Mesenchymal Stem Cells in Organ Transplantation

Immunomodulatory properties of mesenchymal stem cells for application in organ transplantation

Meindert J. Crop

Mesenchymal Stem Cells in Organ Transplantation

Immunomodulatory properties of mesenchymal stem cells for application in organ transplantation

Meindert J. Crop

Cover: "Interaction between mesenchymal stem cells and immune cells"
Microscopical image of mesenchymal stem cells (MSC) cocultured with alloactivated immune cells. This image shows that mesenchymal stem cells bind activated immune cells and may thereby increase the efficacy of their immunosuppressive effects on these cells.

The research described in this thesis was performed at the Department of Internal Medicine, Erasmus University Medical Center, Rotterdam, The Netherlands.

The publication of this thesis was financially supported by:

Astellas Pharma B.V.
Baxter B.V.
BD Biosciences
Greiner Bio-One B.V.
Nederlandse Transplantatie Vereniging
Novartis B.V.
Pfizer B.V.
Roche Nederland B.V.
U-CyTech B.V.

Printed by HAVEKA, Alblasserdam

ISBN/EAN: 978-90-9025763-1

Mesenchymal Stem Cells in Organ Transplantation
Immunomodulatory properties of mesenchymal stem cells for application in organ transplantation

Mesenchymale Stamcellen in Orgaantransplantatie
Immuunmodulatoire eigenschappen van mesenchymale stamcellen voor toepassing in orgaan transplantatie

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. H.G. Schmidt
en volgens besluit van het College van Promoties

De openbare verdediging zal plaatsvinden op
woensdag 3 november 2010 om 15.30 uur

door

Meindert Johannes Crop

geboren te Delft

Promotiecommissie

Promotor:	Prof.dr. W. Weimar
Overige leden:	Prof.dr. W.E. Fibbe Prof.dr. J.N.M. IJzermans Prof.dr. R. Zietse
Copromotor:	Dr. M.J. Hoogduijn

Financial support by the The Dutch Transplantation Society for the publication of this thesis is gratefully acknowledged.

Stellingen behorende bij het proefschrift

Mesenchymal Stem Cells in Organ Transplantation

Immunomodulatory properties of mesenchymal stem cells
for application in organ transplantation

1. Autologous and allogeneic mesenchymal stem cells have comparable immunosuppressive efficacy *(dit proefschrift)*

2. Inflammatory conditions enhance the immunosuppressive effects of mesenchymal stem cells *(dit proefschrift)*

3. Mesenchymal stem cells are lysed by autologous natural killer cells *(dit proefschrift)*

4. Mesenchymal stem cells both activate and control T-cells *(dit proefschrift)*

5. Mycophenolate acid (MPA) does not hamper the immunosuppressive effects of mesenchymal stem cells *(dit proefschrift)*

6. De waarde van proefdier onderzoek wordt overschat.

7. Vetweefsel is een goede bron voor mesenchymale stamcellen

8. De waarde van vetweefsel wordt miskend.

9. Indien vetweefsel de standaard bron wordt voor stamcel therapie, krijgen diëtisten er een extra taak bij.

10. Je kunt een probleem niet oplossen vanuit hetzelfde soort denken dat tot het probleem heeft geleid (Albert Einstein)

11. A cappuccino a day, keeps the headache away

Meindert J. Crop
Rotterdam, 3 november 2010

Er bestaan geen feiten
alleen interpretaties
(*F.W. Nietzsche*)

List of abbreviations:

1-MT	=	1-methyl-L-tryptophan
APC	=	antigen presenting cell
ASC	=	adipose tissue-derived mesenchymal stem cells
CCL	=	chemokines of the C-X-C motif
CML	=	cytotoxicity-mediated kill
CTL	=	cytotoxic T lymphocyte
CXCL	=	ligands for chemokines of the C-X-C motif
ET ratio	=	effector-target ratio
FBS	=	foetal bovine serum
GBP	=	guanylate binding proteins
GVHD	=	graft-versus-host disease
HGF	=	hepatocyte growth factor (HGF)
HLA	=	human leukocyte antigen
HLA-G	=	histocompatibility locus antigen-G
HuS	=	human serum
IFN	=	interferon
ICAM	=	Inter-Cellular Adhesion Molecule
IL	=	interleukin
IMPDH	=	inosine monophosphate dehydrogenase
KTx	=	kidney transplantation
MHC	=	major histocompatibility antigens
MLR	=	mixed lymphocyte reaction
MMF	=	mycophenolate mofetil
MPA	=	mycophenolic acid
MSC	=	mesenchymal stem cells
mTOR	=	mammalian target of rapamycin
MTT	=	1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan
NFAT	=	nuclear factor of activated T-cells
NO	=	nitric oxide
PBMC	=	peripheral blood mononuclear cells
PG-E2	=	prostaglandin-E2 (PGE2)
PHA	=	phytohaemagglutinin
siRNA	=	small interfering RNA
TGF-β1	=	transforming growth factor-β1
TNF	=	tumour necrosis factor
T-regs	=	regulatory T-cells
VCAM	=	vascular cell adhesion molecule

Contents

Chapter 1

Introduction

based on:
Potential of mesenchymal stem cells as immune therapy
in solid organ transplantation

Meindert J. Crop, Carla C. Baan, Willem Weimar
and Martin J. Hoogduijn

Department of Internal Medicine,
Erasmus University Medical Center, Rotterdam, The Netherlands

Transplant International
2009 Apr;22(4):365-76

INTRODUCTION

Kidney transplantation is the only effective treatment for patients with end-stage renal disease. Transplantation of a donor organ, however, leads to recognition of the foreign donor antigens by the recipient's immune system, resulting in rejection of the graft. In addition, ischemia-reperfusion injury leads to the initiation of immune responses. To prevent graft rejection, transplant recipients need to use life-long immunosuppressive medication. These drugs, however, can lead to serious acute and chronic side effects, such as infections, nephrotoxicity, cardiovascular disease and malignancies [1]. As an alternative, cellular therapies may be considered. One of the candidates is the mesenchymal stem cell (MSC). Mesenchymal stem cells have tissue regenerative and immunosuppressive properties [2-4]. These properties make them promising as cellular therapy for tissue regeneration and treatment of immunological disease. In solid organ transplantation, MSC may repair graft injury and may reduce anti-donor reactivity, thereby improving graft function and alleviating the need for immunosuppressive drugs after transplantation. Despite recent clinical trials investigating the use of MSC in treating immune-mediated disease, such as therapy resistant graft-*versus*-host disease (GVHD) and inflammatory bowel disease, the applicability of MSC in solid organ transplantation is still unknown. The present thesis discusses the immunomodulatory properties of MSC and their potential to improve the outcome of solid organ transplantation.

Definition and characteristics of MSC

The definition of mesenchymal stem cells is not precisely defined and still under discussion. In brief, MSC are undifferentiated adult stem cells with self-renewal capacity. The term mesenchymal stem cell is often used interchangeably with the term mesenchymal *stromal* cells as 'stromal' refers to the supportive function of these cells to other cells, such as haematopoietic stem cells or specialized cells. It has been shown that MSC are virtually present in all adult tissues, including bone marrow, adipose tissue, skin, placenta and heart [2, 5-10], from which they can be isolated and expanded *in vitro.* Although bone marrow is the classical source of MSC for research and therapeutic purposes, currently other sources of MSC are investigated. In particular, adipose tissue is of interest as the collection of fat tissue is less invasive for the MSC donor, fat tissue is more easily accessible for the clinician and yields higher cell numbers than bone marrow [11-12]. Cultured MSC are usually identified by a combination of characteristics including their (1) morphology in culture; (2) immunophenotype; (3) differentiation capacity; and (4) immunosuppressive capacity.

MSC in culture are plastic-adherent cells, grow in a monolayer and have a spindle-shaped morphology (Figure 1). Until now, no specific cell surface marker for MSC has been found and MSC are, therefore, identified by a panel of cell-surface markers, including CD29, CD44, CD73 (SH3/4), CD90 (Thy-1), CD105 (SH2), CD106 (VCAM-1), CD166 (ALCAM) and HLA class I [2, 13-14]. In contrast, MSC do not express haematopoietic or endothelial lineage markers such as CD14, CD31 or CD45. In addition, it has been suggested that MSC have a low immunogenic phenotype, i.e. low expression of HLA class I, and absence of HLA class II and co-stimulatory molecules such as CD80 and CD86 [15-17]. This immunophenotype makes MSC potentially immune privileged.

Multilineage differentiation capacity of MSC

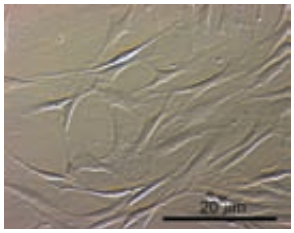


Figure 1. MSC in culture

The first important functional property of MSC is their multilineage differentiation capacity. MSC are progenitor cells for various specialized cell types, such as adipocytes, osteoblasts, chondrocytes and myocytes (Figure 2) [2, 5]. This capacity makes them promising candidates for use in regenerative medicine, for example for the repair of bone [18-19], cartilage [20-21], skin [22-23], and neuronal tissue [24]. In addition, MSC can improve the function of cardiac muscle [25-27] or kidney [28-29]. Currently, clinical trials are investigating the use of MSC in treating heart disease, liver cirrhosis and bone fractures. In addition, clinical application of bioengineered tissues using *in vitro*-differentiated stem cells cultured on scaffolds is promising, as shown for the production of a trachea [30]. Besides the hypothesis that MSC contribute to repair of damaged tissue via their differentiation into more specialized cells themselves, there is now increasing evidence that secretion of trophic factors by MSC that stimulate tissue resident cells is at least as important for the contribution of MSC to tissue regeneration [31-32].

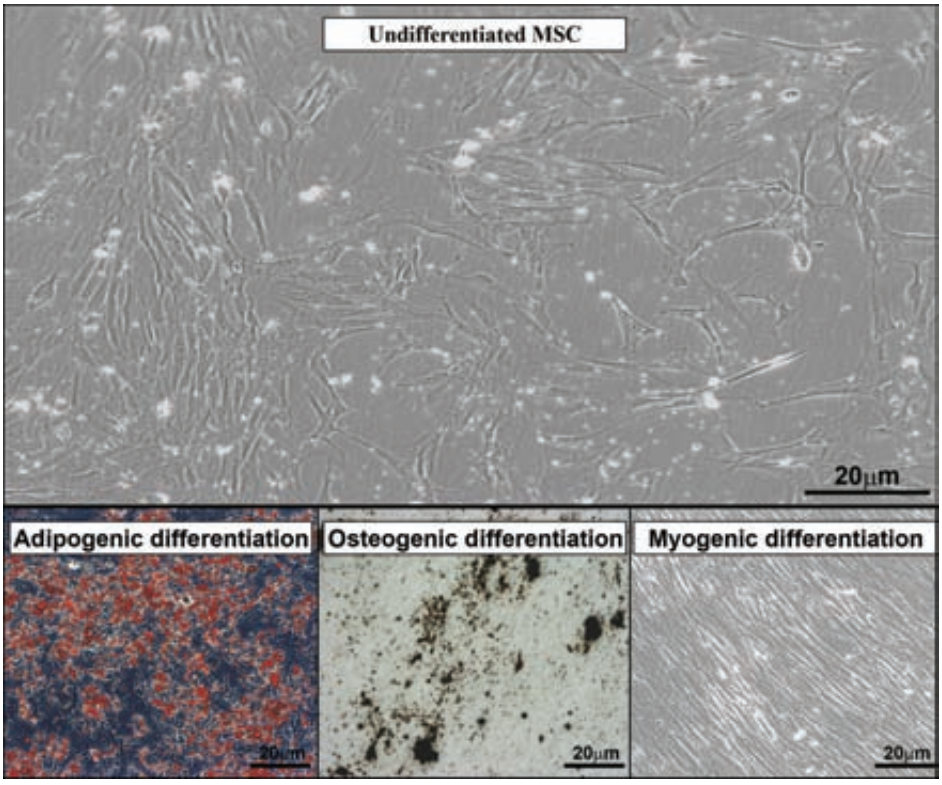


Figure 2. Multilineage differentiation capacity of MSC. MSC were cultured for 21 days under control, adipogenic, osteogenic and myogenic conditions. The figure shows undifferentiated MSC, MSC stained with oil-red-O to detected lipid vesicles after adipogenic differentiation, MSC stained by von Kossa staining to detect calcified minerals after osteogenic differentiation and MSC after induction of myogenic differentiation.

Immunosuppressive capacity of MSC *in vitro*

The second important functional property of MSC is their immunosuppressive capacity (Figure 3). MSC can inhibit the proliferation and production of pro-inflammatory cytokines by activated lymphocytes and maturation of dendritic cells *in vitro* [3-4]. Their effects are dose-dependent, appear to be independent of major histocompatibility complex (MHC) and do not require mediation by antigen-presenting cells (APC) or regulatory T-cells (Tregs) [33]. A variety of mechanisms for the immunosuppressive effects of MSC have been proposed. Inhibition of lymphocyte proliferation by MSC has not been associated with the induction of apoptosis, but is rather dependent on the inhibition of cell division (Figure 4) [4, 33-34].

Experiments using transwell systems showed that the inhibitory effect of MSC on PBMC proliferation was reduced, but not abolished, by the physical separation of MSC and PBMC [33, 35]. This indicates that the immunosuppressive effects are partly mediated by soluble factors. Several soluble factors have been suggested to be involved in the immunosuppressive effect of MSC, such as transforming growth factor-b1 (TGF-β1), hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), IL-10, HLA-G and nitric oxide [36-37]. Blocking each of these factors alone does not completely restore the proliferation of activated immune cells, indicating that multiple factors are involved. MSC also inhibit T-cell proliferation by up-regulating the expression of indoleamine 2,3-dioxygenase (IDO) [3-4, 38-40]. IDO is not constitutively expressed by MSC, but can be induced by exogenous factors such as IFN-γ, and catalyses the conversion of the amino acid tryptophan to kynurenine. Depletion of tryptophan, which is an essential amino acid for T-cells, and formation of the immunosuppressive factor kynurenine, results in inhibition of lymphocyte proliferation.

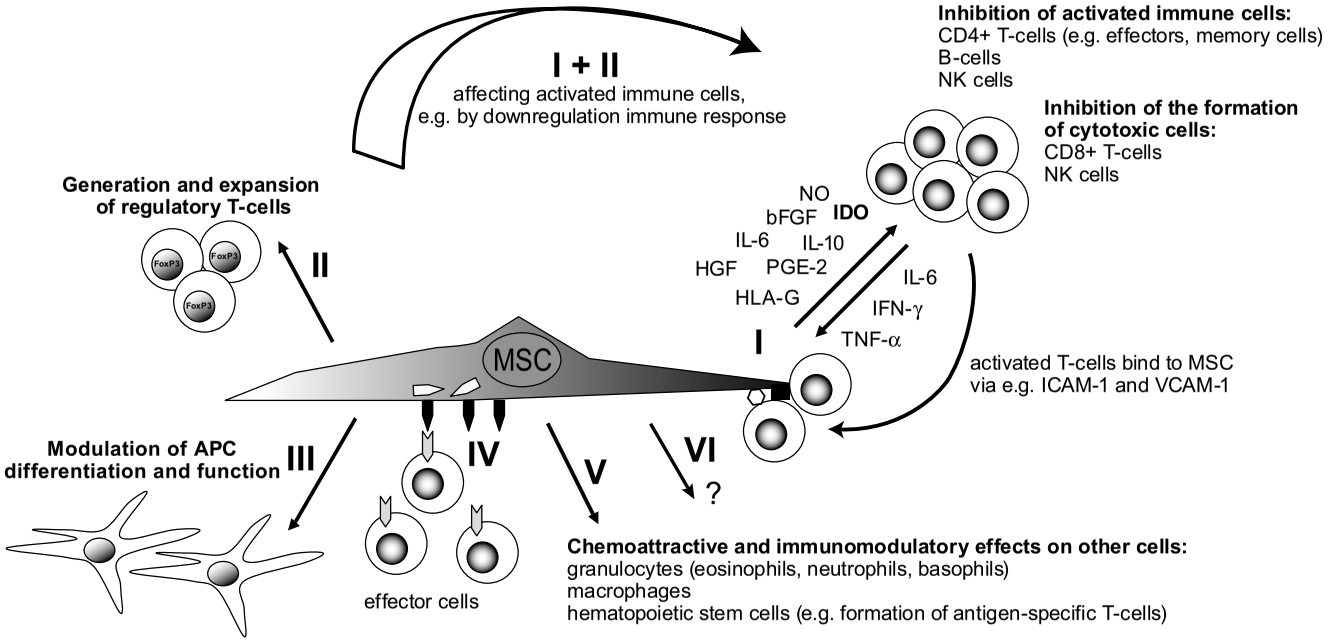
In addition to the secretion of soluble immunosuppressive factors, MSC establish cell-membrane interactions with immune cells, suggesting that non-soluble communication between MSC and immune cells exists [41-42]. MSC express a number of adhesion molecules, including ICAM-1, ICAM-2, VCAM-1, CD72, and LFA-3 that enable physical interaction with T-cells [42-44]. Under inflammatory conditions MSC up-regulate these adhesion molecules, which may increase the capacity of MSC to bind activated T-cells. By keeping activated T-cells in close proximity, the inhibitory effect of soluble mediators generated by MSC may be potentiated [36-37, 41].

Effect of MSC on immune cells

Many studies have demonstrated that MSC inhibit immune responses by affecting various leukocyte subsets. Below, we discuss their specific effects on T-cells, B cells, natural killer (NK) cells, and on other immune cells such as dendritic cells.

Interaction between MSC and T-cells

The effect of MSC on T-cells has been investigated in various experimental settings, showing that MSC modulate the activation, proliferation and function of effector and regulatory T-cell subsets. MSC have a dose-dependent effect on the proliferation and cytokine production (e.g. IL-2, IFN-γ) of alloactivated and mitogen-activated $CD4^+$ T-cells *in vitro* [4, 33, 45-46]. Although MSC strongly suppress the formation of cytotoxic $CD8^+$ T lymphocytes (CTL) in mixed lymphocyte cultures, MSC are not capable of inhibiting the cytolytic activity of activated CTL [47-49]. Conversely, it has been suggested that MSC might be relatively resistant to CTL-mediated lysis [48, 50], which may be explained by the low expression of HLA and co-stimulatory molecules on MSC. In addition to their effects on naive T-cell populations, MSC also inhibit the proliferation of memory T-cells activated by their cognate peptide [4, 33, 48, 51].



Figuur 3. Immunosuppressive effects of MSC

Legend: Immunomodulatory effects of MSC.
The immunomodulatory effects of MSC act on different components of the immune system:

I MSC can inhibit activated lymphocytes. These immunosuppressive effects are mediated by soluble factors and further enhanced by direct cell-cell membrane contact (e.g. via ICAM-I). The immunosuppressive capacity of MSC is enhanced under inflammatory conditions (e.g. by IFNγ)
II MSC can induce regulatory T-cells (Tregs), thereby MSC indirectly affect activated immune cells
III MSC can affect antigen presenting cell (APC) differentiation, maturation, migration and function
IV MSC can act as antigen presenting cells (APC) that are recognized by effector cells
V MSC can secrete chemokines for attraction and modulation of other (immune) cells.
VI (still) unknown mechanisms

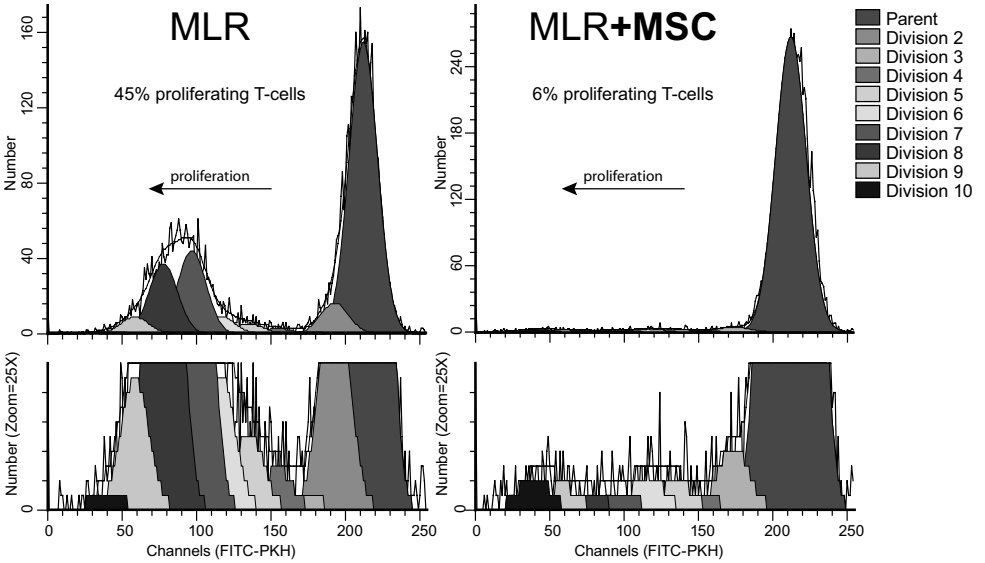


Figure 4. One of the mechanisms of immunosuppression by MSC is the induction of a cell division arrest of activated T-cells.This figure demonstrates that MSC inhibit the proliferation and number of cell divisions of PKH67-labeled responder T-cells after 7 days in mixed lymphocyte reactions (MLR). Analysis was performed by ModFit® software (Verity Software House, USA). Alloactivated T-cells proliferated up to 7-8 cell divisions in the absence of MSC, while alloactivated T-cells were kept in a cell division arrest in the presence of MSC.

In addition to the non-antigen-specific modulation of effector T-cells by MSC through the secretion of soluble factors and cell-membrane interactions, there is evidence that MSC indirectly modulate immune responses through the induction of Tregs. Some studies have reported that MSC increase the proportion of Tregs with strong suppressive capacities in the PBMC population [3, 52-55]. However, the mechanism whereby MSC induce Treg expansion is unclear, as is the specificity of the induced cells. If protocols could be developed whereby MSC were used to induce Tregs in an antigen-specific manner, this would provide MSC with the capacity to inhibit immune responses in an antigen-specific manner.

Interaction between MSC and B cells

The effects of MSC on B-cells have been clarified to a lesser extent than their effects on T-cells. Some studies have shown that MSC effectively inhibit B-cell proliferation, differentiation to plasma cells, and antibody production [56-58]. B-cell proliferation is inhibited by MSC through an arrest in the G0/G1 phase of the cell cycle, and not through apoptosis [56, 59]. On the other hand, some studies found that MSC stimulated polyclonal expansion and antibody production by B-cells [60-61]. Interestingly, it has been suggested that MSC may resemble follicular dendritic cell-like cells, which prevent B-cells from apoptosis and induce their differentiation [62]. This may explain the stimulatory effect of MSC on B cells. The concentration of MSC may be a decisive factor explaining such results: high doses of MSC have an inhibitory effect on B cells, whereas low doses stimulate B-cell activity. Furthermore, the absence or presence of T-cells in the MSC-B cell cultures is of importance as well. Activated T-cells produce IFN-γ that activate the immunosuppressive mechanisms of MSC, which subsequently resulting in an immunosuppressive effect of MSC on B-cells through mechanisms that resemble those used to modulate T-cell activity.

Interaction between MSC and natural killer cells
While MSC suppress IL-2- and IL-15-driven NK-cell proliferation and IFN-γ production [3, 48-49, 63], they do not inhibit the cytotoxic activity of activated NK-cells [48, 64]. The inhibition seems to be independent of cell-membrane contact, and PGE2 and TGF-β appear to play a role the regulation of NK-cell activity [65]. While MSC are not lysed by freshly isolated allogeneic NK-cells, they are susceptible for lysis by IL-15-activated NK-cells [48, 63, 66].

Interaction between MSC and antigen-presenting cells
In addition to directly affecting lymphocytes, MSC modulate immune responses via dendritic cells. MSC can reduce the formation and maturation of both monocyte-derived and $CD34^+$-derived dendritic cells [67-69]. MSC also reduce the expression of co-stimulatory molecules such as CD40, CD83 and CD86 on mature dendritic cells [70] and alter cytokine secretion by stimulating IL-10 [68], IL-12 [70] and TGF-β1 [4] production, while decreasing TNF-α secretion. It has been reported that MSC require activation through IL-1b production by $CD14^+$ monocytes to suppress alloreactive T-cells [35]. Thus, MSC modulate the activation, proliferation and function of dendritic cells, thereby indirectly regulating T- and B-cell activity.

Interaction between MSC and other immune cells
There is little data on the effects of MSC on other immune cells. MSC secrete soluble factors such as monocyte-chemo-attractant protein, macrophage-inflammatory protein, IFN-inducible protein-10 and IL-8, all of which may attract immune cells such as monocytes, macrophages
and neutrophils. An inflammatory environment may affect the secretion of these factors by MSC, thereby changing the attraction of different immune cells. The relevance of the production of these factors by MSC for the attraction and function of monocytes, macrophages and neutrophils is unknown.

Interaction between MSC and haematopoietic stem cells
It has previously been shown in animal and clinical studies that MSC promote haematopoietic engraftment [17, 71-73]. This is likely to relate to the important role of MSC in the haematopoietic niche, where they support haematopoietic stem cell survival and self-renewal [74-75]. It can thus be speculated that MSC have indirect effects on the formation of antigen-specific T-cells by their interference with haematopoietic development.

The differences found by various studies in the immunosuppressive mechanisms of MSC may reflect differences in experimental setups. The type of MSC response to an immune challenge is likely to be dictated by the type, magnitude and timing of this challenge. This should be taken into consideration when MSC are used for treatment of immunologic disease.

Immunosuppressive effects of MSC *in vivo*

Application of MSC for inflammatory disease

In line with the promising results on the immunosuppressive effects of MSC *in vitro*, animal studies demonstrated that MSC treatment can reduce inflammation in various models, such as rheumatoid arthritis, colitis, myocarditis and autoimmune encephalomyelitis [76-80]. In a clinical setting, the application of MSC is currently under investigation in patients with multiple sclerosis, inflammatory bowel disease and rheumatoid arthritis.

Application of MSC in bone marrow and solid organ transplantation

Both regenerative and immunosuppressive properties of MSC may also be beneficial in clinical bone marrow and solid organ transplantation. Furthermore, it has been demonstrated that the administration of MSC not only increased bone marrow engraftment after haematopoietic stem cell transplantation, but also that it reduced conventional therapy-resistant GVHD [71, 81-82]. Recently, clinical trials started to investigate the effect of MSC on GVHD on a larger and more controlled scale.

It can be hypothesized that the application of MSC in solid organ transplantation has the potential to reduces renal ischemia-reperfusion injury, prevent rejection and improve graft function. In a few attempts, the use of MSC in solid organ transplantation has been examined in animal transplant models. Under particular conditions, MSC were shown to prolong the survival of skin, cardiac and liver grafts in primate and rat models [83-86]. Other studies failed to confirm the beneficial effect of MSC on graft survival or showed that, when infused before transplantation, MSC of donor origin may actually accelerate heart and skin graft loss, possibly through HLA recognition and induction of a memory immune response [87-90]. Although it has been claimed that MSC are immune privileged due to their low immunogenic phenotype [91-92], these results indicate allogeneic MSC are recognized and cleared by the recipient's immune system. Clearance by cytotoxic immune cells may also explain why intravenously administered MSC can not be traced within a few days after transplantation [90, 93]. For application of MSC in solid organ transplantation, it is important to further investigate the immunogenicity and survival of administered MSC.

In conclusion, the immunosuppressive and regenerative capacities of MSC make them attractive candidates for numerous clinical applications. Until now, however, little is known of their effects in an end-stage renal disease or kidney transplantation setting. Therefore, the present thesis investigates the potential of MSC in organ transplantation, with special focus on their immunosuppressive effects.

AIM AND OUTLINE OF THE PRESENT THESIS

The aim of this thesis is to investigate the potential of MSC as an immunomodulatory agent to improve the outcome of clinical solid organ transplantation. While bone marrow is the classical source of MSC, the collection bone marrow is invasive. Therefore, alternative sources of MSC are investigated, such as placenta, umbilical cord blood and adipose tissue. In **chapter two** we demonstrate the isolation of MSC from perirenal adipose tissue of living kidney donors, which is a waste product during the kidney donation procedure. The general characteristics of these adipose tissue-derived MSC were compared to those of MSC isolated from bone marrow, spleen and heart tissue. Although the immunosuppressive properties of MSC have been investigated in various experimental settings, it is unknown whether MSC also exert these immunosuppressive effects in a clinical kidney transplantation setting. Thus, in **chapter three** the immunosuppressive effects of kidney donor-derived MSC on recipient anti-donor reactivity before and after transplantation were investigated *in vitro*. Furthermore, we compared the efficacy of autologous and allogeneic MSC to inhibit alloreactivity.

When MSC are applied as cellular therapy in clinical organ transplantation, it is likely that they will be used in combination of immunosuppressive drugs. In **chapter four** we therefore examined the effect of the immunosuppressive drugs tacrolimus, rapamycin and mycophenolic acid (MPA) on MSC functions.

Most studies investigated the effect of MSC on activated immune cells. The effects of inflammatory conditions on MSC are less known and are investigated in **chapter five**. MSC were exposed to two inflammatory conditions, i.e. (1) alloactivated immune cells (MLR) in transwell culture systems or (2) pro-inflammatory cytokines. We performed full gene expression analysis of the treated MSC and examined their immunophenotype and function.

We subsequently investigated how MSC affect non-activated/resting immune cells in **chapter six**. Although it has been claimed that MSC are not immunogenic, there is evidence from animal models that MSC can induce memory responses and that systemically infused MSC can not be traced after administration, which could be the result of recognition of MSC by the recipient's immune system. In **chapter seven** the susceptibility of MSC for lysis by cytotoxic $CD8^+$ T-cells and NK-cells was studied. In **chapter eight** the results presented in this thesis are summarized and discussed.

Finally, in **chapter nine** the properties of MSC in respect to several issues that could be important for clinical application of MSC in kidney transplantation are evaluated.

REFERENCES

1. Marcen R. Immunosuppressive drugs in kidney transplantation: impact on patient survival, and incidence of cardiovascular disease, malignancy and infection. Drugs. 2009 Nov 12;69(16):2227-43.

2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999 Apr 2;284(5411):143-7.

3. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005 Feb 15;105(4):1815-22.

4. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002 May 15;99(10):3838-43.

5. Hoogduijn MJ, Crop MJ, Peeters AM, Van Osch GJ, Balk AH, Ijzermans JN, et al. Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. Stem Cells Dev. 2007 Aug;16(4):597-604.

6. Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol. 2001 Sep;3(9):778-84.

7. da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci. 2006 Jun 1;119(Pt 11):2204-13.

8. In ‘t Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, et al. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells. 2004;22(7):1338-45.

9. Hoogduijn MJ, Crop MJ, Peeters AM, Korevaar SS, Eijken M, Drabbels JJ, et al. Donor-derived mesenchymal stem cells remain present and functional in the transplanted human heart. Am J Transplant. 2009 Jan;9(1):222-30.

10. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002 Dec;13(12):4279-95.

11. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells. 2006 May;24(5):1294-301.

12. Mizuno H. Adipose-derived stem cells for tissue repair and regeneration: ten years of research and a literature review. J Nippon Med Sch. 2009 Apr;76(2):56-66.

13. Mitchell JB, McIntosh K, Zvonic S, Garrett S, Floyd ZE, Kloster A, et al. Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. Stem Cells. 2006 Feb;24(2):376-85.

14. Conget PA, Minguell JJ. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. J Cell Physiol. 1999 Oct;181(1):67-73.

15. Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Alwayn IP, Weimar W, et al. Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients. Transplantation. 2009 Mar 27;87(6):896-906.

16. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells. 2006 Feb;24(2):386-98.

17. Le Blanc K, Ringden O. Immunobiology of human mesenchymal stem cells and future use in hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2005 May;11(5):321-34.

18. Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, et al. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med. 1999 Mar;5(3):309-13.

19. Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, et al. Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med. 2001 Feb 1;344(5):385-6.

20. Ando W, Tateishi K, Hart DA, Katakai D, Tanaka Y, Nakata K, et al. Cartilage repair using an in vitro generated scaffold-free tissue-engineered construct derived from porcine synovial mesenchymal stem cells. Biomaterials. 2007 Dec;28(36):5462-70.

21. Wakitani S, Mitsuoka T, Nakamura N, Toritsuka Y, Nakamura Y, Horibe S. Autologous bone marrow stromal cell transplantation for repair of full-thickness articular cartilage defects in human patellae: two case reports. Cell Transplant. 2004;13(5):595-600.

22. Rasulov MF, Vasilchenkov AV, Onishchenko NA, Krasheninnikov ME, Kravchenko VI, Gorshenin TL, et al. First experience of the use bone marrow mesenchymal stem cells for the treatment of a patient with deep skin burns. Bull Exp Biol Med. 2005 Jan;139(1):141-4.

23. Rasulov MF, Vasilenko VT, Zaidenov VA, Onishchenko NA. Cell transplantation inhibits inflammatory reaction and stimulates repair processes in burn wound. Bull Exp Biol Med. 2006 Jul;142(1):112-5.

24. Phinney DG, Isakova I. Plasticity and therapeutic potential of mesenchymal stem cells in the nervous system. Curr Pharm Des. 2005;11(10):1255-65.

25. Jiang W, Ma A, Wang T, Han K, Liu Y, Zhang Y, et al. Intravenous transplantation of mesenchymal stem cells improves cardiac performance after acute myocardial ischemia in female rats. Transpl Int. 2006 Jul;19(7):570-80.

26. Kocher AA, Schlechta B, Gasparovicova A, Wolner E, Bonaros N, Laufer G. Stem cells and cardiac regeneration. Transpl Int. 2007 Sep;20(9):731-46.

27. Chen SL, Fang WW, Ye F, Liu YH, Qian J, Shan SJ, et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol. 2004 Jul 1;94(1):92-5.

28. Kunter U, Rong S, Djuric Z, Boor P, Muller-Newen G, Yu D, et al. Transplanted mesenchymal stem cells accelerate glomerular healing in experimental glomerulonephritis. J Am Soc Nephrol. 2006 Aug;17(8):2202-12.

29. Morigi M, Imberti B, Zoja C, Corna D, Tomasoni S, Abbate M, et al. Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. J Am Soc Nephrol. 2004 Jul;15(7):1794-804.

30. Macchiarini P, Jungebluth P, Go T, Asnaghi MA, Rees LE, Cogan TA, et al. Clinical transplantation of a tissue-engineered airway. Lancet. 2008 Dec 13;372(9655):2023-30.

31. Bi B, Schmitt R, Israilova M, Nishio H, Cantley LG. Stromal cells protect against acute tubular injury via an endocrine effect. J Am Soc Nephrol. 2007 Sep;18(9):2486-96.

32. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006 Aug 1;98(5):1076-84.

33. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood. 2003 May 1;101(9):3722-9.

34. Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. Blood. 2005 Apr 1;105(7):2821-7.

35. Groh ME, Maitra B, Szekely E, Koc ON. Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. Exp Hematol. 2005 Aug;33(8):928-34.

36. Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, et al. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. Blood. 2007 Jan 1;109(1):228-34.

37. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell. 2008 Feb 7;2(2):141-50.

38. Batten P, Sarathchandra P, Antoniw JW, Tay SS, Lowdell MW, Taylor PM, et al. Human mesenchymal stem cells induce T cell anergy and downregulate T cell allo-responses via the TH2 pathway: relevance to tissue engineering human heart valves. Tissue Eng. 2006 Aug;12(8):2263-73.

39. Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood. 2004 Jun 15;103(12):4619-21.

40. Selmani Z, Naji A, Gaiffe E, Obert L, Tiberghien P, Rouas-Freiss N, et al. HLA-G is a crucial immunosuppressive molecule secreted by adult human mesenchymal stem cells. Transplantation. 2009 May 15;87(9 Suppl):S62-6.

41. Suva D, Passweg J, Arnaudeau S, Hoffmeyer P, Kindler V. In vitro activated human T lymphocytes very efficiently attach to allogenic multipotent mesenchymal stromal cells and transmigrate under them. J Cell Physiol. 2008 March;214(3):588-94.

42. Quaedackers ME, Baan CC, Weimar W, Hoogduijn MJ. Cell contact interaction between adipose-derived stromal cells and allo-activated T lymphocytes. Eur J Immunol. 2009 Dec;39(12):3436-46.

43. Ren G, Zhao X, Zhang L, Zhang J, L'Huillier A, Ling W, et al. Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. J Immunol. 2010 Mar 1;184(5):2321-8.

44. Majumdar MK, Keane-Moore M, Buyaner D, Hardy WB, Moorman MA, McIntosh KR, et al. Characterization and functionality of cell surface molecules on human mesenchymal stem cells. J Biomed Sci. 2003 Mar-Apr;10(2):228-41.

45. Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, et al. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci. 2005;12(1):47-57.

46. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation. 2003 Feb 15;75(3):389-97.

47. Angoulvant D, Clerc A, Benchalal S, Galambrun C, Farre A, Bertrand Y, et al. Human mesenchymal stem cells suppress induction of cytotoxic response to alloantigens. Biorheology. 2004;41(3-4):469-76.

48. Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation. 2003 Oct 27;76(8):1208-13.

49. Spaggiari GM, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood. 2008 Feb 1;111(3):1327-33.

50. Morandi F, Raffaghello L, Bianchi G, Meloni F, Salis A, Millo E, et al. Immunogenicity of human mesenchymal stem cells in HLA-class I-restricted T-cell responses against viral or tumor-associated antigens. Stem Cells. 2008 May;26(5):1275-87.

51. Potian JA, Aviv H, Ponzio NM, Harrison JS, Rameshwar P. Veto-like activity of mesenchymal stem cells: functional discrimination between cellular responses to alloantigens and recall antigens. J Immunol. 2003 Oct 1;171(7):3426-34.

52. Di Ianni M, Del Papa B, De Ioanni M, Moretti L, Bonifacio E, Cecchini D, et al. Mesenchymal cells recruit and regulate T regulatory cells. Exp Hematol. 2008 Mar;36(3):309-18.

53. Maccario R, Podesta M, Moretta A, Cometa A, Comoli P, Montagna D, et al. Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of $CD4^+$ T-cell subsets expressing a regulatory/suppressive phenotype. Haematologica. 2005 Apr;90(4):516-25.

54. Prevosto C, Zancolli M, Canevali P, Zocchi MR, Poggi A. Generation of $CD4^+$ or $CD8^+$ regulatory T cells upon mesenchymal stem cell-lymphocyte interaction. Haematologica. 2007 Jul;92(7):881-8.

55. Casiraghi F, Azzollini N, Cassis P, Imberti B, Morigi M, Cugini D, et al. Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells. J Immunol. 2008 Sep 15;181(6):3933-46.

56. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human mesenchymal stem cells modulate B-cell functions. Blood. 2006 Jan 1;107(1):367-72.

57. Comoli P, Ginevri F, Maccario R, Avanzini MA, Marconi M, Groff A, et al. Human mesenchymal stem cells inhibit antibody production induced in vitro by allostimulation. Nephrol Dial Transplant. 2008 Apr;23(4):1196-202.

58. Asari S, Itakura S, Ferreri K, Liu CP, Kuroda Y, Kandeel F, et al. Mesenchymal stem cells suppress B-cell terminal differentiation. Exp Hematol. 2009 May;37(5):604-15.

59. Tabera S, Perez-Simon JA, Diez-Campelo M, Sanchez-Abarca LI, Blanco B, Lopez A, et al. The effect of mesenchymal stem cells on the viability, proliferation and differentiation of B-lymphocytes. Haematologica. 2008 Jul 18;93(9):1301-9.

60. Rasmusson I, Le Blanc K, Sundberg B, Ringden O. Mesenchymal stem cells stimulate antibody secretion in human B cells. Scand J Immunol. 2007 Apr;65(4):336-43.

61. Traggiai E, Volpi S, Schena F, Gattorno M, Ferlito F, Moretta L, et al. Bone marrow-derived mesenchymal stem cells induce both polyclonal expansion and differentiation of B cells isolated from healthy donors and systemic lupus erythematosus patients. Stem Cells. 2008 Feb;26(2):562-9.

62. Munoz-Fernandez R, Blanco FJ, Frecha C, Martin F, Kimatrai M, Abadia-Molina AC, et al. Follicular dendritic cells are related to bone marrow stromal cell progenitors and to myofibroblasts. J Immunol. 2006 Jul 1;177(1):280-9.

63. Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells. 2006 Jan;24(1):74-85.

64. Maccario R, Moretta A, Cometa A, Montagna D, Comoli P, Locatelli F, et al. Human mesenchymal stem cells and cyclosporin a exert a synergistic suppressive effect on in vitro activation of alloantigen-specific cytotoxic lymphocytes. Biol Blood Marrow Transplant. 2005 Dec;11(12):1031-2.

65. Malygin AM, Meri S, Timonen T. Regulation of natural killer cell activity by transforming growth factor-beta and prostaglandin E2. Scand J Immunol. 1993 Jan;37(1):71-6.

66. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood. 2006 Feb 15;107(4):1484-90.

67. Nauta AJ, Kruisselbrink AB, Lurvink E, Willemze R, Fibbe WE. Mesenchymal stem cells inhibit generation and function of both $CD34^{+}$-derived and monocyte-derived dendritic cells. J Immunol. 2006 Aug 15;177(4):2080-7.

68. Jiang XX, Zhang Y, Liu B, Zhang SX, Wu Y, Yu XD, et al. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. Blood. 2005 May 15;105(10):4120-6.

69. Djouad F, Charbonnier LM, Bouffi C, Louis-Plence P, Bony C, Apparailly F, et al. Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. Stem Cells. 2007 Aug;25(8):2025-32.

70. Zhang W, Ge W, Li C, You S, Liao L, Han Q, et al. Effects of mesenchymal stem cells on differentiation, maturation, and function of human monocyte-derived dendritic cells. Stem Cells Dev. 2004 Jun;13(3):263-71.

71. Lazarus HM, Koc ON, Devine SM, Curtin P, Maziarz RT, Holland HK, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant. 2005 May;11(5):389-98.

72. Maitra B, Szekely E, Gjini K, Laughlin MJ, Dennis J, Haynesworth SE, et al. Human mesenchymal stem cells support unrelated donor hematopoietic stem cells and suppress T-cell activation. Bone Marrow Transplant. 2004 Mar;33(6):597-604.

73. Almeida-Porada G, Porada CD, Tran N, Zanjani ED. Cotransplantation of human stromal cell progenitors into preimmune fetal sheep results in early appearance of human donor cells in circulation and boosts cell levels in bone marrow at later time points after transplantation. Blood. 2000 Jun 1;95(11):3620-7.

74. Valtieri M, Sorrentino A. The mesenchymal stromal cell contribution to homeostasis. J Cell Physiol. 2008 Jul 9.

75. Benvenuto F, Ferrari S, Gerdoni E, Gualandi F, Frassoni F, Pistoia V, et al. Human mesenchymal stem cells promote survival of T cells in a quiescent state. Stem Cells. 2007 Jul;25(7):1753-60.

76. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. Gastroenterology. 2009 Mar;136(3):978-89.

77. Gonzalez-Rey E, Gonzalez MA, Rico L, Buscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut. 2009 Jan 9.

78. Ohnishi S, Yanagawa B, Tanaka K, Miyahara Y, Obata H, Kataoka M, et al. Transplantation of mesenchymal stem cells attenuates myocardial injury and dysfunction in a rat model of acute myocarditis. J Mol Cell Cardiol. 2007 Jan;42(1):88-97.

79. Constantin G, Marconi S, Rossi B, Angiari S, Calderan L, Anghileri E, et al. Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis. Stem Cells. 2009 Oct;27(10):2624-35.

80. Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, et al. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood. 2005 Sep 1;106(5):1755-61.

81. Le Blanc K, Samuelsson H, Gustafsson B, Remberger M, Sundberg B, Arvidson J, et al. Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. Leukemia. 2007 Aug;21(8):1733-8.
82. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet. 2008 May 10;371(9624):1579-86.
83. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol. 2002 Jan;30(1):42-8.
84. Ge W, Jiang J, Baroja ML, Arp J, Zassoko R, Liu W, et al. Infusion of mesenchymal stem cells and rapamycin synergize to attenuate alloimmune responses and promote cardiac allograft tolerance. Am J Transplant. 2009 Aug;9(8):1760-72.
85. Popp FC, Eggenhofer E, Renner P, Slowik P, Lang SA, Kaspar H, et al. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. Transpl Immunol. 2008 Nov;20(1-2):55-60.
86. Wan CD, Cheng R, Wang HB, Liu T. Immunomodulatory effects of mesenchymal stem cells derived from adipose tissues in a rat orthotopic liver transplantation model. Hepatobiliary Pancreat Dis Int. 2008 Feb;7(1):29-33.
87. Inoue S, Popp FC, Koehl GE, Piso P, Schlitt HJ, Geissler EK, et al. Immunomodulatory effects of mesenchymal stem cells in a rat organ transplant model. Transplantation. 2006 Jun 15;81(11):1589-95.
88. Sbano P, Cuccia A, Mazzanti B, Urbani S, Giusti B, Lapini I, et al. Use of donor bone marrow mesenchymal stem cells for treatment of skin allograft rejection in a preclinical rat model. Arch Dermatol Res. 2008 Feb 8;300(3):115-24.
89. Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a non-myeloablative setting. Blood. 2006 May 11;108(6):2114-20.
90. Eliopoulos N, Stagg J, Lejeune L, Pommey S, Galipeau J. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. Blood. 2005 Dec 15;106(13):4057-65.
91. Rasmusson I, Uhlin M, Le Blanc K, Levitsky V. Mesenchymal stem cells fail to trigger effector functions of cytotoxic T lymphocytes. J Leukoc Biol. 2007 Oct;82(4):887-93.
92. Mammolenti M, Gajavelli S, Tsoulfas P, Levy R. Absence of major histocompatibility complex class I on neural stem cells does not permit natural killer cell killing and prevents recognition by alloreactive cytotoxic T lymphocytes in vitro. Stem Cells. 2004;22(6):1101-10.
93. Beggs KJ, Lyubimov A, Borneman JN, Bartholomew A, Moseley A, Dodds R, et al. Immunologic consequences of multiple, high-dose administration of allogeneic mesenchymal stem cells to baboons. Cell Transplant. 2006;15(8-9):711-21.

Chapter 2

Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities

Martin J. Hoogduijn[1], Meindert J. Crop[1], Annemiek M.A. Peeters[1], Gerjo J.V.M. van Osch[2], Aggie H.M.M. Balk[3], Jan N.M. IJzermans[4], Willem Weimar[1], Carla C. Baan[1]

[1]Departments of Internal Medicine, [2]Orthopaedics & Otorhinolaryngology,
[3]Cardiology, and [4]Surgery,
Erasmus University Medical Center, Rotterdam, the Netherlands

Stem Cells and Development
2007 Aug;16(4):597-604

ABSTRACT

Mesenchymal stem cells (MSC) have important tissue repair functions and show potent immunosuppressive capacities *in vitro*. Although usually isolated from the bone marrow, MSC have been identified in other tissues, including the skin and liver. In the present study, we isolated and characterized MSC from human heart, spleen, and perirenal adipose tissue. MSC from these different tissue sites were similar to those derived from bone marrow in that they expressed comparable levels of the cell-surface markers CD90, CD105, CD166 and HLA class I, were negative for CD34, CD45, HLA class II, CD80, and CD86 expression, and were capable of osteogenic and adipogenic differentiation. Like bone marrow-derived MSC, MSC from these different tissue sources inhibited the proliferation of alloactivated peripheral blood mononuclear cells (PBMC), giving 85%, 79%, 79% and 81% inhibition, respectively. Also in line with bone marrow-derived MSC they inhibited proliferative responses of PBMC to phytohaemagglutinin, a non-specific stimulator of lymphocyte proliferation, and reduced memory T lymphocyte responses to tetanus toxoid. The results of this study demonstrate that MSC from various tissues have similar immunophenotypes, *in vitro* immunosuppressive properties, and differentiation potential.

INTRODUCTION

Mesenchymal stem cells (MSC) constitute a low percentage (0.01-0.001%) of low density bone marrow cells [1-2]. Their descendents create at least some of the bone marrow stroma, which provides a supportive environment for haematopoietic stem cell (HSC) proliferation, maturation and homing [3]. Also, and not different from bone marrow stroma generation, upon exposure to the appropriate stimuli, they give rise to a number of mesenchymal lineages, including osteoblasts, adipocytes, chondrocytes, and myocytes, thus providing, when needed, a tissue repair function [2, 4]. In addition there is evidence for an immune regulatory function of MSC. In vitro, MSC have the capacity to inhibit mixed lymphocyte reactions [5-7], probably via mechanisms that are independent of MHC [8] but may involve soluble factors such as transforming growth factor-β1 (TGF-β1) [9] and cell-membrane contact with T lymphocytes [10]. The immunosuppressive properties of MSC have been further evidenced by *in vivo* administration. Skin graft survival in mice was prolonged [11] and graft-*versus*-host disease in humans after bone marrow transplantation was reduced [12].

Although generally considered as bone marrow-residing cells, there is accumulating evidence for a widespread distribution of MSC. Systemic infusion of MSC leads to homing to a wide range of tissues that include kidney, lung, liver, thymus and skin [13]. Recently, MSC have been isolated from peripheral blood [14], and it was shown that their numbers in peripheral blood increase in response to hypoxic stress [15]. However, the biological significance of systemic migration of MSC is unclear. MSC have been isolated from adipose tissue [16], dermis [17], and hair follicles [18], where they may reside from early developmental stages. Recently, a study in mice demonstrated that MSC reside in virtually all organs and tissues [19]. In the present study, we have isolated and expanded MSC from human heart, spleen, and perirenal adipose tissue and compared their immunophenotype, differentiation, and immune regulatory capacity with bone marrow-derived MSC.

MATERIALS AND METHODS

Isolation and culture of human MSC

Heart:

Atrium tissue that is routinely removed from donor hearts during heart transplantation surgery was collected as approved by the medical ethical committee of the Erasmus Medical Center (protocol no. MEC-2006-190) and used for the isolation of MSC. Atrium tissue from 3 hearts was stored in RPMI medium (Invitrogen, UK), minced within 24 hours with a scalpel knife, and digested with sterile filtered 0.5 mg/ml collagenase type IV (Sigma-Aldrich, Germany) in RPMI for 30 min at 37°C under continuous stirring. After two washes in RPMI, the dissociated tissue and cells were transferred to a culture flask and cultured in minimal essential medium-α (MEM-α) (Invitrogen, UK) supplemented with 15% foetal bovine serum (FBS; Biowhittacker, Belgium) and 100 U/ml penicillin and 100 µg/ml streptomycin (1% p/s; Invitrogen, UK). After 3-4 days, the culture medium was refreshed and non-adherent cells removed. Subsequently, medium was refreshed twice weekly and cells trypsinized at subconfluency using 0.05% trypsin-EDTA. Cells were reseeded at 1000 cells/cm^2 to ensure optimal proliferation and used for experiments between passage 2 and 5.

Spleen:
Spleen tissue was obtained from three post-mortem kidney or heart donors and stored in RPMI medium at 4°C. Not more than 48 hours later, the spleen was mashed and splenocytes were washed through a sieve. The residual connective tissue, including the spleen capsule, was then minced with a scalpel knife, incubated with sterile filtered 0.5 mg/ml collagenase type IV in RPMI for 30 min at 37°C under continuous stirring, and treated as described above for the heart tissue.
Perirenal fat tissue:
Adipose tissue surrounding the kidney was removed from three kidney transplant donors during the kidney donation procedure after written informed consent (protocol no. MEC-2006-190). The tissue was mechanically disrupted with a scalpel knife and, after two washes with phosphate-buffered saline (PBS), digested with sterile filtered 0.5 mg/ml collagenase type IV in RPMI for 30 min at 37°C. Medium with serum was then added and the cells and tissue pelleted by centrifugation at 1200 x *g* for 10 min. The pellet was then resuspended in 160 mM NH_4Cl and incubated for 10 min at room temperature to lyse contaminating red blood cells. Subsequently, the cells were centrifuged for 10 min at 1200 x *g*, resuspended in MEM-α with 15% FBS and 1% p/s, filtered through a 100-µm cell strainer, and transferred to culture flasks. The cultures were maintained as described above.
Bone marrow:
Bone marrow was obtained from the femoral shaft or iliac crest of patients undergoing hip replacement treatment after written informed consent (protocol no. MEC-2004-142). MSC were isolated from heparinized femoral shaft marrow aspirate by plating out, and non-adherent cells were removed after 24 hours. Adherent cells were initially cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, The Netherlands), supplemented with 10% FBS, 50 µg/ml gentamycin (Life Technologies), 1.5 µg/ml fungizone (Life Technologies), 1 ng/ml fibroblast growth factor-2 (FGF-2; Instruchemie, the Netherlands), and freshly prepared 25 µg/ml L-ascorbic acid-2-phosphate. At the third passage, the cells were switched to MEM-α supplemented with 1% p/s and 15% FBS and maintained as described above.

Flow cytometry
MSC were removed from culture flasks by incubation in PBS with 5 mM EDTA and 0.2% bovine serum albumin (BSA) at 37ºC. Cell suspensions were incubated in the above buffer with fluorescent-labelled antibodies against CD90, CD105 (R&D systems, UK), CD14, CD34, CD45, HLA class II (BD, USA), CD80, HLA class I (Serotec, UK), CD86 and CD166 (BD Pharmingen, USA) on ice protected from light for 30 min. Isotype control antibodies were used to determine non-specific staining. After two washes in PBS with 5 mM EDTA and 0.2% BSA, flow cytometric analysis was carried out using a four-colour FACSCalibur with Cell Quest Pro software (Becton Dickinson, USA).

Differentiation assays
Osteogenic differentiation
Osteogenic differentiation was induced by culturing confluent MSC cultures in MEM-αsupplemented with 1% p/s, 15% heat-inactivated FBS (56°C, 30 min), 5 mM β-glycerophosphate (Sigma-Aldrich, Germany), 50µg/ml L-ascorbic acid-phosphate (Sigma-Aldrich, Germany), and 10 nM dexamethasone (Sigma-Aldrich, Germany) for 14 days. The presence of calcified nodules, a marker for osteogenic differentiation, was detected by von Kossa staining. Cells were washed with PBS, fixed in cold 4% paraformaldehyde for 5 min, washed once with PBS and twice with distilled water, and incubated in

1% silver nitrate in water on a light box until blackening occurred. The cells were then washed three times with water, incubated with 2.5% sodium thiosulfate in water for 5 min, washed two times with water, and photographed.

Adipogenic differentiation

Adipogenic differentiation was induced by culturing confluent MSC cultures in MEM-α supplemented with 1% p/s, 15% heat-inactivated FBS, 50 µg/ml L-ascorbic acid-phosphate, 500 mM 3-isobutyl-1-methylxanthine (IBMX; Fluka, Germany), and 60 mM indomethacin (Fluka, Germany) for 14 days. The presence of lipid-filled vesicles was detected by oil-red-O staining. Cells were washed with PBS, fixed in 60% isopropanol for 1 min and incubated with filtered 0.3% oil-red-O (Sigma-Aldrich, Germany) in 60% isopropanol for 30 min. After three washes in PBS, the cells were photographed.

Effect of MSC on proliferation of peripheral blood mononuclear cells

Mixed lymphocyte reaction

Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors from the blood bank (Sanquin, Rotterdam). PBMC ($5x10^4$) were seeded in round-bottomed, tissue culture-treated 96-well plates in RPMI medium with 1% p/s and 10% heat-inactivated FBS and stimulated with $5x10^4$ HLA class 1 and 2 mismatched γ-irradiated (40 Gy) PBMC for 7 days in the presence or absence of $1x10^4$ third-party MSC isolated from heart, spleen, adipose tissue, or bone marrow. After 7 days, proliferation was measured by incorporation of [^{3}H]-thymidine (0.5 µCi/well) during 8 hours of incubation.

