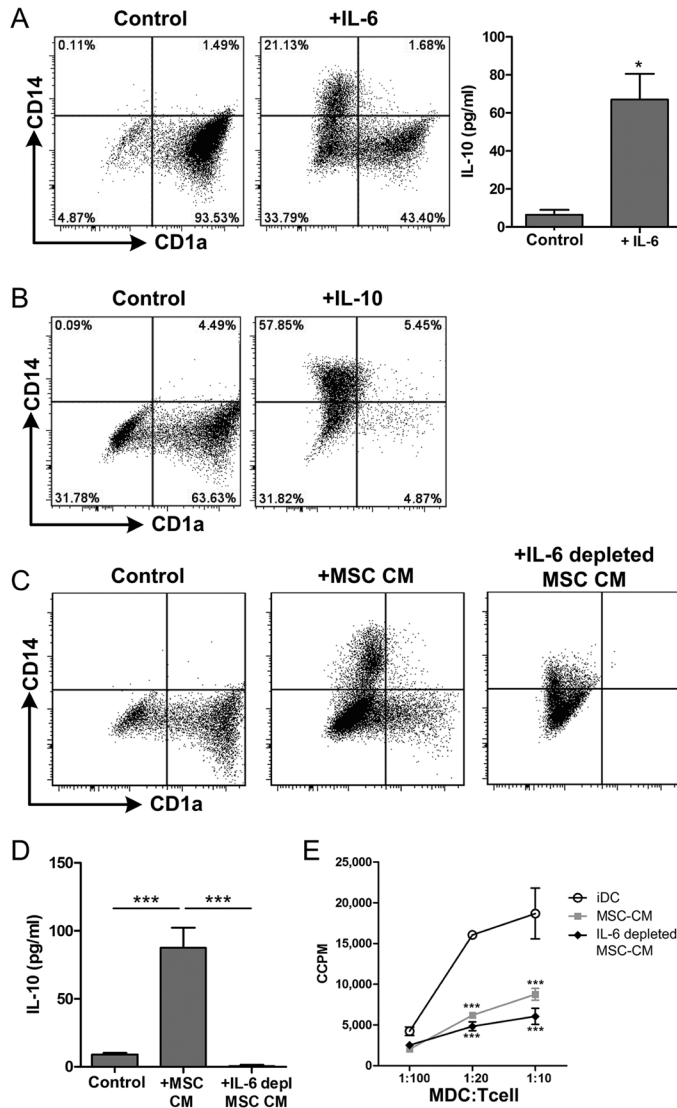


The role of IL-10 and IL-6 in the MSC-mediated inhibition of monocyte differentiation was investigated. (A) Adding a neutralizing antibody against IL-10 did not restore the iDC formation (data are representative dotplots from 2 independent experiments with 2 different MSC donors and 2 different monocyte donors). (B, C) The addition of a neutralizing antibody against IL-6 (2.5 μ g/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 $\pm$ 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 $\pm$ 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.

Figure 5. IL-6 is the key factor in the MSC-induced inhibition of 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 $\pm$ 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,D: data are means $\pm$ 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

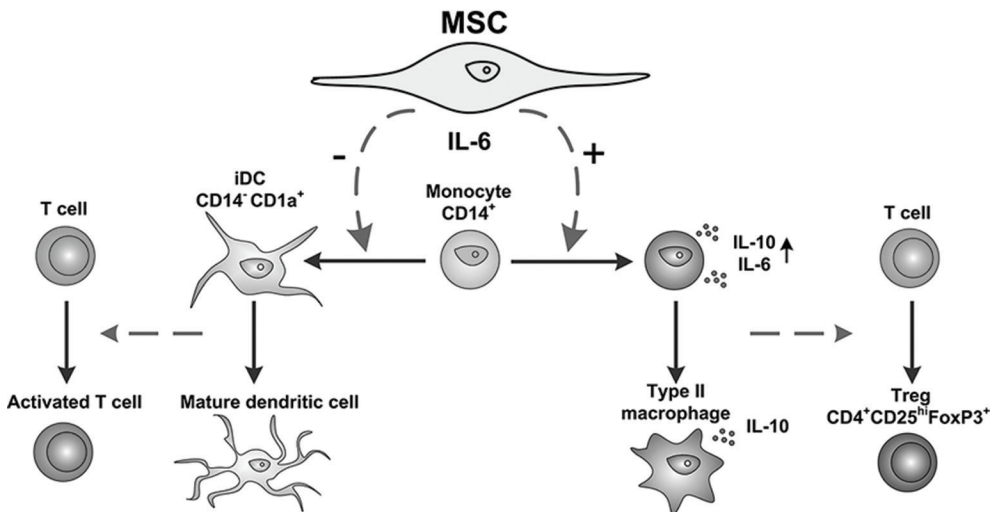
It has been acknowledged that MSC inhibit the differentiation of monocytes towards iDC. The cytokines IL-6 and IL-10 have been described to play a role in the differentiation of monocytes towards immature dendritic cells (iDC) ^{21,22}. However, the role of these cytokines in the MSC-induced inhibition 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.

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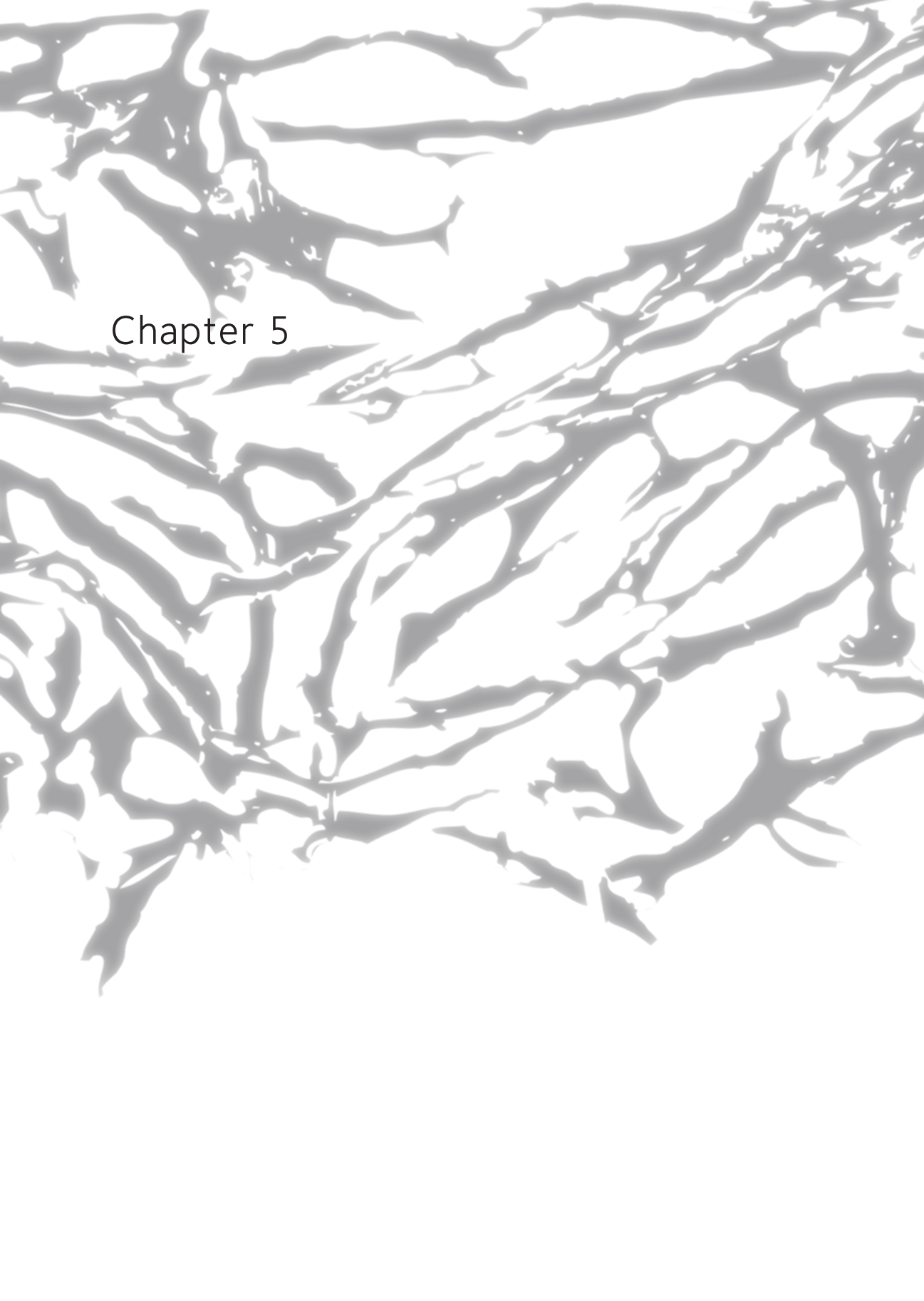
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Chapter 5



MULTIPOTENT STROMAL CELLS INDUCE HUMAN REGULATORY T CELLS THROUGH A NOVEL PATHWAY INVOLVING SKEWING OF MONOCYTES TOWARDS ANTI-INFLAMMATORY MACROPHAGES

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Abstract

Multipotent stromal cells (MSC) have been shown to possess immunomodulatory capacities and are therefore explored as a novel cellular therapy. One of the mechanisms through which MSC modulate immune responses is by the promotion of regulatory T cell (Treg) formation. In this study we focused on the cellular interactions and secreted factors that are essential in this process.

Using an in vitro culture system, we showed that culture-expanded bone marrow derived MSC promote the generation of CD4⁺CD25^{hi}FoxP3⁺ T cells in human PBMC populations and that these populations are functionally suppressive. Similar results were obtained with MSC-conditioned medium, indicating that this process is dependent on soluble factors secreted by the MSC. Antibody neutralization studies showed that TGF- β 1 mediates induction of Tregs. TGF- β 1 is constitutively secreted by MSC, suggesting that the MSC-induced generation of Tregs by TGF- β 1 was independent of the interaction between MSC and PBMC.

Monocyte-depletion studies showed that monocytes are indispensable for MSC-induced Treg formation. MSC promote the survival of monocytes and induce differentiation towards macrophage type 2 cells that express CD206 and CD163 and secrete high levels of IL-10 and CCL-18, which is mediated by as yet unidentified MSC-derived soluble factors. CCL18 proved to be responsible for the observed Treg induction.

These data indicate that MSC promote the generation of Tregs. Both the direct pathway through the constitutive production of TGF- β 1 and the indirect novel pathway involving the differentiation of monocytes towards CCL18 producing, type 2 macrophages are essential for the generation of Tregs induced by MSC.

Introduction

Regulatory T cells (Tregs) are the primary mediators of peripheral tolerance, which maintain immune homeostasis and are moderators of inflammation. Several types of Tregs have been described, including naturally occurring Tregs (nTregs), that develop in the thymus, and induced Tregs (iTregs), that are generated in the periphery and arise from conventional T cells^{1,2}. The mechanisms by which iTregs are induced have not been fully elucidated. The transcription factor forkhead box P3 (FoxP3), which is a key regulatory gene for the development of Tregs³, is expressed in both nTregs and iTregs.

Multipotent stromal cells (MSC) display immunomodulatory capacities, including the capacity to suppress the proliferation of T cells, B cells and IL-2 stimulated NK cells⁴⁻⁶ and to inhibit the differentiation and maturation of dendritic cells⁷. Another important mechanism by which MSC exert their immunomodulatory functions is the induction of Tregs. In *in vivo* studies regarding allergic airway inflammation and heart transplantation, MSC were shown to induce the generation of Tregs^{8,9}. Understanding the mechanisms behind the MSC induced formation of Tregs is important for the application of MSC as a therapeutic agent.

The mechanisms by which MSC induce the generation of Tregs have been studied *in vitro*. These studies involved co-cultures of MSC and PBMC and showed the generation of functionally suppressive CD4⁺CD25^{hi}FoxP3⁺ Tregs¹⁰⁻¹⁴. According to some studies soluble factors are involved, while other studies indicate the requirement of cell-cell contact. The molecular pathways that are involved in the MSC-induced Treg formation have not yet been fully identified. English and colleagues claimed the involvement of TGF- β 1 and PGE2¹², while others found that MSC-induced Treg formation was not reduced by neutralizing antibodies against these factors¹³.

In this study, we explored the cellular and molecular pathways underlying the Treg promoting effect of MSC. We found that the MSC-induced formation of Tregs is driven by TGF- β 1, which is produced by MSC, and by CCL18, which is produced by monocytes upon interaction with MSC.

Materials and methods

Generation of human MSC and PBMC

After obtaining informed consent, adult bone marrow was harvested from healthy donors or orthopedic patients. Mononuclear cells were isolated using a Ficoll-Paque density gradient (1.077 g/cm³) and were plated at $1.3 \times 10^5/\text{cm}^2$ in DMEM-low glucose (Invitrogen Corp., Paisley, UK) supplemented with 10% fetal calf serum (FCS; Greiner Bio-one, Alphen a/d Rijn, The Netherlands) and Penicillin/Streptomycin (P/S; Invitrogen Corp., Paisley, UK). After 3-4 days, non-adherent cells were removed and medium was refreshed every 3-4 days until cells reached approximately 90% confluency. The MSC monolayer was detached using trypsin/EDTA (Invitrogen Corp., Paisley, UK) and reseeded at 4,000/cm² for further expansion. The MSC were characterized by flowcytometric analysis and used in the experiments at passage 2-5.

Human PBMC were isolated from buffy coats from healthy donors obtained from Sanquin Blood Supply (Leiden, The Netherlands) using a Ficoll-Paque density gradient. CD4⁺ T cells were isolated from PBMC by MACS using a cocktail of biotin-labeled antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and CD235a and anti-biotin microbeads followed by separation on a MACS LS column according to the manufacturer's recommendations (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Monocytes were isolated or depleted from PBMC populations by several rounds of plastic adherence or by MACS using CD14 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), followed by MACS LS column separation according to the manufacturer's recommendations.