Response to phytohaemagglutinin (PHA)

PBMC ($5x10^4$) were cultured for 3 days in RPMI with 1% p/s, 10% heat-inactivated FBS, and 1 µg/ml PHA (Murex Biotech, UK) in the presence or absence of $1x10^4$ MSC, and proliferation measured.

Response to Tetanus toxoid

PBMC ($5x10^4$) were cultured for 3 days in RPMI with 1% p/s, 10% heat-inactivated FBS and 7.5 limits of flocculation (lf)/well tetanus toxoid (Tet; RIVM, Netherlands) in the presence or absence of $1x10^4$ MSC and proliferation measured.

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using GraphPad Prism software (GraphPad software, USA). p-values < 0.05 were considered significant.

RESULTS

Isolation and growth of fibroblastic cells from different human tissues

Isolated cells and small pieces of tissue from heart, spleen, and perirenal adipose tissue were transferred to culture flasks. After 3 to 10 days, cells with fibroblast morphology appeared, originating from small pieces of connective tissue (Figure 1A). The number of these cells rapidly increased (Figures 1B and C). The cells from heart, spleen, and adipose tissue were similar in morphology and indistinguishable from cultured bone marrow-derived MSC (Figure 1D). Figure 2 shows the collection, isolation and culture procedure of MSC from perirenal adipose tissue. To investigate whether the isolated cells had MSC characteristics, their cell-surface protein expression, differentiation capacity, and immune modulating abilities were examined.

Flow cytometric characterization of MSC

The immunophenotype of MSC isolated from heart, spleen, and adipose tissue and cultured for two to four passages was characterized by the expression of the cell-surface markers CD90, CD105, CD166, and HLA class I, and the absence of the haematopoietic marker CD34, the leucocyte marker CD45, HLA class II, and the co-stimulatory molecules CD80 and CD86 (Figure 3). MSC that were isolated from the bone marrow showed the same expression profile, and for most markers there were minimal quantitative differences in expression levels of the various markers among MSC from different sources. There were larger differences in the expression of HLA class I. These differences were, however, not significant because there was considerable variation in HLA I expression within the groups. HLA I expression in three cultures of heart MSC was 35%, 54%, and 56%, in spleen MSC 30%, 62%, and 97%, in adipose tissue MSC 81% and 85%, and in bone marrow MSC 98%, 98%, and 25%. In some early-passage cultures of spleen-derived cells, a subset of $CD14^+$ cells was detected, indicative of monocytes, but $CD14^+$ cells disappeared with ongoing passages (result not shown).

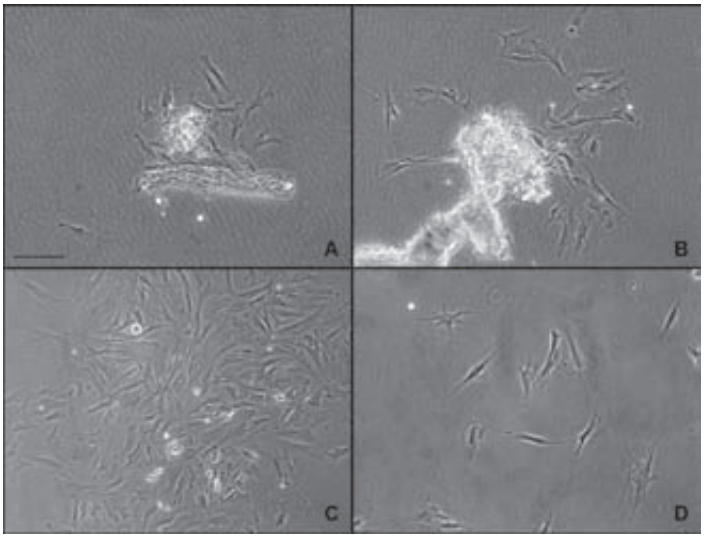


Figure 1. Bright-field microscopic images of cells with fibroblast morphology appearing in spleen cultures at day 4 [A]; day 6 [B] and day 10 [C]. [D] bone marrow-derived MSC.Bar represents 20 μm.

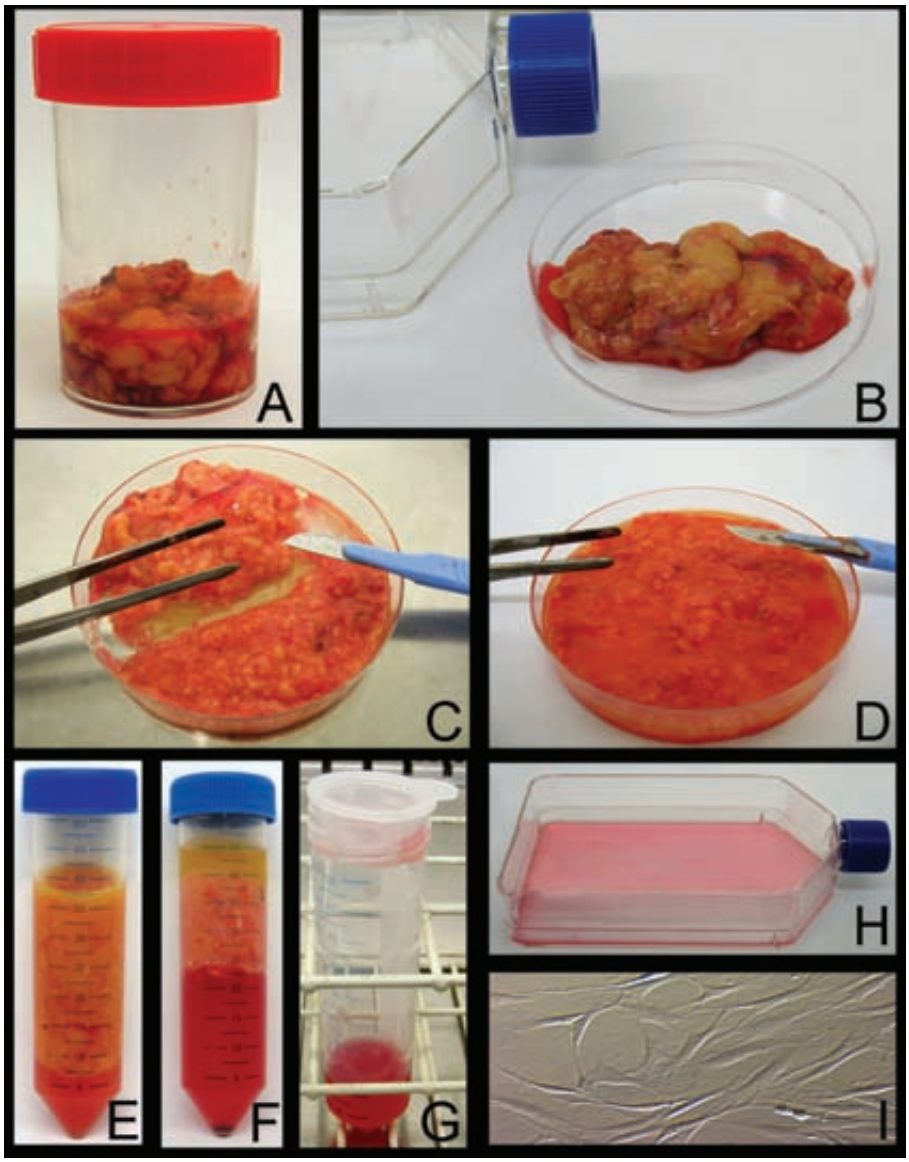


Figure 2. Collection, isolation and culture procedure of MSC from perirenal adipose tissue. [A] During the kidney donation procedure, perirenal adipose tissue was collected in the operation room and transported to the transplantation laboratory. [B-D] Under sterile conditions, perirenal fat was mechanically disrupted. [E] The tissue was then enzymatically digested with sterile 0.5 mg/mL collagenase type IV for 30 minutes at 37oC. [F] Next, the tissue was centrifuged and [G] resuspended in 160mM NH_4Cl in phosphate-buffered saline (PBS) for 10 minutes at room temperature to lyse contaminating red blood cells and filtered. [H] After centrifugation, cells were resuspended in MSC-culture medium and transferred to a culture flask and kept at 37oC, 5% CO_2 and 95% humidity. [I] After several days adherent cells with a spindle-shaped morphology appeared.

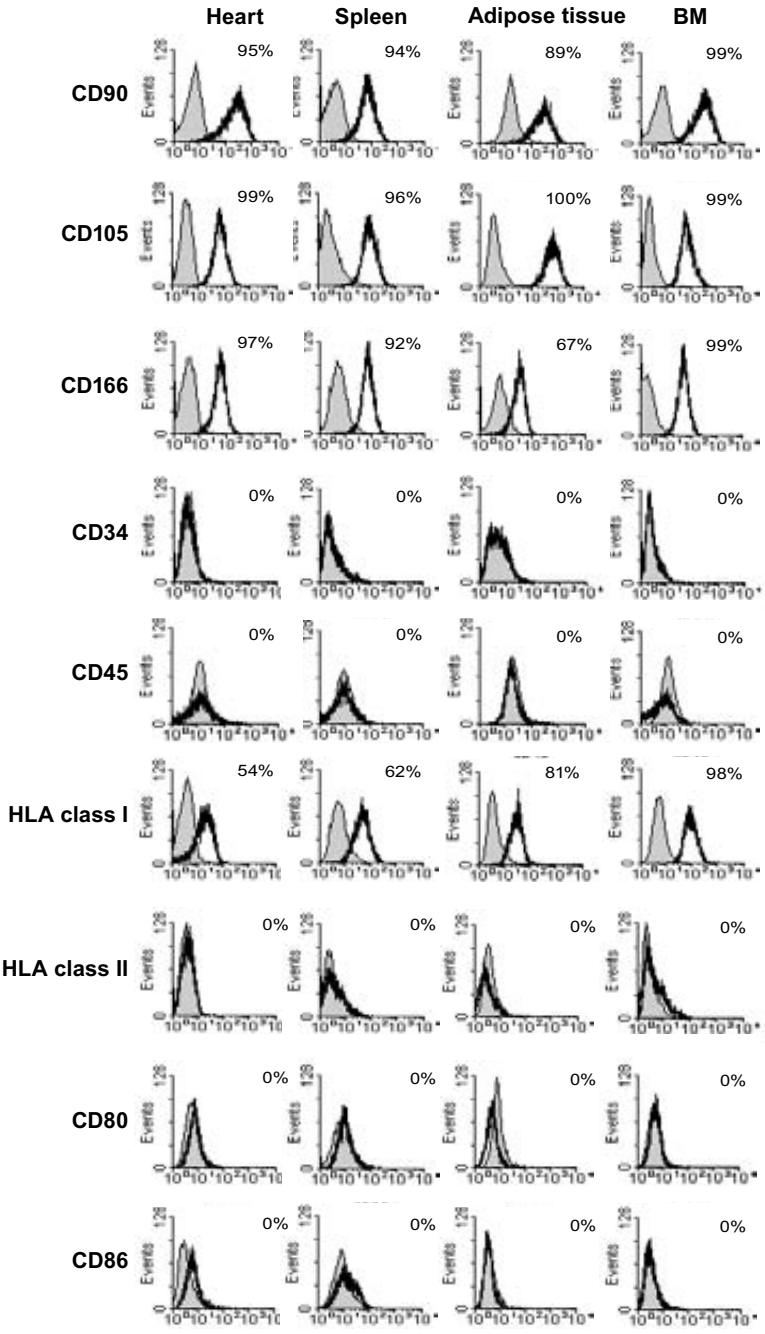


Figure 3. Flow cytometric analysis of the cell surface proteins CD90, CD105, CD166, CD34, CD45, HLA class I and II, CD80 and CD86 on the cells isolated from heart, spleen, adipose tissue and bone marrow. Solid histograms show non-specific staining, open histograms show specific staining for the indicated marker. Experiment carried out three times, median values shown.

Osteogenic and adipogenic differentiation

Culturing of heart, spleen, and adipose tissue-derived MSC in osteogenic medium for 14 days resulted in their differentiation into the osteogenic lineage, which was characterized by the deposition of calcified nodules that stained black by von Kossa staining (Figure 4A-C). Bone marrow-derived MSC developed similar nodules after induction of osteogenic differentiation (Figure 4D).

Induction of adipogenic differentiation of MSC derived from adipose tissue and bone marrow resulted in the development of lipid-filled vesicles, which were stained by oil-red-O after 14 days of culture (Figure 4E-H). Lipid vesicles were smaller in MSC cultures from heart and spleen tissue, which is indicative of an earlier differentiation state.

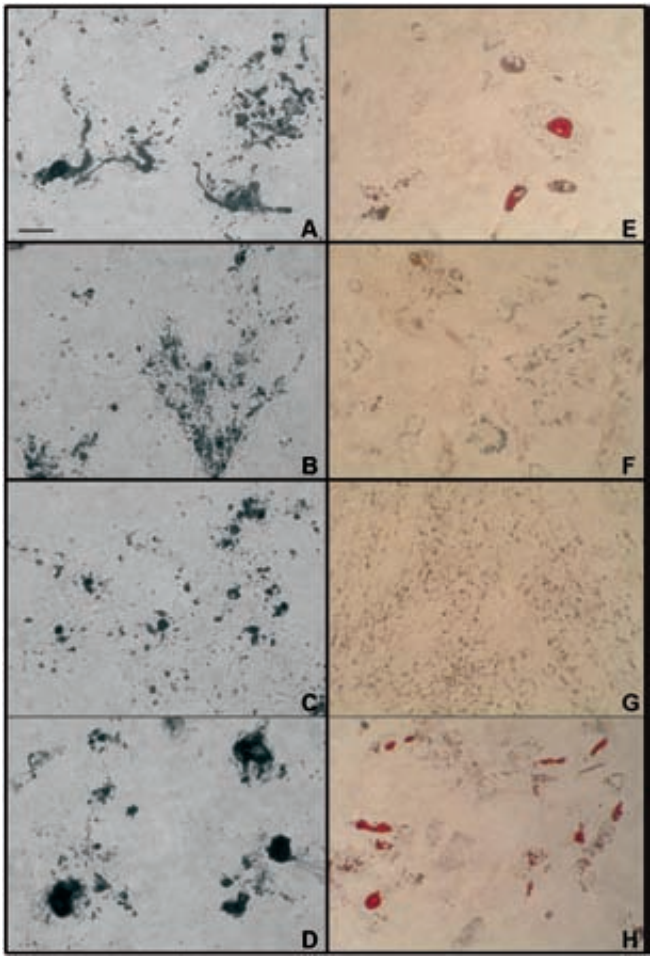


Figure 4. A-D: von Kossa staining for calcified mineral nodules (black) after 14 days of osteogenic differentiation of MSC derived from [A] heart; [B] spleen; [C] adipose tissue; and [D] bone marrow. Figure 4E-H: oil-red-O staining for lipid-filled vesicles (red) after 14 days of adipogenic differentiation of MSC derived from [E] heart; [F] spleen; [G] adipose tissue; and [H] bone marrow. Bar represents 20 μm. Experiment carried out three times, representative images shown.

Effect of MSC from different tissues on PBMC proliferation

Bone marrow MSC inhibit PBMC proliferation. The effect of MSC from different tissues on PBMC proliferation was examined by measurement of [^{3}H]-thymidine incorporation. Stimulation of PBMC with γ-irradiated allogeneic PBMC increased proliferation 31-fold from 891 to 27,514 cpm (Figure 5A). Co-culture with allogeneic MSC from heart, spleen, adipose tissue, or bone marrow in a 1:5 ratio for seven days inhibited PBMC proliferation significantly by on average 85%, 79%, 79% and 81%, respectively (n=3) (Figure 5A). The proliferation of MSC was neglectable (<500 cpm).

Stimulation of PBMC with PHA for 3 days resulted in a more than 200-fold increase in proliferation with [^{3}H]-thymidine incorporation increasing from 392 to 79,869 cpm (n=3). The addition of allogeneic MSC derived from heart, spleen, adipose tissue, or bone marrow in a 1:5 ratio reduced PHA-induced proliferation of PBMC by 73%, 43%, 77% and 94%, respectively (Figure 5B). Bone marrow-derived MSC were significantly more potent in inhibiting PHA responses than MSC derived from the other tissues tested.

Finally, it was tested whether MSC would inhibit a memory T-cell response to Tet. PBMC were stimulated with 7.5 lf/well Tet in the absence or presence of MSC in a 1:5 ratio and proliferation measured after seven days. Tet increased proliferation of PBMC six-fold and co-culture with MSC from heart, spleen, adipose tissue, and bone marrow reduced proliferation by 55%, 50%, and 53%, 52%, respectively (Figure 5C).

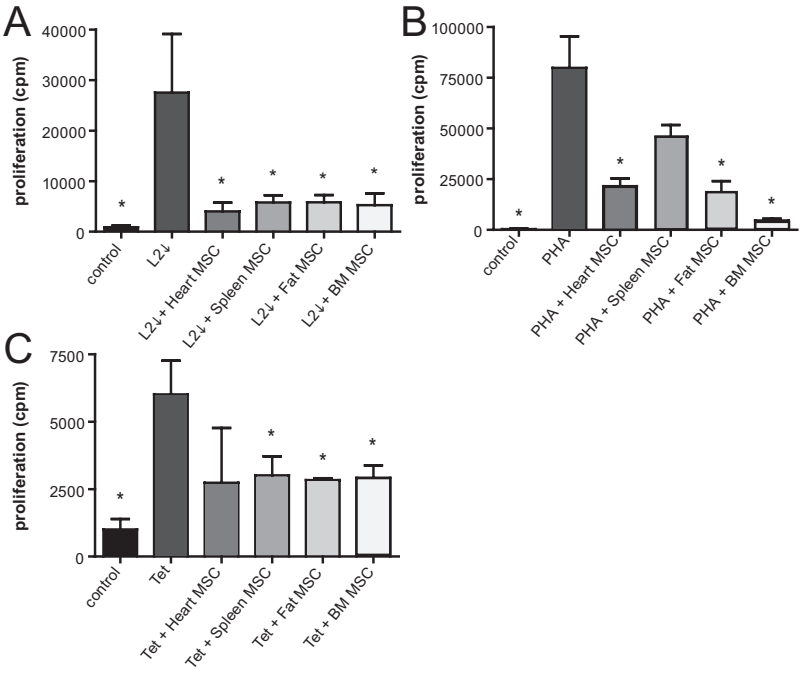


Figure 5. Effect of MSC of different origin on PBMC proliferation measured by [3H]-thymidine incorporation. PBMC were stimulated by [A] γ-irradiated allogeneic PBMC (L2↓); [B] PHA; or [C] Tetanus toxoid (Tet). MSC were added to responder PBMC in a 1:5 ratio. Results shown are the mean values of 3 experiments [Figure A, B] or 2 experiments [Figure C] in triplicate ± SEM. * indicates p-value < 0.05 compared to column 2.

DISCUSSION

In the present study, we isolated and expanded cells with fibroblast morphology from the human heart, spleen, and perirenal adipose tissue. The immunophenotype of these cells was identical to bone marrow-derived MSC described in the literature [2, 20], i.e. the cells were positive for CD90, CD105, CD166 and HLA class I, and negative for HLA class II, CD34, CD45, CD80 and CD86 expression. In addition, as with bone marrow-derived MSC, they differentiated along osteogenic and adipogenic lineages when cultured under the appropriate conditions. Also in line with bone marrow-derived MSC, these cells inhibited the proliferative response of allogeneic PBMC after alloactivation and after stimulation with PHA or the recall antigen Tet. Therefore, we conclude that these cells under test from the human heart, spleen and perirenal adipose tissue are MSC. There were differences among these MSC. For example, heart and spleen-derived MSC showed delayed adipogenic differentiation when compared to bone marrow and adipose tissue-derived MSC. In addition, there were some differences in the capacity of MSC from different body sites to inhibit PBMC proliferation. Spleen MSC, for instance, had less of an effect on PHA-activated PBMC proliferation in relation to MSC from the other tissues. Thus, our results demonstrate that MSC from these tissues have the same functional capacities, but there are differences in their potency.

Earlier studies have shown that MSC are also present in human placenta, umbilical cord blood, and skin and that regardless of the tissue, these MSC possess characteristics that are similar to a large extent to bone marrow MSC [16, 21-23]. The origin of MSC that are present outside the bone marrow is unclear. There is evidence that fibroblast-like stem cells migrate from the bone marrow to other body sites via the blood stream [24-25]. However, other studies have failed to identify MSC from peripheral blood [1, 26]. It is possible that these MSC are end products from embryonic differentiation [27-30] and that they serve to regenerate these mesenchymal tissues when damaged. In any case, their widespread distribution and multifunctionality suggests MSC play an important role in virtually every tissue of the body.

REFERENCES

1. Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM. Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. Br J Haematol. 2003 Apr;121(2):368-74.

2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999 Apr 2;284(5411):143-7.

3. Bonnet D. Biology of human bone marrow stem cells. Clin Exp Med. 2003 Nov;3(3):140-9.

4. Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow.Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation. 1968 Mar;6(2):230-47.

5. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002 May 15;99(10):3838-43.

6. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood. 2003 May 1;101(9):3722-9.

7. Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, et al. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci. 2005;12(1):47-57.

8. Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol. 2003 Jan;57(1):11-20.

9. Groh ME, Maitra B, Szekely E, Koc ON. Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. Exp Hematol. 2005 Aug;33(8):928-34.

10. Augello A, Tasso R, Negrini SM, Amateis A, Indiveri F, Cancedda R, et al. Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. Eur J Immunol. 2005 May;35(5):1482-90.

11. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol. 2002 Jan;30(1):42-8.

12. Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet. 2004 May 1;363(9419):1439-41.

13. Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood. 2003 Apr 15;101(8):2999-3001.

14. Koerner J, Nesic D, Romero JD, Brehm W, Mainil-Varlet P, Grogan SP. Equine peripheral blood-derived progenitors in comparison to bone marrow-derived mesenchymal stem cells. Stem Cells. 2006 Jun;24(6):1613-9.

15. Rochefort GY, Delorme B, Lopez A, Herault O, Bonnet P, Charbord P, et al. Multipotential Mesenchymal Stem Cells are Mobilized into Peripheral Blood by Hypoxia. Stem Cells. 2006 Jun; 24;(6): 2202-8

16. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002 Dec;13(12):4279-95.

17. Toma JG, McKenzie IA, Bagli D, Miller FD. Isolation and characterization of multipotent skin-derived precursors from human skin. Stem Cells. 2005 Jun-Jul;23(6):727-37.

18. Hoogduijn MJ, Gorjup E, Genever PG. Comparative characterization of hair follicle dermal stem cells and bone marrow mesenchymal stem cells. Stem Cells Dev. 2006 Feb;15(1):49-60.

19. da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci. 2006 Jun 1;119(Pt 11):2204-13.

20. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation. 2003 Feb 15;75(3):389-97.

21. Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, et al. Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol. 2001 Sep;3(9):778-84.

22. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, et al. Isolation of mesenchymal stem cells

of fetal or maternal origin from human placenta. Stem Cells. 2004;22(7):1338-45.

23. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells. 2006 May;24(5):1294-301.

24. Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG. Circulating skeletal stem cells. J Cell Biol. 2001 May 28;153(5):1133-40.

25. Kucia M, Wojakowski W, Reca R, Machalinski B, Gozdzik J, Majka M, et al. The migration of bone marrow-derived non-hematopoietic tissue-committed stem cells is regulated in an SDF-1-, HGF-, and LIF-dependent manner. Arch Immunol Ther Exp (Warsz). 2006 Mar-Apr;54(2):121-35.

26. Lazarus HM, Haynesworth SE, Gerson SL, Caplan AI. Human bone marrow-derived mesenchymal (stromal) progenitor cells (MPCs) cannot be recovered from peripheral blood progenitor cell collections. J Hematother. 1997 Oct;6(5):447-55.

27. Gotherstrom C, West A, Liden J, Uzunel M, Lahesmaa R, Le Blanc K. Difference in gene expression between human fetal liver and adult bone marrow mesenchymal stem cells. Haematologica. 2005 Aug;90(8):1017-26.

28. Fan CG, Tang FW, Zhang QJ, Lu SH, Liu HY, Zhao ZM, et al. Characterization and neural differentiation of fetal lung mesenchymal stem cells. Cell Transplant. 2005;14(5):311-21.

29. Zhao Z, Liao L, Cao Y, Jiang X, Zhao RC. Establishment and properties of fetal dermis-derived mesenchymal stem cell lines: plasticity in vitro and hematopoietic protection in vivo. Bone Marrow Transplant. 2005 Aug;36(4):355-65.

30. Zhang L, Hong TP, Hu J, Liu YN, Wu YH, Li LS. Nestin-positive progenitor cells isolated from human fetal pancreas have phenotypic markers identical to mesenchymal stem cells. World J Gastroenterol. 2005 May 21;11(19):2906-11.

Chapter 3

Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients

Meindert J. Crop[1], Carla C. Baan[1], Sander Korevaar[1],
Jan N.M. IJzermans[2], Ian P.J. Alwayn[2], Willem Weimar[1]
and Martin J. Hoogduijn[1]

Departments of [1]Internal Medicine and [2]Surgery,
Erasmus University Medical Center, Rotterdam, The Netherlands

Transplantation
2009 Mar 27;87(6):896-906

ABSTRACT

Human mesenchymal stem cells (MSC) have immunosuppressive capacities. Although their efficacy is currently studied in graft-versus-host disease, their effect on alloreactivity in solid organ transplant patients is unknown. In this study, the immunosuppressive effect of MSC on recipient anti-donor reactivity was examined before and after clinical kidney transplantation.

Anti-donor reactivity was established in pretransplant and posttransplant mixed lymphocyte reactions (MLR) of 14 living-kidney donor-recipient pairs. MSC from donors and third-party controls were added to the MLR in a ratio of 1:5.

MSC were isolated from donor perirenal fat and showed multilineage differentiation potential and the capacity to inhibit lymphocyte proliferation. The immunosuppressive effect of MSC was dose dependent and mediated by cell-membrane contact and soluble factors, including interleukin-10 and indoleamine 2,3-dioxygenase.

Donor-derived MSC significantly inhibited the recipient anti-donor reactivity before and 1 month after transplantation. This effect was independent of human leukocyte antigen (HLA) background of MSC. Flow cytometric analysis showed that MSC inhibited the proliferation of $CD4^+$ and $CD8^+$ T-lymphocyte subsets in pretransplant and posttransplant donor-directed MLR, whereas MSC had no effect on B-cell or natural killer-cell proliferation.

In conclusion, donor MSC significantly inhibited the proliferation of alloactivated recipient T-cells before and after kidney transplantation. We believe these findings should encourage MSC-based intervention in clinical organ transplantation.

INTRODUCTION

In clinical solid organ transplantation, alloreactivity is controlled by immunosuppressive drugs. These drugs, however, can lead to serious side effects. As an alternative, cellular therapies may be considered. One of the candidates is the mesenchymal stem cell (MSC). MSC are progenitor cells for a variety of cell types, such as osteoblasts, adipocytes, chondrocytes, and myocytes [1] and may play a role in tissue repair. In addition, it has been demonstrated that MSC have immunosuppressive effects *in vitro*. They inhibit proliferation and production of pro-inflammatory cytokines by lymphocytes and maturation of dendritic cells [2-5]. The mechanism of immunosuppression by MSC is unclear, but the involvement of factors such as indoleamine 2,3-dioxygenase (IDO), interleukin (IL)-10, transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α) and hepatocyte growth factor (HGF) has been indicated [2, 4, 6]. Blocking each of these factors alone does not restore the proliferation of activated immune cells, indicating that multiple factors are involved.
In a clinical setting, it has been suggested that MSC could be useful for the treatment of graft-versus-host disease [7]. Moreover, the application of MSC for immunological disorders, for example, autoimmune-induced rheumatoid arthritis or inflammatory bowel disease, such as Crohn's disease and ulcerous colitis, is currently under investigation [8]. Furthermore, there are conflicting data from animal transplant models that MSC prolong allograft survival [9-10]. Whether MSC are effective in the prevention and treatment of alloreactivity in a clinical transplantation setting is unknown [11]. Both the choice of MSC source and timing of treatment may be important. Autologous MSC of the recipient may be first choice, as they are not rejected. However, MSC have a low immunophenotype, that is they lack human leukocyte antigen (HLA) class-II and have low HLA class-I expression, which would reduce the clearance of allogeneic MSC [12]. It has also been suggested that allogeneic MSC are more potent immunosuppressors than autologous MSC [13], and there is evidence from animal models that donor-derived MSC may be preferable to inhibit allograft rejection [10]. In addition, the timing of MSC administration may affect the efficacy of MSC treatment, as after transplantation alloreactivity is affected by immunosuppressive medication and the development of memory responses against the donor graft. This study investigated the efficacy and mechanisms of immunosuppression of kidney donor perirenal adipose tissue-derived MSC on alloreactivity before and after human kidney transplantation.

MATERIALS AND METHODS

Living-kidney donors and their recipients

Fourteen non-HLA-identical living-kidney donor-recipient pairs were included (November 2006-May 2007). Their characteristics are described in Table 1 (at the end of this chapter). Immunosuppressive therapy for kidney transplant recipients consisted of tacrolimus, mycophenolate mofetil (MMF) and low-dose steroids. Biopsy-proven acute rejections occurred in 5 of 14 recipients within 1 month after transplantation, median 11 (6-30) days. Histological examination showed three mild tubulointerstitial rejections without evidence of vascular rejection and two severe tubulointerstitial rejections with vascular rejection. Acute rejections were treated with high-dose steroids (solumedrol) or anti-thymocyte globulin (ATG).

During the kidney donation procedure, perirenal fat tissue was surgically removed after written informed consent as approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam (protocol no. MEC-2006-190) and collected in a-minimum essential medium (α-MEM) (Invitrogen, Paisley, Scotland) with 1% penicillin/streptomycin (100 U/mL penicillin and 100µg/mL streptomycin, p/s, Invitrogen).

Isolation of MSC from perirenal fat

MSC were isolated from perirenal fat as described previously [14]. In summary, perirenal fat was mechanically disrupted, enzymatically digested with sterile 0.5 mg/mL collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) in Roswell Park Memorial Institute (RPMI) (culture medium)-Dutch-modified-1640 (Invitrogen) with 200 mM L-glutamine (Biowithaker, Verviers, Belgium) and 1% p/s for 30 minutes at 37°C. Next, the cells were incubated with 160 mM NH_4Cl in phosphate-buffered saline (PBS) for 10 minutes at room temperature. Cells were resuspended in MSC-culture medium, consisting of α-MEM with 1% p/s and 15% fetal bovine serum (FBS, Biowithaker) and transferred to a 175 cm^2 culture flask (Greiner Bio-one, Essen, Germany) kept at 37°C, 5% CO_2 and 95% humidity. MSC were used for experiments between passages 2 to 7. No functional differences were found between MSC from these passages.

Flow cytometric characterization of MSC

Adherent cells were removed from culture flasks by incubation in 0.05% trypsin-EDTA at 37°C and then washed twice with FACSFlow (BD Biosciences, San Jose, CA, USA). Next, cell suspensions were incubated with antibodies against CD14-PerCP, CD34-APC, CD45-PerCP, CD86-FITC, CD166-PE, HLA-DR-FITC (all BD Biosciences), CD40-PE, CD80-PE, HLA-ABC-PE (all Serotec, UK), CD90-APC and CD105-FITC (all R&D systems, Abingdon, UK) at room temperature protected from light for 30 minutes. After two washes with FACSFLOW, flow cytometric analysis was performed using an 8-colour FACSCANTO-II with FACSDIVA Software (BD Biosciences) and FlowJo Software (Tree Star Inc., Palo Alto, CA, USA).

Differentiation of MSC

Perirenal fat-derived MSC were differentiated in osteogenic, adipogenic, and myogenic lineages as described previously [14-15]. After osteogenic differentiation, the deposition of calcified nodules was visualised by von Kossa staining. MSC differentiated in the adipogenic lineage were stained with oil-red-O (Sigma-Aldrich) for identification of lipid droplets.

After 7 days of myogenic differentiation, quantitative expression of desmin was determined by real-time RT-PCR as described previously [16].

Isolation of peripheral blood mononuclear cells

Peripheral blood samples of kidney recipients were collected before transplantation, and 1 month after transplantation. Blood of the living donors was collected before transplantation. Moreover, third-party peripheral blood mononuclear cells (PBMC), not associated with the third-party MSC donors, were collected from buffy coats of healthy blood donors. PBMC were isolated from the collected heparinized peripheral blood by density gradient centrifugation using Ficoll Isopaque (d=1.077, Amersham, Uppsala, Sweden) and frozen –135°C until use.

Mixed lymphocyte reactions

In mixed lymphocyte reactions (MLR), $5x10^4$ recipient PBMC were stimulated by γ-irradiated (40 Gy) donor PBMC or HLA–A, –B and –DR mismatched third-party PBMC in RPMI culture medium+10% heat-inactivated FBS (HI-FBS) or vice versa. Donor-derived MSC or third-party MSC were added to the various MLR at a 1:5 ratio. On day 7, proliferation was measured by incorporation of ^{3}H-thymidine (0.5 μCi/well) during an 8-hour incubation using a b-plate reader (LKB, Bromma, Sweden).

To determine the proliferation capacity of the PBMC, $5x10^4$ cells were stimulated with 1 mg/ml phytohaemagglutinin (PHA; Murex Biotech LTd, Kent, UK) for 3 days and ^{3}H-thymidine incorporation measured. Only results of PBMC with sufficient proliferation capacity (>10,000 cpm) were included. MLR were performed (in round-bottom 96-well plates) in triplicate and medians used for further analysis.

Real-time RT-PCR

Total RNA was isolated and cDNA synthesized as described previously [15]. Quantitative expression of IDO, IL-10, TGF-β and TNF-α was determined by real-time RT-PCR using TaqMan gene expression MasterMix (Applied Biosystems, CA, USA) and an Assay-on-demand for IDO (Hs00158627.m1), IL-10 (Hs00174086.m1), TGF-β (Hs00171257.m1) and TNF-α (Hs99999043.m1) (all Applied Biosystems) on an ABI PRISM 7700 sequence detector (Applied Biosystems). HGF was determined by real-time RT-PCR using primers (F-5'-GGCTGGGGCTACACTGGATTG-3' and R-5'-CCACCATAATCCCCCTCACAT-3') and quantification of sample amplicons by SybrGreen (Sigma-Aldrich) using Taq-DNA-polymerase as described previously [17]. Expression levels were calculated as ratio per 18S RNA or as copies mRNA per 500 ng of total RNA. PBMC-MSC-co-cultures were separated by transwell-membranes (Greiner Bio-one); MSC were cultured in the bottom well, whereas PBMC were kept in the inserts.

Flow cytometric analysis of proliferating lymphocyte subsets

To determine which lymphocyte subsets were inhibited in proliferation by the addition of MSC in MLR, responder recipient PBMC were labeled with PKH67 (Sigma-Aldrich) and the γ-irradiated stimulator donor cells with PKH26 (Sigma-Aldrich) according to the manufacturer's instructions and MSC added in a 1:5 ratio. After 7 days, cells were stained for CD3-AmCyan, CD4-PacificBlue, CD8-APC-Cy7, CD16/56-APC, CD19-PerCP and CD25-PE-Cy7 (all BD Biosciences) and examined by flow cytometry as described above.

Statistical analysis

The effect of MSC on MLR was calculated for each experiment as the fold-change in proliferation. Data were analyzed using the (two-tailed) Wilcoxon signed-rank test. Statistical significance was defined as p less than 0.05.

RESULTS

Isolation and characteristics of MSC from perirenal fat

MSC were isolated from 10 to 50 g of perirenal fat of 14 living-kidney donors. The success rate for the isolation of these cells was 100% (14/14). The cells were adherent and had a spindle-shaped morphology. Flow cytometric analysis demonstrated that the cells expressed CD90, CD105, CD166 and HLA-ABC (MHC-I) and low CD34 and no CD14, CD40, CD45, CD80, CD86 or HLA-DR (MHC-II) (Figure 1A). Culturing of undifferentiated cells for 14 days under adipogenic conditions induced the formation of lipid-filled vesicles that stained red by oil-red-O staining, characteristic for adipocytes (Figure 1B). Induction of osteogenic differentiation of the cells for 14 days resulted in the deposition of mineralised nodules that stained black by von Kossa staining, characteristic for osteoblasts. Culturing under myogenic conditions induced morphological changes (Figure 1B) and increased mRNA expression of desmin by 2.0-fold (Figure 1C). These results confirmed that the isolated cells were MSC.

Immunosuppressive effects of perirenal fat MSC

The MSC showed dose-dependent suppression of proliferation in MLR (Figure 1D). The median (range) ^{3}H-thymidine incorporation of MSC monocultures was 124 (26–450) cpm. Gamma-irradiated MSC inhibited proliferation with comparable capacity (data not shown). MLR performed in transwell systems in which MSC were separated from PBMC by a membrane demonstrated that MSC were still immunosuppressive in the absence of cell contact, although the effect on $CD4^{+}$ and $CD8^{+}$ lymphocyte subsets was greater when direct contact was allowed. MSC strongly inhibited the proliferation of $CD4^{+}$ and $CD8^{+}$ T-cell subsets and had a smaller effect on NK-cells, while proliferation of B-cells was not affected (Figure 1E). Next, we investigated which factors were involved in the immunosuppressive effect of donor MSC in our system and performed real-time RT-PCR analysis of MSC monocultures or MSC co-cultured with MLR (in transwell system). The expression of IDO and IL-10 by MSC co-cultured with MLR was increased 15,000-fold (p=0.008) and 420-fold (p=0.008), whereas the expression of TGF-β and HGF decreased by a factor 1.9 (p=0.008) and 7.6 (p=0.008). The expression of TNF-α did not change (p=0.17) (Figure 1F). Further experiments showed that PBMC in MLR co-cultured with MSC still had proliferative capacity when re-stimulated with PHA and IL-2 (Figure 1G) and in secondary MLR (Figure 1H). Interestingly, PBMC that had been cultured in direct contact with MSC had less proliferative capacity in response to PHA and IL-2.

MSC inhibit pretransplant anti-donor and third-party reactivity

To examine the immunosuppressive effect of donor-derived MSC and third-party-derived MSC on anti-donor reactivity before transplantation, 14 MLR of recipients and their donors were assessed. Characteristics of these donor-recipient pairs are listed in Table 1. Figure 2A shows the effect of MSC on the proliferation of the pretransplant donor-directed MLR. Before transplantation, donor-derived MSC inhibited the proliferation of donor-stimulated recipient PBMC six-fold (2–28) (p<0.001), median (range), whereas third-party MSC inhibited this proliferation five-fold (2–19) (p<0.001). Third-party-directed pretransplant MLR was also inhibited five-fold (2–62) by donor-derived MSC and five-fold (1–19) by third-party-derived MSC, which were not associated with the third-party PBMC (data not shown). There was no correlation between the development of acute rejections within the first month after transplantation and the proliferation in MLR.

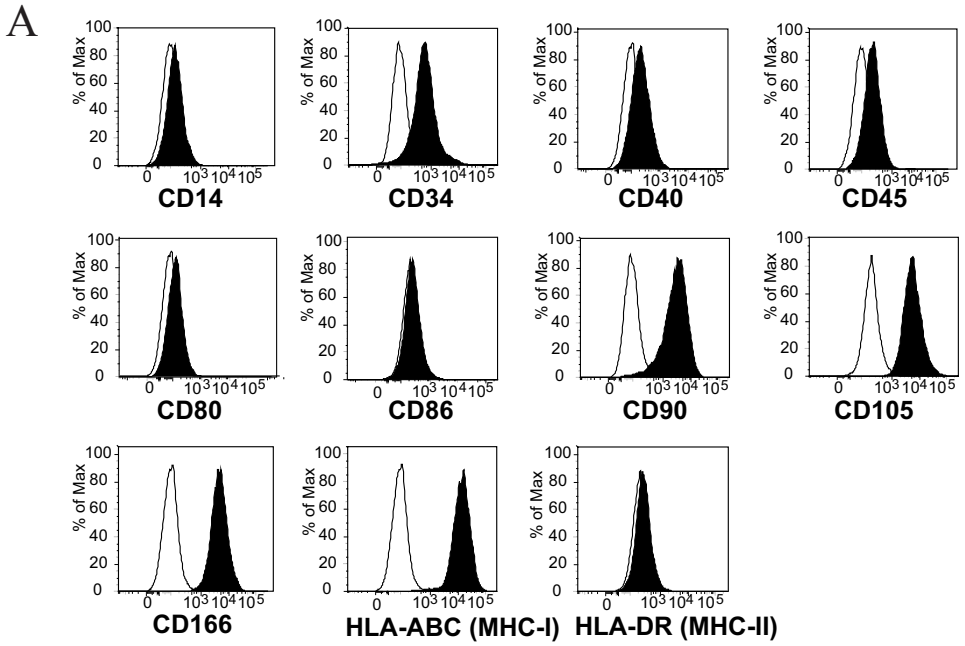
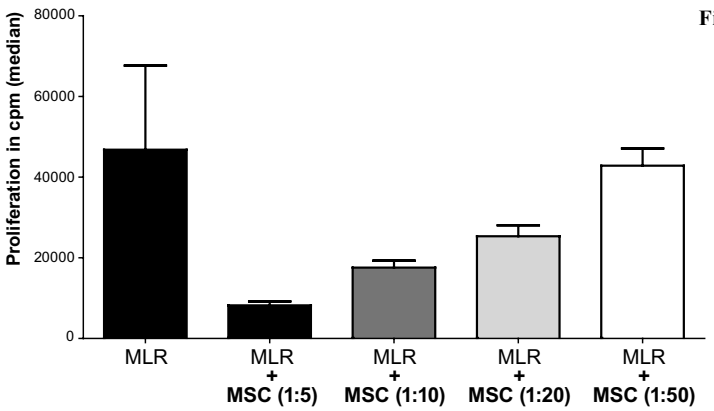


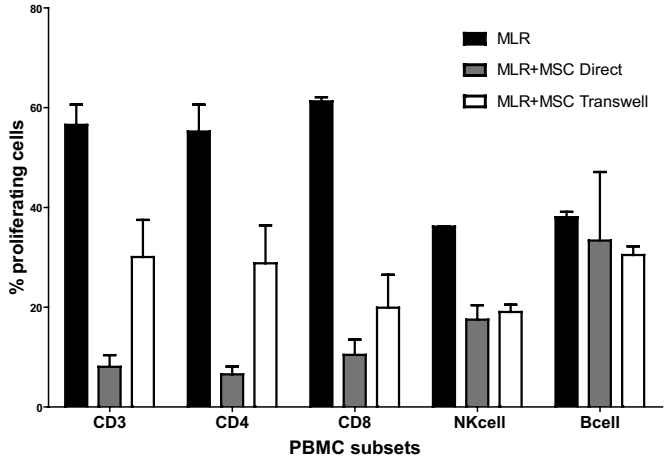
Figure 1 ***(continued)***>>

Figure 1 ***(continued)*** >>

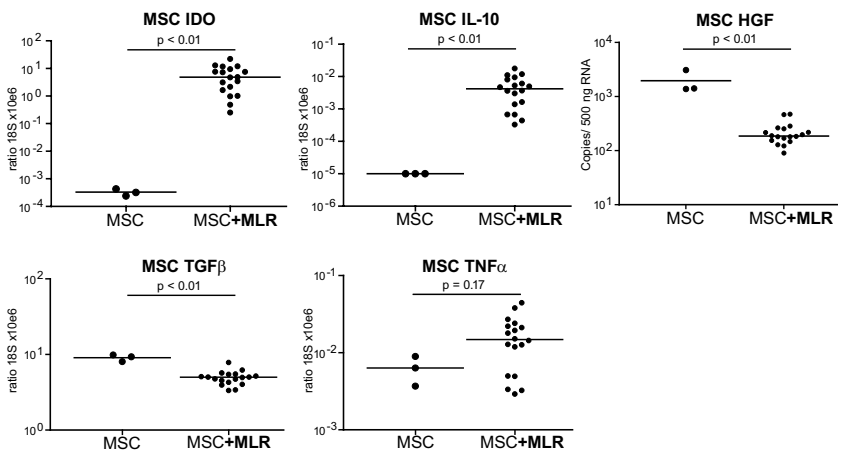
D



E



F



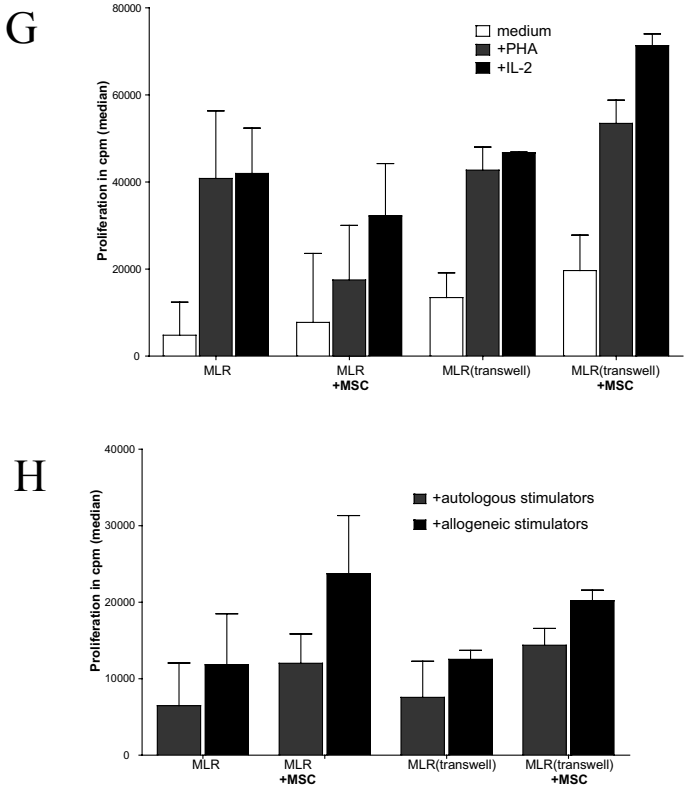


Figure 1. Immunophenotypical and functional characterization of perirenal fat mesenchymal stem cells (MSC). [A] Flow cytometric analysis MSC showed positive expression for CD90, CD105, CD166 and HLA-ABC, intermediate expression for CD34, and an absence of CD14, CD40, CD45, CD80, CD86 or HLA-DR. [B] Microscopic images of undifferentiated MSC, and MSC differentiated into adipogenic, osteogenic and myogenic lineages. After adipogenic differentiation for 14 days, lipid-filled vesicles were stained red with oil-red-O. Osteogenic differentiation for 14 days induced the deposition of mineralised nodules that stained black by von Kossa staining. Myogenic differentiation of MSC for 7 days led to the appearance of cells with a myogenic morphology. [C] Real-time RT-PCR analysis of myogenic differentiated MSC showed increased mRNA expression of desmin (copies/500 ng) compared to undifferentiated MSC (n=2). [D] Dose-dependent immunosuppression by perirenal fat MSC. MSC were added to responder PBMC in MLR at a (1:5), (1:10), (1:20), (1:50) ratio (n=5). [E] Inhibition of the proliferation of lymphocyte subsets by perirenal fat MSC cultured in direct contact or in transwell systems (n=5). [F] Real-time RT-PCR analysis of the expression of IDO, IL-10, TNF-α, TGF-β and HGF by perirenal fat MSC after 7 days in monoculture (MSC) or in transwell-coculture with MLR (MSC+MLR). Data expressed as a ratio to 18S RNA or per 500ng RNA. [G] Proliferation capacity of PBMC isolated after 7 days in primary MLR co-cultured with MSC (in direct-contact and in transwell systems). Proliferation capacity was tested with PHA and IL-2 for 3 days (n=3) and [H] in secondary MLR (7 days) (n=3).

MSC autologous and allogeneic to responder cells in MLR have comparable capacity to inhibit alloreactivity

Next, we investigated whether the origin of MSC (donor or third-party) had effect on their inhibitory effect in MLR. Donor PBMC were used as responder cells, and recipient PBMC (Figure 2B) as stimulator cells in MLR, and donor-derived MSC (=autologous to responder cells) or third-party-derived MSC (=allogeneic to responder cells) added. There was no difference in the inhibitory effect of donor-derived MSC, 13-fold (2–54), or third-party-derived MSC, 13-fold (1–67) (p=0.39). Furthermore, there was no difference between donor-derived and third-party-derived MSC to inhibit third-party PBMC-stimulated donor PBMC proliferation (p=0.68) (data not shown).

MSC inhibit posttransplant anti-donor and anti-third-party proliferation

We then questioned whether MSC could also inhibit posttransplant alloreactivity of kidney transplant recipients. After transplantation, donor-directed proliferation in MLR was lower compared to pretransplantation: 21,026 (9615–56,757) versus 8198 (1216–41,193) cpm (p=0.04). Donor-derived MSC inhibited posttransplant donor-directed reactivity two-fold (0–22) (Figure 3A). Third-party MSC had a comparable effect and inhibited the posttransplant donor-directed proliferation thee-fold (0–24) (Figure 3B). In contrast to donor-directed proliferation in MLR, third-party-directed proliferation of recipient PBMC was not different before and after transplantation (p=0.34). Donor-derived MSC inhibited the posttransplant third-party-directed proliferation by a factor 3 (0–91) (p=0.04) (Figure 3C), and third-party MSC by a factor 4 (0–36) (Figure 3D). There was no difference in the inhibitory capacity between donor and third-party MSC to inhibit anti-donor reactivity (p=0.54) or anti-third-party reactivity (p=0.57).

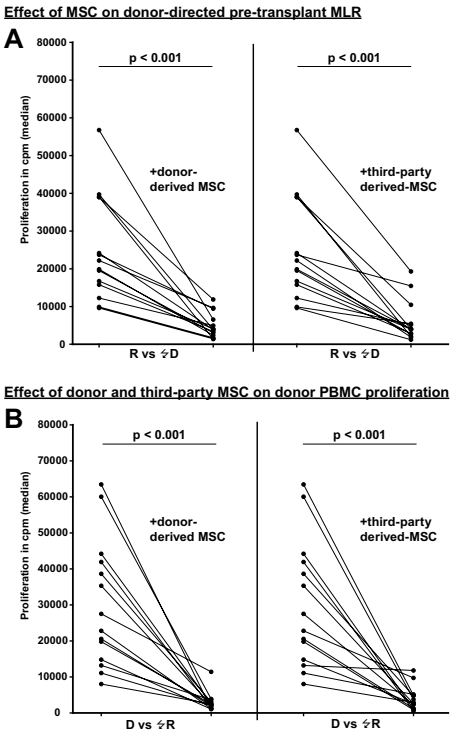


Figure 2. [A] Effect of mesenchymal stem cells (MSC) on donor-directed pretransplant MLR of living-kidney donor-recipient pairs. MSC were added in a 1:5 ratio to responder PBMC. Proliferation was measured by 3H-thymidine incorporation on day 7. Donor and third-party MSC significantly inhibited anti-donor proliferation (n=14). [B] Effect of donor and third-party MSC on donor PBMC proliferation. Donor-derived MSC (=autologous to the responder cells) and third-party-derived MSC (=allogeneic to the responder cells) were added in a 1:5 ratio to alloactivated responder PBMC. Independent of their origin (donor or third-party-derived) MSC inhibited anti-recipient proliferation of donor PBMC (n=14). The median (range) 3H-thymidine incorporation of MSC monocultures was 124 cpm (26±450) for donor-derived MSC and 133 cpm (78±785) for third-party-derived MSC (n=14). R = recipient PBMC; D = donor PBMC; ϟ = γ-irradiation (40 Gy); cpm = counts per minute.

Effect of MSC on proliferation and activation of lymphocyte subsets in posttransplant MLR

To determine which lymphocyte subsets were inhibited in proliferation by MSC, responder recipient PBMC were labeled with PKH67 and γ-irradiated donor PBMC with PKH26 and proliferation analyzed by flow cytometry. Before transplantation, MSC inhibited the number of proliferating T-cells 3.1-fold, subdivided in $CD4^+$ T-cells (2.2-fold) and $CD8^+$ T-cells (2.3-fold), while the proliferation of B-cells (0.7-fold) and NK-cells (1.0-fold) was not inhibited by MSC (Figure 4). After transplantation, the proliferation in donor-directed MLR was lower than in pretransplant MLR, as demonstrated before. MSC inhibited T-cell proliferation 1.5-fold, including a 1.4-fold reduction for $CD4^+$ T-cells and 1.3-fold for $CD8^+$ T-cells. MSC had no effect on B-cell proliferation in posttransplant MLR. NK-cell proliferation was inhibited by a factor 1.2.

Finally, the activation state of $CD4^+$ and $CD8^+$ lymphocyte subsets was reduced by MSC in pretransplant MLR, as measured by CD25 expression (Table 2). In posttransplant MLR, however, the inhibition of T-cell proliferation was accompanied by an increase in CD25 expression. This effect was strongest for $CD4^+$ T-cells.

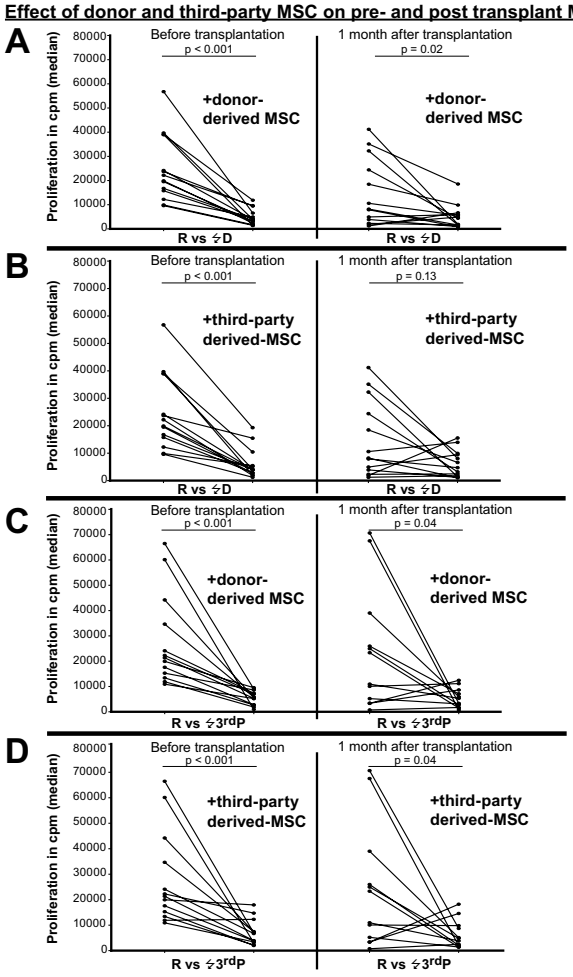


Figure 3. Effect of mesenchymal stem cells (MSC) on the proliferation of donor-directed and third-party-directed pretransplant and posttransplant MLR of recipient PBMC. MSC were added in a 1:5 ratio to recipient PBMC. Proliferation was measured by 3H-thymidine incorporation on day 7. [A] Donor MSC inhibited anti-donor proliferation before (n=14) and after transplantation (n=13). [B] Third-party MSC inhibited anti-donor proliferation before (n=14) and after transplantation (n=13). [C] Donor MSC inhibited anti-third-party proliferation before (n=13) and after transplantation (n=12). [D] Third-party MSC on anti-third-party proliferation before (n=13) and after transplantation (n=12). R = recipient PBMC; D = donor PBMC; 3rdP = third-party PBMC (HLA A-B-DR mismatched with recipient); ⚡ = γ-irradiation (40 Gy); cpm = counts per minute.

DISCUSSION

This study investigated the immunosuppressive capacities of human MSC on alloreactivity in a kidney transplantation setting using recipient and donor PBMC. We found that donor-derived MSC significantly inhibited anti-donor reactivity before and after transplantation. Third-party-derived MSC inhibited alloreactivity at a similar potency. Our results in a human solid organ transplantation setting confirm previous studies that suggested that inhibition of lymphocyte proliferation by MSC is independent of HLA [18-19].