PBMC-MSc co-cultures

To assess the capacity of MSC to induce the generation of Tregs, transwell co-cultures were performed as described earlier¹⁴. In short, PBMC were co-cultured with allogeneic MSC for one week followed by one week of culture in the absence of MSC. As a control experiment, a co-culture of PBMC with autologous MSC was performed. At day 0, 100,000 MSC were plated in 12-wells plates in RPMI medium (Invitrogen Corp., Paisley, UK) containing P/S, L-glutamin (Invitrogen Corp., Paisley, UK) and 10% FCS and in a transwell insert (pore size 0.4 μm , Corning, Inc, Lowell, MA, USA) 500,000 PBMC were added. At day 7, PBMC were harvested from the inserts and washed (day 7-PBMC). Cells were counted and resuspended in fresh medium and cultured for 7 days in the absence of MSC. At day 14, PBMC were harvested (day 14-PBMC), counted and analyzed by flowcytometry.

In addition to the transwell co-cultures, MSC-conditioned medium (CM) was tested for its ability to promote generation of CD4⁺CD25^{hi}FoxP3⁺ T cells. Near-confluent cultures of MSC were cultured with RPMI supplemented with P/S, L-glutamin and 10% FCS for 5-7 days. The cells were harvested and counted and the cell-free culture supernatant was concentrated using 10K Centriprep Centrifugal filters (Millipore Corp, Billerica, MA, USA). PBMC were cultured for 7 days in a 12-wells plate with a CM equivalent of 100,000 MSC supplemented with until 1 ml with fresh RPMI containing P/S and L-glutamin. At day 7, the PBMC were harvested and resuspended in fresh medium and cultured for another 7 days. At day 14, PBMC

were harvested, counted and analyzed by using flow cytometry.

The role of molecular factors in the induction of Treg formation by MSC was assessed by blocking experiments. In the first week of co-culture, antibodies against IL-6 (0.2–1.0 µg/ml), IFN-γ (1.0 µg/ml) and CCL18 (0.5 – 2.0 µg/ml; all from R&D systems Europe Ltd., Abingdon, UK) and an inhibitor of the TFG-β1-receptor (TFG-β1-R) (10 µM, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) were added to the medium. Control experiments were performed by addition of equal concentrations of a non-specific antibody of the same isotype to the co-cultures. The role of HO-1 was determined by adding the inhibiting agent tin protoporphyrin IX dichloride (SnPP, 10 µM, Tocris Bioscience, Bristol, UK).

Monocyte-MSC co-cultures

To directly investigate the effect of MSC on monocytes, 3 day cultures were performed of freshly isolated monocytes in unconcentrated CM diluted with RPMI medium in a ratio 2:1. To reveal an effect of MSC on monocytes, apart from their monocyte survival-enhancing effect, we compared monocytes that were stimulated with CM versus RPMI medium with and without the addition of 5 ng/ml M-CSF (Peprotech EC Ltd. London, UK), neutralizing antibodies against the M-CSF-receptor (concentration [1:100] – [1:500]), the inhibitor of the TFG-β1-receptor (10 µM) or recombinant TFG-β1 (10 ng/ml; BD Biosciences, San Diego, CA, USA). At day 3 of the culture the monocytes were harvested and analyzed for surface marker expression using flow cytometry, counted and assessed for viability using eosine staining. RNA was extracted for gene expression analysis using RNease columns (Qiagen GmbH, Hilden, Germany). Cell-free supernatants were collected and stored at -20°C for analysis of cytokine concentrations.

Functionality assay

For some experiments, PBMC at day 14 of the PBMC-MSC co-culture were separated by FACS (FACSaria III cell sorter; BD Biosciences, San Diego, CA, USA) in a CD25⁺ fraction and a CD25^{hi} fraction. The complete population of PBMC that were cultured in the presence and absence of MSC and the sorted CD25⁺ fraction and a CD25^{hi} fraction PBMC cultured in the presence of MSC were tested for their immunosuppressive capacity in an autologous suppression assay. To this end, autologous CD4⁺CD25⁺ cells were isolated from PBMC (using the CD4⁺CD25⁺ regulatory T cell isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)). CD4 T cells were isolated from PBMC as described above, followed by separation of CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells by CD25 microbeads according to manufacturer's recommendations (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). CD4⁺CD25⁺ T cells (100,000/well) were stimulated with human T-activator CD3/CD28 dynabeads (Invitrogen Corp., Paisley, UK) in a bead:cell ratio 1:5. To this culture, day 14-PBMC, day 14-CD25⁺ or day 14-CD25^{hi} cells were added in a 1:1 ratio with CD4⁺CD25⁺ T cells. After 5 days, the cells were pulsed with [³H]-thymidine (0.5 µCi/well) and incubated for 16 h at 37°C. The cultures were harvested on a glass fiber filter and thymidine incorporation was measured with a liquid scintillation counter (Wallac, Turku, Finland).

Analysis of cytokine production

Cytokine concentrations were measured in the cell-free supernatant of day 14 from the PBMC culture in the presence and absence of CM (IL-10) and in the supernatant from the 3-day monocyte cultures with and without M-CSF and/or CM (IL-10, IL-12, and CCL18). Constitutive production by MSC of the cytokines M-CSF and TGF- β 1 were measured in cell-free culture supernatant of unstimulated MSC. Cytokine concentrations were measured using sandwich ELISA (IL-10, IL-12 and TGF- β 1 and : BD Biosciences, San Diego, CA, USA; M-CSF and CCL18: R&D systems Europe Ltd., Abingdon, UK) or a Bio-Plex Pro Human Cytokine 27-plex panel (Bio-Rad laboratories, Inc, Hercules, CA, USA). Cytokine concentrations were determined using a standard curve. Statistical analysis was performed using a One-way-ANOVA and the differences between groups were analyzed using a paired T-test. A $P < .05$ was considered statistically significant.

Flow cytometry

Antibodies used for flowcytometric analysis of PBMC and monocytes were anti-CD4 PerCP-Cy5.5, anti-CD69 FITC, anti-CD25 PE-Cy7, anti-CD14 PE, anti-CD127 biotin, anti-CD206 APC (BD Biosciences, San Diego, CA, USA), anti-CD25 PE (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), anti-FoxP3 APC (eBioscience, Inc., San Diego, CA, USA), anti-CD163 PerCP-Cy5.5, anti-CD80 PE-Cy7 (Biolegend, Inc., San Diego, CA, USA) and streptavidin-Pacific blue (Invitrogen Corp., Paisley, UK). MSC were analyzed for expression of surface markers using FITC-conjugated and PE-conjugated antibodies against: CD90, CD73, CD45, CD34, HLA-DR, HLA-ABC, CD80 (BD Biosciences, San Diego, CA, USA) and CD105 (AnceCell Corp., Bayport, MN, USA). For surface staining, primary antibodies were added and the cells were incubated for 30 minutes at 4°C in the dark. Cells were washed with PBS/1% albumin, secondary antibodies were added and incubated for 30 minutes at 4°C in the dark. For intracellular FoxP3 staining, the FoxP3 staining buffer kit (eBioscience, Inc., San Diego, CA, USA) was used. All stainings were 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). Mean fluorescence intensity (MFI) was analysed and statistical analysis was performed using a One-way-ANOVA, the differences between groups were analyzed using a paired T-test and a $P < .05$ was considered statistically significant.

Gene expression analysis

To study the effect of MSC on monocytes we performed a microarray analysis on monocytes cultured for 3 days with CM and/or M-CSF. From these monocyte populations, RNA was extracted using the RNeasy micro kit (Qiagen GmbH, Hilden, Germany). For microarray analysis, the RNA was amplified and biotinylated using the Illumina® Totalprep™ RNA amplification kit (Life Technologies Europe BV, Bleiswijk, The Netherlands). The samples were hybridized on a HumanHT-12 v4 Expression BeadChip according to the manufacturer's instructions. Chips were scanned on an Illumina BeadArray 500GX reader and analyzed with BeadStudio software (all from Illumina Inc, San Diego, CA, USA). Gene expression data were

obtained from BeadStudio and processed in R using Bioconductor¹⁵ and the R beadarray package¹⁶, using the BASH algorithm for outlier detection. The remaining bead information was summarized and normalized using quantile normalization. Due to considerable donor variation between the different monocyte sources, differentially expressed genes were determined using the RankProd¹⁷ method, considering a percentage false positive (PFP) rate of 0.05 significant. Gene set enrichment analysis was performed using the Broad Institute GSEA tool¹⁸.

For RT-qPCR analysis cDNA was synthesized with Superscript III RT (Invitrogen GmbH, Hilden, Germany). RT-qPCR analyses were performed on a StepOnePlus real-time PCR system (Applied Biosystems, Fostercity, CA, USA) using SYBR Green reagent (Applied Biosystems). The primer sets used for RT-qPCR are listed in table 1. All RT-qPCR data were normalized to β -actin expression and analyzed using the delta-Ct method.

Table 1. Primers used for RT-qPCR.

Gene	Forward	Reverse
IL-10	5'-CCGAGATGCCTTCAGCAGAG-3'	5'-GGTCTTGTTCTCAGCTTGG-3'
GAS6	5'-GCCTTTCAGGTCTTCGAGGAG-3'	5'-GTCAGGCAGGTTTTGCACG-3'
CCL18	5'-ACAAAGAGCTCTGCTGCCTC-3'	5'-CCCACTTCTTATTGGGGTCA-3'
MMP9	5'-TGGCAGAGGAATACCTGTACC-3'	5'-GAGTAGTTTTGGATCCAATAGG-3'
β -actin	5'-AGGCATCCTCACCCTGAAGTA-3'	5'-CACACGCAGCTCATTGTAGA-3'

Results

Soluble factors produced by MSC promote the formation of CD4⁺CD25^{hi}FoxP3⁺ T cells

The formation of Treg was studied in co-cultures of PBMC and MSC. At day 7 of these co-cultures, PBMC that were cultured in the presence and absence of MSC both contained a population of CD4⁺CD25^{hi}FoxP3⁺ T cells (Figure 1A). At day 14 however, PBMC cultured during the first week in the presence of MSC, contained a significantly higher percentage of CD4⁺CD25^{hi}FoxP3⁺ T cells than PBMC cultured in the absence of MSC (Figure 1A-B; 0.36% versus 10.04%, $n=8$, $P < .001$). The CD4⁺CD25^{hi}FoxP3⁺ T cells did not express the T cell activation marker CD69 and CD127 (supplemental Figure 1), compliant with the reported Treg phenotype¹⁹. The formation of Tregs induced by MSC was observed when PBMC were cultured in the presence of both autologous and allogeneic MSC. Similar results were obtained with MSC conditioned medium (CM) (Figure 1C), indicating that factors secreted by MSC were responsible for this effect.

Cytokine measurements in culture supernatants of MSC and PBMC co-cultures indicated a three-fold increase in IL-10 concentrations in comparison with culture supernatants of PBMC that were cultured in the absence of MSC (Figure 1D; 27.74 pg/ml versus 10.49 pg/ml, $n=4$, $P = .001$). These concentrations fell within the standard curve. This indicates that CM induced the cells in the PBMC population to secrete IL-10.

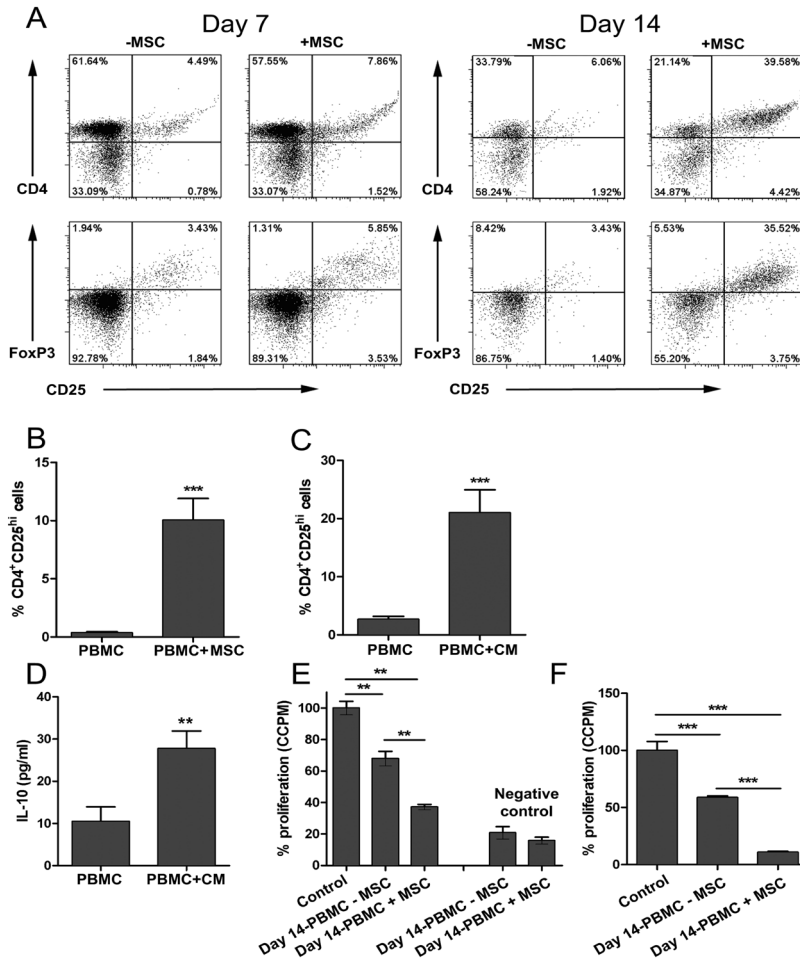
CD4⁺CD25^{hi}FoxP3⁺ T cells induced by MSC exhibit regulatory function

To study the function of the MSC-induced CD4⁺CD25^{hi}FoxP3⁺ T cells, the PBMC populations generated in the absence and presence of MSC were tested for their immunoregulatory capacity. The total PBMC populations obtained at day 14 after co-culture with MSC, exhibited a significantly enhanced capacity to suppress T cell proliferation compared to PBMC populations that were generated in the absence of MSC (Figure 1E). To test specifically whether the CD25^{hi} T cells were responsible for this enhanced immunosuppression, PBMC from day 14 of culture in the presence of CM were separated in a CD4⁺CD25⁻ and a CD4⁺CD25^{hi} T cell fraction and tested in a separate autologous suppression assay. At day 14, the CD4⁺CD25^{hi} T cells showed a significantly higher suppression of T cell proliferation compared to the CD4⁺CD25⁻ T cells (Figure 1F), indicating that the CD4⁺CD25^{hi}FoxP3⁺ T cell population that is formed during co-culture with MSC or CM has regulatory function.

Generation of Tregs is mediated by TGF- β 1

In order to identify the factors involved in the MSC-induced Treg formation, antibody neutralization and inhibitor experiments were performed. Addition of neutralizing antibodies to the TGF- β 1-receptor to the PBMC-MSC co-cultures resulted in a significantly reduced generation of Tregs compared to control cultures without antibodies (4.6%, versus 20.2% $P = .02$, $n = 5$, Figure 2A). MSC constitutively secreted TGF- β 1 as was measured with ELISA in cell free culture supernatant of unstimulated MSC (supplemental figure 2). Addition of neutralizing antibodies

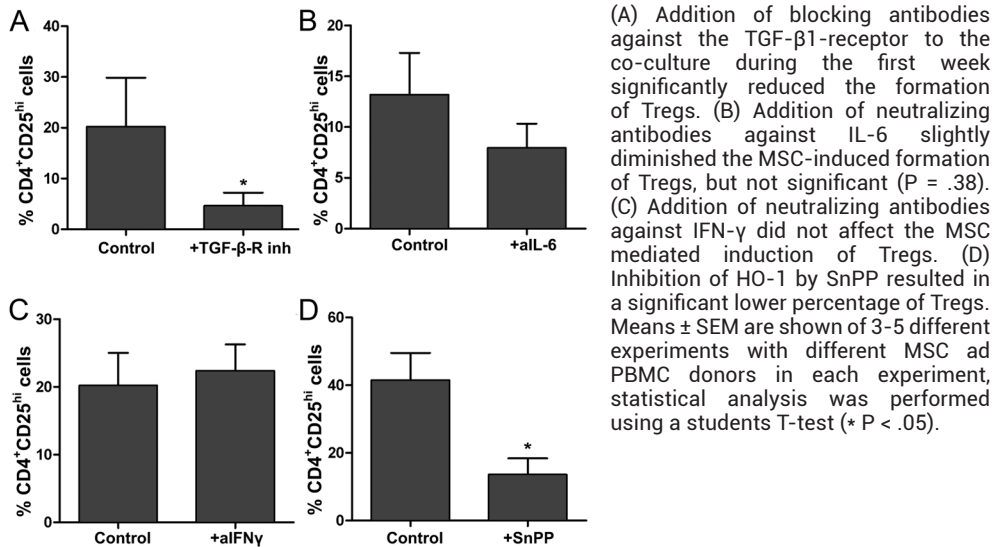
Figure 1. Coculture of MSC with PBMC results in the induction of functional CD4⁺CD25^{hi}FoxP3⁺ T cells.



(A) Representative dotplots of expression of CD4, CD25 and FoxP3 on PBMC on day 7 and 14. (B) The percentage of CD4⁺CD25^{hi} T cells at day 14 is significantly higher in the population that was cultured during the first week in co-culture with MSC compared to PBMC that were cultured without MSC (data are means $\pm$ SEM from 8 different experiments with 9 different MSC and 7 different PBMC. Statistical analysis was performed with a paired T-test; *** $P < .001$). (C) The presence of CM in the PBMC culture obtains similar results as the presence of MSC (data are means $\pm$ SEM from 5 different experiments with 5 different MSC and 2 different PBMC. Statistical analysis was performed with a paired T-test; *** $P < .001$). (D) The IL-10 protein concentrations measured at day 14 were significantly higher in the culture supernatants of PBMC cultures in the presence of MSC compared to cultures in the absence of MSC (data are means $\pm$ SEM from 2 different experiments with 3 different MSC and 2 different PBMC. Statistical analysis was performed using a paired T-test (** $P < .01$). (E,F) Autologous CD25⁺ T cells were stimulated with anti-CD3/CD28 beads (Control) with or without PBMC from the co-culture experiment at day 14 (E) or with sorted CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells from the day 14-PBMC cultured with MSC (F). Day 14-PBMC were also tested separately for their proliferation upon CD3/CD28 stimulation (negative control). Proliferation was measured using 3H-thymidine incorporation after 5 days (data are mean from triplicates $\pm$ SEM). Statistical analysis was performed with a student's T-test (** $P < .01$; *** $P < .001$).

against IL-6 did not result in a significant reduction ($P = .38$) of the MSC-induced generation of Tregs (Figure 2B). As expected, neutralizing antibodies against IFN- γ , which is not constitutively expressed by MSC, did not influence the effect of MSC on the generation of Tregs (Figure 2C). Finally, addition of the HO-1 inhibitor SnPP significantly reduced the population of Tregs at day 14 (13.6%, versus 41.5% $P = .04$, $n = 3$) (Figure 2D).

Figure 2. The induction of Treg formation by MSC is mediated by several factors.



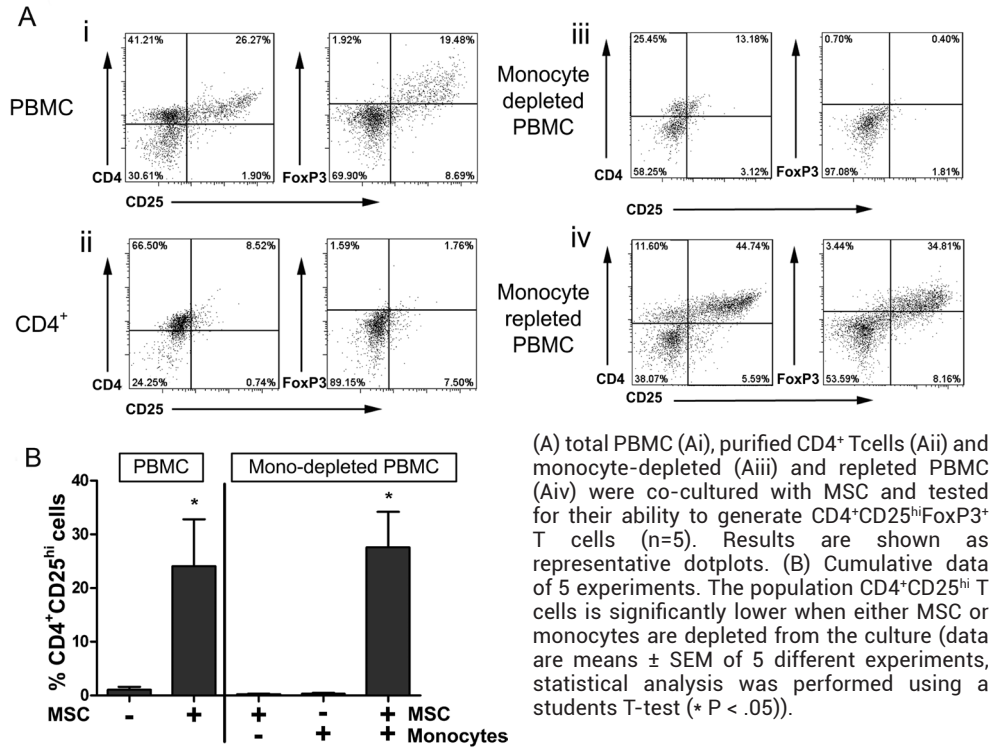
Monocytes are required for induction of CD4⁺CD25^{hi}FoxP3⁺ T cells

Since CD4⁺CD25^{hi}FoxP3⁺ Tregs are derived from the CD4⁺ T cell population ³, we investigated whether a Treg population could be generated by co-culture of purified CD4⁺ T cells with MSC (Figure 3). MSC-induced Tregs could not be generated from the purified CD4⁺ population, as opposed to PBMC, indicating that the PBMC population contains accessory cells mediating the formation of Tregs (Figure 3A). Following depletion of monocytes from the PBMC population, no Treg population could be formed (Figure 3A-B). To exclude the possibility that the depletion procedure interfered with the formation of Tregs, we added monocytes back to the monocyte-depleted PBMC populations. This restored the formation of the Treg population (Figure 3A-B). These data show that monocytes are essential in mediating MSC-induced formation of Tregs.

MSC promote the survival of monocytes

Since monocytes were essential for the MSC-induced Treg formation, we focused on the effect of MSC and CM on monocytes in PBMC-MSC co-cultures and by performing cultures of freshly isolated monocytes stimulated with CM. In the presence of MSC, the PBMC cultures contained a higher percentage of CD14⁺CD16⁺ cells after 4 and 7 days compared to cultures in the absence of MSC (Figure 4A).

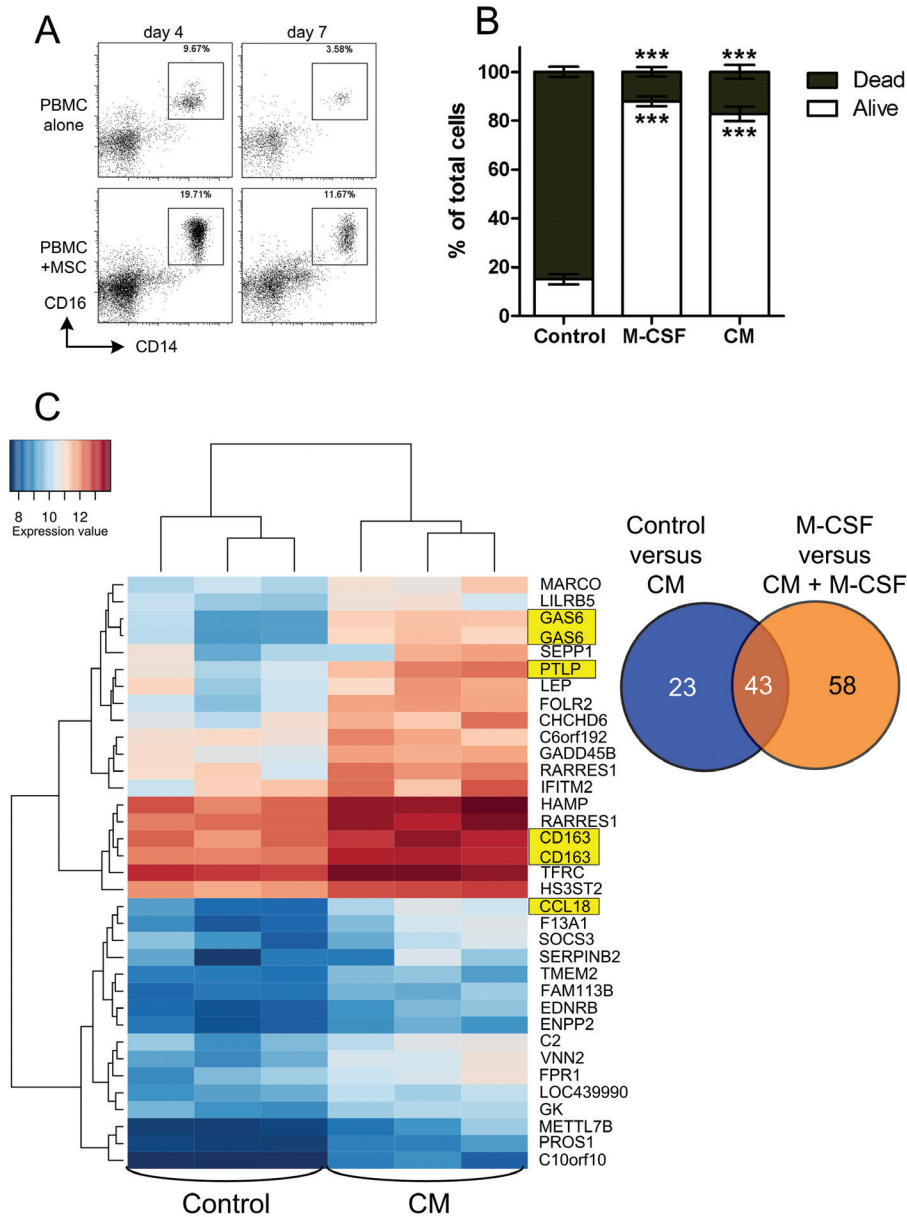
Figure 3. Monocytes are indispensable for the induction of CD4⁺CD25^{hi}FoxP3⁺ T cells.



Staining with Annexin V and CD14 showed that the monocytes that lose CD14 undergo apoptosis and do not differentiate towards dendritic cells (data not shown), indicating that MSC promote survival of monocytes in the culture. This survival-promoting effect was confirmed in 3-day cultures of freshly isolated monocytes in the presence of CM. Similar results were observed in cultures of freshly isolated monocytes stimulated with M-CSF. M-CSF, a factor that is produced by MSC (supplemental figure 2) and is known to promote monocyte survival, did not further enhance the effect of CM on monocyte survival (Figure 4B).