In our experimental model, we found that immunosuppression by MSC was dependent on cell-membrane contact and soluble factors. The strong induction of IDO and IL-10 in MSC co-cultured with MLR suggests that these factors play a role in immunosuppression by MSC, confirming earlier studies indicating that the immunosuppressive capacity of MSC needs to be induced [20]. In contrast to other studies, HGF, TGF-β or TNF-α expression was not increased in our hands [2-3].

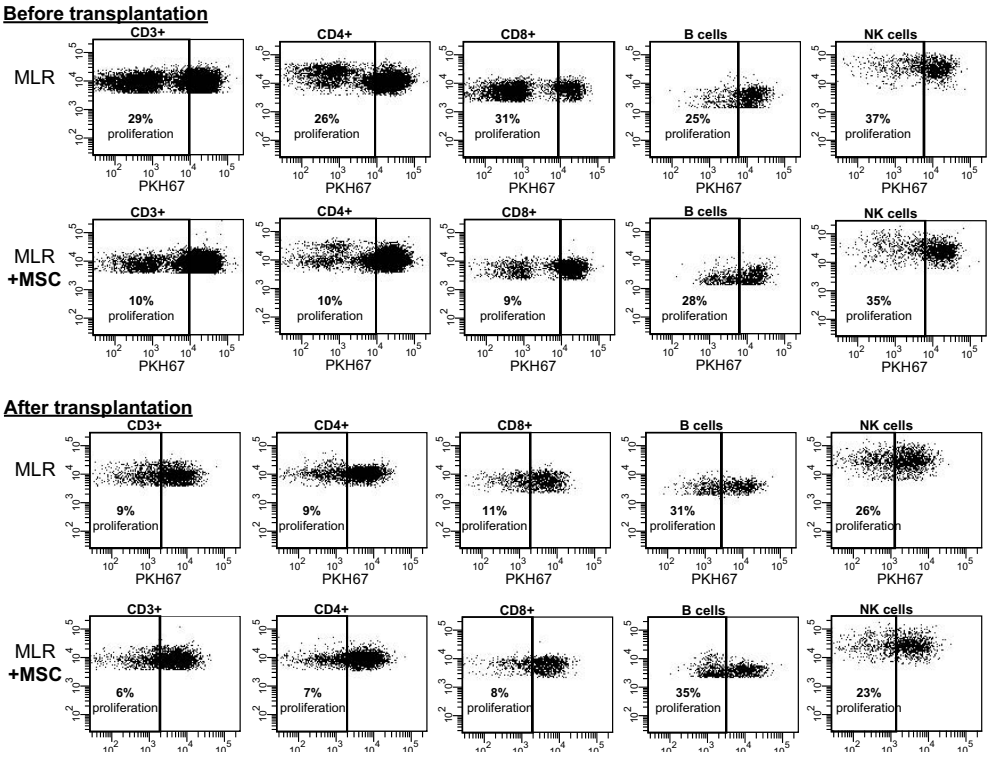


Figure 4. Flow cytometric analysis of the proliferation of recipient lymphocyte subsets in pretransplant and posttransplant donor-directed MLR. One representative example is shown of eight experiments. Proliferation of PKH67-labeled recipient lymphocytes was measured after 7 days. MSC inhibited the proliferation of $CD4^+$ and $CD8^+$ T-lymphocytes in pretransplant MLR, whereas there was no effect on B-cells or NK-cells. MSC were capable of inhibiting the proliferation of T-lymphocytes after transplantation and had a small effect on NK-lymphocyte proliferation.

Analysis of the proliferation of T-, B-, and NK-lymphocyte subsets in pretransplant MLR showed that MSC had a strong inhibitory effect on T-lymphocyte proliferation, whereas they had no effect on NK- and B-lymphocytes. In posttransplant MLR, MSC predominantly inhibited T-lymphocytes. These results indicate that the strong inhibition of PBMC proliferation by MSC that was observed by measurement of ^{3}H-thymidine incorporation was predominantly the result of inhibition of T-cell proliferation. Reports on the effect of MSC on B and NK-lymphocytes are contradicting. For instance, some failed to demonstrate an inhibitory effect of MSC on the proliferation and cytotoxic activity of activated NK-cells [21], while others found that MSC suppress IL-2 and IL-15 driven NK-cell proliferation and IFN-γ production [4, 22-23]. Although we and others [20] found no effect of MSC on B-lymphocyte proliferation in MLR, it has been shown that the proliferation and immunoglobulin production of B-lymphocytes induced by tropic stimuli can be inhibited by MSC [24]. Our results on the effect of MSC on T-lymphocyte proliferation before and after transplantation demonstrate that MSC are effective in targeting the adaptive immune system. This capacity may be important for potential posttransplantation therapy with MSC.

After transplantation, when the recipient's immune system had been exposed to donor-antigen and immunosuppressive drugs, we found lower donor-directed proliferation of recipient PBMC. This was not entirely dependent on immunosuppressive drugs, as third-party-directed proliferation in MLR was less reduced after transplantation. It is attractive to hypothesize that the recipient's immune system adapts to the donor graft and achieves donor-specific hyporesponsiveness. Hyporesponsiveness after transplantation has been reported previously [25-26]. While we found that the proliferation in posttransplant donor-directed MLR decreased, MSC still inhibited this proliferation. This suggests that MSC may be effective after transplantation to control allograft rejection.

Further analysis of the immunosuppressive effect of MSC on PBMC showed that MSC not only strongly inhibit proliferation in pretransplant MLR, but also activation of T-cells. In posttransplant MLR the proliferation of responder T-cells was also inhibited by MSC, but the expression of $CD25^{+}$ on the responder cells was increased. It was recently reported that induced CD25 in effector T-cells is associated with FOXP3 expression but not with regulatory activity [27]. We therefore do not expect that the induced CD25 expression observed in posttransplant MLR correlates with the induction of regulatory cells. The explanation for the increased CD25 expression may lie in an activating effect of MSC on lymphocytes. This effect is obscured by the high proliferation rate of the responder T-lymphocytes in pretransplant MLR, which is associated with a high activation state, but becomes more clearly in posttransplant MLR where the proliferation rate is lower. Interestingly, a recent study, which found that MSC support the survival of T-cells in a quiescent state [28]. It suggests that MSC adapt their immunomodulatory function to the degree of anti-donor reactivity.

Our finding that the immunosuppressive effect of MSC is not antigen-specific suggests that the immunosuppressive capacity of MSC may have a much broader effect than the inhibition of donor-specific T-cells. An additional beneficial effect of MSC may be the reduction of inflammation in the graft due to ischemic injury. Furthermore, it has recently been demonstrated that donor-derived MSC can induce the generation of T-cells with donor-specific regulatory capacity [29]. Therefore, MSC may have potential to be used as anti-rejection and tolerance induction therapy.

Although our results suggest that both autologous and allogeneic MSC can be used for immunosuppression purposes before and after transplantation, it remains possible that allogeneic MSC induce an immune response. It is reported that MSC are hypoimmunogenic and do not activate allogeneic lymphocytes [21, 30-31]. Animal models, however, showed that rejection of allogeneic bone marrow and skin grafts can be accelerated by co-transplantation of syngeneic MSC, possibly by inducing memory T-cell responses [32-33]. It is therefore important to investigate whether MSC can also initiate anti-donor immune responses in humans.

The present study in a clinical living-kidney transplantation setting demonstrates that MSC inhibit alloreactivity before and after transplantation. MSC may be suitable as prevention therapy when applied before or during kidney transplantation, or as anti-rejection therapy after transplantation. In combination with their tissue repair function, the application of MSC in solid organ transplantation may facilitate graft acceptance and function. This needs to be confirmed in a clinical phase-I study.

Table 1. Characteristics of living-kidney transplant donor-recipient pairs and HLA-phenotype of third-party MSC and PBMC used in MLR (n=14)

MLR	HLA-phenotype donor	Donor age (years), sex (M/F)	HLA-phenotype recipient	Recipient age (years), sex (M/F)	Acute Rejection (<1 month)	HLA-phenotype of third-party MSC used in MLR	HLA-phenotype of third-party PBMC used in MLR
1	A2 A31(19) B38(16) B60(40) DR4 DR13(6)	57,F	A11 A68(28) B35 B44(12) DR4 DR13(6)	58,F	+	A1-A68(28) B7 B8 DR15(2) DR17(3)	A24(9) A32(19) B44(12) B27 DR9
2	A3 A26(10) B7 B38(16) DR4 DR17(3)	58,F	A1 A3 B7 B8 DR1 DR15(2)	58,M	+	A2 A31(19) B38(16) B60(40) DR4 DR13(6)	A24(9) A32(19) B44(12) B27 DR9
3	A1 A68(28) B7 B8 DR15(2) DR17(3)	59,F	A1 A2 B52(5) B50(21) DR10 DR15(2)	68,F	–	A1 B35-B37 DR1 DR4	A24(9) A32(19) B44(12) B27 DR9
4	A2 A26(10) B35 B41 DR13(6) DR16(2)	46,F	A29(19) A33(19) B14 B38(16) DR1 DR17(3)	48,M	+	A1 A2 B8 B35 DR3 DR14(6)	A24(9) A32(19) B44(12) B27 DR9
5	A1 A24(9) B57(17) DR7	48,F	A23(9) A26(10) B51(5) DR10 DR11(5)	54,M	–	A11 A68(28) B62(15) B35 DR14(6)	Technical failure
6	A1 A2 B8 B35 DR3 DR14(6)	60,F	A1 A3 B8 B35 DR4 DR17(3)	38,F	–	A1 A24(9) B57(17) DR7	A24(9) A68(28) B44(12) B15 DR13(6) DR9
7	A11 A33(19) B51(5) B62(15) DR7 DR15(2)	38,M	A3 B27 B52(5) DR4 DR15(2)	44,F	–	A2 B7 B27 DR4 DR17(3)	A30(19) A68(28) B8 B39(16) DR1 DR13(6)
8	A1 A2 B7 B62(15) DR4	69,M	A1 A3 B7 DR4 DR15(2)	41,M	+	A3 A26(10) B7 B38(16) DR4 DR17(3)	A24(9) A32(19) B44(12) B27 DR9
9	A2 A24(9) B13 B70 DR4 DR7	37,M	A2 A26(10) B44(12) B38(16) DR13(6) DR7	39,M	–	A11 A33(19) B51(5) B62(15) DR7 DR15(2)	A3 A30(19) B65(14) B18 DR1 DR3
10	A2 B7 B27 DR4 DR17(3)	46,M	A3 A31(19) B35 B51(5) DR1 DR4	54,M	–	A1 A2 B15 B57 DR1	A1 A28 B44(12) B14 DR11(5) DR7
11	A3 A31(19) B56(22) B57(17) DR4 DR7	58,F	A3 A29(19) B39(16) B61(40) DR3 DR7	65,M	+	A1 A2 B15 B57 DR1	A11 A32(19) B51(5) DR16(2) DR11(5)
12	A1 A30(19) B8 B13 DR4 DR7	43,F	A2 A11 B57(17) B60(40) DR4 DR7	33,F	–	A2 A11 B15 B40 DR4 DR12	A3 A24(9) B51(5) B62(15) DR12(5) DR13(6)
13	A36 A74(19) B58(17) B72(70) DR13(6)	56,F	A2 A36 B17 B53 DR13	58,M	–	A1 A24(9) B8 B18 DR1 DR11(5)	A23(9) A33(19) B44(12) B14 DR1 DR7
14	A3 A31(19) B27 B60(40) DR1 DR4	47,F	A3 B7 B37 DR3 DR4	54,M	–	A2 A11 B62(15) B60(40) DR4 DR12(5)	A1 A28 B44(12) B14 DR11(5) DR7

Table 2. Effect of MSC on the activation ($CD25^+$ expression) of responder $CD3^+$ T-cell subsets in pretransplant MLR (n=3) and posttransplant MLR (n=10)

		Before transplantation			After transplantation		
		MLR	MLR +MSC	*Fold-change*	MLR	MLR +MSC	*Fold-change*
$CD3^+$	% proliferating $CD3^+$	29	10	*2.9*	9	6	*1.5*
	% $CD3^+CD25^+$ cells	52	26	*2.0*	6	13	*0.5*
$CD4^+$	% proliferating $CD4^+$	26	10	*2.6*	9	7	*1.3*
	% $CD4_+CD25_+$ cells	58	40	*1.5*	8	17	*0.5*
$CD8^+$	% proliferating $CD8^+$	31	9	*3.4*	11	8	*1.4*
	% $CD8^+CD25^+$ cells	79	36	*2.2*	4	3	*1.3*

Flow cytometric analysis was performed on the PKH67-labeled responder cells after 7 days.

<< Table 1 and 2

REFERENCES

1. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999 Apr 2;284(5411):143-7.

2. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002 May 15;99(10):3838-43.

3. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood. 2003 May 1;101(9):3722-9.

4. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005 Feb 15;105(4):1815-22.

5. Nauta AJ, Kruisselbrink AB, Lurvink E, Willemze R, Fibbe WE. Mesenchymal stem cells inhibit generation and function of both $CD34^+$-derived and monocyte-derived dendritic cells. J Immunol. 2006 Aug 15;177(4):2080-7.

6. Batten P, Sarathchandra P, Antoniw JW, Tay SS, Lowdell MW, Taylor PM, et al. Human mesenchymal stem cells induce T cell anergy and downregulate T cell allo-responses via the TH2 pathway: relevance to tissue engineering human heart valves. Tissue Eng. 2006 Aug;12(8):2263-73.

7. Ringden O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lonnies H, et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. Transplantation. 2006 May 27;81(10):1390-7.

8. Dazzi F, van Laar JM, Cope A, Tyndall A. Cell therapy for autoimmune diseases. Arthritis Res Ther. 2007;9(2):206.

9. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol. 2002 Jan;30(1):42-8.

10. Inoue S, Popp FC, Koehl GE, Piso P, Schlitt HJ, Geissler EK, et al. Immunomodulatory effects of mesenchymal stem cells in a rat organ transplant model. Transplantation. 2006 Jun 15;81(11):1589-95.

11. Crop M, Baan C, Weimar W, Hoogduijn M. Potential of mesenchymal stem cells as immune therapy in solid-organ transplantation. Transpl Int. 2009 Apr;22(4):365-76.

12. Le Blanc K. Immunomodulatory effects of fetal and adult mesenchymal stem cells. Cytotherapy. 2003;5(6):485-9.

13. Maccario R, Moretta A, Cometa A, Montagna D, Comoli P, Locatelli F, et al. Human mesenchymal stem cells and cyclosporin a exert a synergistic suppressive effect on in vitro activation of alloantigen-specific cytotoxic lymphocytes. Biol Blood Marrow Transplant. 2005 Dec;11(12):1031-2.

14. Hoogduijn MJ, Crop MJ, Peeters AM, Van Osch GJ, Balk AH, Ijzermans JN, et al. Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. Stem Cells Dev. 2007 Aug;16(4):597-604.

15. Hoogduijn MJ, Crop MJ, Korevaar SS, Peeters AM, Eijken M, Maat LP, et al. Susceptibility of human mesenchymal stem cells to tacrolimus, mycophenolic acid, and rapamycin. Transplantation. 2008 Nov 15;86(9):1283-91.

16. Hoogduijn MJ, Crop MJ, Peeters AM, Korevaar SS, Eijken M, Drabbels JJ, et al. Donor-derived mesenchymal stem cells remain present and functional in the transplanted human heart. Am J Transplant. 2009 Jan;9(1):222-30.

17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8.

18. Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol. 2003 Jan;57(1):11-20.

19. Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, et al. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci. 2005;12(1):47-57.

20. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells. 2006 Feb;24(2):386-98.

21. Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation. 2003 Oct 27;76(8):1208-13.

22. Maccario R, Podesta M, Moretta A, Cometa A, Comoli P, Montagna D, et al. Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. Haematologica. 2005 Apr;90(4):516-25.

23. Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells. 2006 Jan;24(1):74-85.

24. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human mesenchymal stem cells modulate B-cell functions. Blood. 2006 Jan 1;107(1):367-72.

25. Hendrikx T, Van Gurp E, Mol W, Schoordijk W, Klepper M, Velthuis J, et al. Sequential analysis of $CD4^{+}CD25bright^{+}$ $FoxP3^{+}$ T-cells in renal allograft patients reveals no intrinsic defect in their regulatory capacity [abstract]. Am J Transplant 7[S2]. 2007(308).

26. Bestard O, Cruzado JM, Mestre M, Caldes A, Bas J, Carrera M, et al. Achieving donor-specific hyporesponsiveness is associated with $FOXP3^{+}$ regulatory T cell recruitment in human renal allograft infiltrates. J Immunol. 2007 Oct 1;179(7):4901-9.

27. Allan SE, Crome SQ, Crellin NK, Passerini L, Steiner TS, Bacchetta R, et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int Immunol. 2007 Apr;19(4):345-54.

28. Benvenuto F, Ferrari S, Gerdoni E, Gualandi F, Frassoni F, Pistoia V, et al. Human mesenchymal stem cells promote survival of T cells in a quiescent state. Stem Cells. 2007 Jul;25(7):1753-60.

29. Casiraghi F, Azzollini N, Cassis P, Imberti B, Morigi M, Cugini D, et al. Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells. J Immunol. 2008 Sep 15;181(6):3933-46.

30. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation. 2003 Feb 15;75(3):389-97.

31. Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp Hematol. 2003 Oct;31(10):890-6.

32. Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a non-myeloablative setting. Blood. 2006 May 11;108(6):2114-20.

33. Sbano P, Cuccia A, Mazzanti B, Urbani S, Giusti B, Lapini I, et al. Use of donor bone marrow mesenchymal stem cells for treatment of skin allograft rejection in a preclinical rat model. Arch Dermatol Res. 2008 Feb 8;300(3):115-24.

Chapter 4

Susceptibility of human mesenchymal stem cells to tacrolimus, mycophenolic acid, and rapamycin

Martin J. Hoogduijn[1], Meindert J. Crop[1], Sander S. Korevaar[1], Annemiek M.A. Peeters[1], Marco Eijken[1], Lex P.W.M. Maat[2], Aggie H.M.M. Balk[3], Willem Weimar[1], Carla C. Baan[1]

[1]Departments of Internal Medicine, [2]Thorax Surgery, and [3]Cardiology, Erasmus University Medical Center, Rotterdam, The Netherlands

Transplantation
2008 Nov 15;86(9):1283-91

ABSTRACT

Mesenchymal stem cells (MSC) have multilineage differentiation and immunomodulatory capacities and are potentially useful for therapeutic applications, such as tissue regeneration and control of alloreactivity. MSC are present in most tissues including the transplantable organs. It is therefore unavoidable that MSC will be exposed to immunosuppressive drugs in a clinical transplantation setting. The molecular targets of these drugs are expressed in MSC, but the effect of their inhibition on MSC functioning is unknown.

MSC were isolated and expanded from heart tissue and the effects of the calcineurin inhibitor tacrolimus, the cell cycle inhibitor mycophenolic acid (MPA), and the mTOR inhibitor rapamycin on MSC survival, proliferation, differentiation and immunosuppressive capacity examined.

Short-term exposure to the immunosuppressants did not induce toxicity or apoptosis in MSC, but high-dose tacrolimus induced toxicity after 7 days. MPA and rapamycin inhibited MSC proliferation at therapeutic doses. The immunosuppressants had differential effects on the differentiation capacity of MSC. Tacrolimus reduced the expression of troponin T type 2 and desmin during cardiomyogenic differentiation of MSC, whereas MPA decreased the deposition of calcified minerals during osteogenic differentiation. Rapamycin stimulated lipid production during adipogenic differentiation. Unexpectedly, MSC had adverse effects on the immunosuppressive efficacy of tacrolimus and rapamycin. There was no such effect of MSC on the function of MPA. Pre-incubation of MSC with tacrolimus increased the immunosuppressive capacity of MSC.

In conclusion, the present study demonstrates that therapeutic concentrations of immunosuppressive drugs affect MSC function. MSC affect the efficacy of immunosuppressive medication. These findings are important for potential clinical use of MSC in combination with immunosuppressants.

INTRODUCTION

Mesenchymal stem cells (MSC) are multipotent progenitor cells that reside in the bone marrow and in many other tissues, including adipose tissue, liver, spleen, lung and heart [1-4]. They can be induced to undergo rapid proliferation and differentiation into osteogenic, adipogenic, myogenic, chondrogenic, and other lineages [1, 5-6]. Furthermore, MSC have the ability to inhibit immune responses *in vitro* [7-8] and *in vivo* [9-10]. There are large expectations for the use of MSC for a variety of clinical applications, from regeneration of injured or aged tissues to inhibition of autoimmune responses and allograft rejection. It is foreseen that MSC may be used for clinical purposes in combination with immunosuppressive drugs, because potential MSC transplant recipients are on immunosuppressive medication for containment of autoimmune or allograft responses. Although MSC are considered to be low immunogenic [11], there is evidence that they are susceptible for allogeneic rejection by a competent immune system [12], suggesting a requirement for immunosuppression after allogeneic transplantation of MSC.
After solid organ transplantation, MSC that reside in transplanted kidneys, livers, lungs or hearts, but also the recipient's own MSC, are exposed to a cocktail of immunosuppressive drugs, including calcineurin and/or mammalian target of rapamycine (mTOR) inhibitors and cell cycle blockers [13]. Although intended to inhibit lymphocyte proliferation, the molecular targets for these drugs are also expressed in MSC. Calcineurin, inhibited by tacrolimus and cyclosporine, is expressed in MSC together with its downstream target nuclear factor of activated T-cells (NFAT) and plays a role in osteogenic [14] and chondrogenic differentiation of MSC [15]. The active metabolite of the cell cycle inhibitor mycophenolate mofetil, mycophenolic acid (MPA), inhibits inosine monophosphate dehydrogenase (IMPDH), which is ubiquitously expressed, and the cell proliferation inhibitor rapamycin targets mTOR, which is present in MSC and reported to be involved in osteogenic differentiation of mouse MSC [16]. The presence of the molecular targets of these immunosuppressive drugs in MSC indicates that they may interfere with cellular processes of MSC. The purpose of the present study was to investigate the effects of the calcineurin inhibitor tacrolimus, the IMPDH blocker MPA and the mTOR inhibitor rapamycin on survival, apoptosis, proliferation, differentiation and immunosuppressive capacity of human MSC.

MATERIALS AND METHODS

Cell culture

Donor atrium and ventricular tissue that became available as waste product during heart transplantation surgery at the Departmment of Thorax Surgery of the Erasmus Medical Center was collected for the isolation of MSC (approved by the medical ethical committee of the Erasmus Medical Center, protocol no. MEC-2006-190). The tissue was collected in Roswell Park Memorial Institue (RPMI) culture medium with 4mM L-glutamine, 100U/mL penicillin and 100μg/mL streptomycin (1% p/s) (all Invitrogen, UK) and minced with a scalpel knife within 24 hours. The tissue was then digested with sterile filtered 0.5 mg/mL collagenase type IV (Sigma-Aldrich, Germany) in RPMI for 30 min at 37°C under continuous stirring. After two washes in RPMI, the dissociated tissue and cells were transferred to a culture flask and cultured in minimum essential medium-alpha (MEM-α)(Invitrogen) supplemented with 15% foetal bovine serum (FBS; Biowhittacker, Belgium) and 1% p/s at 37°C, 5% CO_2 and 95% humidity.

After 3 to 4 days, non-adherent cells were removed by refreshment of the culture medium. Subsequently, medium was refreshed twice weekly and cells trypsinized using 0.05% trypsin-EDTA (Invitrogen) at subconfluency and reseeded at 1000 cells/cm^2 to ensure optimal proliferation. The cultures obtained showed immunophenotypical and functional properties characteristic for MSC, as described earlier [4]. The cells were used for experiments between passage number 2 and 7.

Immunosuppressants

Tacrolimus (Astellas, Japan) and rapamycin (Wyeth, NJ, USA) were used from soluble sources. To examine the effect of mycophenolate mofetil, its active metabolite MPA (Sigma-Aldrich) was used.

Measurement of mesenchymal stem cell viability by MTT assay

MSC were seeded in a 96-well plate at 5000 cells/well and incubated with tacrolimus (1, 10, 100 ng/mL), MPA (1, 10, 100 μg/mL) or rapamycin (0.1, 1, 10, 50 ng/mL) in standard culture medium for 24 hours and 7 days. The MTT assay was used to determine viable cell numbers. Briefly, medium was replaced with 100μL fresh medium before the assay, and 25μL of 5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) in PBS was added and incubated for 4 hours at 37°C. Dimethyl sulfoxide (DMSO) (100μL) was then added and absorbance read at 595 nm using a Victor^2 1420 multilabel counter (Wallac, MA, USA).

Detection of apoptosis

MSC were seeded in six-well plates at 200,000 cells/well and incubated with 10 ng/mL tacrolimus, 10 μg/mL MPA or 10 ng/mL rapamycin in standard culture medium for 48 hours and 7 days. The cells were then trypsinized and stained for Annexin V using an Annexin V apoptosis detection kit according to the manufacturer's protocol (BD Bioscience), and analysed on a FACSCanto II flow cytometer (BD Biosciences).

Measurement of cell proliferation

MSC were seeded in 96-well plates at 1000 cells/well and cultured in the presence or absence of tacrolimus (1, 10 ng/mL), MPA (1, 10, 100 μg/mL) or rapamycin (0.1, 1, 10, 50 ng/mL) in standard culture medium for 3 or 7 days. The cells were then pulsed with 0.5μCi ^{3}H-thymidine/well (Amersham, UK), harvested after 8 hours and radionucleotide incorporation measured using a Betaplate counter (LKB, Sweden).

Microarray analysis

Gene expression was analysed using a Miltenyi Biotec human stem cell PIQOR microarray according to the manufacturer's protocol (Miltenyi Biotech, Germany). Expression profiles were determined of four MSC cultures.

Differentiation assays

Osteogenic differentiation

Osteogenic differentiation was induced by culturing confluent MSC cultures in α-MEM supplemented with 1% p/s, 15% heat-inactivated FBS (56°C, 30 min), 5 mM β-glycerophosphate (Sigma-Aldrich, Germany), 50 μg/mL L-ascorbic acid-phosphate (Sigma-Aldrich), and 10 nM dexamethasone (Sigma-Aldrich) for 18 days.

Osteogenic differentiation was quantified by measurement of Ca^{2+} contents of the deposited minerals [17]. In brief, MSC were removed from the culture dish with a rubber policeman in 0.1% triton in PBS, and cell lysates incubated overnight in 0.24 M HCl at 4°C. Ca^{2+} content was calorimetrically determined after addition of 1 M ethanolamine buffer (pH 10.6), 0.35 mM o-cresolphtalein complexone, 19.8 mM 8-hydroxyquinoline and 0.6 mM hydrochloric acid at 595 nm. Results were adjusted for the protein content of the corresponding cell-lysates. Total protein concentration was measured using bicinchoninic acid (BCA) protein assay reagent (Pierce, IL, USA) according to the manufacturer's description.

Cardiomyogenic differentiation

MSC were seeded at 20,000 cells/cm^2 in α-MEM with 15% FBS and 1% p/s. The following day, culture medium was changed to DMEM/F12 (Invitrogen) supplemented with 2% horse serum (Invitrogen). After 7 days the cells were trypsinized, pelleted and kept at –80°C. Total RNA was isolated using the High-Pure RNA Isolation kit (Roche Applied Science, Germany), following the manufacturer's instructions and cDNA synthesized with random primers (Promega, The Netherlands). Quantitative expression of myogenic genes was determined by real-time PCR using universal PCR mix (Invitrogen) and Assay-on-demand products for troponin T type 2 (Hs00165960.m1) and desmin (Hs00157258.m1) (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7700 sequence detector (Applied Biosystems) as described before [18]. Expression levels were expressed as copies mRNA per 500 ng of total RNA.

Adipogenic differentiation

Adipogenic differentiation was induced by culturing confluent MSC cultures in α-MEM supplemented with 1% p/s, 15% heat-inactivated FBS, 50 µg/mL L-ascorbic acid-phosphate (Sigma-Aldrich), 500 µM 3-isobutyl-1-methylxanthine (Fluka, Germany), 60 µM indomethacin (Fluka) and 10 nM dexamethasone (Sigma-Aldrich) for 14 days. Cells were then fixed in 10% formalin for 5 minutes and incubated with fresh 10% formalin for 1 hour. After washing in 60% isopropanol, the cells were incubated in 0.3% oil-red-O (Sigma-Aldrich) solution in 60% isopropanol for 10 minutes to stain lipid droplets. After several washes with H_2O, the oil-red-O was eluted with 100% isopropanol for 10 min and absorption measured at 490 nm on a Victor^2 1420 multilabel counter (Wallac, MA, USA).

Mixed Lymphocyte Reaction

Peripheral blood mononuclear cells (PBMC) ($5x10^4$) were stimulated with $5x10^4$ γ-irradiated (40 Gy) fully HLA A/B/DR mismatched PBMC in round-bottom tissue culture-treated 96-well plates (Nunc, Denmark) in RPMI medium with 1% p/s and 10% heat-inactivated FBS. MSC that were pre-incubated with tacrolimus (1, 10 ng/mL), MPA (1, 10, 100 µg/mL) or rapamycin (0.1, 1, 10, 50 ng/mL) for 48 hours in standard culture medium were trypsinized and washed three times in large volumes of RPMI medium and added at a 1:100 up to a 1:2 ratio ($0.5x10^4$) to the mixed lymphocyte reactions (MLR). At day 7, the cells were pulsed with 0.5 µCi ^{3}H-thymidine (Amersham, UK) per well, harvested after 8 hours and radionucleotide incorporation measured using a Betaplate counter (LKB, Sweden).

Statistical analysis

Data were analysed for significance with Student's t-test by comparing column means with controls using Graphpad Prism software (Graphpad software, USA).

RESULTS

Cultures of MSC were established from heart tissue and were phenotypically and functionally similar to bone marrow-derived MSC, as described earlier [4]. In brief, the cells were positive for CD90, CD105, CD166 and HLA class I expression and negative for CD34, CD45, CD80, CD86 and HLA class II expression. They were capable of osteogenic, cardiomyogenic, and adipogenic differentiation and suppressed allo- and mitogen-stimulated PBMC proliferation.

Effect of tacrolimus, MPA and rapamycin on MSC viability

MSC were incubated with 1, 10 and 100 ng/mL tacrolimus; 1, 10 and 100 μg/mL MPA; and 0.1, 1, 10 and 50 ng/mL rapamycin, concentrations covering the clinical doses of these immunosuppressants. Incubation for 24 hours with the immunosuppressants had no effect on numbers of viable MSC (Figure 1A). After 7 days, 100 ng/mL tacrolimus reduced viable cell numbers significantly (Figure 1B). MPA (100 μg/mL) had a similar but milder effect.

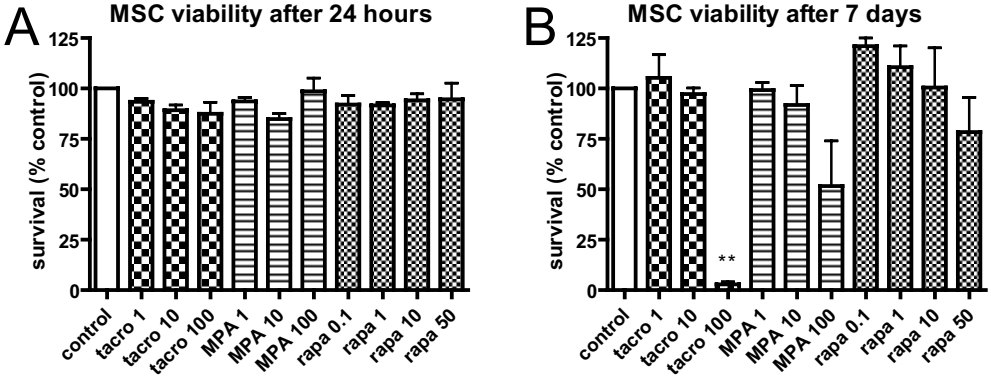


Figure 1. Effect of 24 hours and 7 days exposure to tacrolimus (ng/mL), MPA (μg/mL), and rapamycin (ng/mL) on MSC viability measured by MTT assay expressed as percentage of untreated control. Mean values with SD shown of 4 MSC cultures of passage 3 or 4 measured in five-fold.

Induction of MSC apoptosis by tacrolimus, MPA and rapamycin

The effect of therapeutic concentrations of the immunosuppressants on MSC apoptosis was tested by Annexin V staining. MSC were incubated with tacrolimus (10 ng/mL), MPA (10 μg/mL), or rapamycin (10 ng/mL) for 48 hours or 7 days, then trypsinized and stained for Annexin V. Positive Annexin V staining was detected in 12.7% (mean) of untreated MSC after 48 hours of culture (Figure 2A). The percentages of Annexin V positive cells changed to on 10.3%, 10.7% and 13% after 48 hours treatment with tacrolimus, MPA and rapamycin, respectively, but were not significantly different from untreated cells. After 7 days of culture, Annexin V positive staining increased to 21.5% in untreated cells, and 20.4%, 21.7% and 17.1% in cells treated with tacrolimus, MPA and rapamycin, respectively (Figure 2B). There were no significant differences between the treatments. Raw data of Annexin V staining of five MSC cultures treated for 48 hours and four cultures treated for 7 days are shown in Table 1.

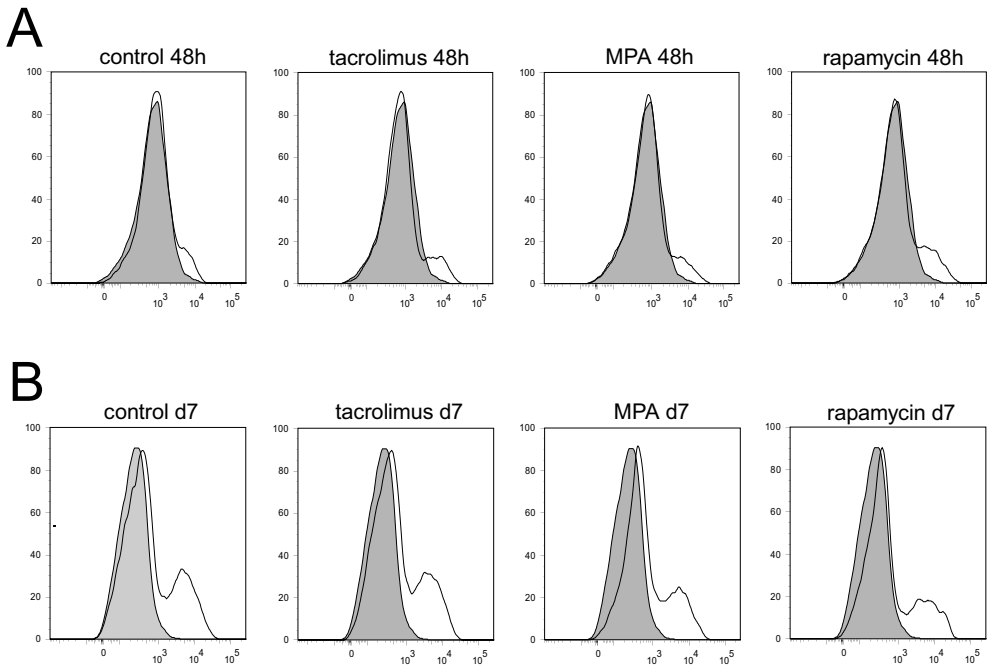


Figure 2. Annexin V staining in untreated MSC and after exposure to 10 ng/mL tacrolimus, 10 µg/mL MPA or 10 ng/mL rapamycin for 48 hours [A] and 7 days [B]. Gray histograms show unstained cells, open histograms show cells stained for Annexin V. Representative example of 5 and 4 MSC cultures (of passages 2, 3, 3, 4 and 4) examined shown. For complete data see Table 1.

Table 1. Percentage of apoptotic MSC after 48 hours or 7 days exposure to immunosuppressants measured by Annexin V staining.

Exposure time	No treatment (%)	Tacrolimus 10 ng/mL (%)	MPA 10 µg/mL (%)	Rapamycin 10 ng/mL (%)
48 hours	14.1	11.3	11.4	15.7
48 hours	17.2	5.9	13.4	11.5
48 hours	18.3	17.9	12.4	15.5
48 hours	4.5	6.9	5.6	6.9
48 hours	9.5	9.3	10.5	15.5
7 days	30.0	26.2	18.1	17
7 days	9.8	8.7	10.8	9.7
7 days	20.8	22.2	33.8	19.4
7 days	25.2	24.6	24.2	22.1

Inhibition of MSC proliferation by tacrolimus, MPA and rapamycin

The intended function of tacrolimus, MPA, and rapamycin is to inhibit lymphocyte proliferation. We examined whether these drugs had the same effect on MSC proliferation after 3- and 7-day incubations. Tacrolimus did not affect MSC proliferation after 3 or 7 days (Figure 3A, B). All concentrations of MPA significantly reduced MSC proliferation after 3 and 7 days, reaching over 90% by concentrations from 10 μg/mL. Rapamycin had a dose-dependent effect on MSC proliferation and had no effect at 0.1 and 1 ng/mL concentrations, but reduced proliferation significantly from 10 ng/mL.

MPA most potently targets IMPDH isoform II. We found strong IMPDH II expression in MSC by microarray analysis, comparable to levels of the MSC marker Thy1 (CD90) (Figure 3C).

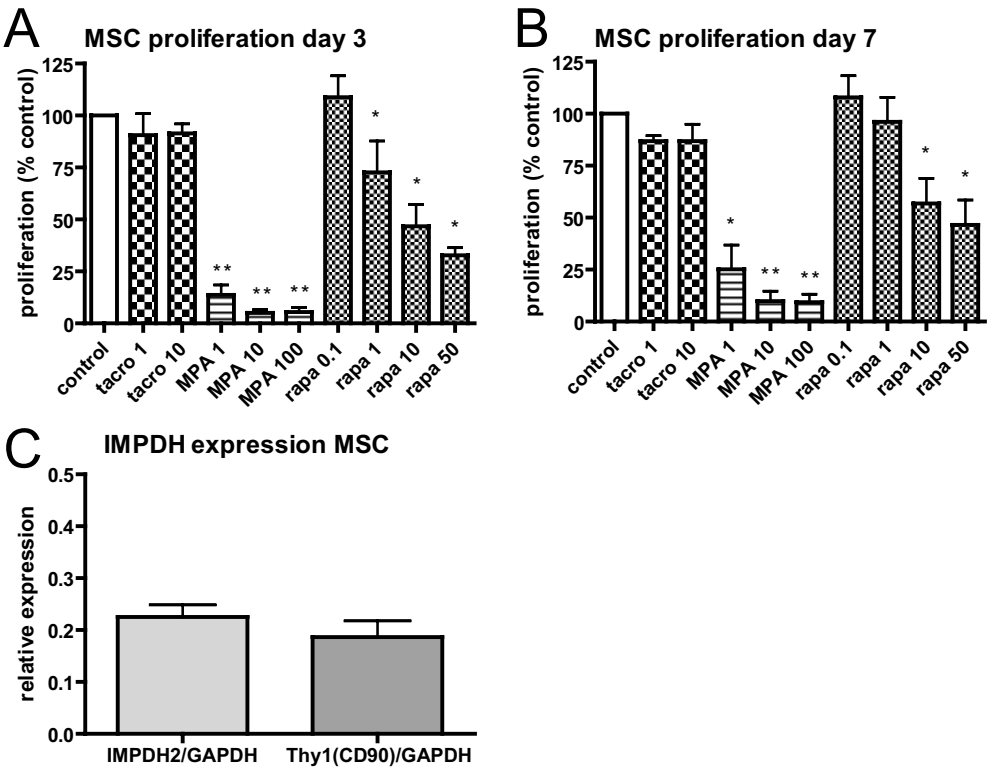


Figure 3. Effect of tacrolimus (ng/mL), MPA (μg/mL), and rapamycin (ng/mL) on MSC proliferation expressed as percentage of untreated controls. MSC were exposed for 3 days [A] or 7 days [B] to immunosuppressants and proliferation measured by 3H-thymidine incorporation. Mean values with SD shown of 3 MSC cultures of passage 3 or 4 measured in five-fold. The expression of IMPDH isoform II was determined in four MSC cultures of passage 3 or 4 and related to GAPDH expression [C]. Expression levels of the MSC marker Thy1 (CD90) are shown to demonstrate the high levels of expression of IMPDH II in MSC. * indicates $p<0.05$, ** $p<0.005$ compared with control.

Effect of tacrolimus, MPA and rapamycin on osteogenic differentiation of MSC

To determine the effect of the immunosuppressants on MSC differentiation, MSC were cultured under osteogenic, myogenic, and adipogenic conditions in the presence of different concentrations of tacrolimus, MPA, or rapamycin. Osteogenic induction resulted in significant increases in the deposition of calcified minerals. Treatment with tacrolimus and MPA tended to decrease Ca^{2+} deposition by MSC, with a significant reduction in calcified mineralization with 10 μg/mL MPA (Figure 4A). Results obtained with 100 μg/mL MPA were not further analysed as 18 days of culture in the presence of this concentration of MPA resulted in strongly reduced cell numbers, hampering the collection of consistent measurements. Rapamycin had no significant effect on Ca^{2+} deposition.

Effect of tacrolimus, MPA and rapamycin on myogenic differentiation of MSC

MSC showed relatively strong expression of troponin T type 2 and desmin before induction of myogenic differentiation. Culturing of MSC under myogenic conditions for 7 days increased the expression of troponin T type 2 by 2.2-fold. Troponin T type 2 expression was significantly reduced by 10 ng/mL tacrolimus (Figure 4B). MPA and rapamycin had no significant effect. Myogenic induction did not increase desmin expression of MSC. Tacrolimus, however, reduced desmin expression, consistent with its effects on troponin T type 2 (Figure 4C).

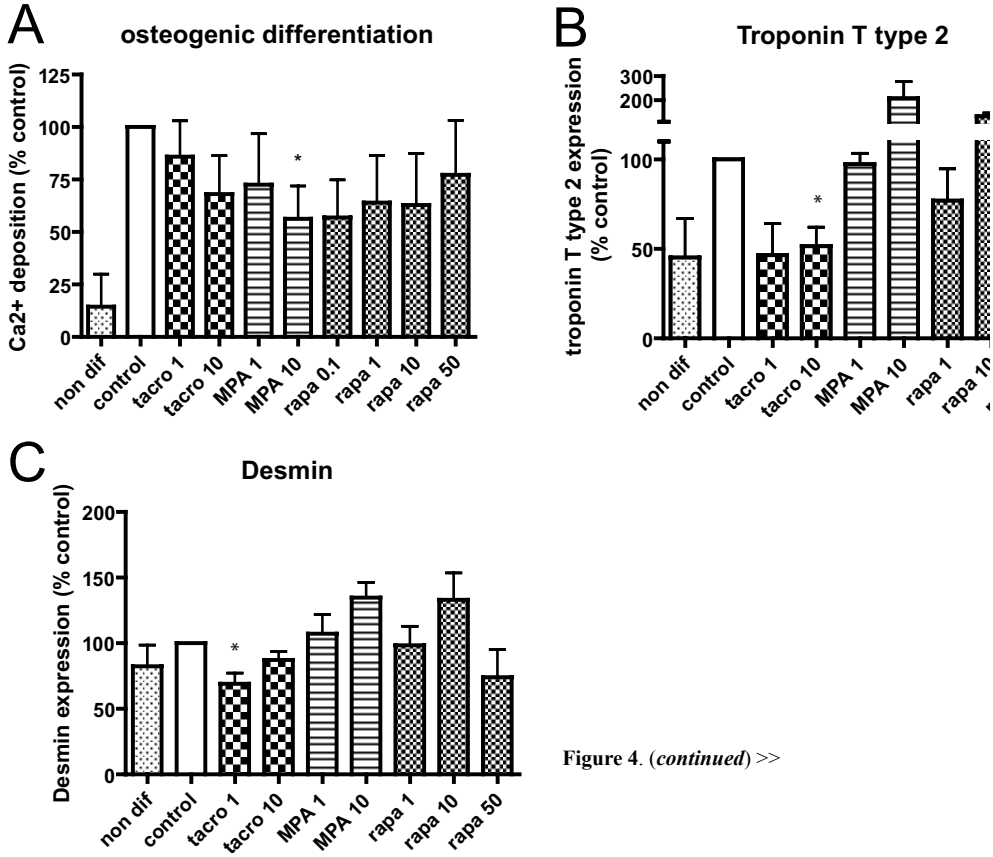
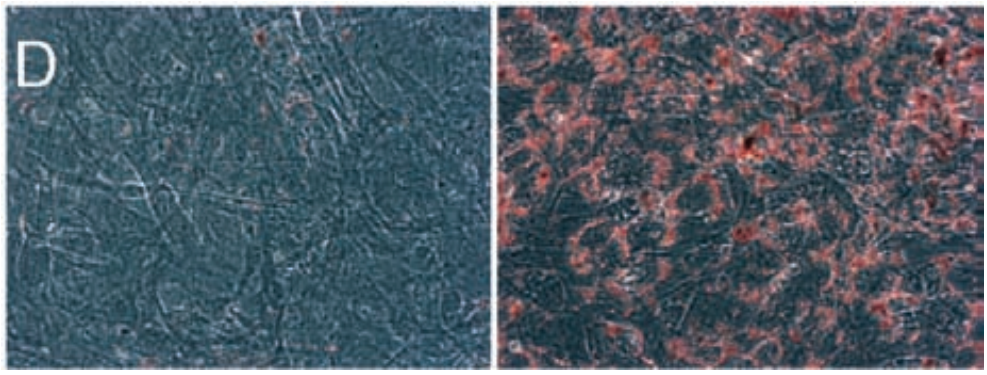


Figure 4. (***continued***) >>

Effect of tacrolimus, MPA and rapamycin on adipogenic differentiation of MSC

Adipogenic differentiation of MSC for 14 days induced the formation of lipid-filled vesicles (Figure 4D). Quantification of lipid contents demonstrated that tacrolimus and MPA had no effect on lipid production, but rapamycin significantly increased lipid production during adipogenic differentiation of MSC (Figure 4E).



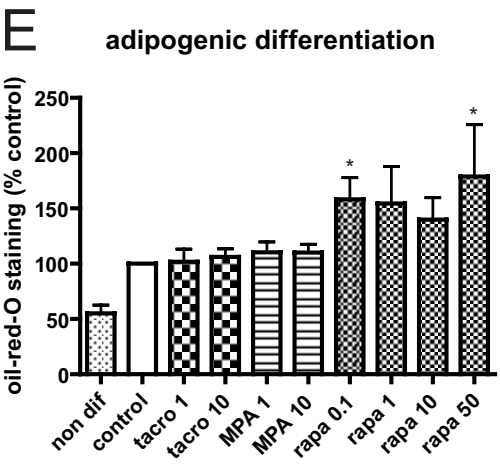


Figure 4 (*continued*). Effect of immunosuppressants on MSC differentiation. MSC were cultured in expansion medium (non dif) or under differentiating conditions (control) in the presence of tacrolimus (ng/mL), MPA (µg/mL), and rapamycin (ng/mL). After 18 days of osteogenic differentiation Ca^{2+} deposition by MSC was measured [A]. Cardiomyogenic differentiation was induced for 7 days and RNA expression of troponin T type 2 [B] and desmin analysed [C]. Adipogenic differentiation of MSC was induced for 14 days and lipid-filled vesicles stained red by oil-red-O staining [D, non-differentiated left, differentiated right] and quantified [E]. Results are shown as percentages of controls of five MSC cultures (A, B, C, passages 3, 3, 4, 4 and 7), or of three MSC cultures (E, passage 3) with SD. * indicates $p<0.05$ compared with controls.

Effect of MSC on the immunosuppressive efficacy of tacrolimus, MPA and rapamycin

MSC have the capacity to inhibit the proliferation of alloactivated PBMC in mixed lymphocyte reactions (MLR). The immunosuppressive capacity of MSC used in this study was tested by adding MSC at 1:100, 1:25, 1:10, 1:5 and 1:2 ratios to MLR and measuring proliferation after 7 days. PBMC proliferation was dose-dependently inhibited by MSC, confirming their immunosuppressive ability (Figure 5A). We then examined whether MSC had additive or adverse effects on the immunosuppressive efficacy of tacrolimus, MPA, and rapamycin. Tacrolimus dose-dependently inhibited PBMC proliferation in MLR from a concentration of 0.01 ng/mL. In the presence of MSC, however, the effect of tacrolimus was reduced and did not reach full inhibition of proliferation even at the highest tacrolimus concentration (Figure 5B). Such an effect of MSC was not seen in combination with MPA. MPA was effective at inhibiting PBMC proliferation from a 0.1 µg/mL concentration. At this concentration of MPA, the presence of MSC induced a further decrease in PBMC proliferation (Figure 5C). Rapamycin dose-dependently inhibited PBMC proliferation from a concentration of 0.01 ng/mL. Compared with tacrolimus, MSC reduced the immunosuppressive efficacy of rapamycin at all concentrations and full inhibition of PBMC proliferation with the highest doses of rapamycin was not reached in the presence of MSC (Figure 5D).

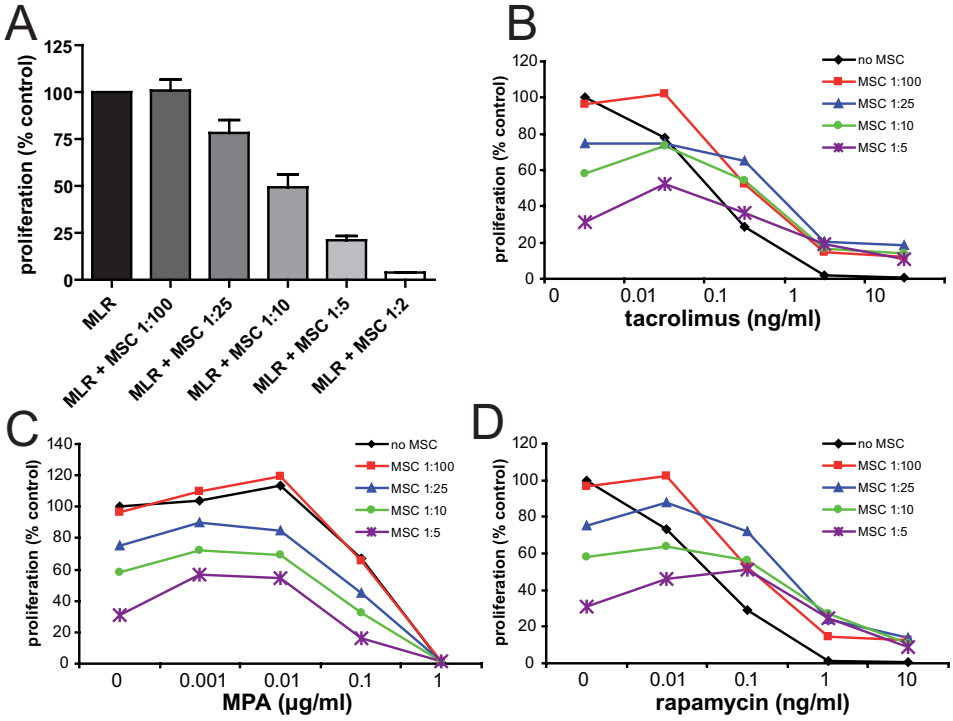


Figure 5. Effect of MSC on the efficacy of immunosuppressants. MSC have immune inhibitory capacity, as demonstrated by the addition of allogeneic MSC of passage 4 at 1:100, 1:25, 1:10, 1:5 and 1:2 ratios to mixed lymphocyte reactions (MLR) [A]. Figures B-D demonstrate the immunosuppressive efficacy of different concentrations of tacrolimus, MPA and rapamycin on MLR in combination with increasing numbers of MSC (ratio 1:100, 1:25, 1:10 and 1:5). MSC of passage 3 or 4 were added at day 0 and proliferation of MLR measured after 7 days. Results of two experiments in three-fold are shown.

Effect of tacrolimus, MPA and rapamycin on immunosuppression by MSC

To test the effect of immunosuppressants on the immune inhibitory effects of MSC, MSC were incubated with 1 and 10 ng/mL concentrations of tacrolimus; 0.1, 1 and 10 ng/mL rapamycin; or 0.1, 1 and 10 μg/mL MPA for 48 hours and after extensive washing added to MLR at a 1:10 ratio. After 7 days proliferation was measured. Untreated MSC significantly inhibited the MLR by 43%. The inhibition of MLR by MSC was increased after pre-incubation of MSC with tacrolimus (Figure 6). Pre-incubation with MPA and rapamycin had no effect on the capacity of MSC to inhibit PBMC proliferation.

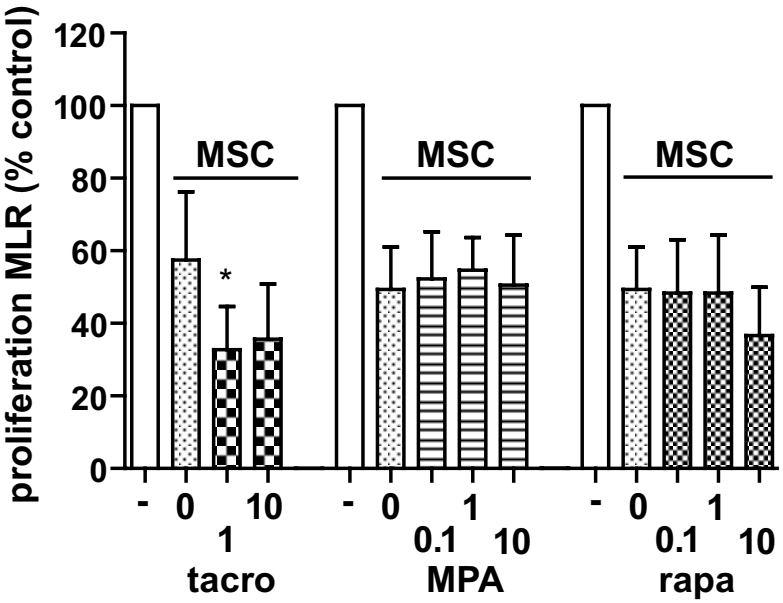


Figure 6. Effect of pre-culture of MSC with immunosuppressants on the immunosuppressive capacity of MSC in MLR. MSC of passage 3 or 4 were pre-incubated for 48 hours with tacrolimus (ng/mL), MPA (μg/mL) and rapamycin (ng/mL) and subsequently added at a 1:10 ratio to MLR. Open bars show MLR without MSC, dotted bars show effect of untreated MSC, other bars show the effect of pre-exposure of MSC to immunosuppressants. Mean values with SD shown of 4 (tacrolimus) and 3 (MPA, rapamycin) experiments measured in three-fold. * indicates $p<0.05$ compared to MLR with untreated MSC.