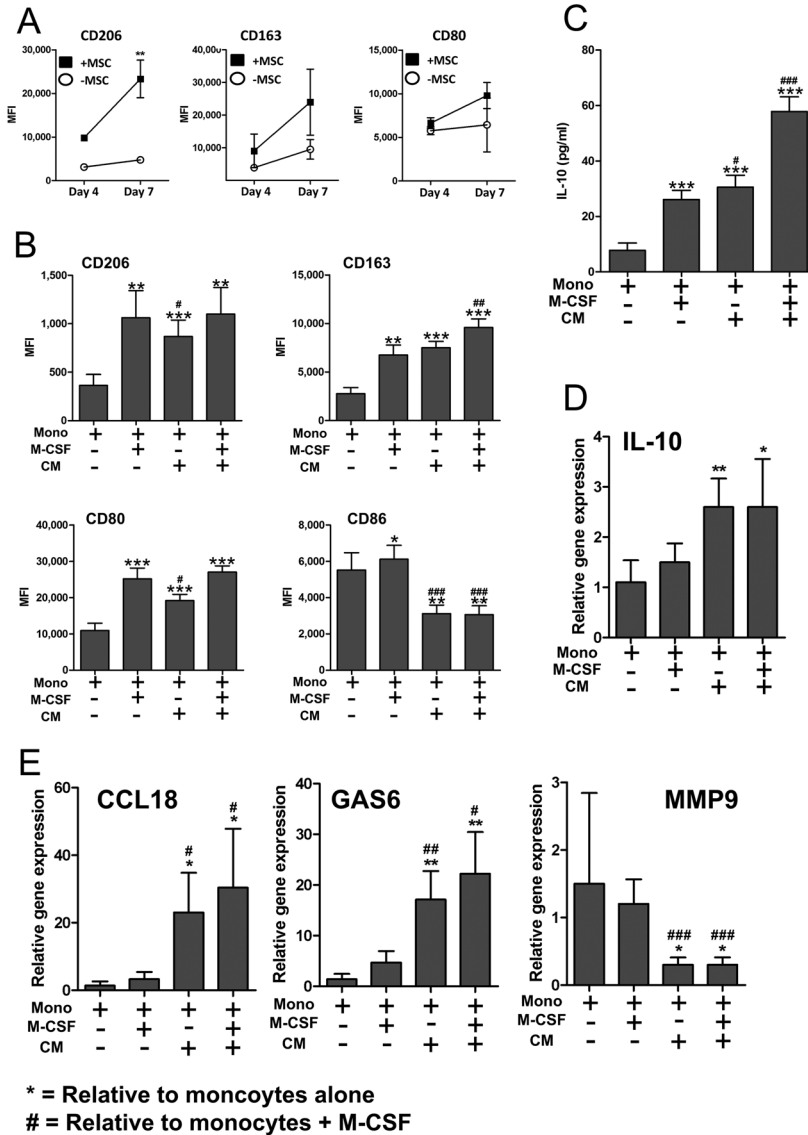
To explore the transcriptional changes in monocytes in response to MSC exposure, we performed a genome wide gene expression profiling of monocytes from 3 different donors. Comparison of the two datasets ('CM stimulated monocytes versus unstimulated monocytes' and 'CM + M-CSF stimulated monocytes versus M-CSF stimulated monocytes'), resulted in 43 overlapping unique genes, that were differentially expressed after stimulation with CM (Figure 4C). Gene set enrichment analysis showed downregulation of apoptosis-related genes²⁰ in monocytes in response to CM, in line with the survival promoting effect of MSC on monocytes (supplemental Figure 3).

Figure 4. MSC promote survival of monocytes.



(A) In the presence of MSC, a higher percentage of CD14⁺CD16⁺ monocytes is present in the PBMC population after 7 days of culture than in PBMC cultured without MSC. (B) Stimulation with either M-CSF or CM increases survival of monocytes in a 3 day culture compared to control cultures (data are means $\pm$ SEM from 3 different MSC; *** $P = .001$). (C) Heatmap representing upregulated genes in monocytes cultured in the presence of CM compared to control cultures of monocytes cultured in the absence CM. The Venn-diagram represents the overlapping differentially regulated genes between the comparison of CM versus control cultures and the CM+M-CSF versus M-CSF cultures.

Figure 5. MSC-CM induces skewing of monocytes towards differentiation into a type-II macrophage.



(A) The mean fluorescence intensities (MFI) of CD206, CD163 and CD80 within the CD14⁺ population are shown (data are means $\pm$ SEM from 2 experiments with 3 different MSC donors; ** $P < .01$). Expression of surface markers on monocytes (B) and IL-10 concentrations in culture supernatant (C) after a 3 days stimulation with M-CSF and/or CM (data are means $\pm$ SEM from 5 different monocyte donors and CM from 2 different MSC; * = compared to control, ** $P < .01$, *** $P < .001$; # = compared to M-CSF, # $P < .05$, ## $P < .01$, ### $P < .001$). (D) IL-10 gene expression is increased in monocytes that were cultured with CM compared to M-CSF and control cultures (data are means $\pm$ SD from 3 different monocyte donors and 2 different MSC donors; * $P < .05$; ** $P < .01$). (E) CCL18, GAS6 and MMP9 gene expression in monocytes that were cultured for 3 days in CM and/or M-CSF (data are means $\pm$ SD from 3 different monocyte donors and 2 MSC donors; * $P < .05$; ** $P < .01$; # $P < .05$, ## $P < .01$, ### $P < .001$).

MSC skew monocytes towards an anti-inflammatory profile

Phenotype analysis

To further study the effect of MSC on monocytes, phenotypic changes of monocytes during co-cultures with MSC were studied. At day 7 of the co-cultures, the expression of the macrophage type 2 markers CD206 and CD163 and of CD80 was increased on monocytes compared to the cultures without MSC (Figure 5A). Further investigation of the effect of MSC on freshly isolated monocytes showed that M-CSF as well as CM increased the expression of the surface markers CD163, CD206 and CD80 on monocytes, indicating a skewing of monocytes towards a macrophage type 2 phenotype (Figure 5B). The expression of CD163 was further increased in the presence of both M-CSF and CM, which shows an additive effect of CM. Expression of CD86 was not changed by M-CSF alone, but was significantly decreased after culture with CM. To investigate the involvement of M-CSF in the effect of CM we added neutralizing antibodies against the M-CSF-receptor (M-CSF-R) to monocytes cultured in the presence of CM. We found that the increased expression of the surface markers CD80, CD163 and CD206 on monocytes cultured in the presence of CM (figure 6A) as well as the increase in survival (figure 6B) was reversed in the presence of neutralizing antibodies against M-CSF-R, showing that M-CSF is responsible for these effects.

Altogether, these surface marker expression data indicate that some of the effects of CM can be explained by M-CSF, but that additional soluble factors, present in the CM, are involved in skewing of monocytes towards type 2 macrophages.

Cytokine secretion

Monocytes were also functionally tested for cytokine secretion after stimulation with CM and/or M-CSF. Stimulation of monocytes with CM or M-CSF resulted in significantly ($P < .001$) increased concentrations of IL-10 in the culture supernatant at day 3. IL-10 concentrations were further increased following stimulation of monocytes with both CM and M-CSF (Figure 5C). Expression levels of IL-10 mRNA were similar in unstimulated monocytes and monocytes that were stimulated with M-CSF (Figure 5D), suggesting that the observed increase in IL-10 concentrations in the culture supernatant was a result of increased monocyte survival. Stimulation of monocytes with CM, however, significantly increased the expression of IL-10 mRNA by monocytes compared to the cultures of monocytes in the absence of CM. The combination of CM and M-CSF did not further increase the mRNA expression of IL-10 in monocytes compared to CM alone, suggesting that the increased IL-10 secretion that was observed after monocyte stimulation with the combination of CM and M-CSF was regulated at a post-transcriptional level. Moreover, the CM-induced increase in IL-10 secretion was not significantly reversed by addition of neutralizing antibodies against M-CSF-R (figure 6C), indicating that the M-CSF is not responsible for the observed IL-10 secretion by monocytes. Taken together, these data indicate that CM induced IL-10 production in monocytes and that CM is superior to M-CSF alone in this effect. IL-12 protein was not detectable in supernatant of monocyte cultures stimulated with CM. Further analysis of cytokine concentrations in the culture supernatant of monocytes showed reduced

Figure 6. M-CSF is partly responsible for the MSC-induced skewing of monocytes towards type-II macrophages.

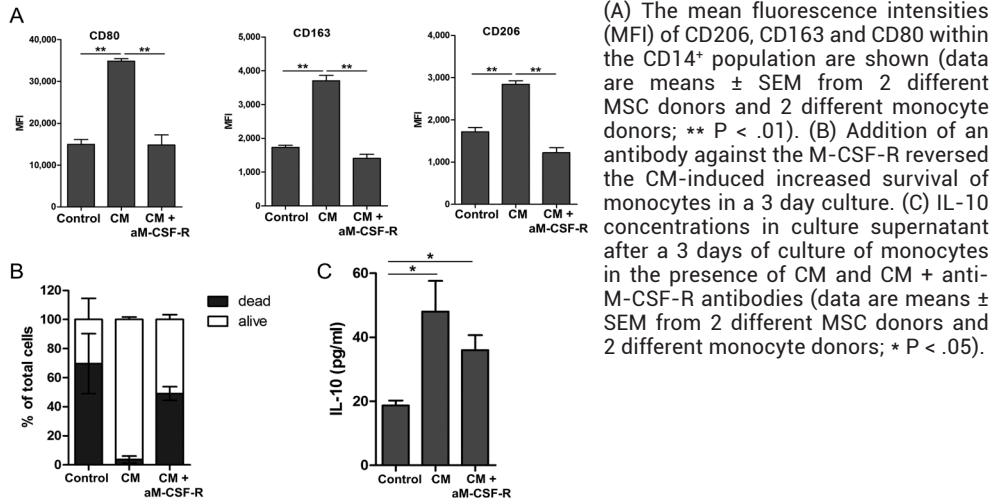
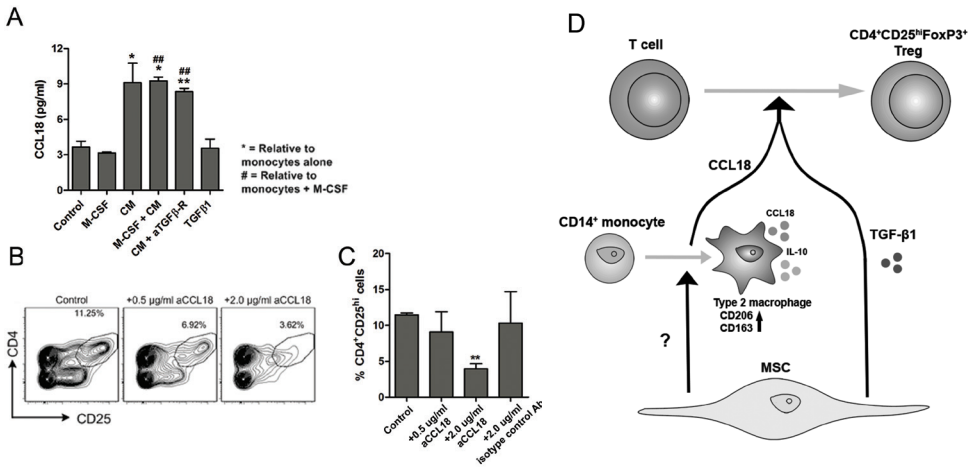


Figure 7. MSC-induced and monocyte-dependent Treg formation is mediated by CCL18.



(A) CCL18 protein concentrations measured with ELISA at day 3 of the monocyte culture are increased when monocytes were stimulated with CM. Addition of an inhibitor of the TGF- β 1-receptor did not reverse the effect of CM and recombinant TGF- β 1 did not induce CCL18 secretion by monocytes. (Data are means of 5 monocyte donors and 2 MSC donors; * = compared to control, * $P < .05$, ** $P < .01$; # = compared to M-CSF, ## $P < .01$). (B) Representative dotplots of expression of CD4 and CD25 on day 14-PBMC from control cultures and from PBMC-CM co-cultures in the presence of neutralizing antibodies against CCL18. (C) The percentage of CD4⁺CD25^{hi} T cells at day 14 is significantly lower in the population that was cultured during the first week in the presence of aCCL18 compared to PBMC that were cultured in control PBMC-CM cultures (data are means $\pm$ SEM from 2 different PBMC. Statistical analysis was performed with a student's T-test; ** $P < .01$). (D) Hypothetical model of Treg formation induced by MSC, both the direct pathway via MSC-derived TGF- β 1 and the indirect pathway via the modulation of monocytes are crucially involved in the MSC-induced Treg formation.

concentrations of the pro-inflammatory cytokines IL-1b, MIP-1a, MIP-1b and RANTES in the culture supernatant of monocytes that were cultured in the presence of CM compared to M-CSF (supplemental Figure 4).

Transcriptional profiling of monocytes

Gene expression profiling of monocytes showed that by CM stimulation of monocytes resulted in upregulation of several genes, including CD163, GAS6, CCL18, PLTP, that are associated with the type 2 macrophage phenotype (Figure 4C). Additionally, gene set enrichment analysis showed that the STAT3 pathway was activated in monocytes that were cultured in the presence of CM ²¹, which is another characteristic of type 2 macrophages (supplemental Figure 3). Microarray results were confirmed and validated by RT-qPCR in samples from other monocyte donors and stimulated with CM from another MSC donor than those used for the microarray analysis. The expression of genes encoding CCL18 and GAS6 was indeed upregulated in monocytes that were stimulated with CM, but not in monocytes that were stimulated with M-CSF (Figure 5E). As expected, MMP9 gene expression was downregulated in response to stimulation with CM (Figure 5E). The changes that were observed in phenotype, cytokine concentrations and transcriptional profile of monocytes stimulated with CM, indicate that CM not only promoted survival of monocytes but also skew monocytes towards type 2 macrophages.

MSC-induced and monocyte-dependent Treg formation is mediated by CCL18

Since CCL18 has been implicated in the generation of Tregs ^{22,23}, we hypothesized that the MSC-induced CCL18 production by monocytes was involved in the MSC-induced Treg induction that was observed in PBMC-MSC co-cultures. Indeed, the culture supernatant of monocytes stimulated with CM contained measurable concentrations of CCL18. These CCL18 concentrations were significantly higher than those in culture supernatant of unstimulated monocytes, which were hardly detectable by ELISA (detection limit of the standard curve was 0.78 pg/ml) (Figure 7A). Since addition of an inhibitor of the TGF- β 1 receptor resulted in an inhibition of the MSC-induced Treg formation, we also tested whether TGF- β 1 was involved in the MSC-induced secretion of CCL18 by monocytes. Addition of a TGF- β 1-receptor inhibitor did not reverse the increase in CCL18 secretion in monocytes induced by CM. Moreover, addition of recombinant TGF- β 1 did not result in CCL18 secretion by monocytes (figure 7A), indicating that the induction of CCL18 secretion by MSC is not mediated by TGF- β 1.

Addition of neutralizing antibodies against CCL18 to the PBMC-CM cultures during the first week resulted in decreased MSC-induced Treg formation (Figure 7B,C). These data indicate that MSC-induced CCL18 promotion by monocytes is responsible for the generation of Tregs.

Discussion

In this study, we explored the factors and cellular interactions involved in the MSC-induced formation of CD4⁺CD25^{hi}FoxP3⁺ Tregs derived from PBMC. We showed that MSC promote the generation of CD4⁺CD25^{hi}FoxP3⁺ T cells with regulatory capacities, which is mediated directly by TGF- β 1. In addition, this is dependent on a novel pathway for the MSC-induced generation of Tregs, for which the presence of monocytes is crucial. We showed that MSC promote monocyte survival and induce skewing of monocytes towards a cell type with an anti-inflammatory, macrophage type 2 phenotype. These cells produce IL-10 and mediate Treg formation through the production of CCL18. We hypothesize that both the TGF- β 1 mediated pathway and the modulation of monocytes are essential for the MSC-induced generation of Tregs.

Despite the variability in the frequency of Tregs generated by co-culture of PBMC with MSC that was observed between experiments, the observation that addition of MSC or CM resulted in a higher frequency of Tregs was a robust finding.

Transwell experiments and the use of CM showed that the MSC-induced Treg formation is dependent on soluble factors secreted by MSC. In contrast to other reports^{24,25}, MSC produce these factors constitutively and do not require activation. It has been reported that TGF- β 1 is involved in the generation and expansion of Tregs and it is constitutively expressed and secreted by MSC²⁶⁻²⁸. Our finding that HO-1 is involved in the MSC-induced Treg formation confirms previous studies²⁹. However, since HO-1 is not a secreted factor, it plays no role in the effects reported in this study.

Here we confirm that in this *in vitro* culture system TGF- β 1 is an essential factor involved in MSC-mediated promotion of Treg induction, indicating that TGF- β 1 produced by MSC might have a direct promoting effect on the formation of Tregs from T cells.

We did not investigate whether the generated Tregs were induced from CD4⁺FoxP3⁺ T cells or whether the natural Tregs preferentially survive or proliferate. Crop et al.¹⁴, however, showed that in this *in vitro* system the CD4⁺CD25^{hi} fraction of the PBMC is highly proliferative during the second week of culture, indicating proliferation of already existing Tregs. An important finding of this study is the crucial role of monocytes in the MSC-induced Treg formation. Other studies showed that monocytes are important mediators for the immunosuppressive effect of MSC on T cell proliferation³⁰. Here we have identified an additional pathway and we show that the MSC-induced formation of Tregs is also mediated by monocytes, revealing an indirect route for MSC to promote Treg formation. The essential involvement of monocyte modulation for MSC-induced Treg formation in addition to the presence of TGF- β 1 also explains why we did not observe Treg formation in co-cultures of purified CD4⁺T cells and MSC.

MSC promoted the survival of monocytes, which was confirmed by gene expression profiling of the monocytes, that showed downregulation of apoptosis-related genes in monocytes in response to CM. M-CSF promotes monocyte survival and is secreted by MSC and we showed that this factor is involved in the promotion of monocyte survival by CM.

In addition to their effect on monocyte survival, we found that MSC skew the differentiation of monocytes towards type 2 macrophages, similar to what has been suggested for fully differentiated macrophages³¹. In accordance, soluble factors derived from MSC induce upregulation of the expression of the surface markers CD206 and CD163 and downregulation of expression of the co-stimulatory molecule CD86. This was confirmed by gene expression profiling, that showed increased gene expression of CD206, CD163, GAS6, PLTP and CCL18. These genes are associated with the immune modulating and anti-inflammatory properties of type 2 macrophages³²⁻³⁵.

The hypothesis that MSC skew monocytes towards type 2 macrophages was further supported by the observed changes in cytokine concentrations. It is well established that type 2 macrophages produce anti-inflammatory cytokines, including IL-10 and CCL18^{22;32;36}. Concentrations of these cytokines were increased in supernatants of monocytes cultured in the presence of CM and antibody neutralization experiments showed that monocyte-derived CCL18 mediated the MSC-induced generation of Tregs. We previously showed that MSC do not produce IL-10 and that soluble factors produced by MSC induce IL-10 production by monocytes³⁷. The data from the current study suggested M-CSF as a candidate factor for this effect. However, addition of neutralizing anti-M-CSF-R antibodies did not prevent IL-10 secretion by monocytes, indicating that other factors produced by MSC are responsible for IL-10 induction.

Antibody neutralization experiments indicated that the increase in CCL18 concentrations that was observed in monocyte cultures in the presence of CM was not dependent on TGF- β 1. Since Martinez et al. showed that alternative stimulation of monocytes results in upregulation of CCL18³⁸, the role of IL-4 and IL-13, cytokines that induce alternative activation of monocytes³⁹, would be of interest for further research on the CCL18-inducing effect of MSC.

Interestingly, type 2 macrophages have been implicated in the formation of Treg in co-culture⁴⁰, which could explain the requirement for monocytes in the MSC-induced Treg formation. Conversely, Tregs are able to induce differentiation of monocytes towards type 2 macrophages⁴¹, thus creating a loop for further amplification of Treg production.

In conclusion we hypothesize that the MSC-induced formation of Tregs relies on multiple direct and indirect mechanisms (Figure 7D). MSC-derived TGF- β 1 might act directly on the T cells to promote their differentiation towards Tregs. In addition, the modulation of monocytes is a crucial mechanism through which MSC indirectly promote Treg formation. MSC promote survival of monocytes and skew monocyte differentiation towards modulatory type 2 macrophages that secrete IL-10 and that induce Treg formation through the production of CCL18. Further studies are required to identify MSC-derived factors that are responsible for the skewing of monocytes. Our results contribute to the understanding of the cellular networks that are involved in MSC-mediated Treg formation and show a crucial role for monocytes/macrophages in MSC-induced immune modulation.

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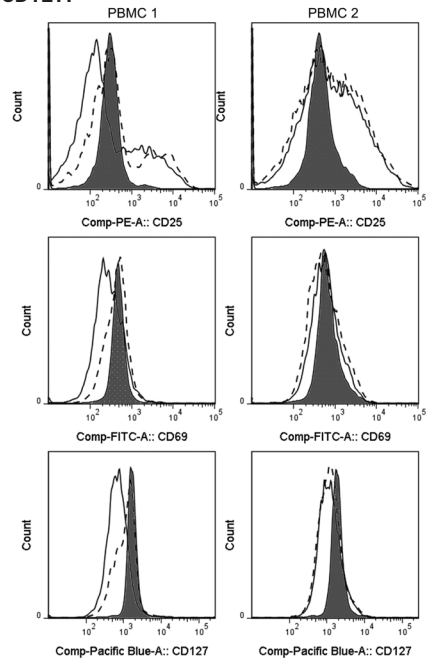
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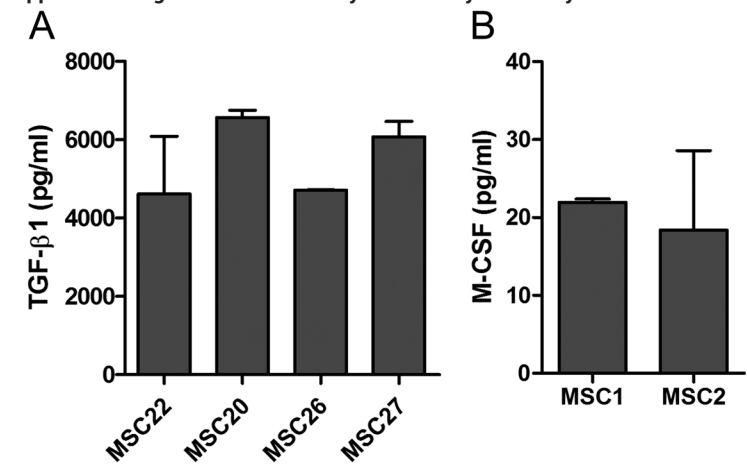
Supplemental figures

Supplemental figure 1. CD4⁺CD25^{hi}FoxP3⁺ T cells do not express T cell activation marker CD69 and CD127.



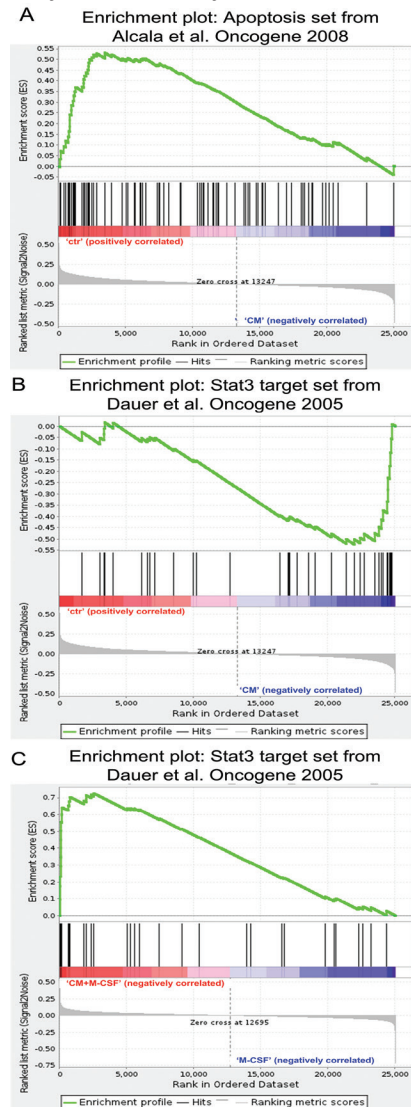
Histograms of two different PBMC populations at day 14. When MSC were present during the first week of MSC-PBMC co-culture, a CD25⁺ cell population emerges while this is not observed when PBMC were cultured without MSC. No increase of CD69 and CD127 expression is seen on the PBMC after co-culture with MSC. Tinted histogram, PBMC alone; straight line, MSC 1; dashed line, MSC 2.

Supplemental figure 2. Constitutively secreted cytokines by MSC.



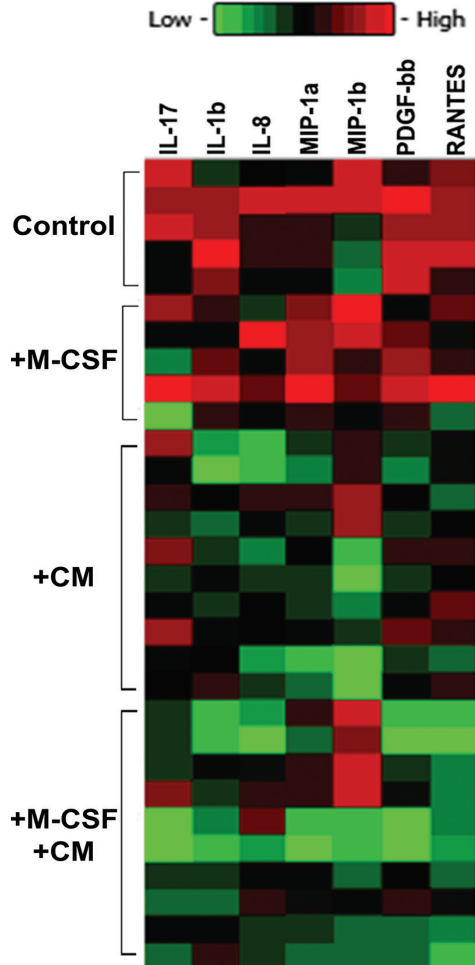
Cytokine concentrations of TGF-β1 and M-CSF were measured in cell culture supernatants of unstimulated MSC. Both cytokines were constitutively secreted by MSC.

Supplemental figure 3. Gene set enrichment analysis of microarray data.