DISCUSSION

Immunosuppressive drugs inhibit lymphocyte proliferation by targeting a number of intracellular signalling cascades that include calcineurin, IMPDH, and mTOR [19]. These targets are not specific for lymphocytes and their presence in other cell types may explain several of the reported side effects of immunosuppressive drugs. The MSC is a relevant cell type to study these effects, because it has fundamental importance for tissue repair processes and if MSC will be used for clinical purposes, this may be in combination with immunosuppressive drugs. The molecular targets for tacrolimus and rapamycin, which bind FK506 binding protein to inhibit calcineurin and mTOR, respectively, and for MPA, which inhibits IMPDH, are expressed in MSC, suggesting that these immunosuppressants may have effects on MSC. Data from the present study confirms this. Clinical doses of the tested drugs did not induce toxicity or apoptosis of MSC, although a 10-fold increased dose of tacrolimus (100 ng/mL) induced cell death after 7 days. There were profound effects of MPA and rapamycin on MSC proliferation. MPA had the strongest effect and nearly abolished MSC proliferation from subclinical dose. The strong inhibition of MSC proliferation by MPA is remarkable, because MPA is a more potent inhibitor of the type II isoform of IMPDH, which is mainly expressed by activated lymphocytes, than of the type I isoform, which is expressed by most other cell types [20]. We found strong expression of IMPDH isoform type II by MSC, which explains the potent effect of MPA on MSC proliferation. The effect of rapamycin on MSC proliferation was milder but significant at a clinical dose. Expansion of MSC is essential for repair processes of injured tissue and the use of mTOR and IMPDH inhibitors has been linked to impaired wound healing [21-22]. In contrast, tacrolimus had no effect on MSC proliferation. In stead, calcineurin inhibition affected differentiation processes in MSC. Tacrolimus tended to inhibit, albeit not significantly, the deposition of calcified minerals during osteogenic differentiation of MSC. Reduced capacity of MSC to deposit calcium would correlate with earlier findings that show an involvement of calcineurin in the regulation of osteoblastic activity [14] and with clinical observations demonstrating that the use of calcineurin inhibitors can lead to bone loss induced by impaired bone formation [23-24]. In addition, tacrolimus reduced the expression of the cardiomyogenic genes troponin T type 2 and desmin by MSC. It is known for some time that calcineurin plays a role in myogenic differentiation [25]. It is important to consider that the use of MSC in combination with calcineurin inhibitors may not be optimal for cardiac regeneration. MPA and rapamycin showed no inhibitory effect on the expression of myogenic genes and in contrast tended to stimulate, albeit not significantly, myogenic gene expression of MSC. A possible increase in myogenic gene expression by MPA and rapamycin may relate to the anti-proliferative effects of the drugs. The myogenic differentiation protocol involves a slowdown of MSC proliferation and this effect is enforced by MPA and rapamycin. Rapamycin furthermore induced the production of lipid vesicles by MSC during adipogenic differentiation, but MPA did not have this effect, suggesting that inhibition of proliferation play no role. mTOR is a known regulator of metabolic pathways [26-27] and our results suggest that inhibition of mTOR by rapamycin stimulates lipid production during adipogenic differentiation of MSC. At this stage, we can only speculate about whether this relates to hyperlipidemia commonly observed in transplant recipients treated with mTOR inhibitors [28-29].

Whereas MPA and rapamycin affected the multilineage differentiation ability of MSC, pre-incubation of MSC with these immunosuppressants had no effect on PBMC proliferation under the experimental conditions used in this study. In contrast, pretreatment of MSC with tacrolimus increased the inhibitory effect of MSC on alloactivated PBMC proliferation. This suggests that inhibition of calcineurin has

a relatively long-lasting effect on the immunomodulatory machine of MSC, perhaps by affecting the secretion of anti-inflammatory cytokines secreted by MSC.

So far we discussed the effects of immunosuppressants on MSC functioning. There is evidence from previous studies that MSC have a synergistic effect with the calcineurin inhibitor cyclosporin on the activation of cytotoxic T-cells [30-31]. In the present study, we observed a contrasting effect. Low as well as high numbers of MSC reduced the efficacy of subclinical as well as clinical doses of tacrolimus and rapamycin to inhibit alloactivated PBMC proliferation. MSC had no adverse effect on the function of MPA. In stead, the effects of MSC and MPA seemed to be cumulative. We do not know why MSC have an adverse effect on the inhibition of PBMC proliferation by tacrolimus and rapamycin. It is possible that a dual role of MSC in the regulation of PBMC is causing this effect. Whereas MSC inhibit the proliferation of activated lymphocytes, there is evidence that they support lymphocytes that are in a quiescent state [32]. In the presence of tacrolimus and rapamycin, the activity of lymphocytes is strongly reduced, which may as a result lead to the stimulation of lymphocyte proliferation by MSC. Because MPA inhibits the process of cell division itself, MSC cannot stimulate the proliferation of lymphocytes in the presence of MPA.

This study demonstrated that immunosuppressants affect MSC that were isolated from heart tissue. Although MSC from different tissues have similar properties, there are quantitative differences between MSC from different sources [4, 33]. It is therefore possible that the results obtained with heart MSC could be different in MSC isolated from bone marrow, considered as the classical source for MSC. Furthermore, there are of course important differences between clinical and *in vitro* conditions. For instance, in the present study, MSC were exposed to immunosuppressants for a maximum of 18 days, whereas transplant recipients need life-long immunosuppression. The effects we detected on MSC could, however, be a good indication for clinical effects that can be expected after long-term exposure to immunosuppressive drugs. Summarizing, this study demonstrates that tacrolimus, MPA, and rapamycin interfere with MSC function, and that MSC affect the efficacy of tacrolimus and rapamycin. These results suggest that the immunosuppressive medication chosen for a particular group of allograft or potential MSC transplant recipients could influence the desired therapeutic effects via their actions on MSC.

REFERENCES

1. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999 Apr 2;284(5411):143-7.

2. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002 Dec;13(12):4279-95.

3. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, Noort WA, Claas FH, Willemze R, et al. Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood. 2003 Aug 15;102(4):1548-9.

4. Hoogduijn MJ, Crop MJ, Peeters AM, Van Osch GJ, Balk AH, Ijzermans JN, et al. Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. Stem Cells Dev. 2007 Aug;16(4):597-604.

5. Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow.Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation. 1968 Mar;6(2):230-47.

6. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 1997 Apr 4;276(5309):71-4.

7. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002 May 15;99(10):3838-43.

8. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood. 2003 May 1;101(9):3722-9.

9. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol. 2002 Jan;30(1):42-8.

10. Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet. 2004 May 1;363(9419):1439-41.

11. Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp Hematol. 2003 Oct;31(10):890-6.

12. Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a non-myeloablative setting. Blood. 2006 May 11;108(6):2114-20.

13. Halloran PF. Immunosuppressive drugs for kidney transplantation. N Engl J Med. 2004 Dec 23;351(26):2715-29.

14. Winslow MM, Pan M, Starbuck M, Gallo EM, Deng L, Karsenty G, et al. Calcineurin/NFAT signaling in osteoblasts regulates bone mass. Dev Cell. 2006 Jun;10(6):771-82.

15. Tomita M, Reinhold MI, Molkentin JD, Naski MC. Calcineurin and NFAT4 induce chondrogenesis. J Biol Chem. 2002 Nov 1;277(44):42214-8.

16. Singha UK, Jiang Y, Yu S, Luo M, Lu Y, Zhang J, et al. Rapamycin inhibits osteoblast proliferation and differentiation in MC3T3-E1 cells and primary mouse bone marrow stromal cells. J Cell Biochem. 2008 Feb 1;103(2):434-46.

17. Eijken M, Koedam M, van Driel M, Buurman CJ, Pols HA, van Leeuwen JP. The essential role of glucocorticoids for proper human osteoblast differentiation and matrix mineralization. Mol Cell Endocrinol. 2006 Mar 27;248(1-2):87-93.

18. Baan CC, van der Mast BJ, Klepper M, Mol WM, Peeters AM, Korevaar SS, et al. Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. Transplantation. 2005 Jul 15;80(1):110-7.

19. Taylor AL, Watson CJ, Bradley JA. Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy. Crit Rev Oncol Hematol. 2005 Oct;56(1):23-46.

20. Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. Immunopharmacology. 2000 May;47(2-3):85-118.

21. Dean PG, Lund WJ, Larson TS, Prieto M, Nyberg SL, Ishitani MB, et al. Wound-healing complications after kidney transplantation: a prospective, randomized comparison of sirolimus and tacrolimus. Transplantation. 2004 May 27;77(10):1555-61.

22. Barbari AG, Stephan AG, Masri MA. Calcineurin inhibitor-free protocols: risks and benefits. Saudi J Kidney Dis Transpl. 2007 Mar;18(1):1-23.

23. Stempfle HU, Werner C, Echtler S, Assum T, Meiser B, Angermann CE, et al. Rapid trabecular bone loss after cardiac transplantation using FK506 (tacrolimus)-based immunosuppression. Transplant Proc. 1998 Jun;30(4):1132-3.

24. Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrugghe B, Nakashima K, et al. NFAT and Osterix cooperatively regulate bone formation. Nat Med. 2005 Aug;11(8):880-5.

25. Delling U, Tureckova J, Lim HW, De Windt LJ, Rotwein P, Molkentin JD. A calcineurin-NFATc3-dependent pathway regulates skeletal muscle differentiation and slow myosin heavy-chain expression. Mol Cell Biol. 2000 Sep;20(17):6600-11.

26. Roh C, Han J, Tzatsos A, Kandror KV. Nutrient-sensing mTOR-mediated pathway regulates leptin production in isolated rat adipocytes. Am J Physiol Endocrinol Metab. 2003 Feb;284(2):E322-30.

27. Chung S, Brown JM, Sandberg MB, McIntosh M. Trans-10,cis-12 CLA increases adipocyte lipolysis and alters lipid droplet-associated proteins: role of mTOR and ERK signaling. J Lipid Res. 2005 May;46(5):885-95.

28. Brattstrom C, Wilczek H, Tyden G, Bottiger Y, Sawe J, Groth CG. Hyperlipidemia in renal transplant recipients treated with sirolimus (rapamycin). Transplantation. 1998 May 15;65(9):1272-4.

29. Lindenfeld J, Miller GG, Shakar SF, Zolty R, Lowes BD, Wolfel EE, et al. Drug therapy in the heart transplant recipient: part II: immunosuppressive drugs. Circulation. 2004 Dec 21;110(25):3858-65.

30. Maccario R, Moretta A, Cometa A, Montagna D, Comoli P, Locatelli F, et al. Human mesenchymal stem cells and cyclosporin a exert a synergistic suppressive effect on in vitro activation of alloantigen-specific cytotoxic lymphocytes. Biol Blood Marrow Transplant. 2005 Dec;11(12):1031-2.

31. Ringden O, Le Blanc K. Mesenchymal stem cells combined with cyclosporine inhibits cytotoxic T cells. Biol Blood Marrow Transplant. 2006 Jun;12(6):693-4.

32. Benvenuto F, Ferrari S, Gerdoni E, Gualandi F, Frassoni F, Pistoia V, et al. Human mesenchymal stem cells promote survival of T cells in a quiescent state. Stem Cells. 2007 Jul;25(7):1753-60.

33. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells. 2006 May;24(5):1294-301.

Chapter 5

Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells

Meindert J. Crop[1], Carla C. Baan[1], Sander S. Korevaar[1], Jan N.M. IJzermans[2], Mario Pescatori[3], Andrew P. Stubbs[3], Wilfred F.J. van IJcken[4], Marc H. Dahlke[5], Elke Eggenhofer[5], Willem Weimar[1], Martin J. Hoogduijn[1]

Departments of [1]Internal Medicine, [2]Surgery, [3]Bioinformatics, and [4]Center for Biomics, Erasmus University Medical Center, Rotterdam, The Netherlands
[5]Department of Surgery, University of Regensburg Medical Center, Regensburg, Germany

Clinical and Experimental Immunology
2010 Sep 15 (in press)

ABSTRACT

There is emerging interest in the application of mesenchymal stem cells (MSC) for the prevention and treatment of autoimmune diseases, graft-*versus*-host disease and allograft rejection. It is, however, unknown how inflammatory conditions affect phenotype and function of MSC. Adipose tissue-derived mesenchymal stem cells (ASC) were cultured with alloactivated PBMC (MLR), with pro-inflammatory cytokines (IFN-γ, TNF-α and IL-6) or under control conditions, and their full genome expression and function examined.

Pro-inflammatory cytokines mostly increased indoleamine-2,3-dioxygenase expression, whereas ASC cultured with MLR showed increased expression of COX-2, involved in prostaglandin E2 production. Both conditions had a stimulatory, but differential, effect on the expression of pro-inflammatory cytokines and chemokines, while the expression of fibrotic factors was decreased only in response to pro-inflammatory cytokines. Functional analysis demonstrated that inflammatory conditions affected morphology and proliferation of ASC, while their differentiation capacity and production of trophic factors was unaffected. The immunosuppressive capacity of ASC was enhanced strongly under inflammatory conditions. In conclusion, ASC showed enhanced immunosuppressive capacity under inflammatory conditions, while their differentiation capacity was preserved. Therefore, *in vitro* preconditioning provides ASC with improved properties for immediate clinical immune therapy.

INTRODUCTION

Mesenchymal stem cells (MSC) are found in a variety of tissues, including bone marrow, skin and adipose tissue [1-3] and can be easily expanded *in vitro*. MSC are thought to have tissue regenerative properties, in the first place via their multilineage differentiation capacity [2] and, perhaps more importantly, via the secretion of trophic factors that may activate local progenitor cells [4]. In addition, MSC have potent immunomodulatory capacity. They inhibit the proliferation of T-cells [5-6] and inhibit dendritic cell maturation [7-8]. These properties make MSC promising for a diversity of clinical applications; for example, for the prevention and treatment of autoimmune diseases and bone marrow rejection. Recently, interest has developed in the use of MSC in solid organ transplantation [9-10]. These conditions are associated with an inflammatory milieu. A restraint for therapeutic use of MSC may, however, be the limited understanding of their characterization and biology under various inflammatory conditions [10-13]. It has been reported that the immunosuppressive effects of ASC are mediated via soluble factors, and enhanced further if direct cell-cell contact between ASC and immune cells was allowed [14]. Different studies have attributed the immunosuppressive effect of MSC to different immunosuppressive factors. These include indoleamine 2,3-dioxygenase (IDO) [15-17], prostaglandin E2 [18], transforming growth factor-β (TGF-β) and hepatocyte growth factor (HGF) [5], HLA-G [19], nitric oxide [20], IL-10 [21] and heme oxygenase [22]. In addition, there is evidence that cell-membrane interactions between MSC and immune cells via the adhesion molecules ICAM-1 or VCAM-1 play a crucial role in the immunomodulatory capacity of MSC [14, 23]. Thus, the immunomodulatory capacity of MSC is a multifactorial process. The activity of these processes may depend on local immunological conditions. It has been demonstrated that in the absence of inflammation, MSC can stimulate lymphocyte survival and proliferation [24]. Under inflammatory conditions a high production of cytokines, such as IFN-γ, TNF-α and IL-6, are largely produced and MSC may respond to these factors by changing their immunomodulatory function [25-27]. Exposure of MSC to IFN-γ has been reported to up-regulate the expression of IDO, TGF-β and HGF [25, 28] and recently, it was demonstrated that IFN-γ-activated MSC are more effective for the treatment of graft-*versus*-host disease [29]. Effective application of MSC in organ transplantation may require potent and immediate immunosuppressive effects. *In vitro* activation of MSC could therefore be beneficial for clinical effectiveness of MSC in organ transplantation.

In the present study, we investigated whether different inflammatory conditions affected the gene expression, phenotype and function of adipose tissue-derived mesenchymal stem cells (ASC). ASC were cultured with alloactivated PBMC (mixed lymphocyte reaction, MLR) or with a cocktail of pro-inflammatory cytokines containing IFN-γ, TNF-α and IL-6, while their functions and full genome expression were examined.

MATERIALS AND METHODS

Isolation and culture of ASC

ASC were isolated and expanded from perirenal adipose tissue of four living healthy kidney donors, as described earlier [30-31]. These donors (3 males, 1 female, mean age 46±7 years) were approved to donate their kidney after routine screening. They did not use immunosuppressive medication.
In brief, perirenal fat was minced and digested with 0.5mg/ml collagenase type IV (Invitrogen, Paisley, UK) in RPMI (Invitrogen) for 30 min at 37°C. The cells were then washed, transferred to culture flasks and kept in MEM-α medium (Invitrogen) with 100U/ml penicillin and 100μg/ml streptomycin (p/s, Invitrogen) and 15% fetal calf serum (Biowhittaker, Verviers, Belgium) at 37°C, 5% CO_2 and 95% humidity. After three days, non-adherent cells were removed and adherent cells continued in cultured. Cultures were refreshed with ASC-culture medium twice a week. At 90% confluence, adherent cells were removed from culture flasks by incubation in 0.05% trypsin-EDTA at 37°C and cells were used for experiments or frozen at –150°C until use. ASC were used for experiments at between passages 2 to 5.
To confirm whether the perirenal fat-derived cells were indeed ASC, they were characterized by flow cytometry, differentiated in osteogenic and adipogenic lineages, and added to mixed lymphocyte reactions (MLR) to test their immunosuppressive capacity as previously described [30-31]. For independent experiments, ASC were used from different ASC donors.

Exposure of ASC to inflammatory conditions

ASC were seeded at 10,000 cells/cm^2 and cultured under two inflammatory conditions for seven days. The first condition consisted of alloactivated PBMC at a ratio of 10:1, in which the PBMC were separated from ASC by a 0.4μm pore size transwell membrane (Greiner Bio-one, Essen, Germany). The second condition consisted of a pro-inflammatory cytokine cocktail containing 50ng/ml IFN-γ (U-Cytech, Utrecht, Netherlands), 20ng/ml TNF-α (PeproTech, London, UK) and 10ng/ml IL-6 (PeproTech).

Measurement of ASC diameter and proliferation

Adherent cells were removed from culture flasks by incubation in 0.05% trypsin-EDTA at 37°C and cells put in cell counting chambers (Bürker). Cells were microscopically photographed (Axiovert 200M; Carl Zeiss, Munich, Germany) at 40x HPF Ph2. Cell diameters were measured using AxioVision software (version 4.7.1) (Carl Zeiss).
Proliferation of ASC cultured under the previously described conditions was determined by counting the living cells manually using cell counting chambers. To avoid contamination of PBMC in ASC-MLR co-cultures, transwell-membrane inserts were used (Greiner Bio-one, Alphen a/d Rijn, The Netherlands).

Flow cytometric characterization of ASC

Adherent cells were removed from culture flasks by incubation in 0.05% trypsin-EDTA at 37°C and then washed twice with FACSFlow (BD Biosciences, San Jose, CA, USA). Next, cell suspensions were incubated with antibodies against CD86-FITC, CD166-PE, HLA-DR-APC-Cy7 (all BD Biosciences), CD40-PE, CD80-PE, HLA-ABC-PE (all Serotec, Oxford, UK), CD90-APC and CD105-FITC (all R&D systems, Abingdon, UK) at RT protected from light for 30 minutes.

After two washes with FACSFLOW, flow cytometric analysis was performed using an 8-colour FACSCANTO-II with FACSDIVA Software (BD Biosciences) and FlowJo Software (Tree Star Inc., Palo Alto, CA, USA).

Differentiation capacity of ASC

Osteogenic differentiation was induced by culturing confluent ASC cultures in α-MEM supplemented with 1% p/s, 15% heat-inactivated FBS, 5mM β-glycerophosphate (Sigma-Aldrich, Munich, Germany), 50μg/ml L-ascorbic acid-phosphate (Sigma-Aldrich), and 10nM dexamethasone (Sigma-Aldrich) for 21 days. Cells were then washed with PBS and fixed in cold 4% paraformaldehyde for 5 min at room temperature. After two washes with H_2O, cells were incubated in 1% silver nitrate in H_2O at room temperature on a light box until blackening occurred. The cells were then washed three times with H_2O, incubated in 2.5% sodium thiosulfate in H_2O for 5 min at room temperature, washed twice with H_2O and photographed.

Adipogenic differentiation was induced by culturing confluent ASC cultures in α-MEM supplemented with 1% p/s, 15% heat-inactivated FBS, 50μg/ml L-ascorbic acid-phosphate (Sigma-Aldrich), 500μM 3-isobutyl-1-methylxanthine (IBMX, Fluka, Buchs, Switzerland), 60μM indomethacin (Fluka) and 10nM dexamethasone (Sigma-Aldrich) for 21 days. Cells were then fixed in 60% isopropanol for 1 min, and incubated in filtered 0.3% oil red O (Sigma-Aldrich) solution in 60% isopropanol for 10 minutes to stain lipid droplets. After several washes with PBS the cells were photographed.

Isolation of PBMC

PBMC were isolated from buffy coats of healthy volunteers using Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden) separation and stored at -135°C until use.

Mixed lymphocyte reactions (MLR)

Immunosuppressive capacity of precultured ASC was tested in MLR. In MLR, $5x10^4$ responder PBMC were stimulated by $5x10^4$ γ-irradiated (40Gy) allogeneic PBMC in RPMI+10% HI-FBS in round-bottom 96-well plates (Nunc, Roskilde, Denmark). ASC were added at the beginning (day 0) or at the end (day 6) of the seven-day MLR to responder cells at a 1:5-ratio. On day 7, proliferation was measured following incorporation of ^{3}H-thymidine (0.5μCi/well) during a 16-hour incubation using a β-plate reader. To determine the proliferation capacity of the PBMC, $5x10^4$ cells were stimulated with 1 μg/ml PHA for 3 days and ^{3}H-thymidine incorporation was measured.

To determine the importance of IDO in the immunosuppressive effect of the ASC precultured under the different conditions, ASC were added to MLR, as describe above, with addition of the IDO1-inhibitor 1-methyl-L-tryptophan (1-MT) (Sigma-Aldrich). 1-MT was prepared by dissolving in 1 M hydrochloric acid and diluted in RPMI+10% heat-inactivated FBS. Finally, the pH of the solution was neutralized by adding 1M sodium hydroxide. The solution was filtered before use.

Isolation of RNA and gene expression analysis

ASC of four healthy donors were seeded at passage four at 10,000 cells/ cm^2. The cells were cultured for seven days under control conditions or with alloactivated PBMC (separated by a transwell membrane), or in the presence of the pro-inflammatory cytokine cocktail. ASC were then harvested by trypsinization and RNA isolated using MINI columns (Qiagen, Valencia, CA, USA). The RNA quality and quantity was assessed using the RNA 6000 Nano kit on a 2100 Bioanalyzer (Agilent, Palo

Alto, CA, USA). Samples with RNA integrity numbers of >8 were selected. 100ng of total RNA was used to prepare sense-strand biotinylated DNA according to the manufacturer's ''Whole transcript sense target labeling'' protocol (Affymetrix, Santa Clara, CA, USA). Hybridization to Affymetrix Human Gene 1.0 ST Arrays (764,885 probe sets, representing 28,869 annotated genes), staining, washing, and scanning (Scanner 3000) procedures were performed as described by Affymetrix (Affymetrix) and performed by Erasmus MC Center for Biomics. Probe set summarization, Array QC, and annotations of the probesets were performed using Affymetrix "Gene Expression Consolle" (Affymetrix, Santa Clara, CA). All of the different QC metrics analyzed met the standards as required by Affymetrix and showed an overall comparability of the signal distribution obtained from the different arrays. Principal Component Analysis was used to asses the underlying structure of the dataset and define correlation relationships among samples. (Partek Inc., St. Louis, USA). Probesets differentially expressed among conditions were identified using the class comparison tool implemented in BRB ArrayTools (National Cancer Institute, Bethesda, USA). Briefly, we identified genes that were differentially expressed among the two classes using a random-variance t-test. The random-variance t-test is an improvement over the standard separate t-test as it permits sharing information among genes about within-class variation without assuming that all genes have the same variance. Genes were considered statistically significant if their p value was less than 0.0001. A stringent significance threshold was used to limit the number of false positive findings. A "per gene" estimate of the false discovery rates among genes passing the test was also computed. The false discovery rate associated with a row of the table is an estimate of the proportion of the genes with univariate p values less than or equal to the one in that row that represent false positives. The Benjamini-Hochberg method for false discovery rate control was used for this estimation [32-33]. Genes passing the test threshold were clustered and displayed as heatmap using Spotfire (Spotfire Inc., Somerville, USA). The change in gene expression of a number of genes (IDO, IL6, IL8, CXCL10) as measured by microarray was confirmed by real-time RT-PCR. In brief, ASC were precultured under control, MLR (in transwell culture systems) or cytokine conditions and trypsinized at day 7. Total RNA was isolated and cDNA synthesized as previously described [34]. Quantitative gene expression was determined using TaqMan Universal PCR Master Mix and assays-on-demand for IDO (Hs 00158027.m1), IL6 (Hs 00174131. m1), IL8 (Hs00174114.m1) and CXCL10 (Hs 00171042.m1) (all Applied Biosystems, CA, USA) on a StepOnePlus (Applied Biosystems).

Statistical analysis

Data were analyzed using paired t-test or Wilcoxon signed-rank test depending on the distribution of the data as tested with Kolmogorov-Smirnov test for normality. Parametric data are expressed as mean±SD, while non-parametric data are expressed as median (interquartile range). Statistical significance was defined as $p<0.05$ (two-tailed).

RESULTS

Effect of inflammatory conditions on ASC gene expression

To investigate the effect of inflammatory conditions on ASC gene expression, ASC were cultured with alloactivated PBMC or pro-inflammatory cytokines and full genome expression analysis carried out by microarray. ASC were cultured for seven days under control conditions and inflammatory conditions; either with alloactivated PBMC (MLR) separated by a transwell membrane or with a pro-inflammatory cytokine cocktail containing IFN-γ, TNF-α and IL-6. The gene expression profiles of ASC derived from four different non-pooled donors showed strong clustering within the different treatment groups, as evidenced in Figure 1 and Table 1. ASC that were cultured in the presence of MLR for seven days showed significant up-regulation of 233 genes and down-regulation of 334 genes compared to ASC cultured under control conditions. ASC that were cultured in the presence of pro-inflammatory cytokines showed significant up-regulation of 635 genes and down-regulation of 296 genes. Hierarchical clustering demonstrated that gene expression changes in response to both inflammatory stimuli only partly overlapped (Figure 1A and 1B), indicating that ASC respond in a significantly different manner to alloactivated PBMC then to pro-inflammatory cytokines. This was evidenced further by the comparison of ASC cultured with MLR with ASC cultured with cytokines, which resulted in the identification of 1080 genes that showed significantly different expression (Figure 1C). The most significant changes in gene expression are described below. In addition, real-time RT-PCR analysis on four relevant genes (IDO, IL6, IL8 and CXCL10) was performed to confirm the data obtained by microarray (data not shown). The pattern of gene expression changes was similar in microarray and RT-PCR analysis. Only the increase in IDO expression in ASC with MLR was a lot larger in the RT-PCR analysis than in the microarray analysis.

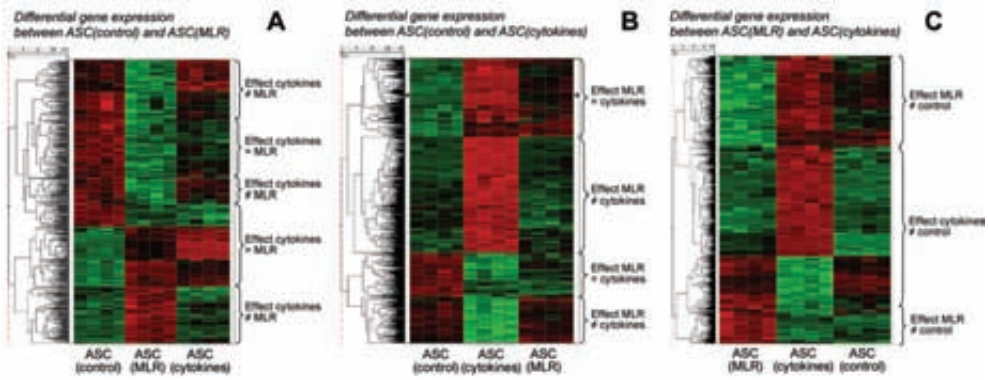


Figure 1. Hierarchical clustering of genes up (red) and down (green) regulated by ASC after culture under inflammatory conditions compared to average gene expression level of all samples, measured by microarray. ASC were cultured under control conditions, with alloactivated PBMC (MLR) or with pro-inflammatory cytokines (n=4). Microarray data is deposited in Gene Expression Omnibus (GEO), number GSE18662 at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18662. [A] Gene expression profile of ASC cultured with MLR compared to control ASC. ASC cultured with MLR showed partial overlap with ASC cultured with pro-inflammatory cytokines. [B] Gene expression profile of ASC cultured with pro-inflammatory cytokines compared to control ASC. ASC cultured with pro-inflammatory cytokines showed partial overlap with ASC cultured with MLR. [C] Gene expression profile of ASC cultured with MLR compared to ASC cultured with pro-inflammatory cytokines. ASC cultured with inflammatory conditions showed partial overlap with control ASC.

Table 1. Gene expression data of ASC cultured under control conditions or with inflammatory conditions.

Genes	Intensity ASC(control) (mean±SD) (log^2)	Intensity ASC(MLR) (mean±SD) (log^2)	Intensity ASC(cytokines) (mean±SD) (log^2)	Fold change intensity ASC(MLR) vs ASC(control)	Fold change intensity ASC(cytokines) vs ASC(control)	Difference ASC(MLR) vs ASC(cytokines) (p<0.0001)
Immunosuppressive factors						
HLA-G	11.8±0.2	12.7±0.2	13.5±0.1	1.8*	3.3*	-
COX-2	8.0±0.4	11.3±0.4	8.5±0.3	10.2	1.5	+
IDO	4.1±0.1	5.6±0.7	12.8±0.1	2.9	394.1*	+
HGF	5.2±0.3	4.6±0.3	7.1±0.3	0.7	3.7*	+
Guanylate binding proteins (GBP)						
GBP1	9.1±0.4	10.9±0.3	12.9±0.1	3.6*	14*	+
GBP2	6.5±0.2	8.9±0.5	11.0±0.1	5.3*	22.2*	+
GBP3	8.2±0.3	9.0±0.8	11.0±0.4	1.8	7.3*	+
GBP4	5.1±0.3	7.7±0.9	11.8±0.1	6.1*	102.0*	+
GBP5	4.5±0.1	6.5±1.1	12.7±0.2	3.9	290.5*	+
GBP6	4.1±0.1	4.6±0.3	6.7±0.4	1.5	6.3*	+
GBP7	4.9±0.1	5.4±0.2	5.6±0.2	1.4	1.7	-
Myxovirus resistance genes						
1	6.2±.0.5	6.8±0.3	10.5±0.2	1.5	19.1*	+
2	6.4±0.5	6.4±0.1	9.7±0.2	1,0	9.7*	+
Pro-inflammatory factors						
IL1-α	5.8±0.6	7.4±0.9	4.0±0.5	3.1	0.3*	+
IL-1β	6.3±0.4	9.1±0.7	5.4±0.2	7.1*	0.5*	+
IL-6	11.9±0.2	13.1±0.1	11.9±0.1	2.2*	1,0	+
IL-8	6.6±0.4	12.1±0.4	8.5±0.4	45.6*	3.7	+
TNF-10	4.5±0.4	5.0±0.2	10.3±0.1	1.4	53.4*	+
TNF-13b	5.2±0.2	5.8±0.2	8.7±0.3	1.5	11.2*	+
IL-33	6.0±0.4	9.3±0.4	6.8±0.4	10.5*	1.8	+
Serum amyloid						
1	5.6±0.3	10.6±0.4	6.6±1.1	31.3*	1.9	+
2	5.9±0.3	10.2±0.3	6.2±0.5	20.0*	1.2	+
HLA class I						
A	12.1±0.1	12.9±0.1	13.7±0.1	1.7*	3.0*	-
B	12.0±0.2	13.0±0.2	13.9±0.1	2.0*	3.5*	-
C	12.0±0.1	12.7±0.1	13.6±0.1	1.7*	3.2*	+
E	10.7±0.1	11.8±0.2	12.7±0.1	2.1*	3.8*	+
F	6.9±0.1	8.0±0.1	9.4±0.1	2.2*	5.9*	+
G	11.8±0.2	12.7±0.2	13.5±0.1	1.8*	3.3*	-
HLA class II						
DPα1	7.4±0.8	7.8±1.0	12.9±0.3	1.3	44.9*	+
DPβ1	6.8±0.4	7.0±0.3	11.7±0.3	1.1	29.4*	+
DQα1	6.0±0.1	6.3±0.2	12.3±1.7	1.2	78.9*	+

* = p< 0.0001

>> **Table 1 (*continued*)**

Genes	Intensity ASC(control) (mean±SD) (log^2)	Intensity ASC(MLR) (mean±SD) (log^2)	Intensity ASC(cytokines) (mean±SD) (log^2)	Fold change intensity ASC(MLR) vs ASC(control)	Fold change intensity ASC(cytokines) vs ASC(control)	Difference ASC(MLR) vs ASC(cytokines)
Chemokine ligands (CXCL)						
CXCL1	7.3±0.4	11.4±0.3	10.0±0.2	17.7*	6.7*	+
CXCL3	6.4±0.2	7.9±0.4	6.4±0.2	2.8	1,0	-
CXCL5	8.3±0.4	11.6±0.7	8.8±0.2	10,0	1.4	+
CXCL6	6.3±0.5	10.7±0.5	9.5±0.3	21.2*	9.4*	-
CXCL9	4.0±0.2	4.1±0.1	11.7±0.1	1.1	209.0*	+
CXCL10	3.7±0.4	6.5±4.8	12.7±0.2	7,0	521.9*	+
CXCL11	4.0±0.3	4.8±0.8	12.0±0.2	1.8	250.7*	+
CXCL12	9.9±0.6	8.9±0.4	11.1±0.3	0.5*	2.3	-
Chemokine ligands (CCL)						
CCL2	10.2±0.3	12.2±0.2	12.4±0.1	3.9*	4.5*	-
CCL5	6.8±0.6	9.6±0.6	11.6±0.4	7.1*	27.4*	+
CCL7	4.9±0.1	6.3±0.4	9.1±0.7	2.5	17.2*	+
CCL8	4.9±0.2	5.8±0.6	10.2±0.9	1.9	40.7*	+
CCL13	6.2±0.4	8.8±0.9	9.8±0.7	5.9*	12.1*	-
CCL20	5.0±0.1	8.1±0.2	5.1±0.4	8.1*	1,0	+
CCL28	5.3±0.1	6.7±0.3	5.9±0.2	2.5*	1.4	-
Collagens						
Iα1	13.9±0.1	13.4±0.1	11.1±0.4	0.7	0.1*	+
Iα2	13.8±0.1	13.5±0.1	12.0±0.3	0.8	0.3*	+
IIα1	7.6±0.1	7.6±0.1	6.7±0.1	1,0	0.5*	+
IIIα1	13.0±0.1	13.4±0.1	10.4±0.4	1.2	0.2*	+
IVα1	11.4±0.2	11.4±0.2	8.9±0.3	1,0	0.2*	+
IVα2	10.3±0.2	10.1±0.3	8.9±0.3	0.9	0.4*	+
Vα1	10.2±0.1	9.4±0.1	7.6±0.1	0.6	0.2*	+
Vα2	11.5±0.1	11.5±0.1	9.4±0.3	1,0	0.2*	+
VIα3	11.2±0.3	10.9±0.2	9.8±0.4	0.8	0.4*	+
XIIα1	12.4±0.1	11.6±0.1	9.6±0.3	0.6	0.1*	+
XIVα1	7.6±0.4	5.8±0.3	5.6±0.2	0.3	0.2*	-
XVα1	10.6±0.6	9.1±0.8	8.0±0.7	0.4*	0.2*	-
XVIα1	8.7±0.1	8.1±0.2	7.6±0.1	0.7	0.5*	-

* = p< 0.0001

Immunosuppressive factors

It is well recognized that multiple factors are involved in the immunosuppressive function of ASC [5, 15, 18-19]. In our hands, there was no up-regulation of the anti-inflammatory factors IL-10, TGF-β, iNOS or heme oxygenase by ASC after culture with MLR or pro-inflammatory cytokines. There was minor up-regulation of HGF (4-fold) and HLA-G (3-fold) (Figure 2A). However, IDO expression was 394-fold increased by ASC cultured with the pro-inflammatory cytokines. The increase in IDO expression was significantly smaller in ASC cultured with MLR (3-fold). In contrast, ASC cultured with MLR had 10-fold increased levels of COX-2, which may result in increased production of anti-inflammatory prostaglandin E2. Increased COX-2 expression was not seen in ASC cultured with pro-inflammatory cytokines.

Exposure of ASC to the inflammatory conditions resulted in strong induction of genes for guanylate binding proteins (GBP) and myxovirus resistance genes, which have antiviral and antimicrobial function. The largest increases were observed for GBP5 (291-fold), GBP4 (102-fold), GBP2 (22-fold) and GBP1 (14-fold) in ASC cultured with pro-inflammatory cytokines (Figure 2B). In addition, ASC cultured with pro-inflammatory cytokines strongly up-regulated the expression of myxovirus resistance genes 1 (19-fold) and 2 (10-fold) (Figure 2C). This increase in expression was not observed in ASC cultured with MLR.

Pro-inflammatory factors

Although ASC can exert immunosuppressive activity, they also express genes for pro-inflammatory factors (Figure 2D). IL-6 was expressed highly under all culture conditions. After exposure of ASC to alloactivated PBMC, we found a 46-fold up-regulation of IL-8, while also the expression of IL-1β (7-fold) and IL-33 (11-fold) increased. In contrast, culture of ASC with pro-inflammatory cytokines up-regulated the expression of tumor necrosis factor superfamily (TNFSF) member 10 and member 13B by a factor 53 and 11, respectively. ASC did not express IL-2. Serum amyloid A1 and A2, factors produced by the liver in response to inflammatory stimuli, showed strongly increased gene expression after culture of ASC with alloactivated PBMC (31-fold and 20-fold, respectively) (Figure 2E), while these factors were not up-regulated in ASC cultured with pro-inflammatory cytokines.

HLA expression

ASC expressed high levels of HLA class I, whereas HLA class II levels were low under control conditions (Figure 2F and 2G). In the presence of alloactivated PBMC, HLA class I expression by ASC was increased slightly (2-fold) and HLA class II expression did not change significantly. In contrast, ASC cultured with pro-inflammatory cytokines up-regulated the expression of HLA class I genes up to 6-fold and HLA class II up to 144-fold.

Chemokines

Next, the effect of inflammatory conditions on the chemo-attractive properties of ASC was examined. Culture of ASC with MLR or pro-inflammatory cytokines induced differential expression of several chemokines. ASC cultured with MLR increased the expression of the neutrophil, monocyte and eosinophil attractants CXCL1 (18-fold) and CXCL6 (21-fold) (Figure 2H). ASC cultured with pro-inflammatory cytokines showed strong increases in the expression of the T lymphocyte attractants CXCL9 (209-fold), CXCL10 (522-fold), CXCL11 (251-fold), whereas the neutrophil, monocyte, eosinophil attractants CXCL1 and CXCL6 showed weaker increases (7-fold and 9-fold).

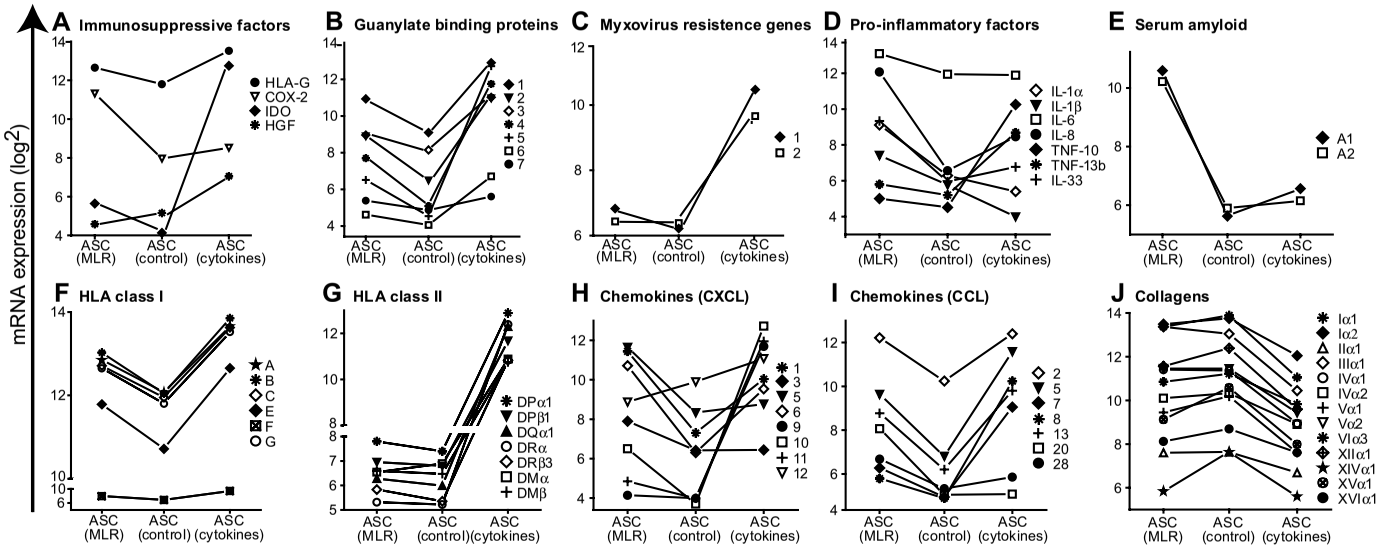


Figure 2. Gene expression of ASC cultured under control conditions, with alloactivated PBMC (MLR) or with pro-inflammatory cytokines for seven days. mRNA expression is shown as log2-transformed median fluorescence intensities of four independent samples as measured by microarray. [A] Immunosuppressive factors; [B] Guanylate binding proteins; [C] Myxovirus resistance genes; [D] Pro-inflammatory factors; [E] Serum amyloid; [F] HLA class I; [G] HLA class II; [H] Chemokine ligands (CXCL); [I] Chemokine ligands (CCL); [J] Collagens.

>> Figure 2

Chemokines of the CCL-motive were also induced specifically by ASC depending on the inflammatory stimulus (Figure 2I). In ASC cultured with MLR the expression of CCL2 (4-fold), CCL5 (7-fold), CCL13 (6-fold), CCL20 (8-fold) and CCL28 (3-fold) was increased significantly compared to control ASC. Culture of ASC with the pro-inflammatory cytokines strongly increased the expression of CCL2 (5-fold), CCL5 (27-fold), CCL7 (17-fold), CCL8 (41-fold) and CCL13 (12-fold), but had no effect on the lymphocyte attractants CCL20 and CCL28.

Fibrosis

To investigate whether inflammatory conditions stimulate ASC to induce fibrosis, we examined the expression of collagens by ASC. Pro-inflammatory cytokines significantly reduced the expression of 13 of a total of 45 types of collagens (Figure 2J). Culture of ASC with MLR reduced expression of collagen type 15α1 only (3-fold). ASC may also induce fibrosis via the secretion of factors such as connective tissue growth factor, TGF-β and platelet-derived growth factor that act on other cell types. The expression of these factors by ASC, however, did not change in response to inflammatory conditions. Furthermore, except from small increases in actin α1 (0.2-fold) and actin γ2 (2-fold) after culture with MLR, no significant changes in gene expression of cytoskeletal proteins such as actins or intermediate filaments were observed in ASC after exposure to pro-inflammatory conditions.

Effect of inflammatory conditions on the phenotype of ASC

Next, functional analysis of ASC cultured under inflammatory conditions was performed. ASC cultured under inflammatory conditions showed morphological changes compared to ASC cultured under control conditions (Figure 3A). ASC cultured under control conditions grew in a monolayer and were distributed equally on the surface of the culture flask, while ASC cultured with alloactivated PBMC clustered in star-shaped formations.

The number of ASC cultured for seven days with MLR increased compared to control ASC cultures (Figure 3B). In contrast, the number of ASC treated with pro-inflammatory cytokines was reduced significantly.

Culture of ASC with MLR or pro-inflammatory cytokines increased significantly the diameter of ASC (Figure 3C). ASC cultured under control conditions had a diameter of 21 (interquartile range 19-25) μm. After culture with MLR, ASC had a diameter of 24 (22-28) μm and treatment of ASC with inflammatory cytokines led to an increase in cell diameter to 29 (25-32) μm.

To investigate whether the immunophenotype of ASC changed after culture with inflammatory factors, flow cytometric analysis was performed (Figure 3D). ASC expressed the characteristic cell surface markers CD90, CD105 and CD166 and the expression of these markers was unaffected by culture of ASC with MLR or pro-inflammatory cytokines. Levels of HLA class I expression by ASC were independent of inflammatory culture conditions. Control ASC were slightly positive for HLA class II (6%), while culture of ASC with MLR or pro-inflammatory cytokines resulted in an increase in HLA class II positive cells of 62% and 86%, respectively. Independently of culture conditions, ASC stained positive for the co-stimulatory molecule CD80 and were weakly positive for CD86. CD40 was not expressed on control or MLR-cultured ASC, but culture of ASC with pro-inflammatory cytokines induced expression of CD40.

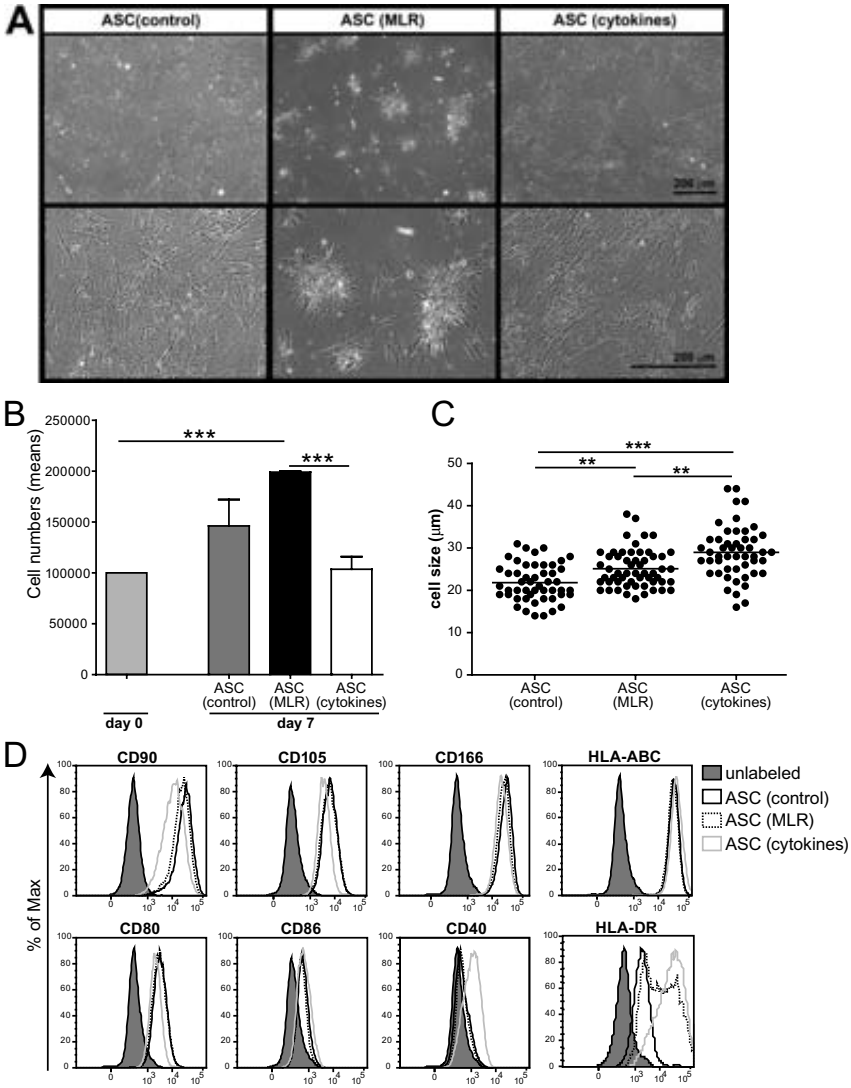


Figure 3. Effect of inflammatory conditions on the phenotype of ASC. ASC were cultured under control conditions, with alloactivated PBMC (MLR) in transwell culture systems, or with pro-inflammatory cytokines. For each independent experiment, ASC were used from different ASC donors. [A] Morphology of ASC. ASC cultured under control conditions and with pro-inflammatory cytokines formed confluent monolayers, while ASC co-cultured with MLR formed star-like clusters of cells. Representative examples shown of four experiments with four different ASC cultures. [B] Proliferation of ASC. Absolute number of viable ASC was counted after seven days in culture. Data represent the mean±SEM of six experiments with six different ASC cultures. [C] Cell size of ASC. ASC cultured with MLR and pro-inflammatory cytokines significantly increased in size compared to ASC cultured under control conditions. Approximately 50 individual trypsinized ASC per culture condition were measured. [D] Flow cytometric analysis of ASC. One representative example of each condition is shown of three independent experiments using three different ASC cultures. * = $p<0.05$; ** = $p<0.01$; ***= $p<0.001$.

Effect of inflammatory conditions on the differentiation capacity of ASC

ASC cultured previously for seven days under inflammatory conditions, were cultured under adipogenic and osteogenic conditions for three weeks (Figure 4). Independent of previous culture conditions, ASC were able to differentiate in adipogenic and osteogenic lineages.

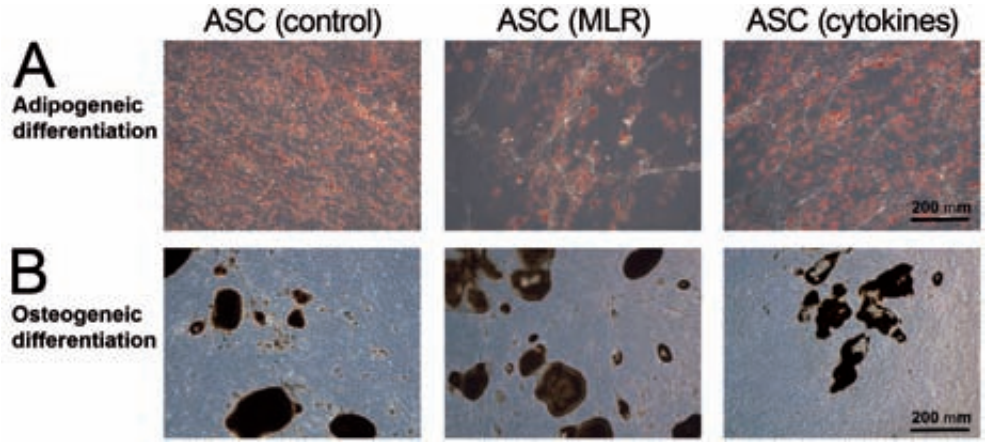


Figure 4. Effect of inflammatory conditions on the differentiation capacity of ASC. ASC cultured under control conditions, with MLR or with pro-inflammatory cytokines were continued in culture for three weeks under osteogenic or adipogenic conditions. One representative example is shown of four experiments using four different ASC donors. [A] Osteogenic differentiation. Deposition of calcified nodules (brown/black) was visualized by von Kossa staining. [B] Adipogenic differentiation. Lipid droplets (red) were stained with oil-red-O.

Effect of inflammatory conditions on the immunosuppressive capacity of ASC

To examine the effect of inflammatory conditions on the immunosuppressive capacity of ASC, pretreated ASC were added to mitogen (PHA)-stimulated or alloactivated PBMC at different concentrations. At an ASC-PBMC ratio of 1:5, ASC inhibited PHA-stimulated PBMC proliferation significantly after three days (Figure 5A). At this ratio, ASC cultured under control conditions inhibited the PHA-stimulated proliferation by 50±26%, ASC precultured with MLR by 59±6% and ASC precultured with pro-inflammatory cytokines by 84±9%. At lower concentrations (1:20 and 1:50), ASC precultured with pro-inflammatory cytokines were still able to inhibit significantly the proliferation of PHA-stimulated PBMC by 36±27% and 20±20%, respectively, whereas ASC cultured under control conditions or with alloactivated PBMC did not show this capacity.

Comparable effects of pretreatment conditions on the immunosuppressive capacity of ASC were observed when precultured ASC were added to MLR for seven days (Figure 5B). At an ASC-PBMC ratio of 1:5, ASC cultured under control conditions inhibited the proliferation of alloactivated PBMC by 44±25%, but this effect disappeared at a 1:20 ratio, and at a ratio of 1:50 they even stimulated the proliferation. ASC cultured previously with MLR inhibited the proliferation by 55±3% (at 1:5-ratio). At lower concentrations (1:20 or 1:50), ASC precultured with MLR had no inhibitory effects. ASC precultured with MLR, however, did not stimulate the proliferation as observed with control ASC.

Pretreatment of ASC with pro-inflammatory cytokines increased further the immunosuppressive capacity of ASC. At a ratio of 1:5 to responder cells, these pretreated ASC inhibited the proliferation in MLR by 76±18%. Their immunosuppressive effect was still present at lower ratios and the proliferation of alloactivated PBMC was inhibited by 42±35% and 32±27% at a ratio of 1:20 and 1:50, respectively.

To examine whether the anti-proliferative effect of ASC was instant, ASC were added on day 6 of a seven-day MLR at a 1:5 ratio (Figure 5C). Addition of control and MLR-precultured ASC did not inhibit, but stimulated, the proliferation of responder cells in MLR by 26±21% and 24±19%, respectively. In contrast, ASC pretreated with pro-inflammatory cytokines inhibited PBMC proliferation by 25±14% during the final day of the seven-day MLR ($p<0.001$). Thus, pretreatment with MLR increased the capacity of ASC to inhibit the proliferation of mitogen and alloactivated PBMC. Pretreatment of ASC with pro-inflammatory cytokines resulted in even stronger and instant immunosuppressive function of ASC.

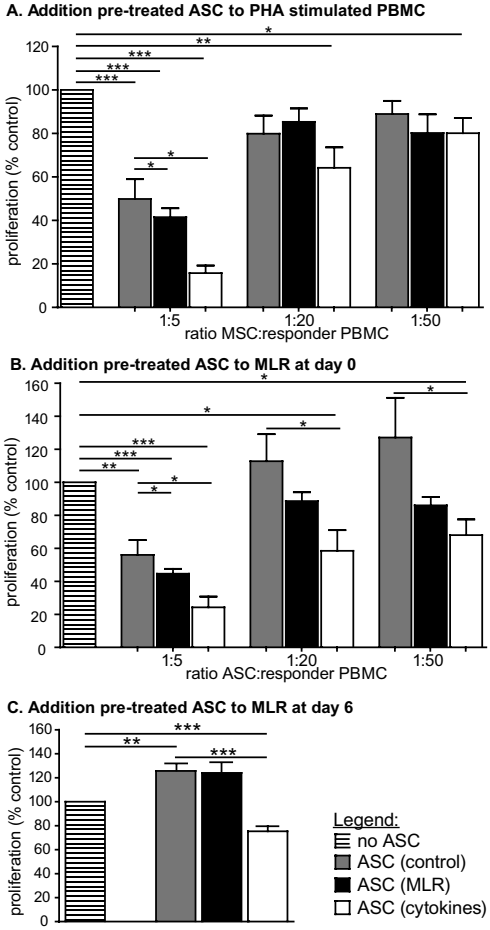


Figure 5. Effect of inflammatory conditions on the inhibition of PBMC proliferation by ASC. ASC were pretreated under control conditions, with alloactivated PBMC (MLR) or with pro-inflammatory cytokines conditions and added to mitogen- or alloactivated PBMC. The proliferation in the test conditions is normalized to the proliferation in the control situation (i.e. MLR without addition of ASC, which is set to 100%). [A] Effect of pretreated ASC on the proliferation of PHA-stimulated PBMC. ASC were added to PBMC at various concentrations (1:5, 1:20, 1:50) (n=8). Proliferation was measured by 3H-thymidine incorporation on day 3. [B] Effect of pretreated ASC added on day 0 to MLR on the proliferation of alloactivated PBMC (MLR). ASC were added to PBMC at various concentrations (1:5, 1:20, 1:50) (n=6). Proliferation was measured by 3H-thymidine incorporation on day 7. [C] Effect of pretreated ASC added on day 6 to MLR. ASC were added to responder PBMC at a 1:5 ratio (n=11). Proliferation was measured by 3H-thymidine incorporation on day 7.