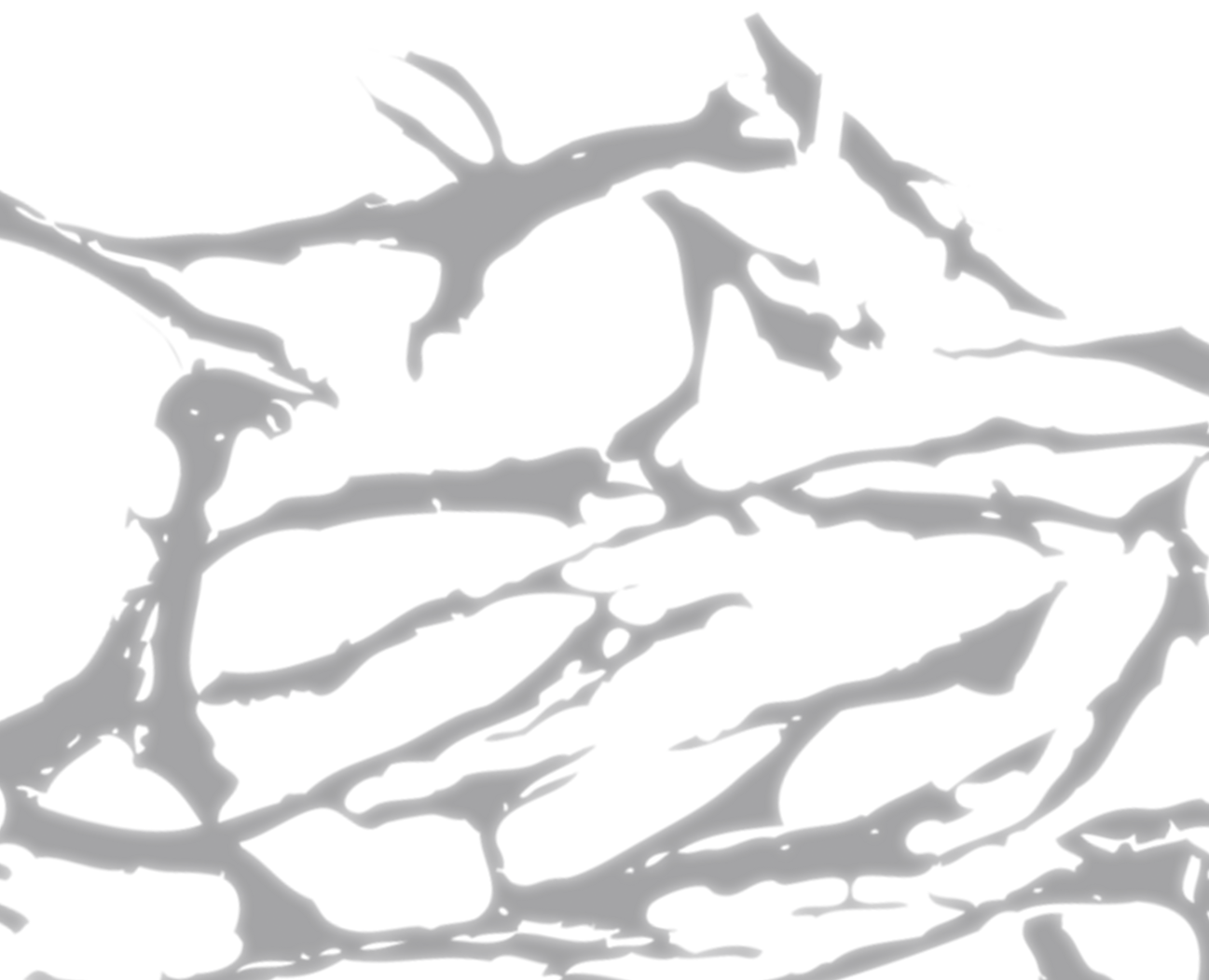
(A) Apoptosis set from Alcala et al. Oncogene 2008: Genes involved in apoptosis are enriched in unstimulated monocytes ('ctr') compared to CM stimulated monocytes ('CM'). Stat3 target set from Dauer et al. Oncogene 2005: Monocytes that are stimulated with CM show activation of the STAT3 pathway. (B) unstimulated monocytes ('ctr') versus CM stimulated monocytes ('CM'), (C) CM+M-CSF stimulated monocytes ('CM+M-CSF') versus M-CSF stimulated monocytes ('M-CSF').

Supplemental figure 4. Stimulation of monocytes with CM results in a decrease of pro-inflammatory cytokine secretion by monocytes.

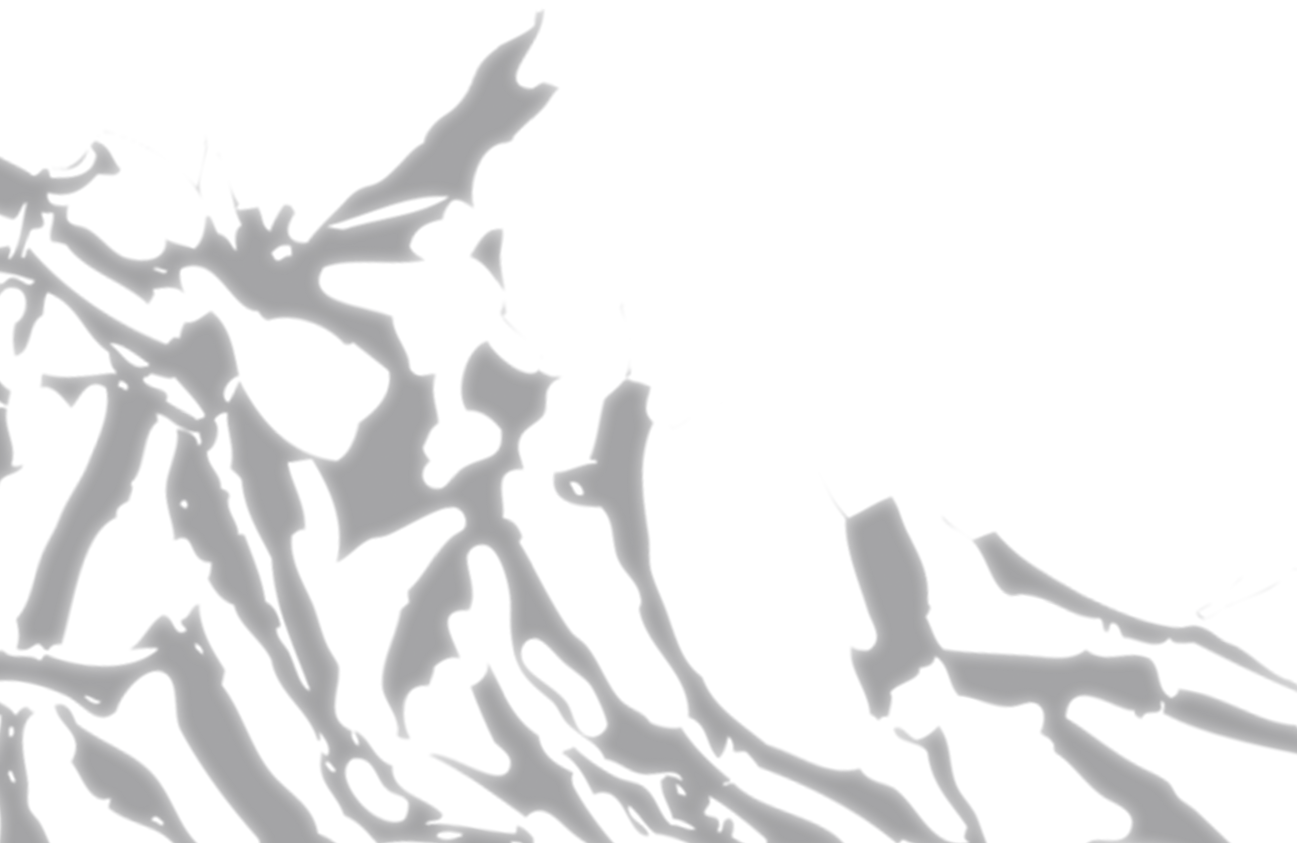


Cytokine concentrations were measured in culture supernatant from monocytes stimulated for 3 days with and without M-CSF and/or MSC-CM using the Bio-plex Pro Human 27-plex panel. Data from cytokines that were decreased in supernatants of monocytes after stimulation with CM are represented as heatmap.

CHAPTER 6



SUMMARY AND DISCUSSION



Summary and discussion

The application of MSC as therapeutic agent to treat a variety of immune related diseases is currently under investigation in preclinical and clinical studies. Although promising results have been reported, more insight in the basic MSC biology and in the mechanisms by which MSC modulate the immune system are necessary to optimize the safe and effective application of MSC in the clinic.

This thesis describes our contributions to the understanding of the immunomodulatory mechanisms of MSC. We also investigated some translational aspects of MSC therapy, aiming at selecting the preferred tissue source for clinical application.

Selecting the optimal MSC population

MSC are rare cells that are found in a variety different tissues ¹. Currently, bone marrow is mainly used as MSC source in clinical trials. In these trials, the infused cell products exist of *ex vivo* expanded MSC populations derived from bone marrow mononuclear cells. Recent studies highlighted methods for prospective isolation of MSC with differential capacities using markers like CD146, CD271 and CD56 ^{2,3}. Fundamental research on MSC subpopulations with specific capacities and on the differences between tissue sources would greatly enhance the possibilities of clinical application, since it may enable making a balanced choice for a specific MSC tissue source or subpopulation for each indication.

The work presented in chapter 2 addresses the variation in proliferative capacity of MSC derived from bone marrow aspirates. We investigated the underlying variation in clonal bone marrow composition in this study and confirmed the clonal heterogeneity within MSC populations, similar to what was described by others ^{4,5}. Heterogeneity in differentiation potential was observed within each MSC population and the frequency of clones with a specific differentiation capacity was different between donors. We directly related the differentiation capacity of clones to their expansion potential. The colonies with osteogenic differentiation capacity exhibited a relatively high proliferation rate and formed larger colonies. The experiments showed that a high variation in phenotypes is present within the primary MSC population and that the osteogenic lineage is most prominent, which is in line with current literature ⁴⁻⁷. We propose a hierarchical model in which colonies with osteogenic potential are more primitive than purely adipogenic colonies and in which cells gradually lose their differentiation potential, concurrently with a loss in proliferation rate.

In **chapter 3** we performed experiments to investigate adipose tissue as an alternative for bone marrow as source for MSC. Comparisons between AT-MSC and BM-MSC were described by others, but our study is the first to compare age-matched donor groups. We systematically compared the *in vitro* immunomodulatory capacities of AT-MSC and BM-MSC. We found that MSC from both sources exhibited immunomodulatory capacities, including their suppression of proliferation of stimulated PBMC and their inhibition of monocyte-to-iDC differentiation. However, on a per cell basis the AT-MSC proved more potent in their immunomodulatory

effects than BM-MSc. This was related to higher levels of cytokine secretion by AT-MSc. Our results indicate that AT-MSc can be considered a promising alternative to BM-MSc for future immunomodulatory clinical applications.

Adipose tissue as a source of MSc has many advantages over bone marrow, including its higher frequency of MSc and its reduced donor burden associated with the tissue collection process, since adipose tissue, that is regarded as waste after liposuction, can be collected without any additional risk for the donor⁸⁻¹⁰. These advantages, together with our results that showed that fewer AT-MSc were needed for effective immunomodulation by MSc than BM-MSc, could eventually lead to a higher feasibility and cost reduction of MSc therapies.

The use of UCB-MSc has been suggested as another alternative to BM-MSc. UCB-MSc are thought to be more primitive than BM-MSc, resulting in high proliferation rates and high multilineage differentiation capacity. This would be interesting for the use of UCB-MSc as regenerative therapy. The disadvantage of UCB is, however, that it contains very low numbers of MSc. We were able to isolate MSc from 43% of UCB (starting with 100-150 ml blood per umbilical cord) that were processed (unpublished data), which is in line with what was reported by others¹¹⁻¹³. Moreover, low numbers of colonies were generated (1 or 2 per sample), resulting in an expanded MSc product consisting of the outgrowth of 1 or 2 clonal populations. This may lead to high variation in expanded UCB-MSc, depending on the capacities of the few clones that were isolated. However, once colonies were generated, UCB-MSc showed a high proliferation rate and could easily be expanded to a high number of cells. On a functional level, UCB-MSc have been reported to have similar differentiation and immunomodulatory capacities as BM-MSc^{12,14}. The low frequency of MSc in UCB limits the use of UCB-MSc for autologous MSc therapy, but the high proliferation frequency of UCB-MSc makes it an interesting tissue source for biobanking purposes.

Factors involved in immunomodulation by MSc

MSc modulate the immune system through their interaction with a variety of immune cells and immunological processes. In the studies described in this thesis we explored the mechanisms and factors that are involved in the inhibitory effect of MSc on the differentiation of monocytes towards iDC and (chapter 4) and in their induction of Treg formation (chapter 5).

We showed that MSc were able to inhibit the differentiation of CD14⁺CD1a⁻ monocytes towards CD14⁺CD1a⁺ iDC. The involvement of IL-10 and IL-6 in immunosuppressive activities by MSc had previously been suggested by others¹⁵⁻¹⁸ and it was reported that monocyte differentiation can be inhibited by these cytokines^{19,20}. In our experiments, neutralization and cytokine-depletion strategies were applied to reveal the cellular source and effect of IL-6 and IL-10. We found that IL-10 is not produced by MSc, but exclusively by monocyte-derived cells (MDC). After exposure to IL-6, this IL-10 production is markedly enhanced. Neutralization of IL-6 diminished the inhibitory effect of MSc and almost completely blocked the IL-10 production by MDC. Since MSc constitutively secrete IL-6, addition of these cells indirectly increased the IL-10 concentration in the culture through skewing of monocytes towards an IL-10 producing cell population. It was reported that IL-10

induces the production of HLA-G by MSC^{21,22}, which indicates a positive feedback loop that could further enhance the immunomodulatory effects of MSC.

The generation of Tregs induced by MSC might be a way in which MSC can induce long-term immunomodulating effects. In the MSC-mediated induction of Treg formation (**chapter 5**), we demonstrated an important role for TGF- β 1, similar to what was suggested by others²³. The formation of Tregs induced by MSC could not be prevented by blocking IL-6 in the culture. Furthermore we found that inhibition of HO-1, which was also reported to mediate the MSC-induced formation of Tregs²⁴, impaired the formation of Tregs. We concluded, however, that the effect of HO-1 can be taken over by other factors, since it is not secreted by MSC and the induction of Treg formation could be accomplished by conditioned medium from unstimulated MSC. We also found that MSC-derived factors induced the skewing of monocytes towards CCL18 producing cells with a type 2 macrophage phenotype and the presence of these monocytes/macrophages was crucial for the MSC-mediated induction of Tregs. The survival of monocytes was promoted by MSC, which was mediated by M-CSF. Furthermore, M-CSF was partly responsible for the MSC-induced skewing of monocytes towards type 2 macrophages with increased expression of CD206 and CD163, but not for the induction of CCL18, which could not be explained by the secretion of TGF- β 1 by MSC. Based on our data we propose that the indirect route via modulation of monocytes and the direct route by secretion of TGF- β by MSC are essential for the induction of Tregs. The MSC-derived factor that is responsible for the skewing of monocytes towards CCL18 producing cells is as yet unknown. Others showed that CCL18 secretion is upregulated when monocytes are alternatively activated²⁵. IL-4 and IL-13 are putative factors that would be interesting to investigate, since these cytokines they could be involved in the alternative activation of monocytes^{26,27}.

Several reports indicated that MSC need to be activated in order to exert their immunomodulatory effects^{16,17,28-30}. The pro-inflammatory cytokines IFN- γ and TNF- α were effectively used to enhance the inhibitory effects of MSC^{30,31}. IFN- γ induces the expression of IDO and it increases the secretion of PGE2, that are both implicated as important mediators of immunomodulation by MSC^{32,33}. In **chapter 4 and 5**, we showed that conditioned medium from unstimulated MSC was sufficient to inhibit monocyte differentiation towards iDC, to promote the formation of Tregs and to skew monocytes towards macrophage type 2 cells. This indicated that, in order to exert these immunomodulatory effects, MSC-activation is not required. We demonstrated that the production of the IL-6 protein by MSC, the effector cytokine for the inhibition of monocytes differentiation towards iDC, was not altered after interaction of MSC with monocytes and/or IL-4 and GM-CSF. However, IL-4 and GM-CSF increased the mRNA levels of IL-6 expressed by MSC, suggesting that activation of MSC might enhance their immunomodulatory effects.

Our observations that activation may not be required for all immunomodulatory effects of MSC fits with the model that was proposed by Singer and Caplan¹⁷. This model differentiates between IFN- γ -dependent and -independent pathways of immunomodulation by MSC. In an inflammatory environment, activated T cells produce IFN- γ and this induces the production of IDO, which inhibits the proliferation of effector T cells. Independent of IFN- γ , MSC constitutively secrete cytokines like

TGF- β , IL-6 and PGE2, which are all involved in the immunomodulatory effects of MSC. Our *in vitro* culture systems of monocyte-to-iDC differentiation and Treg formation lack activated T cells and no IFN- γ is present in the system. Therefore, MSC were not activated in these cultures and we hypothesize that the constitutive secretion of cytokines by MSC was sufficient for the immunomodulatory effects we observed.

In **chapter 3** we studied the MSC-mediated suppression of PBMC proliferation. In this system IFN- γ is produced and this resulted in an increase of IDO mRNA, corresponding to the current literature ^{33,34}. It is conceivable that in such an inflammatory environment, MSC respond by altering their secretion profile, initiating and enhancing their immunomodulatory effects.

Central role for monocytes in immunomodulation by MSC

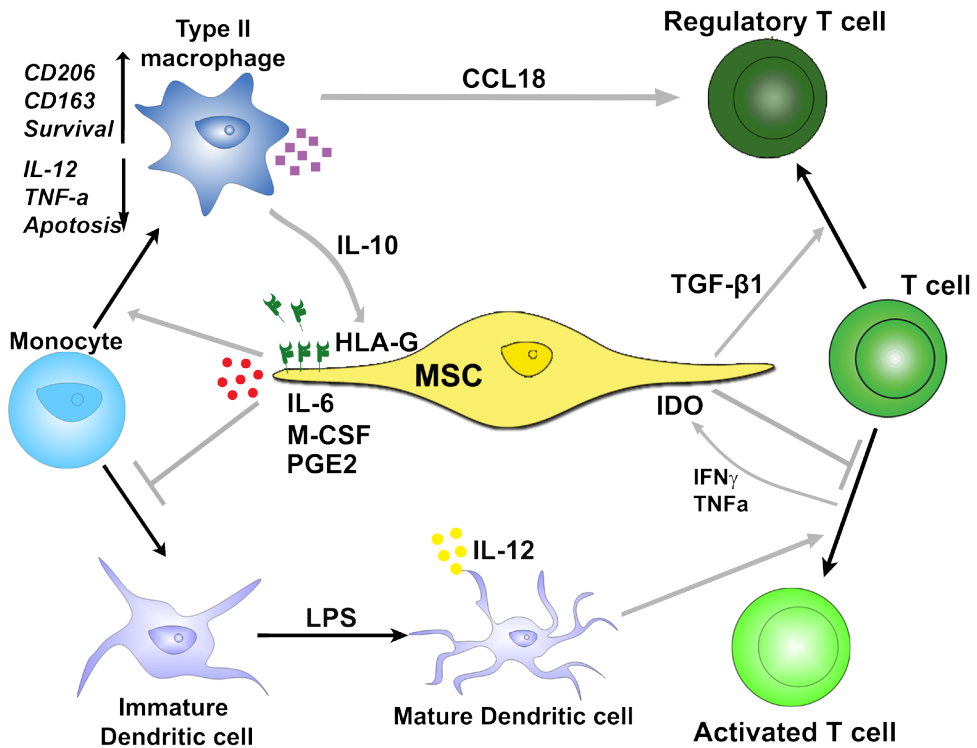
Monocytes are major players of the innate immune response as regulators of inflammation. Monocytes (mostly CD14⁺CD16⁻) ³⁵ circulate in the blood and can migrate into the tissue under inflammatory conditions, where they differentiate towards macrophages or DC ³⁶. Different types of macrophages exist with distinct biological functions. The best described macrophage is the type 1 macrophage (classically activated macrophage) that, activated by IFN- γ and TNF- α , becomes an effector cell that produces pro-inflammatory cytokines and is able to kill and degrade microorganisms. Type 2 macrophages (alternatively activated) on the other hand, are a tolerogenic cell type with an anti-inflammatory role. Type 2 macrophages are activated by various stimuli, including IL-4, IL-13, immune complexes or prostaglandins ^{27,37,38}. The latter type of macrophages can be subdivided in macrophages that play a role in tissue repair and regulatory macrophages with anti-inflammatory activity. Regulatory macrophages upregulate mannose receptors (CD206) ³⁹ and secrete high levels of the anti-inflammatory cytokines IL-10 and low levels of IL-12 ^{27,37,38}. The phenotype of macrophages can change over time due to response to environmental signals. Therefore, Mosser proposed that a spectrum of different shades of activation exists based on macrophage function ³⁸.

An important finding from the work that is presented in this thesis is the major role for monocytes as mediators of the MSC induced immunomodulation. We showed that MSC regulate monocyte differentiation by preventing the differentiation to antigen presenting DC and promoting skewing of monocyte differentiation towards IL-10 producing cells with an impaired ability to stimulate allogeneic T cells. We demonstrated that MSC promote monocyte survival and their skewing towards macrophage type 2 cells, similar as has been suggested by others ^{31,40}. This effect was partly regulated by M-CSF, which is produced by MSC. We showed that MSC induce monocytes to acquire a phenotype that is in accordance to what is described for type 2 macrophages, including upregulation of the surface markers CD206 and CD163, upregulation of PLTP, GAS6 and CCL18 expression and high secretion of IL-10 and low secretion of IL-12. These type 2 macrophage-like cells secrete CCL18, that proved to be responsible for induction of Treg formation.

The importance of monocytes was already described for the MSC-mediated suppression of T cell proliferation ^{29,41-43}. Here we show, that also for the MSC-

mediated induction of Treg formation, the presence of monocytes is crucial. We hypothesize that the MSC-induced skewing of monocytes towards regulatory, anti-inflammatory cells is an essential part of the modulation of the immune response by MSC (figure 1).

Figure 1. hypothetical model of direct and indirect routes of immunomodulation by MSC.



Future perspectives

This thesis supports the role of MSC as powerful modulators of the innate and adaptive immune response. Immune suppression by MSC through modulation of monocytes and macrophages sheds a new light on the possible mechanism of therapeutic application of MSC for immunomodulatory purposes. The involvement of type 2 macrophages in tissue repair might link the immunomodulatory properties of MSC to the possibly advantageous effects of MSC for tissue regeneration. Monocyte modulation might also clarify the observed systemic effects of MSC, despite local entrapment in the lungs after intravenous administration. Systemic effects might be the result of the secretion of immunomodulating factors by MSC that induce skewing of monocytes, that circulate throughout the body, towards anti-inflammatory macrophages. It would be of interest to investigate whether, in certain conditions, administration of MSC conditioned medium or MSC-‘educated’ monocytes might be efficient in exerting the desired immunomodulatory effect.

It is still unclear whether the *in vitro* observed MSC effects are of functional biological relevance *in vivo*. Therefore, it is important to develop relevant preclinical models to investigate MSC therapy and the underlying mechanisms of immunomodulation. In addition, translational issues should be investigated, like ‘what is the optimal time and dose of MSC infusion?’, ‘should MSC be infused locally or systemically?’ and ‘do MSC need to be activated *ex vivo* or *in vivo* after infusion?’.

In conclusion, the fundamental studies presented in this thesis on the immunomodulatory effects of MSC may provide valuable knowledge on how to proceed with applying MSC as a therapy in immune related diseases.

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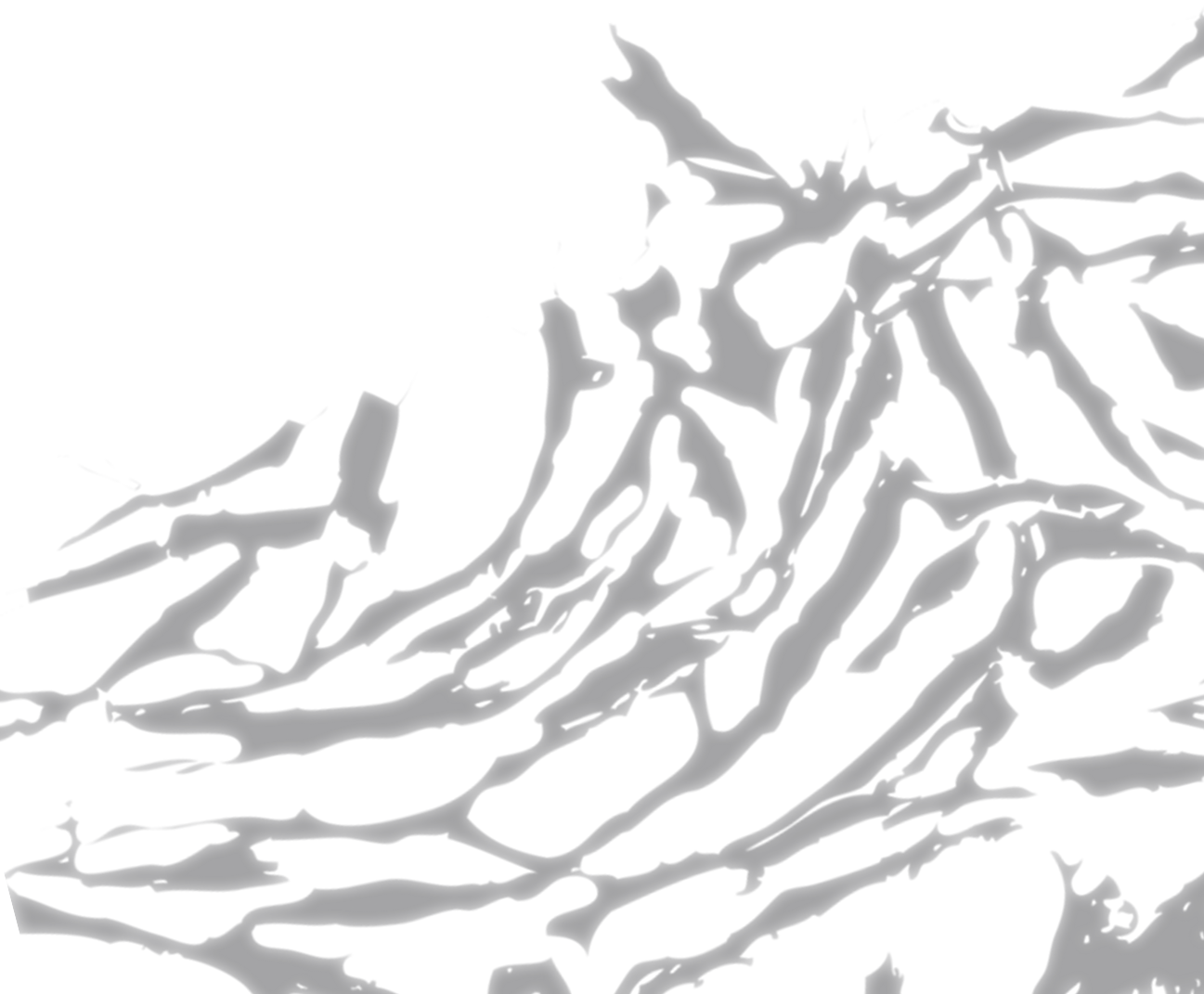
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CHAPTER 7



NEDERLANDSE SAMENVATTING

DANKWOORD

CURRICULUM VITAE

LIST OF PUBLICATIONS



Nederlandse samenvatting

Multipotente stromale cellen (MSC) zijn als eerste geïdentificeerd in het beenmerg in de jaren '60 van de vorige eeuw. Na het in kweek brengen van beenmerg in het laboratorium, worden MSC gekarakteriseerd door hun vermogen om te plakken aan plastic en de capaciteit om uit te rijpen (differentiëren) tot de verschillende mesenchymale weefsels, namelijk bot-, vet- en kraakbeencellen. De International Society for Cellular Therapy (ISCT) heeft de minimale criteria omschreven waaraan een cel moet voldoen om als MSC geïdentificeerd te worden. Deze criteria zijn: 1) de capaciteit om te differentiëren in de richting van bot, vet en kraakbeen en 2) de aanwezigheid of afwezigheid van een bepaalde combinatie van eiwitten op het oppervlak van de cel waarmee MSC zich onderscheiden van andere celtypen. Naast de capaciteit van MSC om te differentiëren naar bot, vet en kraakbeen, kunnen ze ook ontstekingsreacties onderdrukken. Deze functie van MSC als onderdrukkers van het immuunsysteem maakt het interessant om te onderzoeken of MSC gebruikt kunnen worden voor de behandeling van immuun-gerelateerde ziektes, zoals Graft-versus-host ziekte (de afweerreactie die kan ontstaan na een beenmergtransplantatie, waarbij het donorbeenmerg, de 'graft', de ontvanger, de 'host', aanvalt), auto-immuunziekten en ontstekingsziekten als de ziekte van Crohn.