Because of the striking increase in the expression of IDO by ASC cultured with pro-inflammatory cytokines, the importance of IDO as a mediator of the enhanced immunosuppressive capacity of ASC was investigated. Pretreated ASC were added to PHA-stimulated PBMC or MLR in the presence or absence of the IDO inhibitor 1-MT. 1-MT had no effect on the capacity of control and MLR pretreated ASC to inhibit the proliferation of PHA and alloactivated PBMC (Figure 5D and E). However, 1-MT decreased significantly the inhibitory effect of ASC pretreated with pro-inflammatory cytokines. The percentage inhibition of PHA-stimulated PBMC reduced from 84±8% to 64±17% and the inhibition of MLR from 68±20% to 29±45% after addition of 1-MT. The reduction of the immunosuppressive capacity of pro-inflammatory cytokines-activated ASC by 1-MT confirms the involvement of IDO in the increased immunosuppressive activity of ASC.

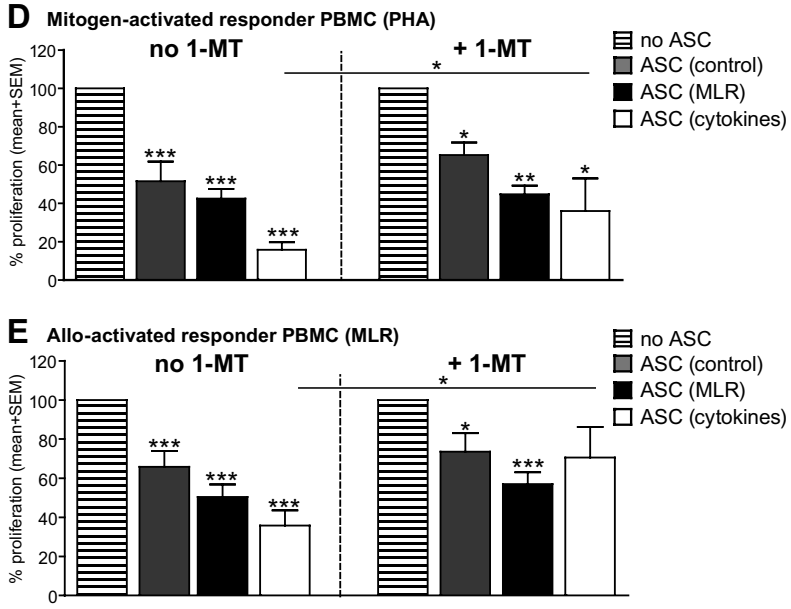


Figure 5 ***(continued)***. Effect of inflammatory conditions on the inhibition of PBMC proliferation by ASC. [D] Effect of IDO1-inhibitor 1-methyl-L-tryptophan (1-MT) on the capacity of pretreated ASC to inhibit the proliferation of PHA-stimulated PBMC (n=4). ASC were added to responder PBMC at a 1:5 ratio. Proliferation was measured by 3H-thymidine incorporation on day 3. [E] Effect of IDO1-inhibitor 1-methyl-L-tryptophan (1-MT) on the capacity of pretreated ASC to inhibit the proliferation in MLR (n=8). ASC were added to responder PBMC at a 1:5 ratio. Proliferation was measured by 3H-thymidine incorporation on day 7. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

DISCUSSION

In the present study we have demonstrated that inflammatory conditions have an important impact on the phenotype and function of ASC. Stimulation of ASC with MLR was used to study the effect of a range of inflammatory cytokines that are associated with immune responses. Stimulation with the pro-inflammatory cytokines IFN-γ, TNF-α and IL-6 represents a controlled and reproducible method of immune activation of ASC. Culture of ASC with alloactivated lymphocytes (MLR) or pro-inflammatory cytokines did not affect their differentiation capacity and production of trophic factors. Both inflammatory conditions, however, affected ASC morphology, proliferation and gene expression of cytokines, chemokines and HLA molecules. These gene expression changes led to increased immunosuppressive capacity of ASC.

Exposure of ASC to MLR or a cocktail of pro-inflammatory cytokines resulted in a change in ASC morphology and distribution in culture. The typical monolayer distribution of ASC changed to a star-shaped clustered distribution of ASC after culture in an inflammatory milieu. This effect was most striking in cultures of ASC in the presence of MLR. The clustering could be the result of differential expression of cell adhesion molecules. Whereas cadherin and selectin expression was not affected, the expression of a number of integrins changed modestly in ASC in the presence of MLR compared to control ASC and ASC cultured with pro-inflammatory cytokines. We also observed that ASC cultured with MLR showed a high proliferation rate, while culture with pro-inflammatory cytokines resulted in ASC with enlarged cell size and dramatically reduced proliferation. These findings indicate that ASC are affected in a different manner by the two inflammatory conditions used.

Inflammatory conditions did not only affect the phenotype of ASC, but also the immunosuppressive function of ASC. Culture of ASC with MLR improved the capacity of ASC to inhibit the proliferation of mitogen or alloantigen-stimulated lymphocytes. Culture of ASC with pro-inflammatory cytokines enhanced the immunosuppressive capacity of ASC even further. In contrast to ASC precultured under control conditions, ASC precultured with pro-inflammatory cytokines were able to inhibit lymphocyte proliferation when added at day 6 of a seven-day MLR. This suggests that pro-inflammatory cytokines activate the immunosuppressive machinery of ASC. This can lead to immediate immunosuppressive activity when ASC are added to an active MLR.
The increased immunosuppressive effect of these ASC was dependent largely upon increased expression of IDO. Blocking IDO reduced the immunosuppressive effect of cytokine-treated ASC to levels found in control ASC, but did not abolish the immunosuppressive capacity completely. This shows that IDO is important for the induced immunosuppressive capacity of ASC treated with cytokines, but less so for the basic immunosuppressive capacity of ASC. As a consequence, other factors must play a role in the immunosuppressive function of ASC, of which several have been reported in the literature, such as HGF, HLA-G and NO [5, 19-20]. We found high expression of HLA-G, TGF-β1 and COX-2, which have been reported to be involved in the immunosuppressive effect of ASC [5, 18-19]. In MLR-cultured ASC we found strong up-regulation of COX-2, which could indicate that prostaglandin E2 is responsible for some of the enhanced immunosuppressive capacity of these cells.

Culture under inflammatory conditions not only changed the expression of anti-inflammatory factors by ASC, but also increased the expression of HLA class I. The expression of HLA class II was increased predominantly by pro-inflammatory cytokines, whereas culture of ASC with MLR had less effect. Up-regulation of HLA makes ASC potentially more immunogenic. This could have consequences for clinical application of ASC of allogeneic origin.
Inflammatory conditions also increased the expression of pro-inflammatory factors and chemokines. The type of pro-inflammatory factors and chemokines produced by ASC depended on the inflammatory condition. Whereas ASC cultured with MLR showed up-regulation of chemokines for, in particular, neutrophils, monocytes and macrophages, culture of ASC with pro-inflammatory cytokines resulted in the up-regulation of chemokines for T lymphocytes. The relevance of the chemoattraction of the different immune cells by ASC is not clear, but could lead to binding of activated immune cells to ASC [23]. Close contact of activate immune cells and ASC may increase the efficacy of the immunomodulatory function of ASC [20, 35]. These results indicate that ASC can exhibit diverse immunomodulatory effects. The local inflammatory milieu is of crucial importance for the balance between the pro- and anti-inflammatory effects of ASC. Furthermore, it determines the mechanisms that ASC employ to execute their immunomodulatory function.

Apart from their immunomodulatory properties, ASC have potential to support tissue regeneration. While this is partially mediated via their differentiation in other cell types [2], there is now increasing evidence that the regenerative effect of ASC is also the result of the production of tropic factors, which stimulate resident progenitor cells [4]. Under inflammatory conditions, ASC maintained the capacity to differentiate in adipogenic and osteogenic lineages. Moreover, inflammatory conditions did not affect the expression of growth factors, such as epidermal growth factor, vascular endothelial growth factor and fibroblast growth factors by ASC, and even increased the expression of stem cell factor. Thus, while ASC gain immunosuppressive capacity under inflammatory conditions, their regenerative capacity is preserved.
A suggested undesired property of ASC is their potential transformation into fibrosis [36]. We found that culture of ASC with MLR had no effect on collagen gene expression, while culture of ASC with pro-inflammatory cytokines induced a down-regulation of the expression of multiple collagens. The expression of connective tissue growth factor, TGF-β and platelet-derived growth factor, which can induce epithelial-mesenchymal transition, was not affected by inflammatory conditions. This suggests that inflammatory conditions do not favor the induction of fibrosis by ASC.

The present study demonstrates that the type of inflammatory stimulus affects the response of ASC. In an alloactivated setting, ASC remain functional and even enhance their immunosuppressive function. Their immunosuppressive activity can be further enhanced by culturing ASC with pro-inflammatory cytokines. This offers the possibility to *in vitro* generate ASC with strong and instant immunosuppressive capacity. The potential regenerative capacity of ASC is not affected by inflammatory conditions and there is no evidence for an increased risk of fibrosis. Therefore, immune activation of ASC could be of benefit for potential clinical immune therapy with ASC.

REFERENCES

1. Toma JG, McKenzie IA, Bagli D, Miller FD. Isolation and characterization of multipotent skin-derived precursors from human skin. Stem Cells. 2005 Jun-Jul;23(6):727-37.

2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999 Apr 2;284(5411):143-7.

3. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 2002 Dec;13(12):4279-95.

4. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006 Aug 1;98(5):1076-84.

5. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002 May 15;99(10):3838-43.

6. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood. 2003 May 1;101(9):3722-9.

7. Nauta AJ, Kruisselbrink AB, Lurvink E, Willemze R, Fibbe WE. Mesenchymal stem cells inhibit generation and function of both CD34+-derived and monocyte-derived dendritic cells. J Immunol. 2006 Aug 15;177(4):2080-7.

8. Uccelli A, Moretta L, Pistoia V. Immunoregulatory function of mesenchymal stem cells. Eur J Immunol. 2006 Oct;36(10):2566-73.

9. Crop M, Baan C, Weimar W, Hoogduijn M. Potential of mesenchymal stem cells as immune therapy in solid-organ transplantation. Transpl Int. 2009 Apr;22(4):365-76.

10. Dahlke MH, Hoogduijn M, Eggenhofer E, Popp FC, Renner P, Slowik P, et al. Toward MSC in solid organ transplantation: 2008 position paper of the MISOT study group. Transplantation. 2009 Sep 15;88(5):614-9.

11. Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol. 2004 Apr;36(4):568-84.

12. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7.

13. Patel SA, Sherman L, Munoz J, Rameshwar P. Immunological properties of mesenchymal stem cells and clinical implications. Arch Immunol Ther Exp (Warsz). 2008 Jan-Feb;56(1):1-8.

14. Ren G, Zhao X, Zhang L, Zhang J, L'Huillier A, Ling W, et al. Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. J Immunol. 2010 Mar 1;184(5):2321-8.

15. Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood. 2004 Jun 15;103(12):4619-21.

16. Popp FC, Eggenhofer E, Renner P, Slowik P, Lang SA, Kaspar H, et al. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. Transpl Immunol. 2008 Nov;20(1-2):55-60.

17. DelaRosa O, Lombardo E, Beraza A, Mancheno-Corvo P, Ramirez C, Menta R, et al. Requirement of IFN-gamma-mediated indoleamine 2,3-dioxygenase expression in the modulation of lymphocyte proliferation by human adipose-derived stem cells. Tissue Eng Part A. 2009 Oct;15(10):2795-806.

18. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005 Feb 15;105(4):1815-22.

19. Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, et al. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce $CD4^{+}CD25highFOXP3^{+}$ regulatory T cells. Stem Cells. 2008 Jan;26(1):212-22.

20. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell. 2008 Feb 7;2(2):141-50.

21. Gao F, Wu DQ, Hu YH, Jin GX, Li GD, Sun TW, et al. In vitro cultivation of islet-like cell clusters from human umbilical cord blood-derived mesenchymal stem cells. Transl Res. 2008 Jun;151(6):293-302.

22. Chabannes D, Hill M, Merieau E, Rossignol J, Brion R, Soulillou JP, et al. A role for heme oxygenase-1 in the immunosuppressive effect of

adult rat and human mesenchymal stem cells. Blood. 2007 Nov 15;110(10):3691-4.

23. Quaedackers ME, Baan CC, Weimar W, Hoogduijn MJ. Cell contact interaction between adipose-derived stromal cells and allo-activated T lymphocytes. Eur J Immunol. 2009 Dec;39(12):3436-46.

24. Benvenuto F, Ferrari S, Gerdoni E, Gualandi F, Frassoni F, Pistoia V, et al. Human mesenchymal stem cells promote survival of T cells in a quiescent state. Stem Cells. 2007 Jul;25(7):1753-60.

25. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells. 2006 Feb;24(2):386-98.

26. Najar M, Rouas R, Raicevic G, Boufker HI, Lewalle P, Meuleman N, et al. Mesenchymal stromal cells promote or suppress the proliferation of T lymphocytes from cord blood and peripheral blood: the importance of low cell ratio and role of interleukin-6. Cytotherapy. 2009;11(5):570-83.

27. Hemeda H, Jakob M, Ludwig A, Giebel B, Lang S, Brandau S. Interferon-gamma and Tumor Necrosis Factor-alpha Differentially Affect Cytokine Expression and Migration Properties of Mesenchymal Stem Cells. Stem Cells Dev. 2010 May;19(5):693-706

28. Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol. 2007 Aug;149(2):353-63.

29. Polchert D, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E, et al. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. Eur J Immunol. 2008 Jun;38(6):1745-55.

30. Hoogduijn MJ, Crop MJ, Peeters AM, Van Osch GJ, Balk AH, Ijzermans JN, et al. Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. Stem Cells Dev. 2007 Aug;16(4):597-604.

31. Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Alwayn IP, Weimar W, et al. Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients. Transplantation. 2009 Mar 27;87(6):896-906.

32. Reiner A, Yekutieli D, Benjamini Y. Identifying differentially expressed genes using false discovery rate controlling procedures. Bioinformatics. 2003 Feb 12;19(3):368-75.

33. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Stat Soc B. 1995;1(57):289–300.

34. Hoogduijn MJ, Crop MJ, Peeters AM, Korevaar SS, Eijken M, Drabbels JJ, et al. Donor-derived mesenchymal stem cells remain present and functional in the transplanted human heart. Am J Transplant. 2009 Jan;9(1):222-30.

35. Augello A, Tasso R, Negrini SM, Amateis A, Indiveri F, Cancedda R, et al. Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. Eur J Immunol. 2005 May;35(5):1482-90.

36. Bellini A, Mattoli S. The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses. Lab Invest. 2007 Sep;87(9):858-70.

Chapter 6

Human adipose tissue-derived mesenchymal stem cells induce explosive T-cell proliferation

Meindert J. Crop[1], Carla C. Baan[1], Sander S. Korevaar[1], Jan N.M. IJzermans[2], Willem Weimar[1], Martin J. Hoogduijn[1]

Departments of [1]Internal Medicine and [2]Surgery,
Erasmus University Medical Center, Rotterdam, The Netherlands

Stem Cells and Development
2010 Apr 5 (in press)

ABSTRACT

Mesenchymal stem cells (MSC) inhibit the proliferation of alloactivated lymphocytes. This effect is primarily dependent on the secretion of anti-inflammatory factors by MSC and is enhanced under inflammatory conditions. MSC, however, also produce factors that can potentially activate resting immune cells. Full understanding of the behaviour of MSC under inflammatory and non-inflammatory conditions is crucial when clinical application of MSC is considered. Human adipose tissue-derived MSC were cultured with non-activated peripheral blood mononuclear cells (PBMC) and activation, proliferation and function of PBMC examined.

Seven days of co-culture with autologous or allogeneic MSC significantly increased the proliferation of PBMC (3-fold). This effect was observed in both direct and transwell co-culture systems. MSC co-cultured with PBMC showed increased mRNA expression of the pro-inflammatory mediators IL-6, IL-8, TNF-α, the growth factors bFGF and VEGFα, and the anti-inflammatory factor IDO. After removal of MSC, PBMC showed a spectacular further increase in proliferation with a maximum of 25-fold after seven days. This increase in proliferation was not seen when PBMC were kept in the presence of MSC. The proliferating fraction of PBMC largely consisted of $CD4^+$ T-cells with high CD25 expression and the proportion of $CD127^{neg}FoxP3^+$ regulatory T-cells significantly increased from 5.0% to 8.5% of total $CD4^+$ T-cells. The expanded T-cells demonstrated normal responses to mitogen or alloantigen stimulation. The $CD25^{pos}$ fraction of these cells had immunosuppressive capacity. In conclusion, MSC can stimulate activation and proliferation of resting T-cells and generate regulatory T-cells. These findings are important when MSC are applied in the clinic.

INTRODUCTION

Over the last decade there has been increasing interest in the immunomodulatory properties of mesenchymal stem cells (MSC). MSC inhibit the proliferation and function of alloactivated lymphocytes and other immune cells [1-2]. The mechanisms of immune modulation by MSC are not fully understood, but partly depend on the secretion of anti-inflammatory factors, such as interleukin-10 (IL-10), indoleamine 2,3-dioxygenase (IDO), nitric-oxide (NO) [3], prostaglandin E2 [1], HLA-G [4], hepatocyte growth factor (HGF) and transforming growth factor-β (TGF-β) [2]. It has also been suggested that MSC modulate immune responses via the generation of regulatory T-cells (T-regs) [1, 5-7].
Animal models have demonstrated that MSC can effectively reduce inflammatory processes, for example in autoimmune encephalitis [8], myocarditis [9], inflammatory bowel disease [10] and glomerulonephritis [11]. In a clinical setting, MSC therapy has been shown to be effective for the treatment of patients with severe therapy-resistant graft-*versus*-host disease (GVHD) [12]. Furthermore, clinical trials are ongoing to determine whether MSC are also effective for the treatment of autoimmune diseases, such as inflammatory bowel diseases, multiple sclerosis and rheumatoid arthritis.

The immunosuppressive properties of MSC make them interesting candidates for cell-based therapy in organ transplantation [13]. Treatment with MSC may provide an immunosuppressive environment that controls anti-donor reactivity. A number of studies with animal transplant models have reported a beneficial effect of MSC on skin [14], heart [6, 15] and liver [16] graft survival.
We recently reported that MSC derived from perirenal adipose tissue of living kidney donors have the capacity to inhibit anti-donor reactivity of kidney transplant patients *in vitro* both before and after transplantation [17]. The immunosuppressive function of MSC is triggered under inflammatory conditions where T-cells produce interferon-gamma (IFN-γ) and tumour-necrosis-factor-alpha (TNF-α) [18-19]. T-cells in a quiescent state, however, are promoted in survival by MSC [20]. Despite many studies investigating the effect of MSC on alloantigen or mitogen-activated immune cells, the effect of MSC on non-activated immune cells remains largely unknown. Understanding the full spectrum of immune modulation by MSC is, however, important when the application of MSC in a clinical setting is considered. In the present study we investigate the effect of human autologous and allogeneic MSC on non-activated immune cells.

MATERIALS AND METHODS

Isolation, culture and differentiation of MSC from perirenal adipose tissue

MSC were isolated from 10-50 grams of perirenal adipose tissue of living kidney donors as previously described [21]. Briefly, perirenal adipose tissue was collected during the kidney donation procedure after written informed consent of the kidney donors as approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam (protocol no. MEC-2006-190). The tissue was collected in RPMI-Dutch-modified-1640 (RPMI, Invitrogen, Paisley, Scotland) with 200μM L-glutamine (Biowithaker, Verviers, Belgium) and 1% penicillin/streptomycin (100U/ml / 100μg/ml, p/s, Invitrogen).

Next, it was mechanically disrupted and enzymatically digested with sterile 0.5mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) in RPMI for 30 minutes at 37°C. Subsequently, the cells were incubated with 160mM NH_4Cl (dissolved in PBS) for 10 minutes at room temperature. Cells were resuspended in MSC-culture medium, consisting of alpha-MEM medium (α-MEM, Invitrogen) with 1% p/s and 15% foetal bovine serum (FBS, Biowithaker), transferred to a 175cm^2 culture flask (Greiner Bio-one, Essen, Germany) and kept at 37°C, 5% CO_2 and 95% humidity. Cultures were refreshed with MSC-culture medium twice a week. At 90% confluence, adherent cells were removed from culture flasks by incubation in 0.05% trypsin-EDTA at 37°C and cells were used for experiments or frozen at –150°C until use. MSC were used for experiments between passages 2 to 5. To confirm whether the perirenal adipose tissue-derived cells were indeed MSC, they were characterized by flow cytometry, differentiated in osteogenic, adipogenic and myogenic lineages, and added to mixed lymphocyte reactions (MLR) to test their immunosuppressive capacity as previously described [17, 21].

Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood samples were collected from living kidney donors and from buffy coats of healthy blood donors. PBMC were isolated from the collected heparinised peripheral blood by density gradient centrifugation using Ficoll Isopaque (δ=1.077, Amersham, Uppsala, Sweden) and frozen at –150°C until use.

Experimental set-up (day 0-7): co-culturing PBMC with MSC

The experimental set-up is shown in Figure 1. At day 0, PBMC were cultured for 7 days with MSC in a (5:1)-ratio in direct or in transwell co-culture systems. In transwell co-culture systems PBMC were separated from MSC by a 0.4-μm-pore-size membrane (Greiner Bio-one); MSC were cultured in the bottom well, while PBMC were kept in the inserts. In 6-well plates, 1x10^6 PBMC were added to 0.20x10^6 MSC, while in 96-well plates 50,000 PBMC were added to 10,000 MSC. After 7 days, proliferation was determined after 16-hour incorporation of ^{3}H-thymidine (0.5μCi/well, Amersham, UK) using a β-plate reader (LKB, Bromma, Sweden). In six out of 56 cases, PBMC in the absence of MSC already showed ^{3}H-thymidine incorporation of 5000 cpm or more (day 7) and these samples were excluded from further analysis as these cells could not be defined as ''non-activated''.

In addition, proliferation was measured after 7 days by flow cytometry after labelling of PBMC on day 0 with PKH67 (Sigma-Aldrich) according to the manufacturer's instructions. Flow cytometric analysis was performed as described below. To investigate whether IL-6 and IL-8 were involved in the stimulatory effect of MSC on T-cell activation and proliferation, neutralizing antibodies against IL-6 (10 μg/ml) and IL-8 (10 μg/ml) (both R&D systems, Minneapolis, MN, USA) were used. Immunoassays (Immulite 1000, Siemens, Deerfield, IL, USA) were performed to determine the protein levels of IL-6 and IL-8 in supernatants of cultures with neutralizing antibodies.

Experimental set-up (day 7-17): continued culture of PBMC previously cultured with MSC

After 7 days, PBMC were collected from PBMC-MSC co-cultures, washed, resuspended in fresh medium (RPMI with p/s, L-glutamine and 10% HI-FBS). There was no evidence for contamination with MSC after removal of PBMC from direct MSC-PBMC co-cultures, as investigated by morphology in culture and flow cytometric analysis. Subsequently, PBMC were continued in culture for 1, 3, 5, 7 and 10 days in the absence of MSC (Figure 1, day 7 to 14). Proliferation was measured by ^{3}H-thymidine incorporation (on experimental day 8, 10, 12, 14 or 17) or flow cytometry (experimental day 14).

Experimental set-up (day 14-21): functional analysis of PBMC previously cultured with MSC
On experimental day 14, PBMC were collected for real-time RT-PCR analysis (as described below). To examine whether these cells were still capable of responding to alloantigens, they were used as responder cells and stimulated with 1 μg/ml phytohaemagglutinin (PHA; Murex Biotech LTd, Kent, UK) for 3 days or with γ-irradiated (40Gy) allogeneic PBMC (added at a 1:1-ratio).
Furthermore, to determine whether the precultured PBMC had immunosuppressive capacity, they were added to MLR at a 1:10-ratio. Finally, the PBMC were sorted for CD25 by auto MACS (as described below). The suppressive capacity of both the $CD25^{pos}$ and the $CD25^{neg}$ fraction was examined by adding the cells to MLR at a 1:5, 1:10 and 1:20 ratio.

Mixed-lymphocyte reactions (MLR)
Immunosuppressive capacity of MSC and PBMC previously cultured with MSC was tested in MLR. In MLR, $5x10^4$ responder PBMC were stimulated by $5x10^4$ γ-irradiated (40Gy) allogeneic PBMC in RPMI+10% HI-FBS in round-bottom 96-well plates (Nunc, Roskilde, Denmark). On day 7, proliferation was measured following incorporation of ^{3}H-thymidine (0.5μCi/well) during a 16-hour incubation using a β-plate reader. To determine the proliferation capacity of the PBMC, $5x10^4$ cells were stimulated with 1 μg/ml PHA for 3 days and ^{3}H-thymidine incorporation was measured. Only results of responder PBMC with sufficient proliferation capacity (>10,000 cpm after stimulation with PHA) were included.

Flow cytometric analysis
PBMC were washed twice with FACSFlow (BD Biosciences, San Jose, CA, USA) and stained with antibodies against CD3-AmCyan, CD4-PacificBlue, CD8-APC-Cy7, CD16/56-APC, CD19-PerCP, CD25-PE-Cy7, CD62L-APC, CD69-PE, CD127-FITC (all BD Biosciences), CCR7-PE (R&D systems, Minneapolis, MN, USA), FASL-APC (Biocarta, San Diego, CA, USA) at room temperature and protected from light for 30 minutes. After two washes with FACSFlow, flow cytometric analysis was performed using an 8-colour FACSCANTO-II with FACSDIVA Software (BD Biosciences). FoxP3-APC staining was carried out as described previously [22].

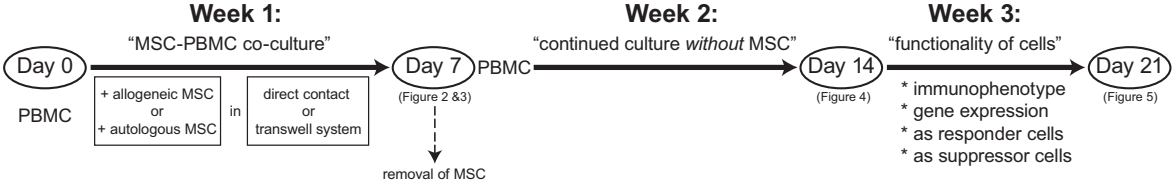


Figure 1. Experimental set-up. PBMC were co-cultured with MSC in direct or transwell co-culture systems during the first week (= week 1). On day 7, MSC were removed from the co-culture systems and culture of PBMC continued in the absence of MSC for another 7 days (= week 2). On experiment day 14, the functionality of the cells previously cultured with MSC was investigated (= week 3).

Isolation of CD25^{pos} cells by autoMACS

On experimental day 14, the CD25^{positive} (CD25^{pos}) cells were isolated from PBMC previously transwell co-cultured with MSC using anti-CD25 microbeads (10 µl /10x10^6 cells) (Miltenyi Biotech, Bergisch Gladbach, Germany), followed by positive selection (POSSELD-program) on the autoMACS (Miltenyi Biotech). To check the purity, both fractions were stained with CD3-FITC, CD4-PerCP and CD25-PE epitope B (clone M-A251, BD Biosciences) and flow cytometric analysis was performed.

Real-time RT-PCR

PBMC and MSC for real-time RT-PCR were obtained from transwell co-culture systems. Total RNA was isolated and cDNA synthesized as previously described [23]. Quantitative gene expression was determined using TaqMan Universal PCR Master Mix and assays-on-demand for IDO (Hs00158627.m1), IL-10 (Hs00174086.m1), TGF-β (Hs00171257.m1), TNF-α (Hs99999043.m1), CD25 (Hs00166229.m1), FoxP3 (Hs00203958), IL-2 (Hs00174114.m1), IL-6 (Hs00174131), IFN-γ (Hs00174143) and IL-8 (Hs00174114.m1) (all Applied Biosystems, CA, USA) on a StepOnePlus (Applied Biosystems). VEGFα and bFGF were determined by real-time RT-PCR using homemade primers (F-5'-GCA-GAC-CAA-AGA-AAG-ATA-GAG-CAA-G-3' and R-5'-CGC-CTC-GGC-TTG-TCA-CAT-3') and (F-5'-GTT-GAC-GGG-GTC-CGG-G-3' and R-5'-GAT-AGA-CAC-AAC-TCC-TCT-CTC-TTC-TGC-3'), respectively. Gene expression levels were normalized to the concentration of 18S per 500ng RNA.

Statistical analysis

Data were analyzed using paired t-test or Wilcoxon signed-rank test depending on the distribution of the data as tested with Kolmogorov-Smirnov test for normality. Parametric data are expressed as means, while non-parametric data are expressed as medians. Statistical significance was defined as $p<0.05$ (two-tailed).

RESULTS

MSC stimulate PBMC proliferation and activation

In contrast to our previous studies [17, 21], in which MSC were added to alloactivated PBMC, the present study investigated the effect of human MSC on non-activated PBMC. The experimental set-up is shown in figure 1. Seven-day co-culture of PBMC with allogeneic MSC significantly increased PBMC proliferation by a factor 4 compared to PBMC cultured without MSC (Figure 2A). This increase in PBMC proliferation was independent of HLA, since also autologous MSC induced a 3-fold increase in PBMC proliferation. There was no significant difference in the proliferative effect of autologous and allogeneic MSC. When PBMC were co-cultured with γ-irradiated MSC, a similar increase in proliferation of the PBMC was observed.

To identify which PBMC subset was stimulated in proliferation in the presence of MSC, flow cytometric analysis of PBMC was carried out. MSC predominantly stimulated the proliferation of $CD4^+$ T-cells and $CD8^+$ T-cells, while co-culture with MSC had no effect on the proliferation of NK and B-cells. Co-culture of PBMC with MSC also significantly increased CD69 and CD25 expression on the proliferating $CD4^+$ and $CD8^+$ T-cells (Figure 2B), while the expression of CD62L, FASL and CCR7 was unchanged in T-cells after culture with MSC. The increased CD25 expression of T-cells co-cultured with MSC was associated with a 2-fold increase in mRNA expression of IL-2 by the PBMC (data not shown). The expression of pro-inflammatory cytokines such as IL-6, IL-8, IFN-γ or TNF-α did not significantly change in PBMC co-cultured with MSC. Expression of the immunosuppressive factor IL-10 decreased by a factor 2 in PBMC co-cultured with MSC ($p=0.0039$).

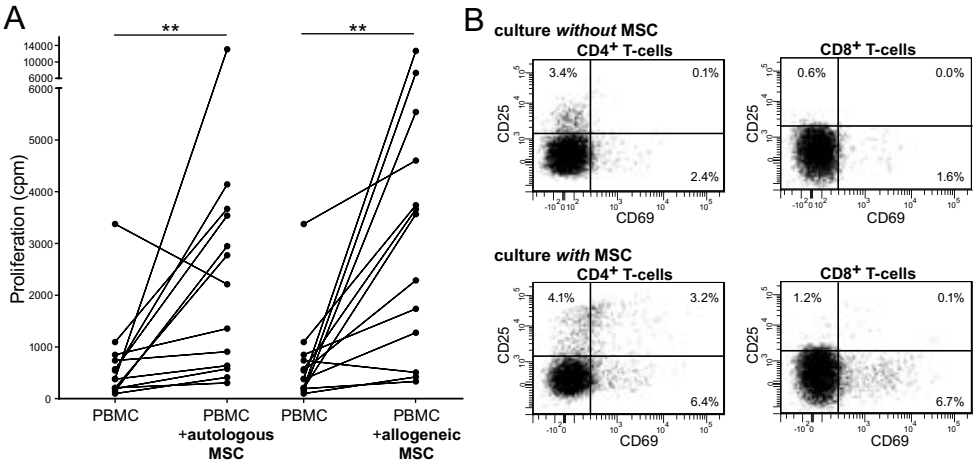


Figure 2. Proliferation and activation of PBMC cultured for seven days in the absence or presence of MSC (in direct culture systems). [A] Proliferation of PBMC co-cultured with autologous and allogeneic MSC measured by 3H-thymidine incorporation (n=13). Proliferation of MSC cultured without PBMC was low, 124 cpm for autologous and 133 cpm for allogeneic MSC. [B] Flow cytometric analysis of CD25 and CD69 expression on $CD4^+$ and $CD8^+$ T-cells that were cultured with or without MSC for seven days. One representative example of five experiments is shown. cpm=counts per minute. ** means $p<0.01$.

The expression of pro-inflammatory, growth and anti-inflammatory factors by MSC is increased in the presence of PBMC

The observed stimulatory effect of MSC on PBMC proliferation was comparable in direct and transwell co-culture systems, which indicates that the effect is mediated by soluble factors. To identify which factors may be responsible, gene expression of pro-inflammatory, growth and immunosuppressive factors by MSC was investigated (Figure 3). MSC significantly increased the expression of IL-8 (527-fold), IL-6 (11-fold), TNF-α (3-fold), VEGFα (3-fold) and bFGF (4-fold) in the presence of PBMC. In addition, the anti-inflammatory factor IDO was 699-fold increased in MSC cultured with PBMC. IL-2, IFN-γ and IL-10 were not detectable.

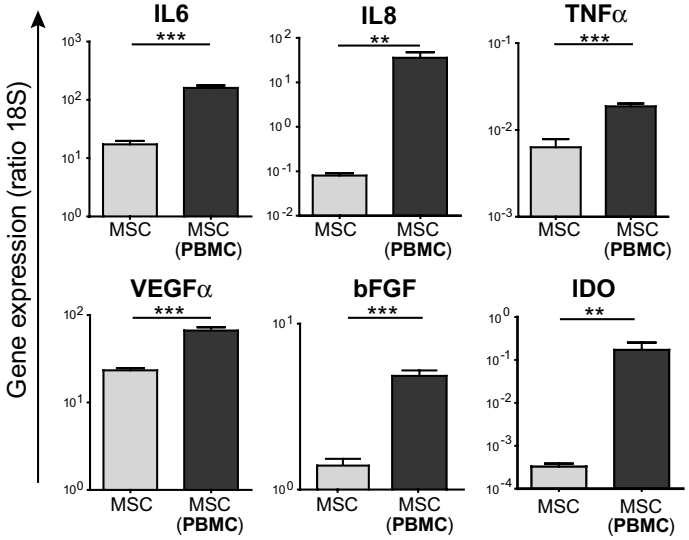


Figure 3. Real-time RT-PCR analysis of MSC co-cultured with PBMC for seven days in transwell co-culture systems. Gene expression levels were normalized to the concentration of 18S RNA per 500ng RNA (n=9). ** means $p<0.01$; *** means $p<0.001$.

T-cell proliferation dramatically increases after removal of MSC

To investigate whether the presence of MSC was needed for continuation of PBMC proliferation, MSC were removed from PBMC-MSC transwell co-culture systems and at the same time PBMC were continued in culture (Figure 1, week 2). PBMC proliferation was measured 1, 3, 5, 7 and 10 days after removal of MSC. Continued culture of the PBMC led to an explosive 25-fold increase in proliferation with a maximum on day 7 after separation of PBMC from MSC (=experiment day 14) (Figure 4A). This extra increase in PBMC proliferation during the second week was not observed when PBMC were kept in the presence of MSC (Figure 4B).

As shown, on experimental day 17, PBMC proliferation declined compared to day 14. To rule out the possibility that this was due to exhaustion of the culture medium, fresh medium was added on day 14 and PBMC proliferation measured on day 17. Refreshment of medium, however, did not improve the proliferation of the PBMC on day 17.

As the expression of IL-6 and especially IL-8 strongly increased in MSC during culture with PBMC, it was investigated whether IL-6 and IL-8 were involved in the stimulatory effect of MSC on resting PBMC.

Therefore, PBMC were co-cultured with MSC in the absence or presence of neutralizing antibodies against IL-6 or IL-8. Blocking IL-6 or IL-8 did not reverse the stimulatory effect of MSC on PBMC proliferation (Figure 4C). Protein levels of IL-6 and IL-8 in supernatants were measured by immunoassays. Anti-IL-8 antibodies strongly reduced the IL-8 concentration in PBMC-MSC co-cultures ($4x10^5$-fold). The production of IL-6 in PBMC-MSC co-cultures was extremely high ($3x10^8$ pg/ml). While anti-IL-6 antibodies reduced the IL-6 levels 7-fold, they were unable to completely remove free IL-6 (Figure 4D).

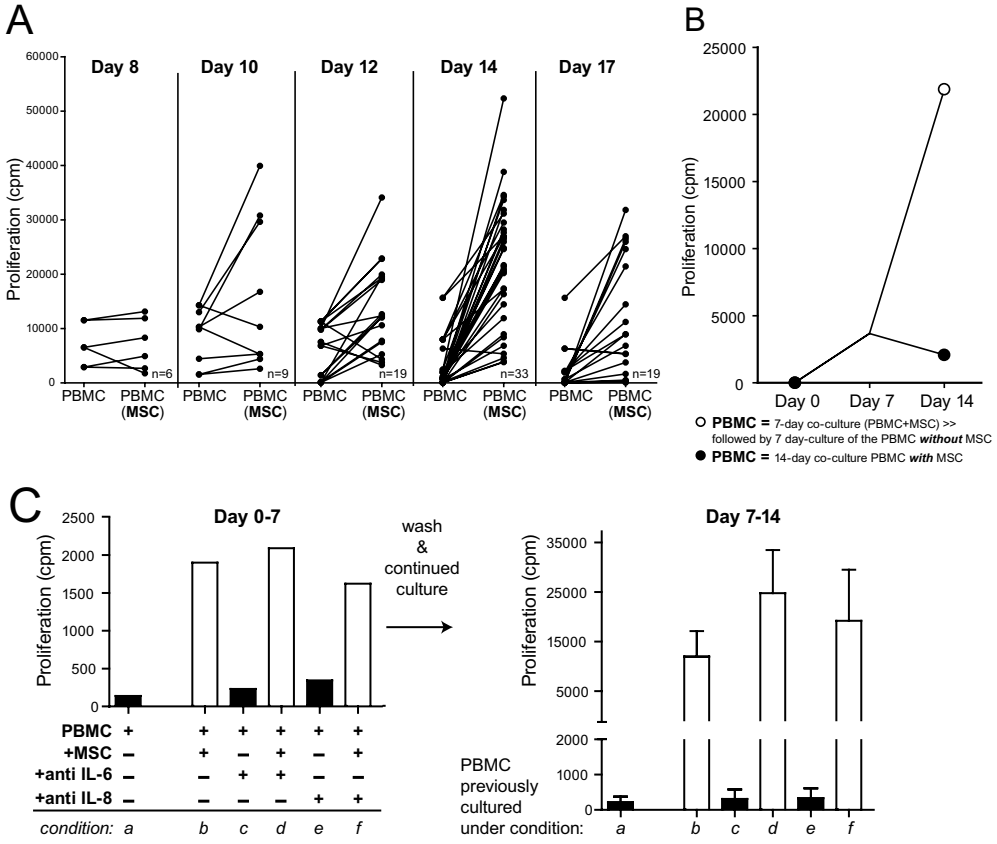
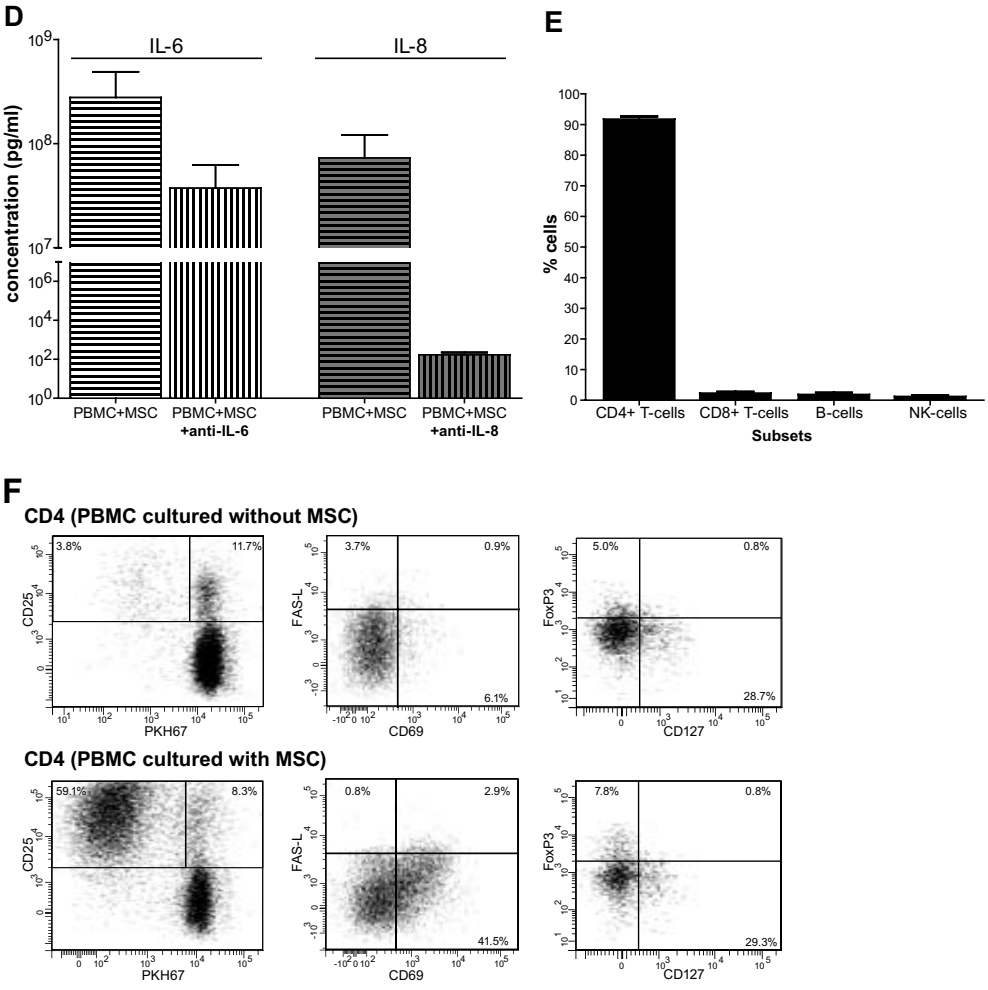


Figure 4. Continued culture of PBMC previously transwell cultured with MSC and after removal of MSC at day 7. [A] Proliferation of PBMC precultured with MSC and then continued in culture for 1, 3, 5, 7 or 10 days without MSC (experimental day 7-17). PBMC were cultured for 14 days without MSC (PBMC), or for seven days with MSC and continued in culture after removal of MSC at day 7 (PBMC+MSC). Proliferation was measured by 3H-thymidine incorporation. [B] Proliferation of PBMC both precultured with MSC for 7 days and subsequently continued in culture without MSC (○), or PBMC which were kept in co-culture with MSC (●). Median is shown of four experiments. Proliferation was measured by 3H-thymidine incorporation at experimental day 14. [C] Proliferation of PBMC in the presence of MSC and neutralizing antibodies against IL-6 and IL-8. PBMC were cultured during experimental week 1 with MSC, anti-IL-6 or anti-IL-8 and 3H-thymidine incorporation measured at day 7. After seven days the PBMC cultured under the various conditions were washed and continued in culture for another week. Again, proliferation was measured by 3H-incorporation (at day 14) (n=2).

MSC induce activation and proliferation of CD4$^+$ T-cells

Flow cytometric analysis was performed to identify which PBMC subset(s) were responsible for the striking increase in proliferation observed seven days after removal of MSC. The proliferating fraction of PBMC expanded by MSC consisted predominantly of CD4$^+$ T-cells (92%) (Figure 4E). The percentages CD8$^+$ T-cells, NK-cells and B-cells did not significantly change. The CD4$^+$ T-cells previously cultured with MSC showed a strong increase in proliferation (as measured by PKH dilution) (Figure 4F). These cells highly expressed CD25 and CD69, while the expression of FAS-L, CD62L and CCR7 was unchanged. Furthermore, the percentage of CD4$^+$CD25^{pos}FoxP3$^+$ and CD127^{neg} was significantly increased by a factor 1.7 compared to PBMC previously cultured without MSC. This indicates that there is expansion of CD4$^+$CD25^{pos}FoxP3$^+$CD127^{neg} T-regs in PBMC previously cultured with MSC.



Expression of pro- and anti-inflammatory genes in PBMC after removal of MSC
Next, PBMC were collected seven days after removal of MSC and RT-PCR analysis performed to characterize the PBMC expanded by MSC (Figure 4G). PBMC expanded by MSC showed significant, but minor, increases in the expression of pro-inflammatory factors IL-2 (3-fold), IL-6 (2-fold), IL-8 (2-fold), TNF-α (2-fold) compared to PBMC not precultured with MSC. There was no significant change in the expression of IFN-γ or the anti-inflammatory factors TGF-β and IDO, while IL-10 expression increased by a factor 2.

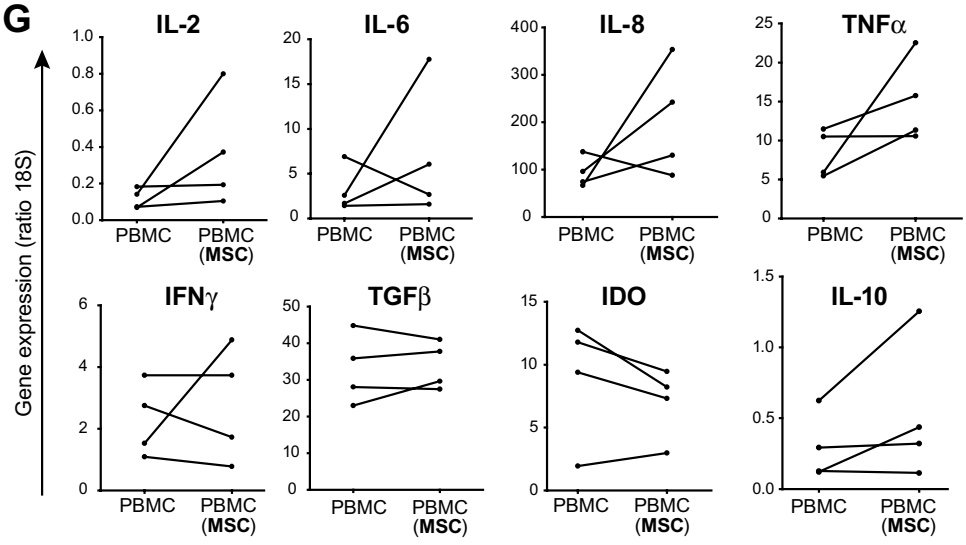


Figure 4 (*continued*). [D] Concentration of IL-6 and IL-8 in supernatants of PBMC-MSC co-cultures in the absence or presence of neutralizing antibodies for IL-6 or IL-8 (n=2). Supernatants were collected from co-cultures at day 7. Data are expressed as mean+SEM. [E] Flow cytometric analysis of the proliferating fraction of PBMC expanded by MSC (day 14). Mean±SD is shown of eight experiments. [F] Flow cytometric analysis of cell proliferation (PKH dilution), CD25, CD69, CD127, FoxP3 and Fas-L expression on $CD4^+$ T-cell subsets of PBMC previously cultured with and without MSC at experimental day 14. One representative example of six experiments is shown. [G] Real-time RT-PCR analysis (day 14) of PBMC previously co-cultured with MSC and then expanded for 7 days (n=4). Gene expression levels were normalized to the concentration of 18S per 500ng RNA. PBMC were cultured for 14 days without MSC (PBMC), or for seven days with MSC and continued in culture after removal of MSC at day 7 (PBMC+MSC). cpm=counts per minute.

PBMC expanded by MSC maintain responsive to mitogen and alloantigen

After one week of culture with MSC, followed by one week of culture without MSC, PBMC were highly proliferative and showed an activated phenotype. To functionally examine the characteristics of these expanded cells, they were stimulated with mitogen (PHA) and alloantigen (γ-irradiated allogeneic PBMC) (Figure 1, week 3). PBMC precultured with MSC showed a good response upon stimulation with PHA (up to 42,000 cpm) ($p=0.005$) and was comparable with the proliferative response of PBMC cultured without MSC. Thus, despite two weeks of culture, the expanded cells still had good proliferative capacity.

PBMC expanded by MSC were then stimulated with γ-irradiated allogeneic PBMC (at a 1:1-ratio). These PBMC showed comparable proliferative responses compared to PBMC previously cultured without MSC. Thus, although the PBMC previously co-cultured with MSC were highly activated and proliferative, the cells were not hyper-reactive upon stimulation with alloantigen.

PBMC expanded by MSC have immunosuppressive capacity

We then investigated whether the expanded PBMC precultured with MSC had immunosuppressive capacity. Therefore, the PBMC were added at a 1:10-ratio to MLR (Figure 5A). PBMC that had been cultured with MSC inhibited the proliferation in MLR by 32% ($p<0.0001$), while PBMC previously cultured without MSC did not show this effect.

The inhibitory capacity of the PBMC was independent of the origin of the cells used in the MLR, indicating that inhibition was not antigen specific. PBMC that were cultured for one week with MSC and added to MLR before their expansion had no immunosuppressive capacity, indicating that the expansion of these cells is crucial for obtaining their immunosuppressive capacity.

The immunosuppressive capacity of PBMC expanded by MSC resides in the $CD25^{pos}$ T-cell fraction

Finally, the previous experiments showed that MSC induced both CD25 expression and immunosuppressive function in PBMC. To examine whether the $CD25^{pos}$ PBMC were responsible for the observed immunosuppressive capacity, PBMC expanded by MSC were sorted in $CD25^{pos}$ and $CD25^{neg}$ fractions and added to MLR at various ratios. Before sorting 51.9% of the PBMC previously cultured with MSC was positive for CD25. After sorting the purity of $CD25^{pos}$ cells was 93.6% (Figure 5B). While the $CD25^{neg}$ fraction had no inhibitory capacity at any ratio and even stimulated the proliferation of alloactivated PBMC, the $CD25^{pos}$ fraction significantly inhibited the proliferation of alloactivated responder cells in MLR by 62% (ratio 1:5), 40% (ratio 1:10) and 21%(ratio 1:20) (Figure 5C).

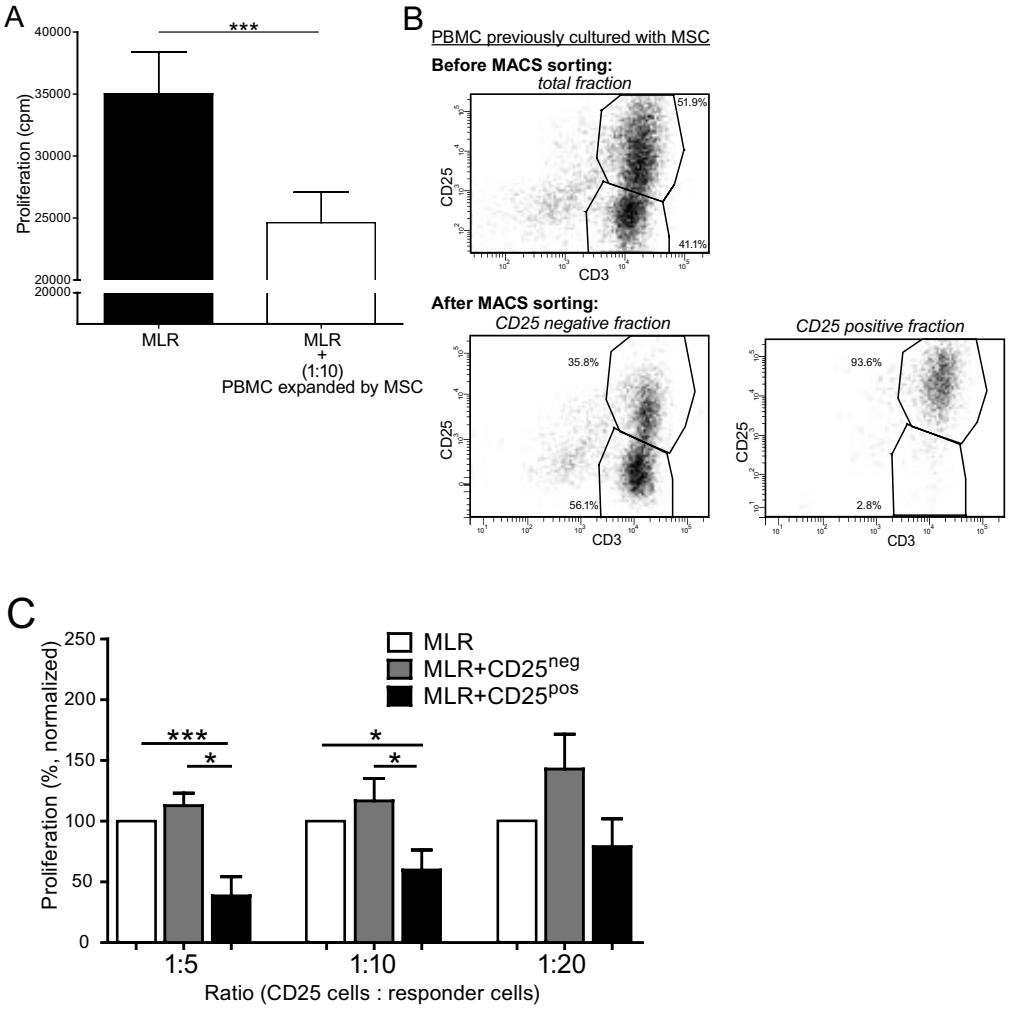


Figure 5. Functionality of PBMC previously transwell cultured with MSC and continued in culture for another seven days without MSC. [A] Immunosuppressive capacity of PBMC precultured with MSC and expanded after removal of MSC. Precultured PBMC were added to MLR at a 1:10 ratio (n=26). Proliferation was measured by 3H-thymidine incorporation at experimental day 21. [B] Purity and intensity of CD25 expression on $CD3^+$ T-cells in PBMC previously cultured with MSC before and after sorting. One representative example is shown of six experiments. [C] Immunosuppressive capacity of CD25pos population of PBMC precultured with MSC and expanded after removal of MSC. Precultured PBMC were sorted in CD25pos and CD25neg fractions and added to MLR at a 1:5, 1:10 and 1:20 ratio (n=6). Proliferation was measured by 3H-thymidine incorporation at experimental day 21. cpm=counts per minute. * means $p<0.05$; *** means $p<0.001$.

DISCUSSION

There is accumulating data on the immunosuppressive effect of MSC demonstrating that MSC inhibit mitogen and alloantigen activated immune cells. The effect of MSC on immune cells under low immunogenic conditions is, however, unknown. It is important to understand the full spectrum of immune regulation by MSC, when MSC are considered for clinical application.
The present study demonstrates that the proliferation of non-activated, "resting", PBMC increases by co-culture with MSC. Removal of MSC leads to accelerated proliferation of in particular $CD4^+$ T-cells, whereas in the continuous presence of MSC PBMC proliferation is kept at a low level. This suggests that MSC activate resting $CD4^+$ T-cells, but, when present, do prevent full activation and proliferation of these cells.
These results are in sharp contrast with previous studies reporting that MSC do not elicit proliferative responses from allogeneic lymphocytes [24-27]. One of the reasons for this disparity is that most groups investigated the effect of MSC on *activated* PBMC, whereas the present study is the first to investigate the effect of MSC on *non-activated* PBMC in detail. Furthermore, this is the first study to examine the effect of removal of MSC from MSC-PBMC co-cultures. It can be speculated that the observed stimulatory effect of MSC on resting T-cells still is present in an activated setting, but that it is covered by the immunosuppressive effect of MSC on activated PBMC.