Het immuunsysteem bestaat uit verschillende soorten cellen (witte bloedcellen) die elk verschillende functies hebben. Belangrijk voor de acute afweerrespons en de het eerste verdedigingsmechanisme van het immuunsysteem zijn de monocytën. Monocytën kunnen uitgroeien tot dendritische cellen, die behoren tot de antigeen-presenterende cellen of, als ze het bloed verlaten om in het weefsel actief te worden, tot macrofagen. Als er ergens een ontsteking is, verplaatsen monocytën zich razendsnel via de bloedcirculatie naar deze ontsteking. Hier verlaten ze de bloedstroom en differentiëren tot macrofagen. Macrofagen kunnen op de ontstoken plek lichaamsvreemde micro-organismen of aangetaste cellen verwijderen. Van macrofagen zijn meerdere subtypes te onderscheiden, afhankelijk van de manier waarop ze geactiveerd worden. M1-macrofagen produceren veel ontstekingsstoffen om het lichaam te verdedigen tegen ziekteverwekkers, maar richten daarbij ook schade aan het omliggende weefsel aan. M2-macrofagen zijn meer gespecialiseerd in herstel van weefselschade en de onderdrukking van de immuunrespons.

Naast de acute immuunrespons, is er ook de adaptieve immuunrespons. Belangrijk hiervoor zijn de T cellen, die zorgen voor de specifieke cellulaire afweerrespons. Deze respons ontstaat als T cellen een molecuul herkennen dat gepresenteerd wordt door een antigeen-presenterende cel. Antigeen-presenterende cellen kunnen een gedeelte van een lichaamsvreemd molecuul (afkomstig van bijvoorbeeld een bacterie, virus of een lichaamsvreemde cel), ofwel een antigeen, op hun oppervlak brengen, waarna dat herkend kan worden door de T cel. Pas als de T cel het antigeen herkent, kan de cellulaire afweerreactie in gang gezet worden, waarbij allerlei stoffen worden uitgescheiden door de T cel waarmee de vreemde cel wordt vernietigd. Er zijn echter ook regulatoire T cellen (Tregs), die de balans van de afweerreactie in de gaten houden en de sterkte van een immuunrespons kunnen

afremmen.

We weten dat MSC interacties aan kunnen gaan met al deze verschillende cellen van het immuunsysteem en op die manier de immuunrespons kunnen beïnvloeden. MSC worden nu al getest en succesvol toegepast om de afstotingsreacties, die kunnen optreden na een beenmerg transplantatie, te onderdrukken. Ook voor de behandeling van ontstekingsziekten als de ziekte van Crohn wordt het toedienen van MSC onderzocht. Wat MSC precies doen in het lichaam na infusie is nog niet helemaal duidelijk. Om patiënten op de juiste manier te kunnen behandelen is het belangrijk dat we meer inzicht krijgen in de interacties en de stoffen die belangrijk zijn voor de werking van MSC. In dit proefschrift is onderzocht wat voor effect MSC precies hebben op T cellen, Tregs, dendritische cellen en monocytten en welke stoffen en mechanismen hierbij belangrijk zijn.

De eerste MSC cellen die gaan groeien na het in kweek brengen van beenmerg, vormen kolonies van cellen en aangenomen wordt dat alle cellen in zo'n kolonie afkomstig zijn uit dezelfde voorlopercel. Tussen de kolonies zitten verschillen in groeisnelheid en de capaciteit tot differentiatie, sommigen zijn bijvoorbeeld alleen in staat te differentiëren naar bot, terwijl andere kolonies het vermogen hebben om te differentiëren naar zowel bot, vet en kraakbeen. Na een periode van kweek (expansie) buiten de patiënt (*ex vivo*) vormen de cellen, afkomstig uit verschillende kolonies, een populatie die een zeer gemengde samenstelling heeft van cellen met verschillende capaciteiten tot differentiatie en groeisnelheid. Er is nog weinig bekend over hoe MSC er precies uitzien als ze in het lichaam zitten en dus welke cellen er aan de basis staan van een cel populatie na een aantal weken kweek. Als we erachter kunnen komen hoe we MSC kolonies met een bepaalde groeisnelheid of differentiatiecapaciteit al heel vroeg kunnen identificeren, zou dit de mogelijkheid geven om een cel populatie te kweken die beter toegespitst is op een bepaalde klinische toepassing, bijvoorbeeld een populatie die heel goed naar bot kan differentiëren voor toepassing als celtherapie voor botreparatie na botbreuken. De studie die wordt beschreven in hoofdstuk 2 van dit proefschrift had als doel om de verschillende typen kolonies waarmee een MSC kweek uit beenmerg begint te onderzoeken. We hebben gekeken naar de differentiatiecapaciteit en groeisnelheid van primaire kolonies en de cellen die hier uit voort komen. De MSC met de capaciteit om naar bot te differentiëren hadden een hogere groeisnelheid dan de cellen die naar vet differentiëren en andersom hadden de cellen die geselecteerd werden voor een hoge groeisnelheid een betere differentiatie capaciteit in de richting van bot dan cellen met een lage groeisnelheid. Wij concluderen dat MSC kolonies met de capaciteit om naar bot te differentiëren primitiever zijn dan de cellen die naar vet differentiëren.

MSC werden als eerste geïdentificeerd in beenmerg, en dat is ook de bron die tot op heden het meeste gebruikt wordt voor klinische studies. Daarnaast worden MSC ook verkregen uit andere weefsels en organen, zoals vet, huid, nier, long, placenta, navelstreng en navelstrengbloed. De efficiëntie om MSC te isoleren uit verschillende weefsels is onderzocht en hieruit bleek het in alle gevallen mogelijk om MSC te isoleren uit beenmerg en vet, terwijl maar 63% van de navelstrengbloed monsters MSC opleverde. Als er MSC geïsoleerd konden worden uit navelstrengbloed hadden deze cellen wel een hogere groeisnelheid vergeleken met MSC die geïsoleerd waren

uit beenmerg of vet. Voor de klinische toepassing van MSC is vet een aantrekkelijke bron, omdat vet een relatief hoog aantal MSC bevat en in de huidige praktijk wordt vet gezien als 'rest-weefsel' na liposuctie ingrepen, waardoor het makkelijk te verkrijgen is. In hoofdstuk 3 hebben we een systematische vergelijking gedaan van beenmerg en vet als bron voor MSC. Hiervoor werden MSC gebruikt die waren geïsoleerd uit beenmerg en uit vet van twee groepen met gelijke leeftijd. We hebben gekeken naar de capaciteit van MSC om het immuunsysteem te onderdrukken. MSC van beide bronnen bleken goed in staat om de deling en activiteit van T cellen te onderdrukken. Daarnaast konden beide typen MSC de differentiatie van monocytten naar dendritische cellen remmen. We vonden ook een verschil tussen de MSC uit beenmerg en uit vet, namelijk dat MSC die geïsoleerd waren uit vet een veel sterker remmend effect op T cellen en dendritische cellen lieten zien dan MSC uit beenmerg. Met andere woorden; om hetzelfde effect te bereiken waren minder vet-MSC nodig dan beenmerg-MSC. Deze potentere activiteit van vet-MSC was waarschijnlijk het resultaat van een hogere metabole activiteit. Ook de uitscheiding van de stoffen die verantwoordelijk zijn voor de onderdrukking van het immuunsysteem (immunomodulerende factoren) was hoger in vet-MSC vergeleken met beenmerg-MSC.

In hoofdstuk 4 en hoofdstuk 5 zijn we dieper ingegaan op de mechanismen die belangrijk zijn voor de immunomodulerende werking van MSC. We hebben onderzocht welke stoffen (cytokinen) belangrijk zijn voor de remming van de differentiatie van monocytten naar dendritische cellen onder invloed van MSC (hoofdstuk 4). De cytokinen IL-6 en IL-10 bleken betrokken bij dit proces en alleen IL-6 werd ook daadwerkelijk geproduceerd en uitgescheiden door MSC. IL-10 (een ontstekingsremmend cytokine) werd alleen geproduceerd door de monocytten en dit was sterk verhoogd na stimulatie van monocytten met MSC. Deze verhoging van IL-10 productie door monocytten was afhankelijk van de IL-6 die door de MSC werd uitgescheiden. De IL-10 producerende cellen, die ontstonden na differentiatie van monocytten naar dendritische cellen in de aanwezigheid van MSC, bleken veel minder goed in de activatie van T cellen dan dendritische cellen die waren ontstaan in de afwezigheid van MSC. MSC remmen dus niet alleen de differentiatie van monocytten naar dendritische cellen maar veroorzaken tegelijkertijd de differentiatie van monocytten naar IL-10 producerende cellen die een dempende werking hebben op het immuunsysteem.

Het stimuleren van de vorming van Tregs, die een dempende werking hebben op de afweerreactie, is een andere manier waarop MSC een modulerende werking zouden kunnen hebben op de immuunrespons. In hoofdstuk 5 hebben we gekeken naar de vorming van Tregs onder invloed van MSC. We concluderen dat het eiwit TGF- β , uitgescheiden door MSC, een belangrijke rol speelt in dit proces. In deze experimenten waren behalve T cellen ook monocytten aanwezig en deze bleken belangrijk te zijn voor de MSC-geïnduceerde vorming van Tregs. Onder invloed van MSC werd de productie van CCL18 door monocytten verhoogd en de aanwezigheid van deze CCL18-producerende monocytten bleek essentieel voor de vorming van Tregs in de kweek. Nadere bestudering van de monocytten liet zien dat monocytten onder invloed van MSC differentieerden naar M2-macrofagen.

De cruciale rol van monocytten voor de verschillende immunomodulerende

activiteiten van MSC is een belangrijk resultaat van de studies die staan beschreven in dit proefschrift. Het was al bekend dat voor de remming van T cel deling en activatie de aanwezigheid van monocysten belangrijk is en hier laten we zien dat ook voor de MSC-geïnduceerde vorming van Tregs monocysten van essentieel belang zijn. Onze hypothese is dat MSC monocysten stimuleren tot de vorming van regulatoire, ontstekingsremmende cellen en dat deze MSC-monocysten interactie een essentieel onderdeel is van de modulatie van de immuunrespons door MSC. De betrokkenheid van monocysten bij het therapeutische effect van MSC zou een verklaring kunnen zijn voor het systemische effect (in het hele lichaam) dat MSC kunnen hebben, terwijl MSC na toediening veelal in de longen blijven hangen. Het is niet ondenkbaar dat MSC in de longen bepaalde stoffen uitscheiden die effect hebben op de monocysten in de bloedcirculatie en op die manier hun invloed uitoefenen. Om dit mechanisme verder te ontrafelen is verder onderzoek noodzakelijk.

De studies beschreven in dit proefschrift zijn allemaal uitgevoerd *in vitro* (in een kweekschtaaltje) en het is nog niet duidelijk hoe relevant deze resultaten zijn voor wat er daadwerkelijk *in vivo* (in het lichaam) gebeurt. Om dit te onderzoeken is het belangrijk dat er preklinische (dier)modellen ontwikkeld worden om MSC therapie en de onderliggende mechanismen te onderzoeken. Daarnaast moeten er translationele vragen beantwoordt worden over het optimale tijdstip en de dosis van MSC toediening en of ze lokaal of systemisch zouden moeten worden toegediend.

De fundamentele studies naar de immunomodulerende effecten van MSC die beschreven staan in dit proefschrift leveren waardevolle kennis op voor verder onderzoek naar de toepassing van MSC als therapie voor immuungerelateerde ziekten.

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Curriculum Vitae

Sara Melief was born on July 4, 1982 in Amsterdam, The Netherlands. In 2000 she graduated from high school at the Revijs Lyceum in Doorn and started studying Biomedical Sciences at Utrecht University. In 2004 she started the research Master program "Experimental and Clinical Neuroscience" at Utrecht University. As part of this Master's program she was involved in a research project on temporal lobe epilepsy, under supervision of dr. Pierre de Graan at the Rudolf Magnus Institute for Neuroscience (Utrecht University). Subsequently, the author carried out a research project on the psychopharmacology of aggression under supervision of prof. Klaus Miczek at Tufts University, Boston, USA. Her Master's thesis was written under supervision of dr. Leonie Boven on migration of antigen-presenting cells in the brain of MS patients at the department of Immunology of the Erasmus University Rotterdam. In addition, the author completed the 6-months 'Management' profile within her Master's program at Utrecht University.

In 2007 Sara obtained her Master's degree and started her research on multipotent stromal cells at the department of Immunohematology and Blood Transfusion at the Leiden University Medical Center under supervision of dr. Helene Roelofs and prof. Wim Fibbe. From April 1, 2013, Sara is employed as post-doc researcher on immunomodulation in melanoma at the department of Clinical Oncology at the Leiden University Medical Center.

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