It is generally considered that T-cell activation and proliferation is initiated by T-cell receptor triggering. In our study, however, we found that the activation was not antigen-dependent as the proliferation of T-cells was stimulated by both autologous and allogeneic MSC and that this effect was present in transwell systems, indicating that the T-cells can be activated by soluble factors only. Apart from the repeatedly reported production of anti-inflammatory factors [1-3], MSC can also produce growth and pro-inflammatory factors, such as basic fibroblast growth factor (bFGF), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), which can explain the stimulatory effect of MSC on PBMC [28]. In the present study, gene expression analysis showed that MSC had particularly increased levels of IL-6 and IL-8 after co-culture with PBMC. Neutralizing IL-8 dramatically reduced the IL-8 concentration in supernatants of PBMC-MSC co-cultures but had no effect on PBMC proliferation, suggesting that IL-8 is not a major player involved in the stimulatory effect of MSC on PBMC.
In addition, reduction of levels of IL-6 by neutralizing antibody in PBMC-MSC cocultures did not prevent activation and proliferation of resting PBMC. However, remaining levels of IL-6 could still be responsible for the stimulatory effect of MSC on PBMC. We can therefore not rule out that the stimulatory effect of MSC on PBMC was independent of IL-6 and this needs further investigation.
Concurrent with the increased expression of IL-6 and IL-8, MSC increased the expression of the anti-inflammatory factor IDO during co-culture with PBMC. This shows the dual effect of MSC on PBMC proliferation, which was further demonstrated by the persisted and even accelerated proliferation of PBMC after removal of MSC. This extra proliferative effect on PBMC was not observed when PBMC were kept in the presence of MSC, suggesting a blockade of PBMC activation and proliferation. Taken together, these results indicate that MSC have a regulatory function to maintain homeostasis in the (local) environment: under inflammatory conditions MSC are immunosuppressive, while MSC stimulate T-cell proliferation under low inflammatory conditions.

The spectacular increase in proliferation of PBMC after removal of MSC was for an overwhelming part dependent on the proliferation of $CD4^+$ T-cells. These cells highly expressed CD25 and CD69, reflecting their activation status. Furthermore, within the $CD4^+$ T-cell population, the proportion of $CD25^{pos}FoxP3^+CD127^{neg}$ T-regs was significantly increased. Our data therefore suggest that MSC favor the expansion of T-regs. Functional analysis showed that the T-cells that expanded after removal of MSC were not anergic and had immunosuppressive function. The $CD25^{pos}$ fraction of the generated cells was responsible for this effect, while the $CD25^{neg}$ fraction had no inhibitory effect on the proliferation of alloactivated cells. Expansion of these cells after removal of MSC was crucial for obtaining this immunosuppressive capacity.

There is currently a trend to apply MSC for an increasing number of applications, including organ transplantation. The relevance of the observed stimulatory effect of MSC on resting T-cells in clinical therapy is unknown. Although the expansion of T-cells by MSC harbors a potential danger, the net effect of MSC was an enhancement of the regulatory capacity of the T-cell compartment. It is important for successful clinical application of MSC that the interaction between MSC, T-regs and other immune cells is studied in further detail under various immunological conditions.

In conclusion, the results of this study give new insights in the mechanisms of immune modulation by MSC under non-inflammatory conditions. Under these conditions, MSC have a stimulating effect on activation and proliferation of T-cells. At the same time, MSC show immune regulatory functions that can control full activation of T-cells. Removal of MSC will lead to further activation and explosive proliferation of T-cells.

REFERENCES

1. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005 Feb 15;105(4):1815-22.

2. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002 May 15;99(10):3838-43.

3. Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, et al. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. Blood. 2007 Jan 1;109(1):228-34.

4. Nasef A, Mathieu N, Chapel A, Frick J, Francois S, Mazurier C, et al. Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G. Transplantation. 2007 Jul 27;84(2):231-7.

5. Di Ianni M, Del Papa B, De Ioanni M, Moretti L, Bonifacio E, Cecchini D, et al. Mesenchymal cells recruit and regulate T regulatory cells. Exp Hematol. 2008 Mar;36(3):309-18.

6. Casiraghi F, Azzollini N, Cassis P, Imberti B, Morigi M, Cugini D, et al. Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells. J Immunol. 2008 Sep 15;181(6):3933-46.

7. Prevosto C, Zancolli M, Canevali P, Zocchi MR, Poggi A. Generation of $CD4^+$ or $CD8^+$ regulatory T cells upon mesenchymal stem cell-lymphocyte interaction. Haematologica. 2007 Jul;92(7):881-8.

8. Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, et al. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood. 2005 Sep 1;106(5):1755-61.

9. Ohnishi S, Yanagawa B, Tanaka K, Miyahara Y, Obata H, Kataoka M, et al. Transplantation of mesenchymal stem cells attenuates myocardial injury and dysfunction in a rat model of acute myocarditis. J Mol Cell Cardiol. 2007 Jan;42(1):88-97.

10. Gonzalez-Rey E, Gonzalez MA, Rico L, Buscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut. 2009 Jul;58(7):929-39

11. Kunter U, Rong S, Djuric Z, Boor P, Muller-Newen G, Yu D, et al. Transplanted mesenchymal stem cells accelerate glomerular healing in experimental glomerulonephritis. J Am Soc Nephrol. 2006 Aug;17(8):2202-12.

12. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet. 2008 May 10;371(9624):1579-86.

13. Crop M, Baan C, Weimar W, Hoogduijn M. Potential of mesenchymal stem cells as immune therapy in solid-organ transplantation. Transpl Int. 2009 Apr;22(4):365-76.

14. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol. 2002 Jan;30(1):42-8.

15. Popp FC, Eggenhofer E, Renner P, Slowik P, Lang SA, Kaspar H, et al. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. Transpl Immunol. 2008 Nov;20(1-2):55-60.

16. Wan CD, Cheng R, Wang HB, Liu T. Immunomodulatory effects of mesenchymal stem cells derived from adipose tissues in a rat orthotopic liver transplantation model. Hepatobiliary Pancreat Dis Int. 2008 Feb;7(1):29-33.

17. Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Alwayn IP, Weimar W, et al. Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients. Transplantation. 2009 Mar 27;87(6):896-906.

18. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells. 2006 Feb;24(2):386-98.

19. Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol. 2007 Aug;149(2):353-63.

20. Benvenuto F, Ferrari S, Gerdoni E, Gualandi F, Frassoni F, Pistoia V, et al. Human mesenchymal stem cells promote survival of T cells in a quiescent state. Stem Cells. 2007 Jul;25(7):1753-60.

21. Hoogduijn MJ, Crop MJ, Peeters AM, Van Osch GJ, Balk AH, Ijzermans JN, et al. Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. Stem Cells Dev. 2007 Aug;16(4):597-604.

22. Sewgobind VD, Kho MM, van der Laan LJ, Hendrikx TK, van Dam T, Tilanus HW, et al. The effect of rabbit anti-thymocyte globulin induction

therapy on regulatory T cells in kidney transplant patients. Nephrol Dial Transplant. 2009 May;24(5):1635-44.

23. Hoogduijn MJ, Crop MJ, Peeters AM, Korevaar SS, Eijken M, Drabbels JJ, et al. Donor-derived mesenchymal stem cells remain present and functional in the transplanted human heart. Am J Transplant. 2009 Jan;9(1):222-30.

24. Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp Hematol. 2003 Oct;31(10):890-6.

25. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation. 2003 Feb 15;75(3):389-97.

26. McIntosh K, Zvonic S, Garrett S, Mitchell JB, Floyd ZE, Hammill L, et al. The immunogenicity of human adipose-derived cells: temporal changes in vitro. Stem Cells. 2006 May;24(5):1246-53.

27. Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, et al. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci. 2005;12(1):47-57.

28. Najar M, Rouas R, Raicevic G, Boufker HI, Lewalle P, Meuleman N, et al. Mesenchymal stromal cells promote or suppress the proliferation of T lymphocytes from cord blood and peripheral blood: the importance of low cell ratio and role of interleukin-6. Cytotherapy. 2009;11(5):570-83.

Chapter 7

Human mesenchymal stem cells are susceptible to lysis by CD8$^+$ T-cells and NK-cells

Meindert J. Crop[1], Sander S. Korevaar[1], Ronella de Kuiper[1], Jan N.M. IJzermans[2], Nicole M. van Besouw[1], Carla C. Baan[1], Willem Weimar[1], Martin J. Hoogduijn[1]

Departments of [1]Internal Medicine and [2]Surgery,
Erasmus University Medical Center, Rotterdam, The Netherlands

(submitted)

ABSTRACT

There is growing interest in the use of mesenchymal stem cells (MSC) to improve the outcome of organ transplantation. The immunogenicity of MSC is, however, unclear and is important for the efficacy of MSC therapy and for potential sensitization against donor antigens. We investigated the susceptibility of autologous and allogeneic MSC for lysis by $CD8^+$ T-lymphocytes and NK-cells in a kidney transplant setting.

MSC were derived from adipose tissue of human kidney donors and were $CD90^+$, $CD105^+$, $CD166^+$ and HLA class I^+. They showed differentiation ability and immunosuppressive capacity. Lysis of MSC by PBMC, FACS-sorted $CD8^+$ T-cells and NK-cells was measured by europium release assay.

Allogeneic MSC were susceptible for lysis by cytotoxic $CD8^+$ T-cells and NK-cells, while autologous MSC were lysed by NK-cells only. NK cell-mediated lysis was inversely correlated with the expression of HLA class I on MSC. Lysis of autologous MSC was not dependent of culturing of MSC in FBS, and MSC in suspension as well as adherent to plastic were lysed by NK-cells. Pretransplant recipient PBMC did not lyse donor MSC, but PBMC isolated 3, 6 and 12 months after transplantation showed increasing lysing ability. After 12 months, $CD8^+$ T-cell mediated lysis of donor MSC persisted, indicating there was no evidence for desensitization against donor MSC.

In conclusion, lysis of MSC is important to take into account when MSC are considered for clinical application. Our results suggest that the HLA background of MSC and timing of MSC administration are important for the efficacy of MSC therapy.

INTRODUCTION

Mesenchymal stem cells (MSC) have beneficial regenerative and immunomodulatory properties that make them promising for use in the treatment and prevention of a variety of disorders, including graft versus host disease and multiple sclerosis [1-3]. Recently, interest has risen in the use of MSC to improve the outcome of organ transplantation [4]. Animal models have demonstrated the ability of MSC to prolong the survival of skin [5], heart [6-7] and islets allografts [8] and to facilitate the recovery of kidney function after acute and chronic injury [9-10]. It is therefore anticipated that MSC therapy in transplant patients may allow a reduction in the dosing of immunosuppressive drugs and thereby limit their side effects, and to aid the repair of ischemia-reperfusion injury of the transplant [11].
The immunomodulatory functions of MSC are largely mediated via soluble factors. Via these factors MSC inhibit T-cell proliferation [12-13], maturation of dendritic cells and differentiation of B lymphocytes [14-16]. Moreover, MSC inhibit the formation of cytotoxic $CD8^+$ T-cells [17] and proliferation and cytotoxic activity of natural killer (NK) cells [18-20]. Reversely, it is less clear how immune cells affect MSC. It is reported that MSC have a low immunophenotype as they express low levels of HLA class I, no HLA class II and co-stimulatory molecules like CD80 and CD86, and that they therefore do not provoke immune responses [21]. This would make MSC transplantable across HLA barriers [22]. In contrast, there is evidence that MSC are immunogenic and can induce memory T-cell responses [23-24]. Furthermore, it has been reported that MSC are susceptible for lysis by NK-cells [17, 20, 25] as MSC express the activating NK cell-receptor ligands NKG2D and UL16 [26]. Interesting in this respect is the fact that intravenously administered MSC have repeatedly been demonstrated to disappear within days after infusion in immunocompetent mice [7]. It is not unlikely that lysis by cytotoxic cells is responsible for the disappearance of MSC [24, 27]. The observation of tumour formation after administration of autologous MSC in immunodeficient mice further strengthens this possibility [28].

It has been demonstrated that exposure of MSC to inflammatory conditions has an important impact on the immunomodulatory function of MSC [29-30]. In concert with the increased immunosuppressive properties of MSC, these conditions also change the immunophenotype of MSC by up regulating the expression of HLA molecules, co-stimulatory molecules and adhesion molecules [31]. This would make allogeneic MSC potentially more susceptible for recognition by $CD8^+$ T-cells. The immunogenicity and survival of MSC after administration is important for the efficacy of MSC therapy. Furthermore, in an organ transplant setting potential priming by donor MSC is of great relevance as it may induce rejection of the donor graft or perhaps desensitize against donor antigens. Therefore in the present study we investigated the susceptibility of autologous and allogeneic adipose tissue-derived MSC for lysis by NK-cells and $CD8^+$ T-cells. In addition, it was examined whether the susceptibility of donor-derived MSC for lysis by recipient cytotoxic cells changed in time after kidney transplantation. The results of this study are important for the choice of MSC and the timing of this therapy in organ transplantation.

MATERIALS AND METHODS

Isolation and culture of perirenal adipose tissue-derived MSC

During the kidney donation procedure, perirenal fat tissue was surgically removed after written informed consent as approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam (protocol no. MEC-2006-190) and collected in α-MEM medium (Gibco BRL, Paisley, UK) with 100 IU/ml penicillin and 100 µg/ml streptomycin (p/s, Gibco BRL, Paisley, UK). Adipose tissue-derived mesenchymal stem cells (ASC) were subsequently isolated as described previously [32-33]. Cultures were kept at 37°C, 5% CO_2 and 95% humidity and refreshed twice weekly with MSC culture medium, consisting of α-MEM (Gibco BRL) with p/s and 15% fetal bovine serum (FBS, Biowithaker, Verviers, Belgium). At 90% confluence, adherent cells were removed from culture flasks by incubation in 0.05% trypsin-EDTA at 37°C and cells were used for experiments described below or frozen at –150°C until further use. MSC were used for experiments between passages 2 to 5. When indicated, MSC were cultured with 50 ng/ml IFN-γ (U-CyTech biosciences, Utrecht, The Netherlands) for seven days.

Silencing of HLA class I expression

HLA class I expression by MSC was modulated by small interfering RNA (siRNA). Seven µl of siRNA for HLA class I (Ambion, Austin, USA) was added to 50 µl Opti-MEM (Gibco BRL). Twelve µl of si-Port (Ambion) was added to 50 µl Opti-MEM. siRNA and si-Port were mixed and incubated for 10 min at room temperature and added to a 6-well plate. Cell suspension in α-MEM with 15% FBS (0.5 ml) was added and incubated for 3 days. Controls were treated with non-coding siRNA.

Flow cytometric characterization of MSC

MSC were trypsinized, washed twice with FACSFlow (BD Biosciences, San Jose, CA, USA) and incubated with antibodies against CD90-APC, CD105-FITC (all R&D systems, Abingdon, UK), CD166-PE (BD Biosciences), HLA class I-ABC-PE, CD86-PE (Serotec) and HLA class II-DR-FITC (BD Biosciences) for 30 minutes. After incubation, cells were washed twice with FACSFlow and flow cytometric analysis performed using an 8-colour FACSCANTO-II with FACSDIVA Software (BD Biosciences).

Differentiation of MSC

Osteogenic differentiation was induced by culturing confluent MSC cultures in α-MEM supplemented with 1% p/s, 15% heat-inactivated FBS, 5mM β-glycerophosphate (Sigma-Aldrich, Munich, Germany), 50µg/ml L-ascorbic acid-phosphate (Sigma-Aldrich), and 10nM dexamethasone (Sigma-Aldrich) for 21 days. Cells were then washed with PBS and fixed in cold 4% paraformaldehyde for 5 min at room temperature. After two washes with H_2O, cells were incubated in 1% silver nitrate in H_2O at room temperature on a light box until blackening occurred. The cells were then washed three times with H_2O, incubated in 2.5% sodium thiosulfate in H_2O for 5 min at room temperature, washed twice with H_2O and photographed.

Adipogenic differentiation was induced by culturing confluent ASC cultures in α-MEM supplemented with 1% p/s, 15% heat-inactivated FBS, 50μg/ml L-ascorbic acid-phosphate (Sigma-Aldrich), 500μM 3-isobutyl-1-methylxanthine (IBMX, Fluka, Buchs, Switzerland), 60μM indomethacin (Fluka) and 10nM dexamethasone (Sigma-Aldrich) for 21 days. Cells were then fixed in 60% isopropanol for 1 min, and incubated in filtered 0.3% oil red O (Sigma-Aldrich) solution in 60% isopropanol for 10 minutes to stain lipid droplets. After several washes with PBS the cells were photographed.

Inhibition of mixed lymphocyte reactions by MSC

The immunosuppressive capacity of MSC was tested in mixed lymphocyte reactions (MLR). $5x10^4$ responder PBMC were stimulated by $5x10^4$ γ-irradiated (40Gy) allogeneic PBMC in RPMI+10% HI-FBS in round-bottom 96-well plates (Nunc, Roskilde, Denmark). MSC were added at various ratios to responder cells. On day 7, proliferation was measured by incorporation of ^{3}H-thymidine (0.5μCi/well) during a 16-hour incubation using a β-plate reader.

Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood samples were collected from living kidney donors and the corresponding kidney transplant recipients. Heparinized blood of kidney transplant recipients was drawn before transplantation, and 3, 6 and 12 months after kidney transplantation. PBMC were isolated by density gradient centrifugation using Ficoll Isopaque (δ=1.077, Amersham, Uppsala, Sweden) and frozen at –150°C until use.

Living kidney transplant recipient and donor pairs

To investigate the cytotoxicity-mediated lysis of MSC in a clinical kidney transplantation setting, non-HLA-identical living kidney donor-recipient pairs were included. Recipient PBMC isolated before transplantation and 3, 6, 12, 16, 18 and 24 months after transplantation were used as effector cells, while PBMC and MSC of the corresponding donor were used as targets in the cell-mediated lysis assays. Immunosuppressive therapy of kidney transplant recipients consisted of tacrolimus, mycophenolate mofetil and low-dose steroids.

Cytotoxicity-mediated lysis by europium release assay

Cytotoxicity-mediated lysis of target cells was determined by europium release assays as described previously [34-35]. In brief, for effector cells PBMC derived from recipients and healthy kidney donors were stimulated with allogeneic γ-irradiated PBMC and 200 IU/mL IL-2 (proleukin; Chiron BV, Amsterdam, The Netherlands) in round-bottom 96-well plates for 7 days at 37°C. PHA blasts and MSC were used as target cells. To obtain PHA blasts, PBMC were stimulated with 200 IU/mL recombinant IL-2 and 2 μg/mL PHA in 24-wells plates (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) for 7 days at 37°C. At day 3 and 6, cell cultures were split in two and refreshed with culture medium containing 200 IU/mL recombinant IL-2. After seven days, target cells were washed and living cells counted using an automatic cell counter system Casy (Schärfe System, Reutlingen, Germany). In addition, 100,000 target cells were used for flow cytometric analysis of HLA class I-ABC. The remaining target cells were labeled with europium-diethylenetriaminepentaacetate (DTPA) (Sigma, St. Louis, MO, USA). Autologous PHA blasts were used as negative control; allogeneic PHA blasts were used as positive control. MSC were cultured under control conditions or with 50 ng/ml IFN-γ for seven days.

At day 7, cytotoxicity-mediated lysis-assays were performed by incubating $5x10^3$ ^{63}Eu-labeled targets with various numbers of effector cells in 96-well plates for four hours. Effector:Target (ET) ratios ranged from 40:1 to 1.25:1. After incubation, the plates were centrifuged and 20 µL of the supernatant was transferred to 96-well plates with low background fluorescence (Fluoroimmunoplate, Nunc, Roskilde, Denmark). Subsequently, 100 µL enhancement solution (PerkinElmer, Groningen, The Netherlands) was added to each well. Released europium was measured in a time-resolved fluorometer (Victor 1420 Multilabel Counter, LKB-Wallac, Turku, Finland). Fluorescence was expressed in counts per second (cps).
Maximum release of europium by target cells was measured by incubation of $5x10^3$ labeled target cells with 1% triton (Sigma-Aldrich, Zwijndrecht, The Netherlands) for four hours. Spontaneous release of europium by target cells was measured by incubation of labeled target cells without effector cells. Percentage leakage was then calculated as (spontaneous release / maximum release)*100%. Mean percentage of leakage was 29±9% for PHA blasts, 25±6% for MSC and 26±7% for IFN-γ pretreated MSC. Finally, the percentage of cytotoxicity-mediated lysis was calculated as: % lysis = [(measured lysis-spontaneous release)/ (maximum release- spontaneous release)]*100%.

Isolation of $CD8^+$ T-cells and $CD16^+56^+$ NK-cells by FACS

PBMC cultured with allogeneic γ-irradiated PBMC and 200 IU/mL IL-2 for 7 days were washed twice with PBS with 1% heat-inactivated FBS and stained with antibodies against CD3-PE, CD4-FITC, CD8-PE-Cy7, CD56-APC, CD19-Amcyan, 7AAD-viaprobe (all BD Biosciences) and CD16-APC (Serotec) at room temperature and protected from light for 30 minutes. Subsequently, cells were sorted using a FACS ARIA-II with FACSDIVA Software (BD Biosciences). The lymphocytes were gated for CD3. The $CD3^+$ cells were then gated for CD8 to obtain $CD8^+$ T-cells. The $CD3^-$ cells were gated for CD16/56 to obtain NK-cells. Purity of sorted $CD8^+$ T-cells and $CD16^+56^+$ NK-cells was □98%. PBMC of healthy kidney donors used as effector cells consisted of 23±10% (mean±SD) $CD8^+$ T-cells and 22±11% NK-cells. Effector cells derived from PBMC of kidney recipients (12 months after transplantation) contained 22±9% $CD8^+$ T-cells and 16±8% NK-cells.

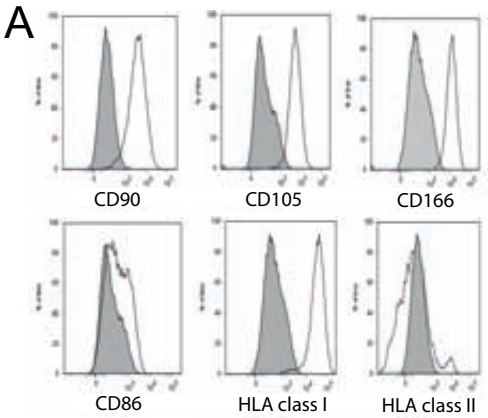
Statistical analysis

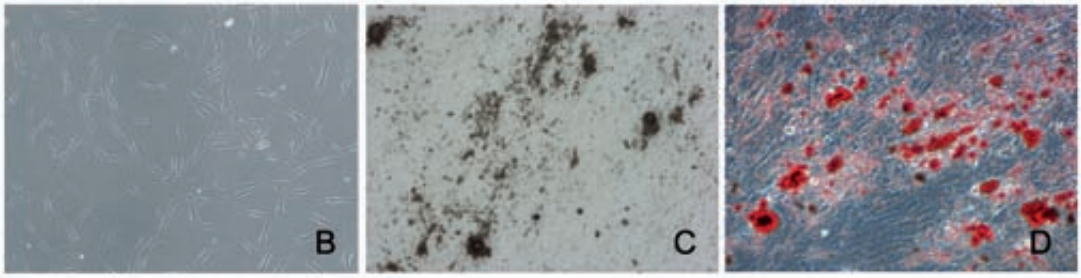
Data was analyzed using two-way ANOVA for repeated measures with Bonferroni post-tests. Distribution of the data was tested with Kolmogorov-Smirnov test for normality. Parametric data are expressed as mean±SD, while non-parametric data are expressed as median (25^{th}-75^{th} percentile, interquartile range). Statistical significance was defined as $p<0.05$ (two-tailed).

RESULTS

Characterization of MSC

The cells isolated from perirenal adipose tissue showed an immunophenotype typical for MSC with positive expression of CD90, CD105, CD166, HLA class I-ABC, and very low expression of CD86 and HLA class II-DR (Figure 1A), as described earlier [33]. They were adherent with a spindle-shaped morphology (Figure 1B) and showed the capacity to differentiate into the osteogenic lineage, as demonstrated by von Kossa staining for calcified nodules (Figure 1C) and the adipogenic lineage, as demonstrated by Oil red O staining for lipid vesicles (Figure 1D). Typically for MSC, they showed the capacity to dose-dependently inhibit the proliferation of alloactivated PBMC (Figure 1E).





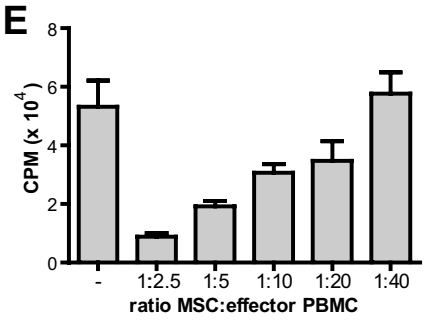


Figure 1. Characterization of adipose tissue-derived MSC. [A] Immunophenotype of MSC determined by flow cytometry. Grey histograms depict unstained MSC, open histograms depict MSC stained for the indicated marker. [B] Bright-field image of cultured MSC (magnitude 100x). [C] Von Kossa staining of osteogenic differentiated MSC. Black staining indicates calcified nodules. [D] Oil red O staining of adipogenic differentiated MSC. Red staining indicates lipid-filled vesicles. [E] Effect of MSC on alloactivated PBMC proliferation. MSC show dose-dependent inhibitory effect on PBMC proliferation.

Allogeneic MSC are susceptible for cytotoxicity-mediated lysis by CD8$^+$ T-cells and NK-cells

To investigate the susceptibility of MSC for lysis by cytotoxic immune cells, lysis of MSC was compared to lysis of PHA blasts. Over 95% of MSC and PHA blasts generated from PBMC of healthy kidney donors expressed HLA class I-ABC. Cytotoxic effector cells were generated from PBMC of healthy kidney donors by culture in the presence of γ-irradiated allogeneic PBMC and 200 IU/ml IL2 for one week. Effector cells lysed allogeneic PHA blasts in a dose-dependent manner (Figure 2A). Allogeneic MSC were lysed in a comparable fashion. To investigate whether lysis of allogeneic PHA blasts and MSC was mediated by CD8$^+$ T-cells or NK-cells, effector cells were sorted in CD3$^+$/CD8$^+$ T-cells and CD3$^-$/CD16$^+$56$^+$ NK-cells. PHA blasts were lysed by CD8$^+$ T-cells, but not by NK-cells (Figure 2B). MSC, in contrast, were lysed by both CD8$^+$ T-cells and NK-cells (Figure 2B). Lysis of MSC by allogeneic NK-cells was most efficient (58% of MSC at an ET ratio of 1:40 vs 41% of MSC by allogeneic CD8$^+$ T-cells at the same ET ratio).

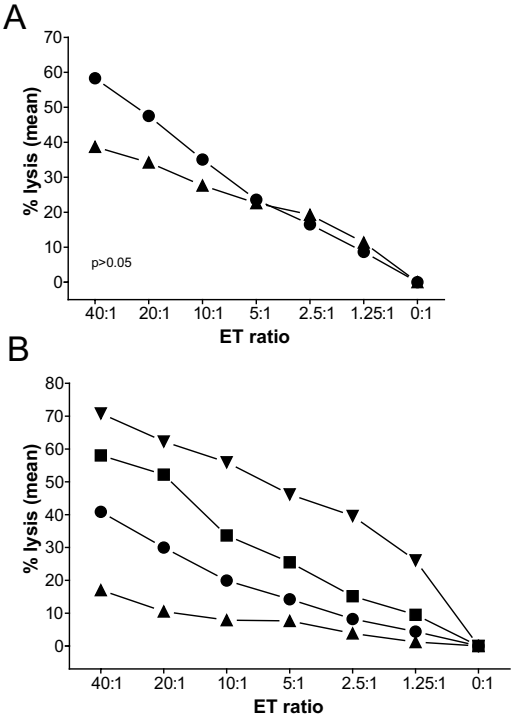


Figure 2. Cytotoxicity-mediated lysis of PHA blasts and MSC. [A] Cytotoxicity-mediated lysis of PHA blasts (▲) (n=15) and MSC (●) (n=5) by allogeneic effector cells of healthy kidney donors. MSC are lysed by allogeneic PBMC with comparable or even greater efficacy than PHA blasts. [B] Cytotoxicity-mediated lysis of MSC by purified allogeneic CD8$^+$ T-cells (●) and NK-cells (■) of healthy kidney donors (n=4) and lysis of PHA blasts by purified allogeneic CD8$^+$ T-cells (▼) and NK-cells (▲) of healthy kidney donors (n=3). Purity of CD8$^+$ T-cell and CD16$^+$56$^+$ NK cell fractions was ≥98%. ET-ratio = effector: target ratio.

IFN-γ enhances CD8$^+$ T-cell-mediated lysis, but reduces NK cell-mediated lysis of allogeneic MSC

To investigate whether the susceptibility of MSC for cytotoxicity-mediated lysis was affected by inflammatory conditions, MSC were cultured for 7 days with IFN-γ. Culturing of MSC with IFN-γ increased the mean fluorescence intensity (MFI) of HLA-ABC 4.7-fold (Figure 3A). The susceptibility of allogeneic MSC for NK cell-mediated lysis was decreased by IFN-γ treatment (Figure 3B), while lysis of MSC by CD8$^+$ T-cells was increased (Figure 3C).

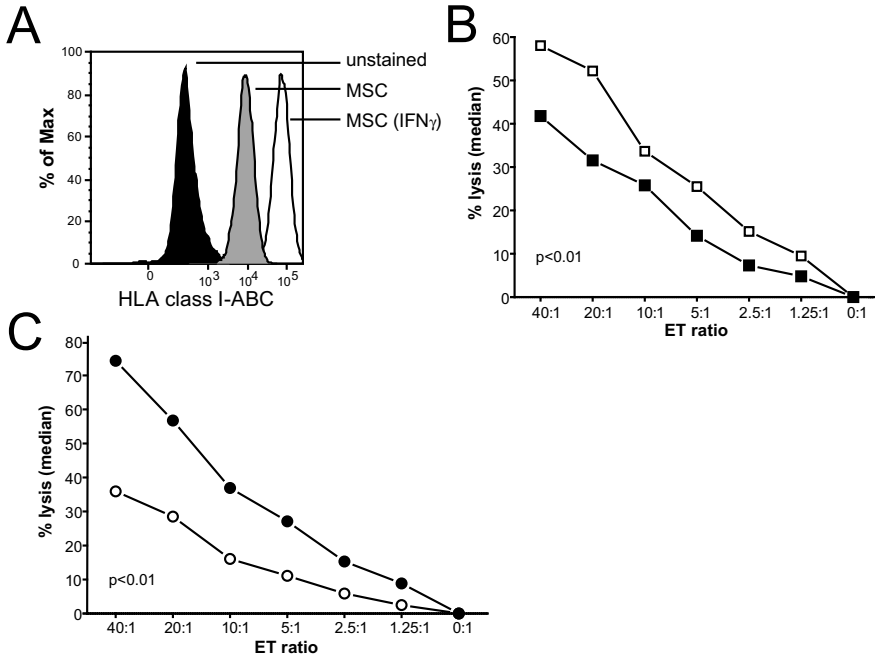


Figure 3. Effect of IFN-γ on cytotoxicity-mediated lysis of MSC. [A] Flow cytometric detection of HLA class I-ABC expression on MSC after seven days of culturing under control conditions or with IFN-γ (50 ng/ml). Representative example shown of n=16. [B] Cytotoxicity-mediated lysis of allogeneic MSC cultured under control conditions (□) or with IFN-γ (■) by purified NK-cells of healthy kidney donors (n=4). [C] Cytotoxicity-mediated lysis of allogeneic MSC cultured under control conditions (○) or with IFN-γ (●) by purified $CD8^+$ T-cells of healthy kidney donors (n=4). ET-ratio = effector: target ratio.

Autologous MSC are susceptible for cytotoxicity-mediated lysis by NK-cells

After demonstrating that MSC were effectively lysed by allogeneic $CD8^+$ T-cells and NK-cells, we questioned whether MSC were susceptible for lysis by autologous cytotoxic cells. Cytotoxic effector cells were not capable of lysing autologous PHA blasts at any ET-ratio, but lysed 27±20% of autologous MSC at an ET-ratio of 40:1 (Figure 4A). FACS purification of $CD8^+$ T-cells and NK-cells showed that lysis of autologous MSC was the result of NK cell cytotoxic activity (Figure 4B).

To exclude the possibility that the observed lysis of autologous MSC was the result of recognition of bovine proteins by NK-cells due to culture of MSC in foetal bovine serum (FBS), MSC were isolated from adipose tissue and cultures established and maintained in either medium containing 15% FBS or 15% human serum (HuS). Autologous effector cells lysed MSC cultured in FBS as efficiently as MSC cultured in HuS (52±25 vs. 49±20 %lysis at an ET-ratio of 40:1) (Figure 4C). These results indicate that culturing of MSC in FBS is not the cause of their susceptibility to cytotoxicity-mediated lysis.

To examine the role of HLA class I on NK cell lysis of autologous MSC, HLA expression was increased by treatment of MSC with IFN-γ for seven days or decreased by culturing MSC with siRNA for HLA class I for three days. Culturing MSC with siRNA for HLA class I strongly reduced HLA class I-ABC expression on MSC and only 13% of MSC showed positive expression, while the MFI reduced 2.8-fold (Figure 4D).

In agreement with the findings with allogeneic MSC, the lysis of autologous MSC by NK-cells was decreased after IFN-γ treatment of MSC to 50±2% (ET ratio of 40:1) (Figure 4E). MSC cultured with siRNA for HLA class I showed significantly increased susceptibility of MSC for autologous NK cell-mediated lysis (Figure 4F).

The fate of MSC after infusion is currently unclear. One possibility is that MSC home to sites of injury where they adhere to local stroma or epithelial cell layers. To determine whether adherent MSC would remain susceptible for lysis by autologous NK-cells, MSC were seeded in 6-well plates and purified NK-cells added. Autologous NK-cells showed potent capacity to lyse adherent MSC (92% lysis at an ET ration) (Figure 4G). IFN-γ treatment strongly reduced the susceptibility of adherent MSC for lysis by autologous NK-cells to 52% lysis.

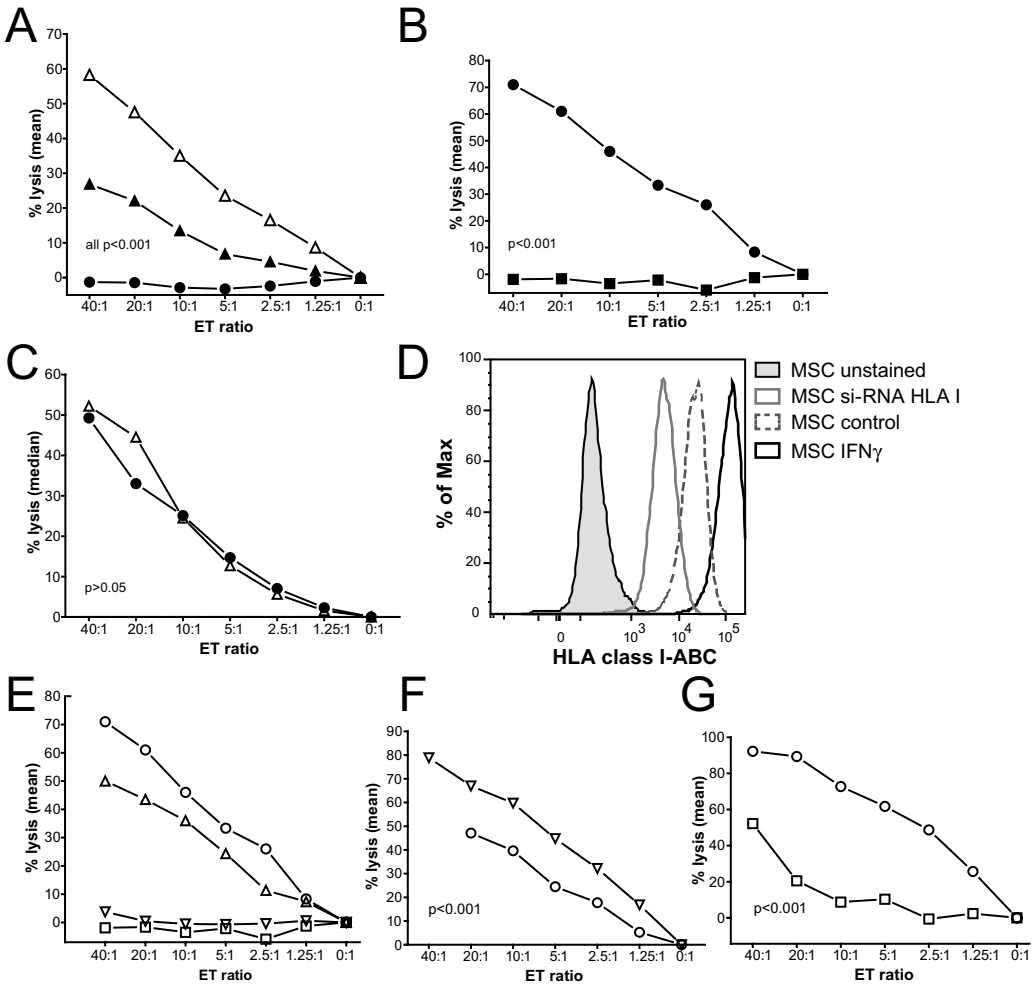


Figure 4. Cytotoxicity-mediated lysis of autologous MSC. [A] Cytotoxicity-mediated lysis of autologous MSC (▲) (n=12), allogeneic MSC (Δ) (n=5) and of autologous PHA blasts (●) (n=15) by effector cells of healthy kidney donors. [B] Cytotoxicity-mediated lysis of autologous MSC by purified $CD8^+$ T-cells (■) or NK-cells (●) by effector cells of healthy kidney donors (n=3). [C] Cytotoxicity-mediated lysis of autologous MSC isolated and cultured in foetal bovine serum (FBS)(Δ) or in human serum (HuS)(●) by effector cells of healthy kidney donors (n=4). [D] Flow cytometric detection of HLA class I-ABC expression on MSC cultured under control conditions, MSC cultured with siRNA for HLA class I-ABC for 3 days, or MSC cultured with IFN-γ (50 ng/ml) for seven days. Representative example shown of n=3. [E] Cytotoxicity-mediated lysis of IFN-γ–treated MSC by purified autologous NK-cells (Δ) or $CD8^+$ T-cells (∇) of healthy kidney donors and of control MSC by autologous purified NK-cells (○) or $CD8^+$ T-cells (□) (n=3). [F] Cytotoxicity-mediated lysis of control MSC (○) and of MSC treated with siRNA for HLA class I (∇) by purified autologous NK-cells of healthy kidney donors (n=2). [G] Cytotoxicity-mediated lysis of adherent autologous MSC (○, n=4) or IFN-γ–pretreated MSC (□, n=3) by NK-cells of healthy kidney donors. ET-ratio = effector: target ratio.

Kidney donor-derived MSC are lysed by recipient cytotoxic immune cells

The immunogenicity and survival of MSC is an important issue for their application in organ transplantation. As we found that MSC are susceptible for $CD8^+$ T-cell and NK cell-mediated lysis, we wanted to examine this in a kidney transplant setting using donor MSC and kidney recipient immune cells isolated at various time points after kidney transplantation. Cytotoxic effector cells of recipients isolated before transplantation showed poor capacity to lyse donor MSC (5±5% lysis at an ET ratio of 40:1). Recipient cytotoxic cells isolated 3, 6 and 12 months after transplantation showed increasing capacity to lyse MSC in time after transplantation (Figure 5A). Lysis of donor MSC 12 month after transplantation was for 33 (9-57)% dependent on $CD8^+$ T-cell-mediated lysis while the $CD8^+$ T-cell depleted cell fraction was responsible for 55 (46-68)% of MSC lysis (Figure 5B). This indicates that the recovered lysis of donor MSC after transplantation is for a major part dependent on NK cell cytotoxicity.

Finally, we examined whether there was evidence for the development of hypo- or hyper-responsiveness (sensitization) of kidney transplant recipients against donor MSC after transplantation by looking at the capacity of recipient $CD8^+$ T-cells to lyse donor MSC. As we found that non-$CD8^+$ T-cell-mediated lysis of donor MSC was recovered 12 months after transplantation, we examined whether the balance between $CD8^+$ T-cell-mediated and non-$CD8^+$ T-cell-mediated lysis of donor MSC was shifted more than 12 months after transplantation compared to non-transplant healthy kidney donors. $CD8^+$ T-cell-mediated lysis of donor MSC remained well below the lysis of donor MSC by $CD8^+$ T-cell depleted cells in 3 recipients that were 16, 18 and 24 months after transplantation (Figure 5C). These results suggest that there is no evidence for hypo- or hyper-responsiveness against donor MSC long after kidney transplantation.

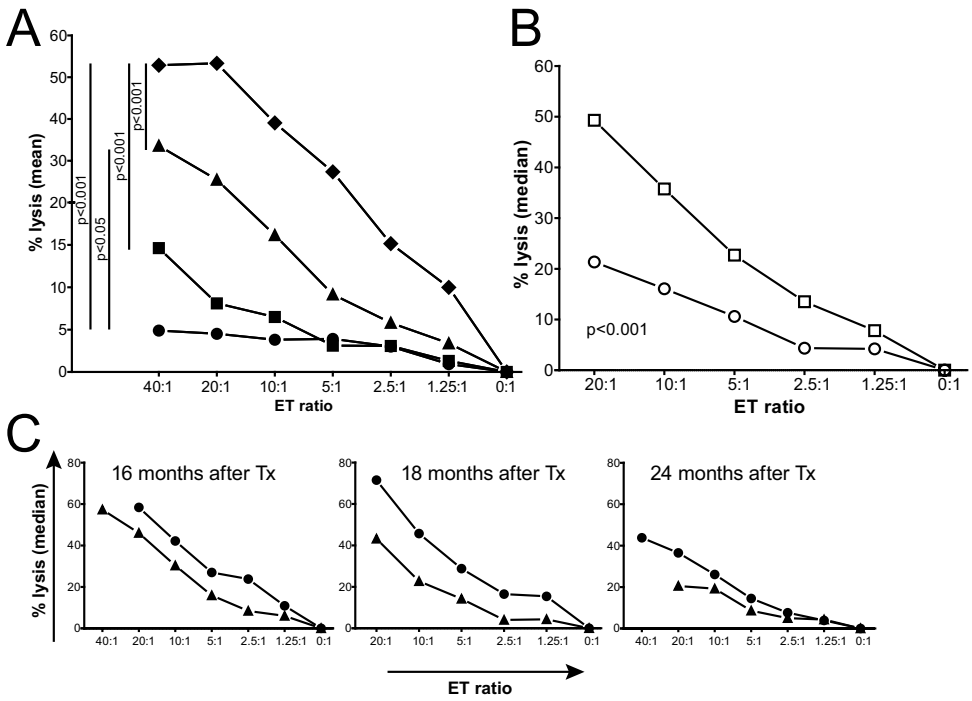


Figure 5. Cytotoxicity-mediated lysis of MSC in a kidney transplantation setting

[A] Cytotoxicity-mediated lysis of kidney donor-derived MSC by recipient immune cells collected before transplantation (●, n=5), and 3 months (■, n=5), 6 months (▲, n=8) and 12 months (♦, n=9) after transplantation. [B] Cytotoxicity-mediated lysis of kidney donor-derived MSC by purified $CD8^+$ T-cells (○) and $CD8^+$ T-cell-depleted (□) recipient cells isolated 12 months after transplantation (n=6). [C] Cytotoxicity-mediated lysis of kidney donor-derived MSC by purified $CD8^+$ T-cells (▲) and $CD8^+$ T-cell-depleted (●) recipient cells isolated 16 months (n=1), 18 months (n=1) and 24 months (n=1) after transplantation (n=6). ET-ratio = effector: target ratio. Tx = transplantation.

DISCUSSION

In an increasing number of clinical trials the use of MSC for immune therapy is studied. Trials investigating the use of MSC in organ transplantation have recently initiated [11]. Although the use of autologous MSC may be preferable to avoid recognition of MSC by the immune system, the use of "off-the-shelf" allogeneic MSC is more practical. The immunogenicity and survival of autologous and allogeneic MSC is, however, unknown and is important for the efficacy of clinical trials. In particular, it may have implications in an organ transplant setting where cross-reactivity between MSC and the organ transplant may induce allograft rejection. In the present study we investigated the susceptibility of autologous and allogeneic MSC cultured under control and inflammatory conditions for lysis by cytotoxic $CD8^+$ T-cell and NK-cells. Subsequently, the susceptibility of donor-derived MSC for lysis by kidney transplant recipient cytotoxic cells isolated before and after kidney transplantation was examined.

Several studies reported that MSC can inhibit the formation of cytotoxic cells [17-18, 36]. Data on the susceptibility of MSC for lysis by cytotoxic cells is, however, contradictory. It has been suggested that MSC are immune privileged and that they are not susceptible for lysis by cytotoxic immune cells [18, 21]. Others reported that MSC are susceptible for cytotoxicity-mediated lysis, especially by NK-cells [37-38]. In our hands, allogeneic adipose tissue-derived MSC were highly susceptible for cytotoxicity-mediated lysis, at a rate comparable to allogeneic PHA blasts. Whereas allogeneic PHA blasts were lysed by $CD8^+$ T-cells only, allogeneic MSC were lysed by $CD8^+$ T-cells and NK-cells. This difference may lie in expression of activating NK cell receptor ligands on MSC and PHA blasts of which several are found on MSC [26, 37]. Autologous MSC were not lysed by $CD8^+$ T-cells, but were susceptible for lysis by NK-cells. Expression levels of HLA class I molecules modulated the susceptibility of MSC for NK cell lysis, as down-regulation of HLA class I increased lysis of MSC by NK-cells, whereas up-regulation of HLA class I by IFN-γ was associated with protection of MSC. We ruled out the possibility that lysis of MSC was the result of recognition of bovine antigens due to culture of MSC in FBS by establishing and maintaining MSC cultures in medium with human serum. Culturing in human serum had no effect on lysis of MSC.

The fate of MSC after intravenous infusion is not yet clear. One possibility is that MSC home to specific tissue sites or to sites of injury, and adhere to the stroma or epithelial cell layers at these sites. To find out whether homing would protect MSC from lysis by NK-cells, we added autologous NK-cells to adherent MSC monolayers. Under these conditions, MSC remained susceptible for lysis by NK-cells, suggesting that homed MSC can still be cleared by NK-cells.
Our finding that both autologous and allogeneic MSC are susceptible for lysis by cytotoxic cells may explain why MSC seem to disappear shortly after infusion [4, 27, 39]. Nevertheless, lysis of autologous MSC remains remarkable. We previously demonstrated that MSC of donor origin can be isolated from heart biopsies up to six years after heart transplantation in human [40]. These heart transplant patients were obviously under immunosuppression, suggesting that the lysis of MSC may be preventable with immunosuppressive drugs.

It has been reported previously that exposure of MSC to inflammatory conditions affects immunophenotype and enhances the immunosuppressive function of MSC [30, 41-42]. This is particularly relevant when MSC are used as clinical immune therapy. Pro-inflammatory cytokines, such as IFN-γ, increase the expression of HLA class I and II subtypes on MSC. Pre-treatment of autologous and allogeneic MSC with IFN-γ made them less susceptible for NK mediated lysis, whereas it increased $CD8^+$ T-cell-mediated lysis of allogeneic MSC as a result of elevated HLA class I expression. In line with the results obtained from healthy individuals, we found that kidney transplant recipient-derived cytotoxic $CD8^+$ T-cells and NK-cells were capable of lysing donor-derived MSC. Recipient cytotoxic cells isolated with increasing time after transplantation were increasingly efficient in lysing MSC compared to recipient cells isolated before transplantation. The lack of lysis of MSC by recipient immune cells isolated prior to transplantation could reflect the poor renal function and general health of the patients, while the increasingly efficient lysis of MSC after transplantation may be the result of recovery of the renal function or improvement of the recipient's immune system and tapering of immunosuppressive drugs after transplantation. We also found that $CD8^+$ T-cell-mediated lysis of donor MSC more than a year after transplantation remained well below the lysing efficiency of NK-cells, similar as found in healthy kidney donors, indicating that hyper-responsiveness against donor MSC may not occur.

In conclusion, our results suggest that controlling MSC lysis by cytotoxic immune cells may be important for successful MSC therapy in organ transplantation. Recent data shows that blocking of CD16 on NK-cells inhibits lysis of MSC [43]. Other approaches to improve MSC survival may include *in vitro* modulation of MSC to reduce their immunogenicity. Pre-clinical models should be used to find out whether such approaches affect the balance between the efficacy and safety of MSC therapy.

REFERENCES

1. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet. 2008 May 10;371(9624):1579-86.

2. Freedman MS, Bar-Or A, Atkins HL, Karussis D, Frassoni F, Lazarus H, et al. The therapeutic potential of mesenchymal stem cell transplantation as a treatment for multiple sclerosis: consensus report of the International MSCT Study Group. Mult Scler. 2010 Apr;16(4):503-10.

3. Giordano A, Galderisi U, Marino IR. From the laboratory bench to the patient's bedside: an update on clinical trials with mesenchymal stem cells. J Cell Physiol. 2007 Apr;211(1):27-35.

4. Dahlke MH, Hoogduijn M, Eggenhofer E, Popp FC, Renner P, Slowik P, et al. Toward MSC in solid organ transplantation: 2008 position paper of the MISOT study group. Transplantation. 2009 Sep 15;88(5):614-9.

5. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol. 2002 Jan;30(1):42-8.

6. Casiraghi F, Azzollini N, Cassis P, Imberti B, Morigi M, Cugini D, et al. Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells. J Immunol. 2008 Sep 15;181(6):3933-46.

7. Popp FC, Eggenhofer E, Renner P, Slowik P, Lang SA, Kaspar H, et al. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. Transpl Immunol. 2008 Nov;20(1-2):55-60.

8. Ding Y, Xu D, Feng G, Bushell A, Muschel RJ, Wood KJ. Mesenchymal stem cells prevent the rejection of fully allogenic islet grafts by the immunosuppressive activity of matrix metalloproteinase-2 and -9. Diabetes. 2009 Aug;58(8):1797-806.

9. Morigi M, Introna M, Imberti B, Corna D, Abbate M, Rota C, et al. Human bone marrow mesenchymal stem cells accelerate recovery of acute renal injury and prolong survival in mice. Stem Cells. 2008 Aug;26(8):2075-82.

10. Semedo P, Palasio CG, Oliveira CD, Feitoza CQ, Goncalves GM, Cenedeze MA, et al. Early modulation of inflammation by mesenchymal stem cell after acute kidney injury. Int Immunopharmacol. 2009 Jun;9(6):677-82.

11. Hoogduijn MJ, Popp FC, Grohnert A, Crop MJ, van Rhijn M, Rowshani AT, et al. Advancement of mesenchymal stem cell therapy in solid organ transplantation (MISOT). Transplantation. 2010 Jul 27;90(2):124-6.

12. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005 Feb 15;105(4):1815-22.

13. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002 May 15;99(10):3838-43.

14. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human mesenchymal stem cells modulate B-cell functions. Blood. 2006 Jan 1;107(1):367-72.

15. Tabera S, Perez-Simon JA, Diez-Campelo M, Sanchez-Abarca LI, Blanco B, Lopez A, et al. The effect of mesenchymal stem cells on the viability, proliferation and differentiation of B-lymphocytes. Haematologica. 2008 Jul 18;93(9):1301-9.

16. Asari S, Itakura S, Ferreri K, Liu CP, Kuroda Y, Kandeel F, et al. Mesenchymal stem cells suppress B-cell terminal differentiation. Exp Hematol. 2009 May;37(5):604-15.

17. Angoulvant D, Clerc A, Benchalal S, Galambrun C, Farre A, Bertrand Y, et al. Human mesenchymal stem cells suppress induction of cytotoxic response to alloantigens. Biorheology. 2004;41(3-4):469-76.

18. Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation. 2003 Oct 27;76(8):1208-13.

19. Spaggiari GM, Capobianco A, Abdelrazik H, Becchetti F, Mingari MC, Moretta L. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood. 2008 Feb 1;111(3):1327-33.

20. Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells. 2006 Jan;24(1):74-85.

21. Rasmusson I, Uhlin M, Le Blanc K, Levitsky V. Mesenchymal stem cells fail to trigger effector functions of cytotoxic T lymphocytes. J Leukoc Biol. 2007 Oct;82(4):887-93.

22. Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated

mesenchymal stem cells. Exp Hematol. 2003 Oct;31(10):890-6.

23. Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a non-myeloablative setting. Blood. 2006 May 11;108(6):2114-20.

24. Badillo AT, Beggs KJ, Javazon EH, Tebbets JC, Flake AW. Murine bone marrow stromal progenitor cells elicit an in vivo cellular and humoral alloimmune response. Biol Blood Marrow Transplant. 2007 Apr;13(4):412-22.

25. Pende D, Cantoni C, Rivera P, Vitale M, Castriconi R, Marcenaro S, et al. Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. Eur J Immunol. 2001 Apr;31(4):1076-86.

26. Poggi A, Prevosto C, Zancolli M, Canevali P, Musso A, Zocchi MR. NKG2D and natural cytotoxicity receptors are involved in natural killer cell interaction with self-antigen presenting cells and stromal cells. Ann N Y Acad Sci. 2007 Aug;1109:47-57.

27. Eliopoulos N, Stagg J, Lejeune L, Pommey S, Galipeau J. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. Blood. 2005 Dec 15;106(13):4057-65.

28. Fiorina P, Jurewicz M, Augello A, Vergani A, Dada S, La Rosa S, et al. Immunomodulatory function of bone marrow-derived mesenchymal stem cells in experimental autoimmune type 1 diabetes. J Immunol. 2009 Jul 15;183(2):993-1004.

29. Polchert D, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E, et al. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. Eur J Immunol. 2008 Jun;38(6):1745-55.

30. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells. 2006 Feb;24(2):386-98.

31. Cao Y, Luetkens T, Kobold S, Hildebrandt Y, Gordic M, Lajmi N, et al. The cytokine/chemokine pattern in the bone marrow environment of multiple myeloma patients. Exp Hematol. 2010 Jul 6.

32. Hoogduijn MJ, Crop MJ, Peeters AM, Van Osch GJ, Balk AH, Ijzermans JN, et al. Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. Stem Cells Dev. 2007 Aug;16(4):597-604.

33. Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Alwayn IP, Weimar W, et al. Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients. Transplantation. 2009 Mar 27;87(6):896-906.

34. van Besouw NM, van der Mast BJ, de Kuiper P, Smak Gregoor PJ, Vaessen LM, JN IJ, et al. Donor-specific T-cell reactivity identifies kidney transplant patients in whom immunosuppressive therapy can be safely reduced. Transplantation. 2000 Jul 15;70(1):136-43.

35. Bouma GJ, van der Meer-Prins PM, van Bree FP, van Rood JJ, Claas FH. Determination of cytotoxic T-lymphocyte precursor frequencies using europium labeling as a nonradioactive alternative to labeling with chromium-51. Hum Immunol. 1992 Oct;35(2):85-92.

36. Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, et al. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. Stem Cells. 2008 Jan;26(1):212-22.

37. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood. 2006 Feb 15;107(4):1484-90.

38. Yen BL, Chang CJ, Liu KJ, Chen YC, Hu HI, Bai CH, et al. Brief report--human embryonic stem cell-derived mesenchymal progenitors possess strong immunosuppressive effects toward natural killer cells as well as T lymphocytes. Stem Cells. 2009 Feb;27(2):451-6.

39. Togel F, Hu Z, Weiss K, Isaac J, Lange C, Westenfelder C. Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. Am J Physiol Renal Physiol. 2005 Jul;289(1):F31-42.

40. Hoogduijn MJ, Crop MJ, Peeters AM, Korevaar SS, Eijken M, Drabbels JJ, et al. Donor-derived mesenchymal stem cells remain present and functional in the transplanted human heart. Am J Transplant. 2009 Jan;9(1):222-30.

41. Najar M, Rouas R, Raicevic G, Boufker HI, Lewalle P, Meuleman N, et al. Mesenchymal stromal cells promote or suppress the proliferation of T lymphocytes from cord blood and peripheral blood: the importance of low cell ratio and role of interleukin-6. Cytotherapy. 2009;11(5):570-83.

42. Hemeda H, Jakob M, Ludwig A, Giebel B, Lang S, Brandau S. Interferon-gamma and Tumor Necrosis Factor-alpha Differentially Affect Cytokine Expression and Migration Properties of Mesenchymal Stem Cells. Stem Cells Dev. 2010 May;19(5):693-706

43. Jewett A, Arasteh A, Tseng HC, Behel A, Arasteh H, Yang W, et al. Strategies to rescue mesenchymal stem cells (MSCs) and dental pulp stem cells (DPSCs) from NK cell mediated cytotoxicity. PLoS One. 2010;5(3):e9874.

Chapter 8

Summary and Discussion

SUMMARY

End-stage renal disease (ESDR) is gradually but inevitably leading to dialysis. Kidney transplantation is the only effective treatment for patients with ESRD, but requires life-long use of immunosuppressive medication to prevent graft rejection. Although immunosuppressive drugs are effective in preventing graft loss, they can have severe side effects, such as allowing the occurrence of cardiovascular diseases, opportunistic infections, development of malignancies and nephrotoxicity [1-2]. There is therefore an ongoing search for new approaches for immunosuppression. These include the development of cellular therapies. One of the candidate cell types are mesenchymal stem cells (MSC) (**Chapter 1**). This thesis investigates the immunomodulatory properties of mesenchymal stem cells for application in organ transplantation and in particular in kidney transplantation.

MSC and are virtually present in all adult tissues [3]. They can be relatively easily isolated and expanded *in vitro* for clinical use and appear as spindle-shaped, adherent cells in culture (Figure 1).
The classical source of MSC is bone marrow. Because the collection of bone marrow is invasive, painful and yields variable numbers of MSC, currently alternative sources of MSC are investigated.

In **Chapter 2** we demonstrated the isolation of MSC from human spleen, heart and perirenal adipose tissue and compared their phenotype and function with bone marrow-derived MSC. We found that MSC derived from the different tissues had comparable characteristics to bone marrow MSC. Especially adipose tissue may be a good alternative source for MSC, as it can be obtained under local anesthesia through a simple, little invasive surgical procedure and provides more consistent numbers of MSC than bone marrow.

MSC have two interesting properties that can be beneficial in solid organ transplantation. Firstly, MSC have the potential to differentiate into more specialized cell types, such as adipocytes or osteoblasts, but probably many others such as renal cells [4-6]. Furthermore, MSC secrete a diversity of trophic factors that stimulate proliferation and differentiation of tissue resident progenitor cells [7]. In solid organ transplantation MSC therapy may therefore repair ischemia-reperfusion injury of the graft and enhance graft function.

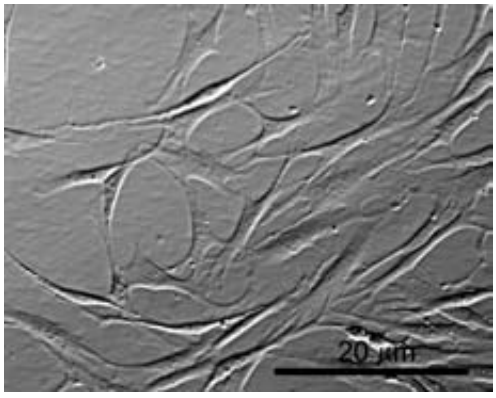


Figure 1. Mesenchymal stem cells in culture

Secondly, MSC have immunomodulatory properties. MSC demonstrate potent immunosuppressive capacity by inhibiting the proliferation and antibody production of mitogen and alloactivated T and B lymphocytes and by preventing the differentiation of antigen-presenting cells *in vitro* [8-10]. These immunomodulatory effects are partly mediated via soluble mechanisms, such as via the depletion of tryptophan by indolamine-2,3-dioxygenase (IDO), and via the secretion of anti-inflammatory factors such as prostaglandin E2, TGF-β, IL-10, HLA-G, hepatocyte growth factor, and nitric oxide [11-15]. In addition, MSC establish cell contact interactions with immune cells that may enhance the immunomodulatory effects of MSC [16-17]. In a kidney transplantation setting, we found that MSC significantly suppressed donor-stimulated proliferation of transplant recipient peripheral blood mononuclear cells (PBMC), especially of T-cells (**chapter 3**). Autologous, donor and third-party MSC had comparable efficacy to inhibit alloreactivity, suggesting that the inhibition of PBMC proliferation by MSC is HLA independent. MSC were furthermore able to effectively suppress donor-directed mixed lymphocyte reactions after kidney transplantation, which shows that MSC are capable of inhibiting memory responses.

If MSC are used as cellular therapy in solid organ transplantation, it is likely that they will be given in conjunction with immunosuppressive medication. In **chapter 4**, the effect of the immunosuppressants tacrolimus, rapamycin and mycophenolic acid (MPA) on MSC immunophenotype and functionality was examined. Clinical concentrations of these immunosuppressive drugs did not induce toxicity or apoptosis in MSC. The differentiation capacity of MSC was, however, differentially affected by the different drugs. Unexpectedly, MSC hampered the immunosuppressive efficacy of tacrolimus and rapamycine. There was no adverse effect of MSC on the function of MPA. These data therefore suggest that MPA is the best immunosuppressant to combine with MSC therapy in transplant patients.

In contrast to most studies that investigate the immunomodulatory effects of MSC on activated immune cells, we examined in **chapter 5** the effect of inflammatory conditions on MSC. MSC were exposed to two inflammatory conditions; alloactivated PBMC (MLR) separated from MSC in transwell culture systems or pro-inflammatory cytokines (IFN-γ, TNF-α and IL-6), and examined the immunophenotype and function of MSC. We found that the inflammatory conditions differentially affected the immunophenotype and proliferation capacity of MSC. While the regenerative properties of MSC were not affected, the immunosuppressive effects of MSC were strikingly boosted after exposure to inflammatory conditions. In particular the expression of IDO was stimulated by pre-treatment of MSC with pro-inflammatory cytokines. Increased expression of IDO was correlated to enhanced immunosuppressive effects of MSC, while blocking of IDO reduced the immunosuppressive effect of MSC. Furthermore, gene expression analysis also showed that both inflammatory conditions increased the expression of genes for HLA class I and II and for a variety of chemokines in a differential manner. This suggests in the first place that MSC adapt a potentially more immunogenic phenotype under inflammatory conditions and secondly that the phenotype and function of MSC is differentially affected by different inflammatory conditions. Consecutively, these results indicate that *in vitro* modulation of MSC can be used to modulate MSC function before clinical application.

Whereas the immunosuppressive effects of MSC on *activated* immune cells are intensively investigated, we demonstrated in **chapter 6** that MSC stimulate the activation and proliferation of *resting, non-activated* T-cells. MSC of autologous origin had the same effect as allogeneic MSC, indicating the activating effect of MSC is independent of HLA. Further experiments demonstrated that removal of MSC from co-cultures with resting PBMC resulted in a striking increase in the proliferation of T-cells. An increased proportion of these T-cells demonstrated a regulatory T-cell phenotype, i.e. $CD4^+CD25^+FoxP3^+CD127^{low}$, and had immunosuppressive capacity. These results indicate that by stimulation of the proliferation of resting T-cells MSC can generate functional regulatory T-cells.
Despite the promising properties of MSC demonstrated in *in vitro* studies, there are a number of issues concerning the survival of MSC after in vivo administration that need to be clarified before clinical application of MSC. Firstly, it is unclear whether MSC are recognized by cytotoxic $CD8^+$ T-cells and risk the development of memory responses. Furthermore, it is unclear how the innate immune system tolerates infused culture expanded MSC.

In **chapter 7** the susceptibility of MSC for lysis by $CD8^+$ T-cells and natural killer (NK) cells was examined. In contrast to several studies demonstrating that MSC are not immunogenic, we found that allogeneic MSC were efficiently lysed by both cytotoxic $CD8^+$ T-cells and NK-cells. Surprisingly, autologous MSC were also susceptible for lysis by NK-cells. Pre-treatment of MSC with IFN-γ increased the expression of HLA class I on MSC and led to increased $CD8^+$ T-cell-mediated lysis, but reduced NK-cell-mediated lysis. In a kidney transplantation setting, recipient's cytotoxic immune cells were able to lyse donor-derived MSC with increased efficacy in time after transplantation, probably by recovering of the immune system after transplantation. These results are important should be taken into account for timing of MSC therapy.

In conclusion, the immunosuppressive and regenerative capacities of MSC make them attractive candidates for numerous clinical applications, including end-stage renal disease and kidney transplantation. MSC therapy has the potential to target various aspects of organ transplantation, from stretching the life-span of progressively deteriorating organs and delay transplantation, preservation of procured organs, reduction and repair of ischemia-reperfusion injury, to treatment of acute rejection and reduction of chronic allograft injury by sparing long-term anti-rejection drugs and eventually promoting tolerance.

It is clear that more basic research is needed to understand how MSC can most effectively be applied in organ transplantation. Well-controlled clinical trials can greatly accelerate the process. Early safety trials should take the opportunity to look at mechanistic effects of MSC in clinical organ transplantation accompanied by proper biomarker evaluation. Several phase I trials testing the safety of MSC infusion before and after liver and kidney transplantation are currently in preparation and a small number have recently started. Very preliminary results indicate that MSC therapy in transplantation patients is feasible and safe. Hopefully, new data from the first trials will confirm this, while ongoing pre-clinical studies can further address the impact of MSC cell source, timing and *in vitro* modulation to identify the optimal efficacy of MSC therapy. The coming years will be critical for the implementation of the lessons learned from basic and clinical studies in MSC therapy in solid organ transplantation.

REFERENCES

1. Marcen R. Immunosuppressive drugs in kidney transplantation: impact on patient survival, and incidence of cardiovascular disease, malignancy and infection. Drugs. 2009 Nov 12;69(16):2227-43.

2. Boots JM, Christiaans MH, van Hooff JP. Effect of immunosuppressive agents on long-term survival of renal transplant recipients: focus on the cardiovascular risk. Drugs. 2004;64(18):2047-73.

3. da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci. 2006 Jun 1;119(Pt 11):2204-13.

4. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999 Apr 2;284(5411):143-7.

5. Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow.Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation. 1968 Mar;6(2):230-47.

6. Herrera MB, Bussolati B, Bruno S, Fonsato V, Romanazzi GM, Camussi G. Mesenchymal stem cells contribute to the renal repair of acute tubular epithelial injury. Int J Mol Med. 2004 Dec;14(6):1035-41.

7. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006 Aug 1;98(5):1076-84.

8. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002 May 15;99(10):3838-43.

9. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood. 2003 May 1;101(9):3722-9.

10. Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, et al. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci. 2005;12(1):47-57.

11. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood. 2005 Feb 15;105(4):1815-22.

12. Groh ME, Maitra B, Szekely E, Koc ON. Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. Exp Hematol. 2005 Aug;33(8):928-34.

13. Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, et al. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. Blood. 2007 Jan 1;109(1):228-34.

14. DelaRosa O, Lombardo E, Beraza A, Mancheno-Corvo P, Ramirez C, Menta R, et al. Requirement of IFN-gamma-mediated indoleamine 2,3-dioxygenase expression in the modulation of lymphocyte proliferation by human adipose-derived stem cells. Tissue Eng Part A. 2009 Oct;15(10):2795-806.

15. Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol. 2007 Aug;149(2):353-63.

16. Quaedackers ME, Baan CC, Weimar W, Hoogduijn MJ. Cell contact interaction between adipose-derived stromal cells and allo-activated T lymphocytes. Eur J Immunol. 2009 Dec;39(12):3436-46.

17. Ren G, Zhao X, Zhang L, Zhang J, L'Huillier A, Ling W, et al. Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. J Immunol. 2010 Mar 1;184(5):2321-8.

DISCUSSION

In the present thesis the potential of MSC for application in solid organ transplantation as an alternative for current drug-based immunosuppressive regimens was investigated. The immunosuppressive properties of MSC may reduce immune reactivity, while at the same time their regenerative properties may improve graft function by repairing damage of the transplanted organ. For these perspectives several topics relevant for the use of MSC in kidney transplantation were investigated. We studied the tissue source for MSC, the immunosuppressive capacity of autologous and allogeneic MSC before and after kidney transplantation, the effect of concurrent immunosuppressive drugs or inflammatory conditions on the phenotype and function of these cells. Finally, we examined whether *in vitro*-expanded MSC are susceptible for lysis by cytotoxic immune cells. The results of these studies are important to understand the biology and function of MSC, which may contribute to the clinical application of MSC in solid organ transplantation.

1. Mesenchymal stem cells

A recurrent topic in MSC research is the poor definition of MSC due to lack of a specific MSC marker. MSC consist of a heterogeneous pool of cells with comparable characteristics and their distinction from fibroblasts is difficult [1-2]. Better characterization of MSC is recommended, which would facilitate the comparison of results between studies. Heterogeneity of MSC in culture, however, does not hamper the promising results obtained from *in vitro*, animal and human studies and is not necessarily disadvantageous for application of MSC in organ transplantation as cellular (immune) therapy. There is, however, a possibility that various subsets within the MSC pool possess different functions, which may be more or less suitable for use of MSC as immune modulators.

It has been reported that MSC can be isolated from virtually all adult tissues [3]. The question remains what the physiological role of MSC in these tissues is. It can be speculated that tissue-resident MSC contribute to tissue regeneration and (immunological) homeostasis via secretion of various immunomodulatory and trophic factors. These functions may in fact be shared by all stromal cells, including fibroblasts [1, 4].

2. Tissue source of MSC

Traditionally, MSC are isolated from bone marrow. Currently, this source is used for most clinical applications. Although bone marrow is a reliable source of MSC, bone marrow procurement procedures are painful and invasive, and may yield relatively low and variable numbers of MSC upon processing [5]. Several other sources for MSC are investigated, such as umbilical cord blood, placenta or adipose tissue [6-7]. Subcutaneous adipose tissue is a suitable source of MSC that can be obtained under local anaesthesia through a simple, less invasive surgical procedure that provides repeatable access to large quantities of tissue. As we demonstrated in **Chapter 2**, adipose tissue-derived MSC have comparable phenotype, multilineage differentiation capacity and immunosuppressive properties compared to MSC derived from bone marrow [8-9]. Therefore, several clinical studies using adipose tissue as source for MSC are currently in the planning phase. In our opinion, adipose tissue seems to be an ideal substitute for bone marrow for the isolation of MSC. Although the functional properties of the MSC derived from these different sources appear to be similar, it is important to consider that MSC derived from various tissues are not identical and may differ in (tissue-specific) gene expression that may have subtle effects on their function [5, 10].

3. Immunosuppressive effects of MSC; potential in kidney transplantation?

One of the main properties of MSC is their immunosuppressive capacity. Several studies reported that MSC inhibit the proliferation and function of activated immune cells. In **Chapter 3** we demonstrated that MSC can also potently inhibit anti-donor reactivity in a kidney transplantation setting *in vitro*. In addition, MSC were able to inhibit anti-donor responses after transplantation, suggesting that donor-directed memory responses can be inhibited. The immunosuppressive effects were independent of the HLA background of the MSC, as autologous and allogeneic MSC showed comparable efficacy to inhibit alloreactivity. The immunosuppressive effects were mediated via soluble immunosuppressive factors, such as transforming growth factor-b1 (TGF-β1), hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), IL-10, HLA-G and nitric oxide [11-12]. Blocking each of these factors alone does not completely restore the proliferation of activated immune cells, indicating that multiple factors are involved. Also the expression of IDO is an important mechanism of MSC to inhibit T-cell proliferation. Thus, based on the immunosuppressive effect of MSC *in vitro*, there is no preference in the use of recipient (autologous), donor or third-party MSC for immune modulation. Nevertheless, HLA-background of MSC may be important for other issues, for example for the immunogenicity of MSC and for induction of memory responses as we demonstrated (**Chapter 7**).

4. Effect of immunosuppressive drugs on MSC

When immune therapy with MSC is used in solid organ transplantation, the immunosuppressive drugs used by the recipients may affect the function of MSC. In **Chapter 4** we therefore investigated the effect of tacrolimus, mycophenolic acid (MPA) and rapamycin on MSC [13]. While the molecular targets of these drugs are expressed in MSC, their effect on MSC and in particular their immunosuppressive capacity is largely unknown. We found that clinical doses of these drugs induced no toxicity in MSC, but that the proliferation of MSC was inhibited by MPA and rapamycin. Strikingly, MSC in combination with MPA showed enhanced immunosuppressive capacity. The synergistic effect between MSC and low doses of MPA found *in vitro* was confirmed by others and it has been shown in a rat model that the combination of MSC and MPA could induce long term acceptance of heart allografts [14-15]. In contrast, we found that tacrolimus and rapamycin antagonized the immunosuppressive effects of MSC, because these drugs inhibit the activation of immune cells and thereby remove the pro-inflammatory trigger for MSC activation needed to exert their immunosuppressive function. These findings were later confirmed by another group [14]. In addition, Buron et al. found that the glucocorticoid dexamethasone did not modify the inhibitory effects of MSC. The interaction between MSC and cyclosporine A is less clear. Some *in vitro* and animal studies showed that a combination of MSC and cyclosporine A was more immunosuppressive [16-17], while others found no, or even adverse, effects of combining cyclosporine A and MSC [18-19]. Based on these data, it can be hypothesized that addition of MSC to the immunosuppressive drug regimen consisting of MPA and steroids, allows dose reduction without affecting the efficacy of the therapy and seems preferential for application in clinical organ transplantation.

5. *In vitro* pre-treatment of MSC to modulate MSC functions

MSC are responsive to their environment and adapt their function to local circumstances [20]. Therefore, pre-treatment of MSC *in vitro*, i.e. modification of culture medium by specific factors, offers the possibility to modulate the properties of MSC. Evidence from experimental studies convincingly demonstrated that pre-treatment of MSC with IFN-γ strongly enhances their immunosuppressive effect, which allows more efficient immune therapy with MSC [20-22]. Furthermore, IFN-γ pre-treatment of MSC reduces their susceptibility for lysis by NK-cells, as we showed in **chapter 5**. Pre-treatment may therefore prolong the presence of administered MSC, which may result in increased efficacy of MSC treatment.

Besides modulation of their immunosuppressive effects, the differentiation capacity of MSC can be modulated *in vitro* [23-24]. This may be beneficial for applications that require that MSC are (pre) differentiated in more specific cell types. Interestingly, MSC *in vitro* differentiated into adipogenic, osteogenic or chondrogenic lineages might preserve their immunosuppressive capacity [25-26]. This suggests that MSC contribute to tissue regeneration and at the same time control immune reactivity. Modulation of the properties of MSC offers the opportunity to adapt the function of MSC for use in specific clinical applications. In organ transplantation both enhanced regenerative and immunosuppressive function may be beneficial, as fewer cells may be required and potential side effects may be reduced by guiding the function of the MSC. Pre-activation or pre-differentiation of MSC therefore adds a new aspect to MSC therapy.

Another advantage of *ex vivo* expansion of MSC is that MSC-derived culture medium can be obtained, because MSC-conditioned medium could have therapeutic value [27-28]. However, it is unlikely that conditioned medium can fully replace the effects of MSC, because MSC establish cell-membrane interactions with for example activated immune cells resulting in more effective inhibition of the proliferation or function of these cells [29]. The potential of MSC-derived products is still under investigation.

6. MSC exert dual immunomodulatory functions

We and others clearly showed that MSC have potent immunosuppressive effects on activated immune cells. Alternatively, we demonstrated in **Chapter 6** that MSC can also stimulate and control the activation and proliferation of resting T-cells, thereby generating cells with immunosuppressive capacity. Thus besides the direct immunosuppressive effects of MSC on activated T-cells themselves, they also indirectly affect activated T-cells via regulatory T-cells (T-regs). Furthermore, it has been reported that MSC have a supportive function and can prevent lymphocytes from apoptosis [30]. Based on the current data, it can therefore be hypothesized that the balance between the regenerative and immunosuppressive functions of MSC *in vivo* strongly depends on the local conditions [20, 31-32]. Under inflammatory conditions, MSC employ immunosuppressive mechanisms in addition to the production of trophic factors that may be important for tissue repair [33]. On the other hand, under non-inflammatory conditions MSC are supportive to immune cells and support their differentiation.

7. Immunogenicity of MSC

7.1 Susceptibility of MSC for lysis by cytotoxic immune cells

Some studies claim that MSC are immune privileged due to their low immunophenotype, i.e. low or absent expression of HLA molecules and co-stimulatory molecules. MSC would therefore not provoke proliferation of allogeneic lymphocytes [25, 34], and escape lysis by cytotoxic immune cells [35-36]. Theoretically, the low immunogenicity of MSC would reduce the clearance of allogeneic MSC by the immune system after infusion, suggesting that transplantation of MSC across MHC HLA barriers may be possible. In contrast to many studies, we demonstrated in **chapter 7** that allogeneic MSC are susceptible for lysis by $CD8^+$ T-cells. Lysis of allogeneic MSC by $CD8^+$ T-cells was even further increased after up-regulation of HLA class I expression by IFN-γ pre-treatment of MSC. Moreover, allogeneic MSC were susceptible for $CD16^+56^+$ NK cell-mediated lysis. As expected, autologous MSC were not susceptible for lysis by $CD8^+$ T-cells, but, to our surprise, they were susceptible for lysis by NK-cells. We furthermore demonstrated that IFN-γ enhanced HLA class I expression on MSC resulted in reduced lysis of MSC by NK-cells, whereas the NK cell-mediated lysis increased after reducing the expression of HLA class I on MSC by small interfering RNA (siRNA). Therefore, lysis of MSC is at least partly dependent on the expression of HLA class I on MSC. In particular the non-classical molecules HLA-E and HLA-G have been suggested to be involved [37-39]. Our finding that autologous MSC are lysed by cytotoxic immune cells is difficult to interpret in view of MSC biology *in vivo*. A possibility might be that NK-cell mediated lysis prevent uncontrolled proliferation of MSC, for example leading to the formation of sarcomas [40]. Alternatively, it can be speculated that MSC cultured *ex vivo* have a different phenotype compared to tissue resident MSC, which make them more susceptible for lysis by NK-cells.

Furthermore, MSC in suspension as used in the clinic for intravenous administration could have changed characteristics compared to MSC present in tissue and those MSC could be recognized as foreign and subsequently killed. Lysis of autologous MSC suggests that HLA matching is not sufficient to avoid clearance of administered MSC and that control of NK cell lysis may be important for optimisation of MSC therapy.

The susceptibility of MSC for $CD8^+$ T-cell and NK cell-mediated lysis were confirmed in a kidney transplantation setting. Recipient $CD8^+$ cytotoxic T-cells and NK-cells efficiently lysed donor-derived MSC. The capacity to lyse donor-derived MSC increased in time after transplantation: pretransplant immune cells did not lyse donor MSC, while immune cells isolated 3, 6 and 12 months after transplantation showed increasing ability to lyse MSC. After 12 months, the lysing ability of recipient cytotoxic cells was fully recovered. While this could be explained by recovery of the recipients' immune system and tapering of the immunosuppressive drugs during the first year, it may also result from sensitization against donor-antigens. However, both the NK cell and $CD8^+$ T cell-mediated lysis of donor MSC increased after transplantation, maintaining the balance between NK cell and $CD8^+$ T cell-mediated lysis of MSC. This suggests that there was no $CD8^+$ T-cell sensitization or desensitisation against donor MSC.

Our cytotoxicity data indicate that infused MSC, both of autologous and allogeneic origin, are recognized and subsequently cleared by the recipient's immune system, and thereby reject the claim that MSC are immune privileged. Moreover, clearance of MSC by cytotoxic cells may explain the observations in animal models, in which infused cells can not be traced within a few days after infusion [15, 41].

The question is whether clearance of MSC is beneficial or disadvantageous for MSC therapy. Clearance of MSC may be beneficial when only a temporary therapeutic effect is desired and it would omit the risk for tumour formation on the long-term, while rapid clearance of MSC shortens the time of MSC effects, leading to reduced efficacy.

7.2 Choice between autologous or allogeneic MSC

One of the major questions concerning the clinical application of MSC is whether MSC of autologous or allogeneic origin should be used. This is especially important for their application in organ transplantation. In an organ-transplantation setting, there is theoretically a choice of MSC from three origins; MSC can be derived from the recipient, from the donor, or from a third-party. In planned living-organ transplantation, there is an opportunity to isolate and expand MSC of recipient or donor origin; in contrast, the transplantation of organs from deceased donors would limit the options to recipient and "of-the-shelf" third-party MSC.

Although it has been suggested that allogeneic MSC are better immunosuppressors [16], we, like others, found that autologous and allogeneic MSC have a comparable immunosuppressive capacity *in vitro* [42-44]. Furthermore, in clinical studies in which patients with GVHD are treated with MSC, no differences in the effects of autologous, haploidentical and third-party MSC have been observed. This indicates that, based on the immunosuppressive effect of MSC, there is no preference in the use of recipient (autologous), donor or third-party MSC for treatment of transplant patients. The HLA background of MSC may, however, be important for the immunogenicity of MSC and for the induction of memory responses. It has, indeed, been demonstrated in murine models that pretransplant infusion of allogeneic MSC can induce memory T-cell responses [45]. Priming with donor MSC may thus potentially sensitize the recipient to donor antigens, and thereby accelerate graft rejection [45-46]. To avoid cross-reactivity between administered MSC and the graft, MSC derived from the recipient or MSC that are mismatched with donor HLA could be used in clinical studies.

Nevertheless, recognition of donor MSC by the host immune system does not necessarily lead to sensitization. It is also possible that recognition of donor MSC prior to transplantation desensitizes the recipient to donor antigens. Such a mechanism would resemble the effect seen in the past with the use of pretransplant donor blood transfusions, which in some cases led to donor-specific hypo-responsiveness [47]. If MSC have the capability of inducing donor hypo-responsiveness, this is likely to rely on a balanced dosing and timing of MSC administration. The clinical consequence of the application of MSC with a different HLA background needs further investigation.

In summary, MSC have regenerative and immunosuppressive properties that could be beneficial for improving the outcome of solid organ transplantation, in particular kidney transplantation. Several aspects of these cells, however, need further investigation in order to develop effective MSC therapy for improvement of current standard therapy.

REFERENCES

1. Haniffa MA, Wang XN, Holtick U, Rae M, Isaacs JD, Dickinson AM, et al. Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. J Immunol. 2007 Aug 1;179(3):1595-604.

2. Haniffa MA, Collin MP, Buckley CD, Dazzi F. Mesenchymal stem cells: the fibroblasts' new clothes? Haematologica. 2008 Dec 23.

3. da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci. 2006 Jun 1;119(Pt 11):2204-13.

4. Jones S, Horwood N, Cope A, Dazzi F. The antiproliferative effect of mesenchymal stem cells is a fundamental property shared by all stromal cells. J Immunol. 2007 Sep 1;179(5):2824-31.

5. Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells. 2006 May;24(5):1294-301.

6. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001 Apr;7(2):211-28.

7. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, et al. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells. 2004;22(7):1338-45.

8. Puissant B, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, et al. Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. Br J Haematol. 2005 Apr;129(1):118-29.

9. Hoogduijn MJ, Crop MJ, Peeters AM, Van Osch GJ, Balk AH, Ijzermans JN, et al. Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. Stem Cells Dev. 2007 Aug;16(4):597-604.

10. Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, Krause U, et al. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. Exp Hematol. 2005 Nov;33(11):1402-16.

11. Sato K, Ozaki K, Oh I, Meguro A, Hatanaka K, Nagai T, et al. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. Blood. 2007 Jan 1;109(1):228-34.

12. Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell. 2008 Feb 7;2(2):141-50.

13. Hoogduijn MJ, Crop MJ, Korevaar SS, Peeters AM, Eijken M, Maat LP, et al. Susceptibility of human mesenchymal stem cells to tacrolimus, mycophenolic acid, and rapamycin. Transplantation. 2008 Nov 15;86(9):1283-91.

14. Buron F, Perrin H, Malcus C, Hequet O, Thaunat O, Kholopp-Sarda MN, et al. Human mesenchymal stem cells and immunosuppressive drug interactions in allogeneic responses: an in vitro study using human cells. Transplant Proc. 2009 Oct;41(8):3347-52.

15. Popp FC, Eggenhofer E, Renner P, Slowik P, Lang SA, Kaspar H, et al. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. Transpl Immunol. 2008 Nov;20(1-2):55-60.

16. Maccario R, Moretta A, Cometa A, Montagna D, Comoli P, Locatelli F, et al. Human mesenchymal stem cells and cyclosporin a exert a synergistic suppressive effect on in vitro activation of alloantigen-specific cytotoxic lymphocytes. Biol Blood Marrow Transplant. 2005 Dec;11(12):1031-2.

17. Le Blanc K, Rasmusson I, Gotherstrom C, Seidel C, Sundberg B, Sundin M, et al. Mesenchymal stem cells inhibit the expression of CD25 (interleukin-2 receptor) and CD38 on phytohaemagglutinin-activated lymphocytes. Scand J Immunol. 2004 Sep;60(3):307-15.

18. Inoue S, Popp FC, Koehl GE, Piso P, Schlitt HJ, Geissler EK, et al. Immunomodulatory effects of mesenchymal stem cells in a rat organ transplant model. Transplantation. 2006 Jun 15;81(11):1589-95.

19. Zhang W, Qin C, Zhou ZM. Mesenchymal Stem Cells Modulate Immune Responses Combined With Cyclosporine in a Rat Renal Transplantation Model. Transplant Proc. 2007 Dec;39(10):3404-8.

20. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells. 2006 Feb;24(2):386-98.

21. Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol. 2007 Aug;149(2):353-63.

22. Polchert D, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E, et al. IFN-gamma activation of mesenchymal stem cells for treatment and

prevention of graft versus host disease. Eur J Immunol. 2008 Jun;38(6):1745-55.

23. Castano-Izquierdo H, Alvarez-Barreto J, van den Dolder J, Jansen JA, Mikos AG, Sikavitsas VI. Pre-culture period of mesenchymal stem cells in osteogenic media influences their in vivo bone forming potential. J Biomed Mater Res A. 2007 Jul;82(1):129-38.

24. Jaquiery C, Schaeren S, Farhadi J, Mainil-Varlet P, Kunz C, Zeilhofer HF, et al. In vitro osteogenic differentiation and in vivo bone-forming capacity of human isogenic jaw periosteal cells and bone marrow stromal cells. Ann Surg. 2005 Dec;242(6):859-67, discussion 67-8.

25. Le Blanc K, Tammik C, Rosendahl K, Zetterberg E, Ringden O. HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp Hematol. 2003 Oct;31(10):890-6.

26. Liu H, Kemeny DM, Heng BC, Ouyang HW, Melendez AJ, Cao T. The immunogenicity and immunomodulatory function of osteogenic cells differentiated from mesenchymal stem cells. J Immunol. 2006 Mar 1;176(5):2864-71.

27. Parekkadan B, van Poll D, Suganuma K, Carter EA, Berthiaume F, Tilles AW, et al. Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. PLoS One. 2007;2(9):e941.

28. Walter MN, Wright KT, Fuller HR, Macneil S, Johnson WE. Mesenchymal stem cell-conditioned medium accelerates skin wound healing: An in vitro study of fibroblast and keratinocyte scratch assays. Exp Cell Res. 2010 Mar 3.

29. Quaedackers ME, Baan CC, Weimar W, Hoogduijn MJ. Cell contact interaction between adipose-derived stromal cells and allo-activated T lymphocytes. Eur J Immunol. 2009 Dec;39(12):3436-46.

30. Benvenuto F, Ferrari S, Gerdoni E, Gualandi F, Frassoni F, Pistoia V, et al. Human mesenchymal stem cells promote survival of T cells in a quiescent state. Stem Cells. 2007 Jul;25(7):1753-60.

31. Najar M, Rouas R, Raicevic G, Boufker HI, Lewalle P, Meuleman N, et al. Mesenchymal stromal cells promote or suppress the proliferation of T lymphocytes from cord blood and peripheral blood: the importance of low cell ratio and role of interleukin-6. Cytotherapy. 2009;11(5):570-83.

32. Bocelli-Tyndall C, Bracci L, Schaeren S, Feder-Mengus C, Barbero A, Tyndall A, et al. Human bone marrow mesenchymal stem cells and chondrocytes promote and/or suppress the in vitro proliferation of lymphocytes stimulated with the cytokines IL 2, IL 7 and IL 15. Ann Rheum Dis. 2008 Jul 22.

33. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006 Aug 1;98(5):1076-84.

34. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation. 2003 Feb 15;75(3):389-97.

35. Maccario R, Podesta M, Moretta A, Cometa A, Comoli P, Montagna D, et al. Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. Haematologica. 2005 Apr;90(4):516-25.

36. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, et al. Human mesenchymal stem cells modulate B-cell functions. Blood. 2006 Jan 1;107(1):367-72.

37. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood. 2006 Feb 15;107(4):1484-90.

38. Selmani Z, Naji A, Zidi I, Favier B, Gaiffe E, Obert L, et al. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. Stem Cells. 2008 Jan;26(1):212-22.

39. Selmani Z, Naji A, Gaiffe E, Obert L, Tiberghien P, Rouas-Freiss N, et al. HLA-G is a crucial immunosuppressive molecule secreted by adult human mesenchymal stem cells. Transplantation. 2009 May 15;87(9 Suppl):S62-6.

40. Tolar J, Nauta AJ, Osborn MJ, Panoskaltsis Mortari A, McElmurry RT, Bell S, et al. Sarcoma derived from cultured mesenchymal stem cells. Stem Cells. 2007 Feb;25(2):371-9.

41. Eliopoulos N, Stagg J, Lejeune L, Pommey S, Galipeau J. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. Blood. 2005 Dec 15;106(13):4057-65.

42. Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol. 2003 Jan;57(1):11-20.

43. Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, et al. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci. 2005;12(1):47-57.

44.Crop MJ, Baan CC, Korevaar SS, Ijzermans JN, Alwayn IP, Weimar W, et al. Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients. Transplantation. 2009 Mar 27;87(6):896-906.

45. Nauta AJ, Westerhuis G, Kruisselbrink AB, Lurvink EG, Willemze R, Fibbe WE. Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a non-myeloablative setting. Blood. 2006 May 11;108(6):2114-20.

46.Grinnemo KH, Mansson A, Dellgren G, Klingberg D, Wardell E, Drvota V, et al. Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium. J Thorac Cardiovasc Surg. 2004 May;127(5):1293-300.

47. Waanders MM, Roelen DL, Brand A, Claas FH. The putative mechanism of the immunomodulating effect of HLA-DR shared allogeneic blood transfusions on the alloimmune response. Transfus Med Rev. 2005 Oct;19(4):281-7.

Chapter 9

Mesenchymal stem cells as immune therapy in solid organ transplantation;

Potential pitfalls and future directions

based on:
Potential of mesenchymal stem cells as immune therapy
in solid organ transplantation

Meindert J. Crop, Carla C. Baan, Willem Weimar and Martin J. Hoogduijn

Department of Internal Medicine, Erasmus University Medical Center,
Rotterdam, The Netherlands

Transplant International
2009 Apr;22(4):365-76

INTRODUCTION

The present thesis investigated the immunomodulatory properties of MSC to explore their potential as immune therapy in solid organ transplantation as an alternative for the current drug-based immunosuppressive regimens. The immunosuppressive properties of MSC may reduce immune reactivity, but at the same time their regenerative properties may improve graft function by repairing damage of the transplanted organ. Although the effects of MSC obtained from *in vitro* and animal studies seem promising, their applicability in clinical organ transplantation is still unknown. Furthermore, several issues need to be addressed if MSC therapy in solid organ transplantation is considered, such as dosing, timing and route of administration. It is crucial to investigate the role of the HLA-background of the MSC source to find out whether autologous (recipient), donor-derived or third-party MSC should be preferred as cellular therapy for immunomodulatory purposes in solid organ transplantation. Based on the results of the present thesis and current literature, these and other issues relevant for the application of MSC as therapy in clinical organ transplantation are discussed below.

POTENTIAL PITFALLS AND FUTURE DIRECTIONS

1. Dosing of MSC

An important issue in the clinical application of MSC involves establishing the dosing that will achieve the best results, but does not induce toxicity. *In vitro* studies show immunosuppressive effects at (MSC:PBMC)-ratios of (1:10) or higher *in vitro* [1], while these high concentrations of MSC can not be achieved *in vivo*. Furthermore, animal studies demonstrated that the majority of MSC disappear within days after infusion. This could be the result of distribution to other organs, of increased clearance of MSC by immune cells or due to cell loss caused by mechanical stress after infusion of MSC. In a human setting, there is some experience with dosing of MSC in the treatment of bone marrow transplant patients with severe GVHD. A multicenter trial showed that doses of $0.5x10^6$ to $9x10^6$ cells per kg body weight did not lead to adverse side effects [2-3]. In this study, doses from $0.8x10^6$ were found to be effective to reduce GVHD, but no clear dose-dependent effect was obtained. In breast cancer patients that underwent myeloablation, infusion of $1x10^6$ and $2.2x10^6$ MSC per kg body weight showed no toxic effects [4]. Trials in Crohn's disease patients are currently testing doses of $2x10^6$ and $8x10^6$ bone marrow MSC per kg body weight, or amounts of $600x10^6$ and $1200x10^6$ MSC per patient (clinicaltrials.gov). In solid organ transplantation the safety and optimal dose of MSC is unknown.

If local administration of MSC is considered in organ transplantation, it is unclear whether the number of MSC should be calculated per kg body weight or whether the number of MSC per target organ should be calculated. As MSC migrate poorly in tissue, repeated multi-focal injections with MSC may be required to obtain an effective dose of MSC in the graft.

In view of dosing it is also crucial to consider the susceptibility of MSC for lysis by cytotoxic immune cells, especially NK-cells as demonstrated in Chapter 7. While most studies do not investigate this effect or neglect it, cytotoxicity-mediated lysis of administrated MSC can dramatically reduce the number of MSC and thus the efficiency of the therapy. We found that even autologous MSC were killed by NK-cells, therefore in an autologous transplant setting this phenomenon should be taken into account. In addition, it was demonstrated that pre-treatment of MSC with IFN-γ resulted in reduced susceptibility for lysis by NK-cells, which (party) depended on the expression of HLA class I molecules. It can therefore be speculated that *in vitro* pre-treatment of MSC may therefore reduce, yet not avoid, the clearance of the cells after administration. Currently animal studies are being performed to investigate the effect of IFN-γ treatment of MSC and immunogenicity *in vivo*.

2. Timing of MSC administration

Another important issue that needs to be considered in planning a clinical study in solid organ transplantation is the moment of infusion. Choices in time (before or after transplantation) or disease phase (acute versus chronic rejection) should be made. Timing of administration of cells depends on the aim of the cellular therapy. If the aim of MSC therapy is tolerance induction, donor-MSC should be administered before transplantation. By this, donor-derived MSC may induce long-term allograft acceptance, as shown previously in murine heart transplantation models and a rat hind-limb allograft model after bone marrow transplantation [5-7]. Most experimental and clinical studies, however, do not study tolerance induction by MSC, but investigate the direct immunosuppressive effects of MSC on activated immune cells in various *in vitro* studies and animal models.

As a result, there is currently little evidence whether and how tolerance induction by MSC works, and whether this is a special MSC characteristic or just dependent of the pretransplant presentation of donor-antigen.
Besides tolerance induction, MSC may be used for anti-rejection therapy, as MSC possess immunosuppressive properties. Their immunosuppressive effects are even further enhanced under inflammatory conditions as shown by our group and others [8-10].
Furthermore, it can be speculated that addition of MSC to the conventional treatment may reduce the need for immunosuppressive drugs and could allow tapering of immunosuppressive medication. As shown in Chapter 4, the commonly used immunosuppressive drugs (MPA, tacrolimus and rapamycin) in kidney transplantation did not induce apoptosis or toxicity in MSC. We also found that MPA, in contrast to tacrolimus and rapamycin, did not hamper the immunosuppressive effects of MSC. A treatment with MPA may therefore be preferred above tacrolimus or rapamycin.

Application of MSC therapy for acute versus chronic rejection?
If MSC are applied as anti-rejection therapy, the question is whether MSC are most effective in treatment of acute or chronic graft rejection. Most experimental studies focus on the effect of MSC on activated immune cells *in vitro* or acute inflammatory processes, such as (autoimmune) inflammatory disease models, for example colitis or rheumatoid arthritis [11-12]. Little is known about the efficacy of MSC in acute versus chronic graft rejection in solid organ transplant. In acute rejection very active immune responses are ongoing, which activate the immunosuppressive mechanism in MSC, leading to strong immunosuppressive effects by MSC. This suggests that the application of MSC in acute rejection may be effective.

In vitro studies have shown that MSC successfully suppress lymphocyte activity in the afferent phase and not in the cytotoxic phase of an allo-response [13-15]. In addition, data from animal models have shown that MSC were effective immune suppressors when infused before the onset of inflammatory processes [6], and that they could prevent the development of autoimmune encephalitis in a murine model [16]. Moreover, in the same model, MSC were also effective when administered at the peak phase of the disease, suggesting that they can also ameliorate active inflammatory processes. This was further demonstrated in patients with severe drug-resistant GVHD whose clinical condition was improved by treatment with MSC [3, 17]. Consequently, MSC based-therapy may be effective in preventing anti-donor reactivity and in reducing active rejection in organ transplantation.
In addition to application in acute inflammatory processes, MSC therapy could be used in chronic inflammatory processes, such as in chronic allograft rejection or biopsy-proven sub-clinical rejection. In organ transplantation, immune modulation by MSC in an early phase after transplantation may result in reduced damage of the graft which may improve graft function in the long-term. Only a few studies investigated the effect of MSC in chronic inflammatory diseases such as chronic experimental autoimmune encephalomyelitis [18-19]. Many studies, however, investigate the potential of MSC in acute inflammatory diseases, and to much lesser extent to the effects of MSC on chronic immune processes. In view of long term immunomodulatory effects of MSC, the reported induction of regulatory T-cells by MSC is relevant. Via the induction of T-regs, the immunosuppressive effect of MSC may remain present long after the disappearance of MSC and in this way contribute to long term immunosuppression that could serve to dampen chronic inflammatory processes.
These data suggest that timing of MSC administration may be crucial for the efficacy of the therapy.

3. Route of administration and migration of MSC

MSC used for immune therapy in solid organ transplantation can be administered via two routes: systemic or local. It is, however, unknown *where* MSC exert their potential immunoregulatory effects. This could be in the target organ or perhaps in the lymph nodes. As a result it is unknown whether it is more effective to administer MSC locally (into the target organ, i.e. the graft) or systemic. The choice of administration depends on the clinical effect that is aimed for, for example the regenerative effects of MSC may be more effective if MSC are infused locally in the damaged target organ, as a result of the local production of trophic factors by MSC [20].

Until now intravenous infusion has been the route of administration of choice for all clinical studies with MSC for immunomodulatory purposes. The few clinical studies performed so far suggest that intravenous administration of MSC is feasible and safe. To date, no severe adverse reactions have been recorded in humans after MSC administration, both in terms of immediate, infusion-related toxicity and of late effects [2-3, 21]. For, tissue repair purposes, however, MSC have injected in various local tissues such as in muscle (e.g. in myocardial infracted hearts) or in skin (e.g. in burn/irradiation wounds or treatment-resistant diabetic wounds) [22-24].

Although MSC express homing receptors [25], it is unclear whether such receptors would lead systemically administered MSC to the target organ. Data from a number of studies suggests that intravenously injected MSC home to sites of inflammation [26-30], while other studies demonstrated systemic intravenous route of administration was not appropriate for MSC to reach their target site [31-32].

An important point to consider is that *in vitro* expansion of MSC changes the expression of cell surface markers on MSC and thereby may affect the homing behaviour of MSC. Sackstein et al. demonstrated that modification of the cell surface molecule CD44 on *ex vivo* expanded MSC can influence the migration of MSC after infusion [33]. This is of great interest for the specific delivery of MSC for clinical applications. However, other studies reported that selective homing of MSC to lymphoid tissues [34] or sites of inflammation [29, 35] is limited, and that MSC get trapped in lungs and liver by space constrictions [36].

To increase the number of MSC in the target organ, intra-arterial infusion can be considered [37-39]. In a rat kidney transplantation model intra-arterial infusion of MSC prevented acute cellular rejection [40]. In our own group we studied the effect of renal arterial infusion of labelled MSC in pigs (unpublished data). We, however, did not find traces of labelled MSC in the kidney one hour after infusion. We then hypothesized that MSC may prefer a inflammatory environment by the up-regulation of adhesion molecules and production of pro-inflammatory cytokines. We, therefore, induced 45 minutes of hypoxia in the kidney and subsequently infused labelled MSC via the renal artery. Again, no labelled MSC were found in the kidney. Although this was only a pilot study, these results do not support the hypothesis that arterial infusion increases the number of MSC. Interestingly, infusion of MSC via the renal artery in a small animal model demonstrated that MSC remain in the kidney, but mal-differentiate into adipocytes in within weeks after administration [41]. Furthermore, it has been reported that infusion of MSC via coronary artery can lead to micro-infarcts in the pigs heart [41-42].

Therefore, as most experience with MSC as immune therapy has been gained with intravenous administration of MSC, it seems most safe to perform the initial trials in organ transplantation with intravenous administered MSC.

4. Potential risks of MSC

Besides the promising results of MSC in experimental and clinical studies, there are also several issues concerning potential risks and safety of MSC therapy that need to be discussed. For example effects of *ex vivo* expansion, systemic immune suppression, ectopic tissue formation and malignant transformation of MSC. Furthermore, while MSC are relatively modestly present within tissues under physiological conditions and are particularly rare in the circulation, MSC used for clinical applications are infused intravenously in high numbers. Currently, more than 1,000 patients have received MSC and there have not been reported acute or intermediate-term side effects of MSC [3, 43]. Long-term follow-up studies, however, are certainly required to fully address safety issues associated with MSC therapy.

4.1 Infusion-related and acute allergic reactions

As the lungs are the major filters for intravenous administered cells, MSC are expected to accumulate predominantly in the lungs. This could potentially lead to effects on breathing. These or allergic side effects, however, have not been observed in humans.

4.2 In vitro expansion of MSC

It is important to consider the potential functional differences between tissue-resident MSC and *in vitro*-expanded MSC and to consider the non-physiological aspects of MSC therapy. *In vitro* expansion of MSC may affect the properties of MSC, for example proliferation rate and exposure to culture media, including bovine serum. Below examples of these concerns are discussed.

4.2.1 chromosomal abnormalities

There is some evidence that chromosomal abnormalities develop in MSC after long-term expansion *in vitro*, e.g. culturing MSC for more than 25 passages. In particular, continued culturing of MSC after senescence increases the risk for genetic changes [44-49]. Other studies, however, found no or neglectable aberrations occurring in culture [50-51]. Furthermore, it has been reported that murine MSC frequently have chromosomal abnormalities and lead to tumour formation, while human MSC have a stable karyotype [52-53]. The tendency for MSC to undergo malignant transformation could vary according to the source (e.g. bone marrow, adipose tissue), species (e.g. human vs. mice) and culture conditions. Thus, for safety reasons, the use of high-passage "senescent" MSC for clinical applications should be, and can easily be, avoided. Moreover, karyotyping of *ex vivo*-expanded MSC is recommended before clinical administration.

4.2.2. In vitro expansion of MSC; need for bovine serum free conditions?

Until now most clinical trials using *ex vivo*-expanded MSC, cultured their MSC in medium with foetal bovine serum. Although most experience has been gained using these culture conditions and no allergic reactions against bovine antigens have occurred, there is a potential risk of priming recipients with bovine antigens [54] or transferring bovine-related disease. This problem is easily to overcome as bovine-free MSC medium is now commercially available.

4.3 Systemic immunosuppressive effect: increased risk for infections due to suppression of the host's immune system?

Other safety issues that involve systemic administration of MSC are the potential modulation of the overall immune system as the immunosuppression by MSC is not antigen-specific. Consecutively, systemic suppression may potentially reduce patients' resistance to infections or support the development of pre-existing tumours [46]. The occurrence of opportunistic infections and tumours have already been reported for immunosuppressive drugs, but are still unclear for MSC. In baboons treated with multiple administrations of high dose allogeneic MSC, alloreactive immune responses were affected, but there was no compromising of the overall immune system [55]. In contrast, in a considerable number of patients that has been treated with MSC for several diseases, the incidence of infections seemed high [3]. Unfortunately, until now almost all the clinical studies reported are non-placebo controlled studies. Thus, it is too early to conclude that MSC therapy leads to an increased risk for infections.

4.4 Ectopic tissue formation

4.4.1 Calcifications, adipogenesis and fibrosis

Another concern is the multilineage differentiation potential of MSC, which may not always be beneficial, as it may lead to the formation of the "wrong" cell types. It has been reported that treatment of mice with myocardial infarcts with MSC induced increased calcification of the heart [56] or formation of adipose tissue in the kidney [41]. Alternatively, differentiation of MSC in the wrong cell type may lead to tumour formation. Until now, this has not been observed in a human setting.

Perhaps more likely is the induction of fibrosis by MSC, as MSC are progenitors of fibroblast-like cells and therefore may be associated with tissue fibrosis. Although some evidence for this is provided by a recent clinical study in which local administration of progenitor-enriched adipose tissue in breasts induced fibrosis [57], it is unclear whether *ex vivo*-expanded MSC will act in the same way. Studies in animal models showed that MSC infusion did not worsen, yet ameliorated fibrosis [28, 58].

4.4.2 Tumour formation

Because there is evidence that tumours are sites of inflammatory cytokine and chemokine production, MSC may home to, and immunomodulate, the tumour environment [59]. There is evidence that MSC actively migrate to, and proliferate in, tumours [60-61]. MSC were found to contribute to the tumour-associated stroma within sites of inflammation and were in close proximity with tumour cells as part of the remodelling process. Furthermore, there are also theories that stromal tumours, e.g. sarcomas [62], originate from developmental abnormalities in progenitor cells, possibly MSC. Other animal and clinical studies, however, did not find an increased incidence of these tumours [3, 53, 63]. Although these preliminary results suggest that the use of MSC is safe in respect to tumour formation, again, it is too early to conclude and longer follow-up is needed.

4.5 Dual functions of MSC

We and others have shown that the immunomodulatory function of MSC strongly depends on the local environment. This can be referred to as the "dual" immunomodulatory effect of MSC: immunosuppression versus immune stimulation [64-66]. In an inflammatory condition MSC employ their immunosuppressive mechanisms, such as activation of IDO and secretion of pre-dominantly anti-inflammatory factors, while in a non-inflammatory setting MSC can stimulate immune activation. Immune activation in a transplantation setting is a potential risk as induction of immune reactivity may stimulate graft rejection. Since this is an important issue, we demonstrated in this thesis that indeed MSC modestly stimulated the proliferation of lymphocytes, but kept the proliferation of activated lymphocytes under control. We subsequently found that MSC could also generate regulatory T-cells from *resting* T-cells. Under non-inflammatory conditions MSC may rather employ functions to support other (more specialized) cells than to bring about their immunosuppressive functions. It can therefore be speculated that administration of MSC in patients with low of no immune reactivity, such as in the absence of rejection, does not lead to accelerated rejection due to the activation of immune cells by MSC.

CONCLUSION

There is accumulating evidence from *in vitro*, animal and some clinical studies that MSC have tissue repair and immunosuppressive properties which are promising for treatment of various diseases. It is, however, currently unknown whether these capacities are functional or beneficial in a human organ transplantation setting. We believe that MSC have unique properties that allow them to prevent damage of the transplant, improve graft acceptance and its function. We therefore subscribe to the current high hopes regarding their use in cell therapy, but future research and clinical trials in organ transplantation will show whether this is justified. In addition, it is important not to overestimate the potential therapeutic effects of MSC, since almost all the clinical studies reported so far are non-placebo-controlled studies. The complexity of the MSC suggests the introduction of successful MSC therapy in organ transplantation is going to be a long-term process of trial and error.

Although multiple clinical trials are ongoing for various applications, the safety and feasibility of MSC infusion also needs to be confirmed in kidney transplantation. To avoid interference of MSC therapy with the transplantation, phase I clinical study can be performed in kidney patients with end-stage renal disease (ESRD). If the infusion is safe, the application of MSC in a clinical kidney transplantation setting can be investigated.

This thesis presents some of the first steps in exploring the possibilities for the use of MSC for clinical immune therapy in solid organ transplantation.

REFERENCES

1. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002 May 15;99(10):3838-43.

2. Lazarus HM, Haynesworth SE, Gerson SL, Rosenthal NS, Caplan AI. Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. Bone Marrow Transplant. 1995 Oct;16(4):557-64.

3. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet. 2008 May 10;371(9624):1579-86.

4. Koc ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI, et al. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol. 2000 Jan;18(2):307-16.

5. Casiraghi F, Azzollini N, Cassis P, Imberti B, Morigi M, Cugini D, et al. Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells. J Immunol. 2008 Sep 15;181(6):3933-46.

6. Popp FC, Eggenhofer E, Renner P, Slowik P, Lang SA, Kaspar H, et al. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. Transpl Immunol. 2008 Nov;20(1-2):55-60.

7. Pan H, Zhao K, Wang L, Zheng Y, Zhang G, Mai H, et al. Mesenchymal stem cells enhance the induction of mixed chimerism and tolerance to rat hind-limb allografts after bone marrow transplantation. J Surg Res. 2010 May 15;160(2):315-24.

8. Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol. 2007 Aug;149(2):353-63.

9. Polchert D, Sobinsky J, Douglas G, Kidd M, Moadsiri A, Reina E, et al. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. Eur J Immunol. 2008 Jun;38(6):1745-55.

10. DelaRosa O, Lombardo E, Beraza A, Mancheno-Corvo P, Ramirez C, Menta R, et al. Requirement of IFN-gamma-mediated indoleamine 2,3-dioxygenase expression in the modulation of lymphocyte proliferation by human adipose-derived stem cells. Tissue Eng Part A. 2009 Oct;15(10):2795-806.

11. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells. Arthritis Rheum. 2009 Apr;60(4):1006-19.

12. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. Gastroenterology. 2009 Mar;136(3):978-89.

13. Angoulvant D, Clerc A, Benchalal S, Galambrun C, Farre A, Bertrand Y, et al. Human mesenchymal stem cells suppress induction of cytotoxic response to alloantigens. Biorheology. 2004;41(3-4):469-76.

14. Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation. 2003 Oct 27;76(8):1208-13.

15. Potian JA, Aviv H, Ponzio NM, Harrison JS, Rameshwar P. Veto-like activity of mesenchymal stem cells: functional discrimination between cellular responses to alloantigens and recall antigens. J Immunol. 2003 Oct 1;171(7):3426-34.

16. Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, et al. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood. 2005 Sep 1;106(5):1755-61.

17. Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet. 2004 May 1;363(9419):1439-41.

18. Kassis I, Grigoriadis N, Gowda-Kurkalli B, Mizrachi-Kol R, Ben-Hur T, Slavin S, et al. Neuroprotection and immunomodulation with mesenchymal stem cells in chronic experimental autoimmune encephalomyelitis. Arch Neurol. 2008 Jun;65(6):753-61.

19. Constantin G, Marconi S, Rossi B, Angiari S, Calderan L, Anghileri E, et al. Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis. Stem Cells. 2009 Oct;27(10):2624-35.

20. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006 Aug 1;98(5):1076-84.

21. Koc ON, Day J, Nieder M, Gerson SL, Lazarus HM, Krivit W. Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH). Bone Marrow Transplant. 2002 Aug;30(4):215-22.

22. Henning RJ, Burgos JD, Vasko M, Alvarado F, Sanberg CD, Sanberg PR, et al. Human cord blood cells and myocardial infarction: effect of dose and route of administration on infarct size. Cell Transplant. 2007;16(9):907-17.

23. Poh KK, Sperry E, Young RG, Freyman T, Barringhaus KG, Thompson CA. Repeated direct endomyocardial transplantation of allogeneic mesenchymal stem cells: safety of a high dose, "off-the-shelf", cellular cardiomyoplasty strategy. Int J Cardiol. 2007 May 2;117(3):360-4.

24. Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. Stem Cells. 2007 Oct;25(10):2648-59.

25. Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells. 2007 Nov;25(11):2739-49.

26. Chapel A, Bertho JM, Bensidhoum M, Fouillard L, Young RG, Frick J, et al. Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome. J Gene Med. 2003 Dec;5(12):1028-38.

27. Herrera MB, Bussolati B, Bruno S, Morando L, Mauriello-Romanazzi G, Sanavio F, et al. Exogenous mesenchymal stem cells localize to the kidney by means of CD44 following acute tubular injury. Kidney Int. 2007 Aug;72(4):430-41.

28. Ortiz LA, Gambelli F, McBride C, Gaupp D, Baddoo M, Kaminski N, et al. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. Proc Natl Acad Sci U S A. 2003 Jul 8;100(14):8407-11.

29. Wu GD, Nolta JA, Jin YS, Barr ML, Yu H, Starnes VA, et al. Migration of mesenchymal stem cells to heart allografts during chronic rejection. Transplantation. 2003 Mar 15;75(5):679-85.

30. Chen J, Li Y, Wang L, Zhang Z, Lu D, Lu M, et al. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. Stroke. 2001 Apr;32(4):1005-11.

31. Schrepfer S, Deuse T, Reichenspurner H, Fischbein MP, Robbins RC, Pelletier MP. Stem cell transplantation: the lung barrier. Transplant Proc. 2007 Mar;39(2):573-6.

32. Fischer UM, Harting MT, Jimenez F, Monzon-Posadas WO, Xue H, Savitz SI, et al. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. Stem Cells Dev. 2009 Jun;18(5):683-92.

33. Sackstein R, Merzaban JS, Cain DW, Dagia NM, Spencer JA, Lin CP, et al. Ex vivo glycan engineering of CD44 programs human multipotent mesenchymal stromal cell trafficking to bone. Nat Med. 2008 Feb;14(2):181-7.

34. Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood. 2003 Apr 15;101(8):2999-3001.

35. Grimm PC, Nickerson P, Jeffery J, Savani RC, Gough J, McKenna RM, et al. Neointimal and tubulointerstitial infiltration by recipient mesenchymal cells in chronic renal-allograft rejection. N Engl J Med. 2001 Jul 12;345(2):93-7.

36. Gao J, Dennis JE, Muzic RF, Lundberg M, Caplan AI. The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. Cells Tissues Organs. 2001;169(1):12-20.

37. Behr L, Hekmati M, Fromont G, Borenstein N, Noel LH, Lelievre-Pegorier M, et al. Intra renal arterial injection of autologous mesenchymal stem cells in an ovine model in the postischemic kidney. Nephron Physiol. 2007;107(3):p65-76.

38. Walczak P, Zhang J, Gilad AA, Kedziorek DA, Ruiz-Cabello J, Young RG, et al. Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia. Stroke. 2008 May;39(5):1569-74.

39. Zonta S, De Martino M, Bedino G, Piotti G, Rampino T, Gregorini M, et al. Which is the most suitable and effective route of administration for mesenchymal stem cell-based immunomodulation therapy in experimental kidney transplantation: endovenous or arterial? Transplant Proc. 2010 May;42(4):1336-40.

40. De Martino M, Zonta S, Rampino T, Gregorini M, Frassoni F, Piotti G, et al. Mesenchymal stem cells infusion prevents acute cellular rejection in rat kidney transplantation. Transplant Proc. 2010 May;42(4):1331-5.

41. Kunter U, Rong S, Boor P, Eitner F, Muller-Newen G, Djuric Z, et al. Mesenchymal stem cells prevent progressive experimental renal failure but maldifferentiate into glomerular adipocytes. J Am Soc Nephrol. 2007 Jun;18(6):1754-64.

42. Moelker AD, Baks T, Wever KM, Spitskovsky D, Wielopolski PA, van Beusekom HM, et al. Intracoronary delivery of umbilical cord blood derived unrestricted somatic stem cells is not suitable to improve LV function after myocardial infarction in swine. J Mol Cell Cardiol. 2007 Apr;42(4):735-45.

43. Le Blanc K, Ringden O. Immunomodulation by mesenchymal stem cells and clinical experience. J Intern Med. 2007 Nov;262(5):509-25.

44. Rubio D, Garcia-Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC, et al. Spontaneous human adult stem cell transformation. Cancer Res. 2005

Apr 15;65(8):3035-9.

45. Bochkov NP, Voronina ES, Kosyakova NV, Liehr T, Rzhaninova AA, Katosova LD, et al. Chromosome variability of human multipotent mesenchymal stromal cells. Bull Exp Biol Med. 2007 Jan;143(1):122-6.

46. Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood. 2003 Nov 15;102(10):3837-44.

47. Rosland GV, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, et al. Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. Cancer Res. 2009 Jul 1;69(13):5331-9.

48. Wang Y, Huso DL, Harrington J, Kellner J, Jeong DK, Turney J, et al. Outgrowth of a transformed cell population derived from normal human BM mesenchymal stem cell culture. Cytotherapy. 2005;7(6):509-19.

49. Miura M, Miura Y, Padilla-Nash HM, Molinolo AA, Fu B, Patel V, et al. Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. Stem Cells. 2006 Apr;24(4):1095-103.

50. Meza-Zepeda LA, Noer A, Dahl JA, Micci F, Myklebost O, Collas P. High-resolution analysis of genetic stability of human adipose tissue stem cells cultured to senescence. J Cell Mol Med. 2008 Mar-Apr;12(2):553-63.

51. Bernardo ME, Zaffaroni N, Novara F, Cometa AM, Avanzini MA, Moretta A, et al. Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. Cancer Res. 2007 Oct 1;67(19):9142-9.

52. Foudah D, Redaelli S, Donzelli E, Bentivegna A, Miloso M, Dalpra L, et al. Monitoring the genomic stability of in vitro cultured rat bone-marrow-derived mesenchymal stem cells. Chromosome Res. 2009;17(8):1025-39.

53. Aguilar S, Nye E, Chan J, Loebinger M, Spencer-Dene B, Fisk N, et al. Murine but not human mesenchymal stem cells generate osteosarcoma-like lesions in the lung. Stem Cells. 2007 Jun;25(6):1586-94.

54. Sundin M, Ringden O, Sundberg B, Nava S, Gotherstrom C, Le Blanc K. No alloantibodies against mesenchymal stromal cells, but presence of anti-fetal calf serum antibodies, after transplantation in allogeneic hematopoietic stem cell recipients. Haematologica. 2007 Sep;92(9):1208-15.

55. Beggs KJ, Lyubimov A, Borneman JN, Bartholomew A, Moseley A, Dodds R, et al. Immunologic consequences of multiple, high-dose administration of allogeneic mesenchymal stem cells to baboons. Cell Transplant. 2006;15(8-9):711-21.

56. Breitbach M, Bostani T, Roell W, Xia Y, Dewald O, Nygren JM, et al. Potential risks of bone marrow cell transplantation into infarcted hearts. Blood. 2007 Aug 15;110(4):1362-9.

57. Yoshimura K, Aoi N, Suga H, Inoue K, Eto H, Sato K, et al. Ectopic Fibrogenesis Induced by Transplantation of Adipose-Derived Progenitor Cell Suspension Immediately After Lipoinjection. Transplantation. 2008 Jun 27;85(12):1868-9.

58. Semedo P, Correa-Costa M, Cenedeze MA, Malheiros DM, Antonia Dos Reis M, Shimizu MH, et al. Mesenchymal Stem Cells Attenuate Renal Fibrosis Through Immune Modulation and Remodeling Properties in a Rat Remnant Kidney Model. Stem Cells. 2009 Dec;27(12):3063-73.

59. Hall B, Dembinski J, Sasser AK, Studeny M, Andreeff M, Marini F. Mesenchymal stem cells in cancer: tumor-associated fibroblasts and cell-based delivery vehicles. Int J Hematol. 2007 Jul;86(1):8-16.

60. Studeny M, Marini FC, Champlin RE, Zompetta C, Fidler IJ, Andreeff M. Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. Cancer Res. 2002 Jul 1;62(13):3603-8.

61. Karnoub AE, Dash AB, Vo AP, Sullivan A, Brooks MW, Bell GW, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature. 2007 Oct 4;449(7162):557-63.

62. Naka N, Takenaka S, Araki N, Miwa T, Hashimoto N, Yoshioka K, et al. Synovial Sarcoma is a Stem Cell Malignancy. Stem Cells. 2010 Jun 1.

63. Wakitani S, Okabe T, Horibe S, Mitsuoka T, Saito M, Koyama T, et al. Safety of autologous bone marrow-derived mesenchymal stem cell transplantation for cartilage repair in 41 patients with 45 joints followed for up to 11 years and 5 months. J Tissue Eng Regen Med. 2010 Jul 5.

64. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells. 2006 Feb;24(2):386-98.

65. Najar M, Rouas R, Raicevic G, Boufker HI, Lewalle P, Meuleman N, et al. Mesenchymal stromal cells promote or suppress the proliferation of T lymphocytes from cord blood and peripheral blood: the importance of low cell ratio and role of interleukin-6. Cytotherapy. 2009;11(5):570-83.

66. Bocelli-Tyndall C, Bracci L, Schaeren S, Feder-Mengus C, Barbero A, Tyndall A, et al. Human bone marrow mesenchymal stem cells and chondrocytes promote and/or suppress the in vitro proliferation of lymphocytes stimulated with the cytokines IL 2, IL 7 and IL 15. Ann Rheum Dis. 2008 Jul 22.

Chapter 10

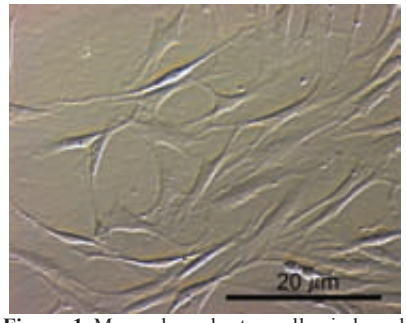
Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

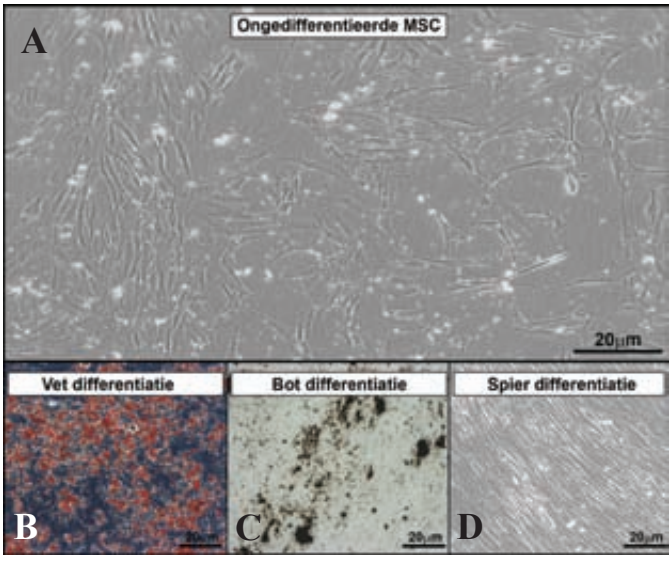
Door een aangeboren afwijking of verworven ziekte kunnen mensen steeds slechter functionerende nieren krijgen, waardoor ze in veel gevallen moeten gaan dialyseren om de filter functie van de nier over te nemen en de schadelijke stoffen uit het lichaam te verwijderen. De nier heeft echter veel meer functies die niet door dialyse vervangen kunnen worden. Daarnaast is dialyse een lichamelijk en psychologisch zware belasting voor de nierpatiënt. De enige definitieve behandeloptie voor patiënten met eindstadium nierfalen is dan ook niertransplantatie. Tijdens de transplantatie wordt het zieke orgaan van de ontvanger vervangen door een gezond orgaan van een donor. Het immuunsysteem van de ontvanger ziet het nieuwe orgaan echter als lichaamsvreemd en zal pogen het transplantaat af te stoten. Om een dergelijke afstotingsreactie te voorkomen moet een ontvanger van een transplantaat levenslang behandeld worden met medicijnen die het immuunsysteem onderdrukken, zogenaamde immunosuppressieve medicijnen. Deze medicatie kan het immuunsysteem onder controle houden en daarmee het risico op afstoting van het orgaan verminderen. Helaas heeft deze medicatie allerlei (ernstige) bijwerkingen, zoals een verhoogd risico op hart- en vaatziekten, infecties, kanker en, paradoxaal genoeg, tast ze ook de nierfunctie aan [1-2]. Daarom wordt er uitgebreid onderzoek gedaan naar nieuwe behandelmethoden die effectiever de afstotingsreactie kunnen remmen. Hierdoor zou de hoeveelheid immunosuppressieve medicijnen verlaagd of zelfs gestopt kunnen worden, zodat het risico op bijwerkingen verlaagd wordt.
Eén van de veelbelovende nieuwe behandelingen is zogenaamde cellulaire (immuun)therapie, waarbij cellen met speciale eigenschappen aan de patiënt worden toegediend om immuunreacties te remmen. Eén van de cellen die geschikt lijken is de zogenaamde mesenchymale stamcel (MSC) (**Hoofdstuk 1**). Hierover gaat dit promotieonderzoek.

MSC zijn grillig gevormde cellen met lange uitlopers in kweek (Figuur 1). Ze kunnen worden geïsoleerd uit vrijwel alle weefsels van zowel embryo's als volwassenen [3]. De klassieke weefselbron voor MSC is beenmerg. Omdat beenmergpuncties pijnlijk en belastend zijn en het beenmerg zeer gevarieerde hoeveelheden cellen oplevert, wordt tegenwoordig ook de bruikbaarheid van andere weefselbronnen onderzocht. In **Hoofdstuk 2** werden mesenchymale stamcellen geïsoleerd uit milt-, hart- en vetweefsel en de eigenschappen van deze MSC zijn vergeleken met MSC geïsoleerd uit beenmerg. Hoewel er subtiele, weefselspecifieke verschillen kunnen zijn, vonden wij dat de algemene kenmerken en eigenschappen van MSC uit de verschillende weefsels vergelijkbaar zijn. In ons onderzoek werden ook stamcellen uit zogenaamd perirenaal vetweefsel van nierdonoren geïsoleerd. Perirenaal vetweefsel is gelokaliseerd rondom de nier en komt bij nierdonatie vrij als afvalproduct. In ons onderzoek bleek echter dat perirenaal vet een zeer rijke bron is voor mesenchymale stamcellen. Tevens kunnen stamcellen uit buikvet geïsoleerd worden; voor klinische studies kan dit een toegankelijke en betrouwbare stamcelbron zijn.
Mesenchymale stamcellen kunnen relatief makkelijk uit weefsels worden geïsoleerd en in kweek gebracht worden (zie figuur Hoofdstuk 2). Het kweken van MSC in het laboratorium heeft het grote voordeel dat de celaantallen vergroot kunnen worden en dat de kweekcondities beïnvloed kunnen worden. Op deze manier kunnen voldoende cellen worden verkregen voor toediening bij klinische toepassingen.

Mesenchymale stamcellen hebben eigenschappen die mogelijk gunstig zijn bij de behandeling van nierfalen of transplantatie patiënten. Allereerst hebben ze de interessante eigenschap dat ze kunnen veranderen van min of meer naïeve, ongedifferentieerde, "pluripotente" cellen in gespecialiseerde weefselcellen, zoals botcellen, vetcellen, spiercellen en mogelijk ook niercellen (Figuur 2) [4-6]. Dit veranderingsproces wordt differentiatie genoemd. Hoewel het verloop van dit proces in het lichaam nog onduidelijk is, zouden MSC met deze eigenschap mogelijk beschadigde weefsels in het lichaam kunnen herstellen. Bovendien maken MSC allerlei groeifactoren die de groei van weefselcellen kan verbeteren, bijvoorbeeld de weefselcellen in het transplantaat [7].

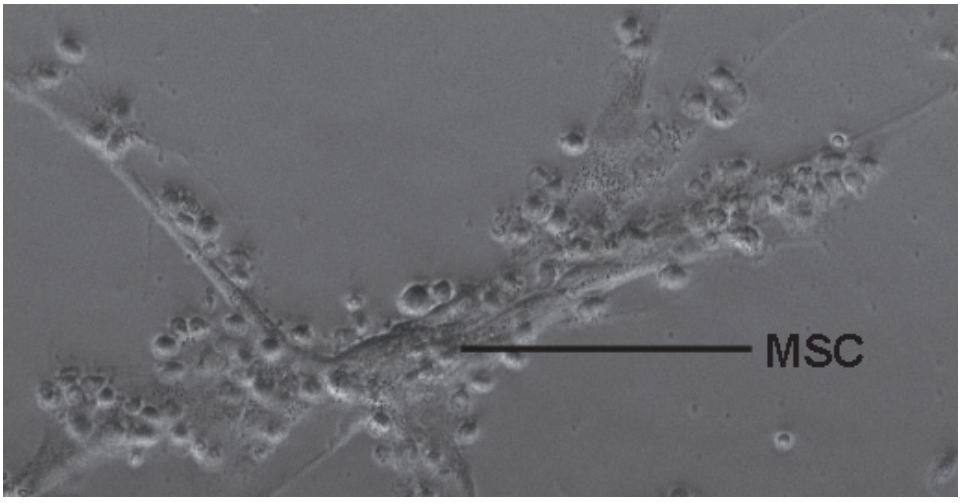


Figuur 1. Mesenchymale stamcellen in kweek



Figuur 2: Differentiatie capaciteit van mesenchymale stamcellen. Mesenchymale stamcellen hebben de interessante eigenschap, dat ze in het laboratorium onder speciale kweekcondities in enkele weken van min of meer naïeve, ongedifferentieerde, 'pluripotente' cellen [A] kunnen differentiëren naar gespecialiseerde weefselcellen, zoals bot-, vet-, spier-. [B] Differentiatie naar bot wordt zichtbaar gemaakt met een Von Kossa kleuring wat kalkafzettingen zwart maakt. [C] Differentiatie van MSC naar vetcellen kan worden zichtbaar gemaakt door de vetblaasjes die door de vetcellen gemaakt zijn, rood te kleuren. [D] Differentiatie naar spiercellen kan morphologisch gevolgd worden en tevens kan de hoeveelheid gevormde spiereiwitten gekwantificeerd worden.

Hoewel de "reparatie" eigenschap van MSC zeer interessant is, heeft dit promotieonderzoek zich gericht op een andere interessante eigenschap van MSC, namelijk hun zogenoemde immunosuppressieve functies. Immunosuppressie betekent letterlijk onderdrukking van de (natuurlijke) afweerreactie. Hoewel immuuncellen, ofwel witte bloedcellen, ons lichaam beschermen tegen indringers van buitenaf, zoals tegen bacteriën en virussen, zullen in geval van transplantatie de immuuncellen zich tegen de cellen in het lichaamsvreemde niertransplantaat richten. De afstotingsreactie die volgt is, zoals eerder gezegd, ongewenst en resulteert in afstoting van het getransplanteerde orgaan. Er is toenemend bewijs dat mesenchymale stamcellen de mogelijkheid hebben om de proliferatie en functie van geactiveerde immuun cellen te remmen. Vooral een bepaald type witte bloedcellen, namelijk zogenaamde T-lymfocyten, kunnen in hun celdeling en functie beïnvloed worden door MSC. Momenteel wordt veel onderzoek gedaan naar de verschillende mechanismen die betrokken zijn bij de onderdrukking van immuun cellen door MSC. Hoewel er nog veel onderzoek nodig is, is het inmiddels wel duidelijk dat de immunosuppressieve effecten van MSC grotendeels berusten op de productie van oplosbare immunosuppressieve factoren [8-10]. De productie van deze factoren door MSC kan worden opgeschroefd indien de mesenchymale stamcellen in aanraking komen met geactiveerde immuun cellen (Figuur 3) [11-12].



Figuur 3. Geactiveerde immuuncellen worden gebonden door mesenchymale stamcellen, zodat, naar men denk, de MSC nog effectiever de geactiveerde cellen kunnen inactiveren.

Verder is gevonden dat ook enzymen belangrijk zijn voor de immunosuppressieve functie van MSC, in het bijzonder het enzym indolamine-2,3-dioxygenase (IDO) wordt hierbij genoemd [13-14]. IDO zorgt ervoor dat tryptofaan, een voedingsstof voor T-lymfocyten, onttrokken wordt uit de omgeving, zodat de groei van deze cellen geremd wordt. Een ander mechanisme waarmee MSC (indirect) immuun reacties zouden kunnen beïnvloeden is dat ze de ontwikkeling van zogenaamde regulatoire T-lymfocyten (T-regs) stimuleren. T-regs zijn T-lymfocyten die andere geactiveerde witte bloedcellen kunnen remmen. Samengevat, kunnen we zeggen dat MSC zowel directe en indirecte manieren hebben om immuun cellen te beïnvloeden.

In **Hoofdstuk 3** worden experimenten beschreven die uitgevoerd zijn waarbij een niertransplantatie werd nagebootst in het laboratorium (zgn. *in vitro* experimenten). Hiertoe reageerden witte bloedcellen van de ontvanger met witte bloedcellen van de donor, waarbij de proliferatie van de ontvanger cellen als surrogaat uitleespunt werden gebruikt voor de afstotingsreactie in het lichaam van de ontvanger. Het bleek dat MSC zeer goed de proliferatie van geactiveerde T-lymfocyten, en daarmee dus de afstotingsreactie, konden remmen. Bovendien kon de afstotingsreactie van de ontvanger gericht tegen de donor cellen, zowel vóór als na transplantatie, significant geremd worden. Aangetoond werd dat het voor deze immunosuppressieve effecten niet uitmaakte of de mesenchymale stamcellen afkomstig waren van de ontvanger zelf, van de nier donor of van een vreemde stamcel donor.
Indien MSC gebruikt gaan worden in de kliniek is het waarschijnlijk dat ze aanvankelijk worden gebruikt in combinatie met de immunosuppressieve medicijnen die niertransplantatie patiënten momenteel krijgen. Daarom werd onderzocht wat de effecten zijn van de immunosuppressieve medicijnen tacrolimus, rapamycine en mycofenolaat mofetil (MMF) op de functies van MSC (**Hoofdstuk 4**). Bovendien zitten in MSC namelijk de aangrijpingspunten van deze immunosuppressieve medicijnen, zodat mogelijk de functie van MSC beïnvloed kan worden. Bij klinische doseringen bleek de medicatie niet giftig voor MSC. Blootstelling aan de verschillende medicijnen beïnvloedden echter wel de differentiatie capaciteit van MSC. Verder toonden we aan dat de immunosuppressieve effecten van MSC verminderd werden indien MSC blootgesteld werden aan rapamycine of tacrolimus. Verrassend genoeg bleek dat de combinatie van MSC en MMF de immunosuppressieve effecten juist vergrootte. Op basis hiervan lijkt MMF het beste immunosuppressieve medicijn om MSC therapie mee te combineren in niertransplantatie patiënten.
Hoewel vrijwel alle onderzoeksgroepen die onderzoek doen naar de immunosuppressieve eigenschappen van stamcellen zich concentreren op de effecten van MSC op geactiveerde immuun cellen, onderzochten we in **Hoofdstuk 5** het omgekeerde, namelijk wat het effect van geactiveerde immuun cellen –oftewel de inflammatoire omstandigheden- op MSC eigenschappen en functies is. We vonden dat blootstelling van MSC aan inflammatoire reacties, eigenschappen van de MSC beïnvloedden. Het meest opvallend was dat - terwijl de differentiatie eigenschappen niet beïnvloed werden - de immunosuppressieve eigenschappen enorm versterkt werden door voorbehandeling van MSC onder inflammatoire condities. Door deze interessante bevinding kunnen we speculeren dat de vermeerdering en voorbehandeling van MSC met inflammatoire factoren in het laboratorium het mogelijk maakt om de immunosuppressieve eigenschappen van MSC al te activeren voordat de MSC ingespoten worden voor klinische behandeling. Op deze manier zouden een grote hoeveelheid stamcellen met nog sterkere immunosuppressieve eigenschappen gemaakt kunnen worden.
In tegenstelling tot het immunosuppressieve effect van MSC op *geactiveerde* immuun cellen, bleek ook dat MSC niet-geactiveerde immuun cellen kunnen activeren (**Hoofdstuk 6)**. Het grootste gedeelte van deze cellen die geactiveerd worden gaan zich echter ontwikkelen tot immuun cellen die immunosuppressieve eigenschappen hebben, de eerdergenoemde T-regs.
Een zeer belangrijke vraag bij de klinische toepassing van cel therapie is of MSC ook immuun reacties kunnen opwekken, met andere woorden of ze immunogeen zijn. Het al dan niet herkend worden door het immuun systeem van de ontvanger is van belang voor de duur van de aanwezigheid van MSC na toediening. Daarnaast zou het herkennen van lichaamsvreemde donor MSC door het immuunsysteem van de ontvanger kunnen leiden tot het opwekken van een “geheugenreactie” bij de immuun cellen, een proces wat sensitisatie wordt genoemd. Het gevolg van sensitisatie zou kunnen zijn dat de immuun cellen de donor nier herkennen als lichaamsvreemd en deze aanvallen. Indien dit het geval is, zou het

geven van stamcellen van de nierdonor aan de ontvanger ervoor zorgen dat het nier transplantaat van de donor versneld afgestoten kan worden. Allereerst vonden we, in tegenstelling tot veel studies, dat MSC immunogeen zijn en dus herkend worden door immuun cellen, namelijk door de zogenaamde cytotoxische T-cellen ($CD8^+$ T-cellen) en natural killer (NK) cellen (**Hoofdstuk 7**). Als gevolg van deze herkenning werden de MSC gedood, zogenoemd gelyseerd. Verder bleek dat lichaamsvreemde MSC door zowel $CD8^+$ T-cellen als door NK cellen gelyseerd werden. Lichaamseigen stamcellen werden echter niet gedood door $CD8^+$ T-cellen. Tot onze verrassing werden ook MSC van de ontvanger zelf, dus lichaamseigen cellen, herkend en gelyseerd door NK cellen, maar niet door $CD8^+$ T-cellen. Hoe dit komt weten we niet, maar het zou kunnen komen doordat het kweken van MSC in het laboratorium de vorm van de cellen verandert, zodat ze vervolgens als vreemd worden herkend. Deze resultaten zijn belangrijk om mee te nemen in de klinische toepassing van MSC in de kliniek.

Concluderend kunnen we zeggen dat, op basis van de resultaten van onze experimenten en de huidige literatuur, mesenchymale stamcellen beschadigde nieren zouden kunnen herstellen en tegelijkertijd de afstotingsreactie zouden kunnen onderdrukken. Hoewel de resultaten uit de laboratorium- en dierstudies veelbelovend zijn, moet de effectiviteit van mesenchymale stamcellen in klinische orgaan transplantatie nog bewezen worden. Daarnaast is er nog veel onbekend over de basale werking van MSC en hun functie in het lichaam. Dit vraagt om verder onderzoek. **Hoofdstuk 9** beschrijft daarom op basis van de huidige literatuur en de resultaten van dit promotieonderzoek de mogelijkheden voor toepassing van mesenchymale stamcellen bij orgaan transplantatie, en dan met name bij niertransplantatie. Er worden enkele overwegingen en valkuilen genoemd die daarbij relevant kunnen zijn. Voorbeelden hiervan zijn veiligheid, tijdsplanning en effectiviteit van MSC behandeling. Voor succesvolle ontwikkeling van een nieuwe therapie, zoals mesenchymale stamcel therapie, is de interactie tussen laboratorium experimenten en goed opgezette klinische studies cruciaal. In de komende jaren zal duidelijk worden of de op dit moment veelbelovende mesenchymale stamcellen een succesvolle nieuwe behandeling zullen worden voor patiënten met nierziekten of niertransplantatie.

REFERENTIES

1. Marcen R. Immunosuppressive drugs in kidney transplantation: impact on patient survival, and incidence of cardiovascular disease, malignancy and infection. Drugs. 2009 Nov 12;69(16):2227-43.

2. Boots JM, Christiaans MH, van Hooff JP. Effect of immunosuppressive agents on long-term survival of renal transplant recipients: focus on the cardiovascular risk. Drugs. 2004;64(18):2047-73.

3. da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci. 2006 Jun 1;119(Pt 11):2204-13.

4. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999 Apr 2;284(5411):143-7.

5. Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow.Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation. 1968 Mar;6(2):230-47.

6. Herrera MB, Bussolati B, Bruno S, Fonsato V, Romanazzi GM, Camussi G. Mesenchymal stem cells contribute to the renal repair of acute tubular epithelial injury. Int J Mol Med. 2004 Dec;14(6):1035-41.

7. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006 Aug 1;98(5):1076-84.

8. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002 May 15;99(10):3838-43.

9. Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood. 2003 May 1;101(9):3722-9.

10. Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, et al. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci. 2005;12(1):47-57.

11. Quaedackers ME, Baan CC, Weimar W, Hoogduijn MJ. Cell contact interaction between adipose-derived stromal cells and allo-activated T lymphocytes. Eur J Immunol. 2009 Dec;39(12):3436-46.

12. Ren G, Zhao X, Zhang L, Zhang J, L'Huillier A, Ling W, et al. Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. J Immunol. 2010 Mar 1;184(5):2321-8.

13. DelaRosa O, Lombardo E, Beraza A, Mancheno-Corvo P, Ramirez C, Menta R, et al. Requirement of IFN-gamma-mediated indoleamine 2,3-dioxygenase expression in the modulation of lymphocyte proliferation by human adipose-derived stem cells. Tissue Eng Part A. 2009 Oct;15(10):2795-806.

14. Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol. 2007 Aug;149(2):353-63.

Chapter 11

Appendix

Dankwoord
PhD portfolio
List of publications

DANKWOORD

Na deze drieënhalf jaar van promotie onderzoek ben ik vele ervaringen rijker. Heel bewust heb ik na mijn studie geneeskunde gekozen om promotie onderzoek te gaan doen. De interesse voor onderzoek was al sinds mijn doctoraalscriptie gewekt en het leek me een mooie kans om op een andere manier naar de geneeskunde te kijken. Enkele maanden voor mijn afstuderen als arts heb ik een open sollicitatie gestuurd naar de afdeling Interne geneeskunde met de vraag of er een interessant onderzoek was om in te stappen. Binnen de afdeling niertransplantatie was toen heel recent een project gestart om te kijken of mesenchymale stamcellen nuttig konden zijn bij niertransplantatie. Bij mijn sollicitatie werd duidelijk dat het gehele onderzoek nog opgezet moest worden en dat de richting waarin het onderzoek zich zou ontwikkelen nog niet duidelijk was. Dit deed mij echter geenszins afschrikken. Integendeel, het motiveerde mijn juist om de (immunosuppressieve) eigenschappen van mesenchymale stamcellen en de mogelijkheden bij orgaantransplantatie uit te zoeken. Ik denk dat we inmiddels een zeer mooie en vernieuwde (onderzoeks)weg ingeslagen zijn, die niet alleen tot een heel aantal mooie publicaties heeft geleid, maar ook een wetenschappelijke basis heeft gelegd voor potentiële toepassing van mesenchymale stamcellen in de kliniek.

Hoewel onderzoek doen iets volledig anders is dan het klinische werk als arts ben ik nog steeds van mening dat het voor een professionele arts essentieel is om affiniteit met en begrip van wetenschappelijk onderzoek te hebben. Het toepassen van resultaten behaald door onderzoek in de dagelijkse praktijk maakt het mogelijk de patiëntenzorg steeds weer te verbeteren, de zogenaamde “evidence-based” geneeskunde. Naast basale biologie en laboratoriumtechnieken heb ik veel geleerd over praktisch, gestructureerd experimenten bedenken, uitvoeren en analyseren. Ik heb hierbij veel geleerd van de collega’s met wie ik samen heb gewerkt. Hieronder staan enkele mensen genoemd die ik graag in het bijzonder wil bedanken. Ook diegenen die niet expliciet genoemd worden, maar waarmee ik leerzame en leuke momenten heb beleefd: bedankt!

Allereerst wil ik mijn promoter Prof. dr. W. Weimar bedanken. Beste Willem, bedankt dat je mijn promotor wilde zijn. Door mijn open sollicitatie kwam ik bij jou terecht, omdat er op jouw afdeling net een nieuwe onderzoekslijn opgestart werd onder leiding van Martin Hoogduijn. Hoewel de titel veelbelovend “mesenchymale stamcellen en transplantatie” luidde, was de inhoud daarvan nog grotendeels blanco. Bovendien was het onbekend of het onderzoek een succes zou worden.
Eén van je eerste vragen waarmee ik tijdens mijn werkbespreking geconfronteerd werd, was of “mesenchymale stamcellen niet gewoon fibroblasten zijn”, en of “fibroblasten dezelfde resultaten geven in een MLR? heb je dat getest?”. Ik denk dat we er na vier jaar onderzoek nog steeds niet met zekerheid kunnen zeggen of de ene cel heel anders is dan de andere cel. Toch hebben we mooie resultaten behaald met deze fibroblast-achtige MSC (of zijn het dan toch MSC-achtige fibroblasten?!). Van onze besprekingen heb ik onder andere geleerd dat de titel en het abstract de belangrijkste zijn van een manuscript. Dit heeft zich bevestigd door het feit dat ik nog nooit zoveel e-mails heb ik ontvangen met het verzoek om een PDF op te sturen als van het manuscript met in de titel “*Explosive* T-cell proliferation”; een titel waar we hard over hebben gelachen, en *catchy* is de titel dus zeker! Ook heb ik geleerd dat de rest van een manuscript zo duidelijk moet zijn dat reviewers mijn manuscript begrijpen zonder dat ze mijn (soms erg uitgebreide) toelichting erbij hebben, die ik wel bij onze besprekingen kon geven.

Ook weet ik dat het inzicht van de ene week, niet hetzelfde inzicht van de week erna hoeft te zijn ("… moet die titel niet veranderen in …"?, "o, je bedoelt dat wat er vorige week stond"). "Voortschrijdend inzicht" is een mooie term hiervoor. Bedankt ook dat ik mijn resultaten op zoveel congressen mocht presenteren, een leerzame en leuke ervaring. De "één-boodschap" presentaties zal ik zeker onthouden, hoewel het me soms door mijn enthousiasme toch niet lukt minder te vertellen.
Dank ook voor het mogelijk maken van de labweekenden, ook namens mijn vrouw Eefje. Het waren elke keer leuke en gezellige momenten met elkaar. Ik heb veel geleerd op het Transplantatie laboratorium en ik denk dat dit een mooi begin is geweest voor mijn toekomst waarin hoop ik onderzoek en onderwijs een belangrijke rol mogen spelen naast het klinische werk.

Mijn promotie zou zeker niet zo leuk, humoristisch, inspirerend en voorspoedig zijn gelopen zonder mijn co-promotor Dr. M.J. Hoogduijn. Beste Martin, inspirerende baas, trouwe labonderzoeker en goede vriend. Ik wil je heel hartelijk bedanken voor je begeleiding. Onze inzichten kwamen vrijwel altijd goed overeen. Je hebt me veel geleerd over het opzetten van experimenten, het pipetteren en het schrijven van mijn manuscripten. Hoewel de eerste kennismaking nogal een verrassing voor je was (Meindert: "ik ben je nieuwe AIO!"; Martin: "oh, krijg ik een nieuwe AIO dan"?!), is onze samenwerking in de afgelopen jaren zeer succesvol gebleken en heeft dit ook nog eens geleid tot een goede vriendschap. Dat ik je bij het squashen af en toe met het balletje raakte, kwam meer door mijn geringe squash ervaring dan uit frustraties opgedaan in de werksituatie. Ook op congressen hebben we veel leuke momenten beleefd. Ik hoop nog veel contact met je te houden, zowel wetenschappelijk als vriendschappelijk.

Carla, alweer een kleine vier jaar geleden leerde ik je kennen tijdens het sollicitatie gesprek. Hoewel het voor mij als dokter zeker even wennen was om basaal wetenschappelijk onderzoek te gaan doen ("pipetteren"), heb ik het heel leerzaam gevonden om op je "lab" bezig te zijn. Gelukkig is het praktische werk prima gelukt. Onze samenwerking kan ik als direct, vriendelijk en kritisch beschrijven. Bedankt voor je begeleiding, je goede aanvullende ideeën en opbouwende kritiek. Ik denk dat de uitbreiding van het transplantatie lab met onderzoek naar MSC veel mogelijkheden biedt naast de andere interessante onderzoekslijnen.

Mijn paranimfen: Jasper Brugts en Ramin Vafadari. Twee mannen die mij zullen bijstaan op een belangrijke dag: een dag waar ik vier jaar naar toe hebt gewerkt. Super bedankt Jasper, of moet ik Dr. Brugts zeggen! We kennen elkaar sinds de opleiding geneeskunde, en werden samen met Sietse ook wel de drie musketiers genoemd. Altijd samen hard studeren, maar ook veel plezier maken, zoals wintersport. Wij tweeën hebben na het artsdiploma besloten wetenschappelijk onderzoek te gaan, iets wat ons allebei goed ligt denk ik. Samen zijn we nu ook in het Albert Schweitzer ziekenhuis met de patiëntenzorg bezig. Leuk om onze levenslijnen zo dicht bij elkaar te zien. Als collega en als vriend hoop ik nog samen veel te beleven.
Ramin, mijn achterbuurman in het AIO hok: we hebben de afgelopen jaren een goede band opgebouwd die zich kenmerkt door werkinhoudelijk opbouwende kritiek, maar bovenal door droge humor en vriendschap. Voor jouw onderzoek heb ik bij heel wat mensen bloed afgetapt. Nu ik weg ben, ben ik blij dat je eindelijk zelf geleerd hebt om bloed te prikken. Ik vind het bijzonder dat mijn paranimf wil zijn.

Collega AIO's. Allen met een andere achtergrond, variërend van biologie, geneeskunde, gezondheidswetenschappen en farmacologie. Ik denk dat we een hele leuke groep vormden waarin we ook laagdrempelig met elkaar konden overleggen, plezier maakten en zeker ook kritisch konden zijn.
Beste Varsha, Dr. Sewgobind inmiddels, jij was mijn "bling-bling" buurvrouw met een uitgesproken voorkeur voor roze en goud. Ik heb het erg gezellig samen met je gevonden, hoewel we af en toe ook pittige discussies over statistiek hadden. Dat we het afgelopen jaar beiden als "oudste" AIO's in het AIO hok zaten om "het boekje" af te ronden, gaf zeker een gevoel van saamhorigheid. Ook op congressen (o.a. Boston) was je een trouwe supporter bij mijn presentaties. Ik vond het bijzonder om jouw "supporter" (para-nimf) bij jouw promotie te zijn. Nu heb je dan toch het basale onderzoek verlaten en komen er geen regulatoire T-cellen meer voor in jouw presentaties: je bent (zoals wel enigszins verwacht) in de farmaceutische industrie terecht gekomen. Heel veel succes daar!
Anja, als hardwerkende AIO kom je er wel; je begon vaak nog vroeger dan ik, wat op zich al een hele prestatie was, omdat je helemaal uit Breda moet komen! Het is erg leuk dat je met jouw onderzoek "de interactie tussen Tregs en MSC" onderzoekt, aangezien het onderzoek naar deze twee cel typen belangrijke onderzoekslijnen in het transplantatie laboratorium zijn. De grote vraag blijft, ben je een "Tregs-*believer*" of een "*MSC-believer*"? Ik ben benieuwd welke cel de race gaat winnen!
Martijn, samen zijn we een begonnen met iets nieuws op ons lab: renale tubulus epitheel cellen (TEC's). Hoewel de groei van TEC's in het begin moeilijker was dan de groei van schimmels, hoop ik dat je mooie resultaten vindt. Erg gezellig dat je meedeed met vrijwel alle activiteiten die georganiseerd werden, van borrels tot bowling! We hebben heel wat plezier gehad.
Anne, collega dokter. Je kwam als meest recente AIO ons AIO hok binnen en werd mijn achterbuurvrouw. Hoewel je aanvankelijk wat moeilijk je draai kon vinden, hoop ik dat je onderzoek leuke resultaten gaat opleveren. En je weet het, je mag altijd nog aansluiten bij het "stamcel groepje", zoals je dat noemde. Tot snel weer in de kliniek; het Albert Schweitzer ziekenhuis in Dordrecht misschien? Dat zou gezellig zijn.
De laatste AIO die ik expliciet wil noemen is Marieke. Beste Marieke, je solliciteerde naar het onderzoekproject dat mijn werk zou gaan vervolgen, nog voordat je afgestudeerd was. Waar ik alleen maar basaal wetenschappelijk experimenten deed, zal jij waarschijnlijk de meer klinische toepassing gaan mee maken. Hoewel ik daar ook graag direct bij betrokken had willen zijn, ben ik heel blij dat jij het "stamcel-stokje" over hebt genomen. Het is spannend hoe het (klinische) stamcel onderzoek gaat lopen. Ik weet echter zeker dat je met jouw enthousiasme er alles aan zal doen om mooie resultaten te verkrijgen. Het is noemenswaardig dat door jouw contacten bij de chirurgie, de hoeveelheid vetweefsel die verwerkt werd tot stamcellen significant gestegen is. Van perirenaal of subcutaan vet van de ontvanger, tot subcutaan vetweefsel van de donor; jij weet aardig wat potjes vet op de OK te regelen. Heel veel succes, ik blijf graag op de hoogte van de stamcelletjes!

Dan zijn er verder nog enkele oud-collega's die ik wil bedanken: Thijs, Jeroen en Esmé. Bedankt voor het inwerken aan het begin van mijn promotie. In de AIO kamer en op congressen waren we er voor elkaar. Bedankt voor jullie vrolijke noot, kopje filter koffie en ook voor de opbouwende kritiek. Ook Monique Quaedackers wil ik graag bedanken voor onze leuke momenten samen, niet alleen in de kantine (zgn. "lunchen met Mo en Ho") – trek er dan maar 10 minuten extra voor uit"), cappuccino na de lunch, maar ook op congres. Je hebt een hele overstap gemaakt door als "hardcore basic scientist" docent te worden, maar ik ben blij dat je het zo naar je zin hebt.

Collega's op het transplantatie lab. Ik wil jullie allen graag bedanken voor de leuke tijd die ik heb gehad. Van borrels, bowlinguitjes en paaslunches tot labweekenden, het was erg gezellig met elkaar. Als kersverse dokter zonder enige pipetteer ervaring kwam ik op het lab. Mede dankzij jullie inwerken en hulp, heb ik erg mooie experimenten kunnen doen. Hoewel ik hier niet alle ervaringen kan opschrijven, heb ik het erg naar mijn zin gehad op het lab: van 's morgens 7.30 tot soms laat in de avond!".
In het bijzonder wil ik Sander bedanken. Door jouw nauwgezetheid en gedrevenheid vond ik onze samenwerking erg aangenaam. Ik ben heel blij dat jij als echte "Kill Bill" de Europium-"kill"-assays weer aan de praat gekregen hebt. We hebben erg mooie resultaten behaald en jouw naam staat met trots bij de artikelen. Bedankt voor je inzet, ook al werd het soms wat later op vrijdagmiddag. Ook het samen monteren van de film ter ere van de promotie van Esmé was erg gezellig, alhoewel "nog een paar kleine dingetjes, en dan is het klaar" toch wel enkele uurtjes extra tijd kostten.

De resultaten in dit promotieboekje zijn mede mogelijk gemaakt door de transplantatie chirurgen van het Erasmus MC onder leiding van Prof. Jan IJzermans. Heel hartelijk dank voor het verzamelen van het "potje met vet"….en ja, alle mogelijke woordspelingen en liedjes zijn daar al over gemaakt. Ook arts-onderzoeker Nienke Dols die soms op de OK aanwezig was en mijn "redding" dat het vet niet per ongeluk weggegooid werd, wil ik hartelijk bedanken. Bovendien kwamen we elkaar geregeld tegen bij congressen, zoals in Boston en Vancouver.

Luc van der Laan en Abdulla Pan, beiden ook ervaren onderzoekers in het stamcel veld, waarbij jullie focus niet lag op de toepasbaarheid van MSC in de niertransplantatie, maar in de levertransplantatie. Spannend hoe de MSC zich in de orgaantransplantatie verder gaan ontwikkelen. Met mijn "wilde" idee tijdens de koffie pauze, is inmiddels een mooi onderzoek opgestart. Deze "uitvinding" is nu een Europees geaccepteerd patent. Hopelijk volgt binnenkort een mooie paper met alle resultaten. We houden contact. Ik ben benieuwd hoe het zich verder ontwikkelt.

Graag wil ik op deze manier ook Prof. Zietse bedanken. Beste Bob, jij hebt me door je rustige, opbouwende en soms zelfs komische kritiek door mijn eerste wetenschappelijke tekst gesleept. De eerste zinnen waren soms lastig en soms zelfs komisch. Het wordt "slepen" kunnen we wel heel letterlijk nemen bij ons artikel over de "dysmorfjes". Dat artikel was een zeer lange tijd een blok aan mijn/ons been. Ik denk echter dat we er uiteindelijk toch nog een erg leuk stuk van hebben gemaakt. Bedankt voor je inspiratie en geduld. Ook met Ewout Hoorn heb ik in de context van "hypokaliëmie" fijn samengewerkt. Ewout, samen met Bob sta je aan het begin van mijn onderzoekscarrière. Het was geweldig om met jullie te mogen samenwerken. Nu moeten we misschien nog echt eens een keer afspreken om samen te gaan tennissen!

Verder wil ik de mensen op de D-vleugel hartelijk bedanken voor hun hulp bij mijn onderzoek; o.a. de internisten / nefrologen, de onderzoekers, de (research)verpleegkundigen en de secretaresses. Speciaal wil ik ook jou, Saïda, secretaresse van professor Weimar, bedanken voor je hulp, maar ook voor je geduld. Geduld voor als ik weer eens om een "nog even tussendoor afspraak" kwam vragen; of omdat mijn bespreking met de professor weer uitliep, omdat we alleen nog maar de "laatste paar dingetjes" van de paper hoefden door te spreken voordat het manuscript klaar was. Uiteraard duurde dit altijd langer dan gepland!

Anderen van het Erasmus MC die ik graag wil bedanken zijn de assistenten van de nierpoli. Dankzij jullie heb ik een mooie database opgebouwd. Bedankt voor het prikken van alle patienten onder vermelding van “5 hep bb Tx lab meindert”).

Dit proefschrift zou niet mogelijk zijn geweest als nierontvangers en nierdonoren geen toestemming zouden hebben gegeven om hun vetweefsel te gebruiken voor het onderzoek. Ik wil hen daar heel hartelijk voor danken. Sommigen waren niet geheel tevreden met de hoeveelheid vetweefsel die we gebruikten: i.p.v. enkele grammen wilden ze graag kilo’s afstaan! Misschien een idee voor een vervolgproject, in navolging van de afdeling experimentele cardiologie: eerst een volledige liposuctie doen om vervolgens stamcellen uit het vetweefsel te halen?

Lieve vrienden en familie. Lieve pap en mam. In de afgelopen jaren ben ik met dingen bezig geweest die soms nauwelijks voor jullie te begrijpen waren en waarbij Engelse termen, zoals “multilineage differentiation capacity”, “immunomodulatory effects”, and “mixed lymphocyte reactions”, de revue passeerden. Mijn promotieonderzoek was wat dat betreft soms een “ver-van-je-bed” show, en zo anders dan het “echte” dokter zijn. Toch ben ik blij met de interesse en steun die ik van jullie heb gekregen, want soms was het best pittig. Nu ligt het resultaat voor jullie. Hoewel ik natuurlijk de patiëntenzorg erg gemist heb, is mijn promotietraject een leerzame en interessante tijd geweest en heeft mij zeker verrijkt.
De afgelopen periode was erg hectisch door de combinatie tussen het klinische werk in het ziekenhuis en het afronden van mijn proefschrift. Daarom hoop ik dat ik de komende periode weer meer tijd voor jullie te hebben.

Lieve Sietse, mijn tweelingbroer. Hoewel onze levens heel parallel gelopen zijn tot en met het artsexamen, hebben we daarna ieder onze eigen weg gekozen. Jij bent gaan werken op de Spoedeisende hulp in Zeeland en ik startte met mijn promotieonderzoek. Twee totaal verschillende werelden: van acute patiëntenzorg tot stamcellen kweken in het laboratorium. Ondanks dat we ons afzonderlijk verder ontwikkeld hebben, blijf jij mijn trouwe broer en grootste vriend. Ik ben blij dat je samen met Lieke gelukkig bent.

Bovenal wil ik mijn lieve vrouw Eefje bedanken. Jij hebt mij altijd gesteund en gaf me rust en zekerheid als ik weer laat thuis was of lang door moest werken. Vooral de laatste periode was erg intensief. Jouw nuchterheid en praktische insteek zorgden ervoor dat ik ook afstand kon nemen van het onderzoek. Hierdoor bleef ik met beide benen op de grond en kon ik mijn onderzoek ook hopelijk enigszins begrijpelijk overbrengen naar mijn omgeving. Door het afronden van mijn promotieonderzoek en het starten van mijn werk in de kliniek zijn onze werk-gerelateerde gesprekken een stuk beter op elkaar af gestemd. Bovendien ben ik door jou als diëtist gespitst om bij patiënten ook op de voedingsstatus te letten. Naast een zorgzame en lieve vrouw, kan je erg lekker koken. Elke avond, na een lange werkdag, is het weer genieten en het spreekwoord “liefde van de man gaat door de maag” had zomaar één van mijn stellingen kunnen zijn! Je muzikaliteit vind ik geweldig. Ik geniet ervan om naar je piano spel en zang te luisteren. Je bent een fantastische, veelzijdige, en zelfstandige vrouw; ik houd veel van jou!

Meindert

PHD PORTFOLIO

Name PhD student:	Meindert Johannes Crop
Erasmus MC Department:	Internal Medicine – Transplantation
Research School:	Molecular Medicine (MolMed) Graduate school
PhD period:	October 2006 – May 2010
Promotor:	Prof. Dr. Willem Weimar
Supervisor (co-promotor):	Dr. Martin J. Hoogduijn

1. PhD training

Courses

2010 Feedback geven – Albert Schweitzer Ziekenhuis, Dordrecht
2010 Basic Life Support / Advanced Life Support – Albert Schweitzer Ziekenhuis, Dordrecht
2010 Brain training (speed reading, mind mapping) – MT company BV
2010 Geavanceerde beeldvormende technieken voor dokters, MolMed, Erasmus MC, Rotterdam
2009 Communicatie rond donatie, Nederlandse Transplantatie Stichting
2009 Partek Training Course micro-arrays, MolMed, Erasmus University, Rotterdam
2009 Mini-course Patient-related Research, Erasmus Medical Center, Rotterdam
2008 Overview course molecular techniques, MolMed, Erasmus University, Rotterdam
2008 Classical Methods in Biostatistics, NIHES, Erasmus University Rotterdam
2008 Literature Search, Central Library Erasmus Medical Center, Rotterdam
2008 European Transplant Fellow (EFTW) – Workshop presentation skills
2006 Biomedical English writing and communication, MolMed, Erasmus University, Rotterdam
2006 Irradiation Hygiene Expertise 5A/B, Erasmus University, Rotterdam

(Inter)national conferences and presentations		*presentation*
2010	International Transplantation Society (ITS) congress, Vancouver, Canada	2xmini-orals
2010	Annual Meeting Bootcongres NTV, Rotterdam, The Netherlands	2x oral
2010	Wetenschapsdagen Interne Geneeskunde Erasmus MC, Antwerpen, Belgium	oral
2009	Annual Meeting ATC, Boston, USA	oral
2009	Annual Meeting Bootcongres NTV, Zeewolde, The Netherlands	oral
2009	Annual Meeting NND (NfN), Veldhoven, The Netherlands	oral
2009	MolMed Day, Erasmus MC, Rotterdam, The Netherlands	oral
2009	Wetenschapsdagen Interne Geneeskunde Erasmus MC, Antwerpen, Belgium	oral
2009	Annual Meeting ESOT, Paris, France	2x oral*
2009	ISSCR meeting, Barcelona, Spain	poster
2009	Basic Science meeting ESOT, Bruxelles, Belgium	poster
2008	MolMed Day, Erasmus MC, Rotterdam, The Netherlands	poster
2008	Meeting European Society of Hematology (ESH), Cannes, France	poster
2008	International Transplantation Society (ITS) congress, Sydney, Australia	mini-oral&poster*
2008	Annual Autumn NfN, Utrecht, The Netherlands	
2008	Annual Meeting NTV	oral
2008	Annual Meeting NND Dutch Society of Nephrology, Veldhoven, The Netherlands	oral
2008	PLAN Research day, Rotterdam, The Netherlands	oral
2007	Annual Autumn NfN, Utrecht, The Netherlands	poster

2007	Annual Meeting ESOT, Prague, Czech Republic	oral
2007	Annual Meeting NVVI, Noordwijkerhout, The Netherlands	poster
2007	Annual Meeting Bootcongres NTV, Zeewolde, The Netherlands	oral
2007	Wetenschapsdagen Interne Geneeskunde Erasmus MC, Goes, The Netherlands	poster

** Presentations given by Dr. Martin Hoogduijn*

Abbreviations:

NTV = Nederlandse Transplantatie Vereniging (Dutch Transplantation Society)
ATC = American Transplant Congress (American Transplantation Society)
ESOT = European Society of Transplantation
ISSCR = International Society of Stem Cell Research
ITS / TTS = International Transplantation Society / The Transplantation Society
MolMed = Molecular Medicine Graduate school
NND = Nederlandse Nefrologie Dagen (Dutch Society of Nephrology)
NfN = Nederlandse Federatie voor Nefrologie (Dutch Federation of Nephrology)
NVVI = Nederlandse Vereniging voor Immunology
PLAN = Platform Aio's/post-docs Nefrologie (PLAN)

Scientific Awards

2010	Annual Meeting Bootcongres NTV	*congress grant*
2009	Genzyme award for best oral presentation	*best oral presentation*
2009	American Transplant Congress	*young investigator award*
2009	Novartis Pharma Transplantation Advisory Board	*travel grant ATC*
2007	Genzyme award for best oral presentation	*best oral presentation*
2007	European Transplant Congress	*young investigator award*
2010	Novartis Pharma Transplantation Advisory Board	*travel grant ISOT*

Memberships

2010 - Jonge Nederlandse Internisten Vereniging (JNIV)
2006 - 2010 Nederlandse Transplantatie Vereniging (NTV)
2006 - 2010 European Society of Transplantation (ESOT)
2006 - 2010 Nederlandse Federatie voor Nefrologie (NfN)
2006 - 2010 International Society of Stem Cell Research (ISSCR)
2006 - 2010 The Transplantation Society (TTS)

Scientific boards / committees

2009 Nephrological Issues in Experimental Research (NIER) (www.nier.eu); *editor*
2007-2009 Nederlandse Federatie voor Nefrologie – sectie Wetenschap
2007 Organizer of the PhD information market Erasmus MC, Rotterdam
2006-2009 Platform Landelijke AIO's/post-docs Nefrologie (PLAN); *chairman (2007-2009)*

2. Teaching activities

2010	Lecture for medical students	2010
2010	Supervising medical student Master's thesis	2009
2007	Lecture for medical students	2007

PATENTED SCIENTIFIC INVENTION

Patent (WO2010053350): “Inhibition of viral infection and replication by mesenchymal stem cells (MSC) and MSC-derived products”.
Available at http://ep.espacenet.com/ or https://register.epoline.org/espacenet/application;jsessionid=92E6F963091BC8A77515CF5E6B5DBEAF.RegisterPlus_prod_0?number=EP08876226&tab=main

Inventors: Luc J. W. van der Laan, Qiuwei (Abdulla) Pan, Meindert J. Crop

Content: This invention is related to the field of viral control, more specifically to the control of viral replication, especially of chronic viruses and particularly hepatitis C virus (HCV) and hepatitis B (HBV).

Summary:
The invention now concerns a method for inhibition of a viral infection comprising exposing said virus to mesenchymal stem cells (MSC), preferably said virus is a hepatitis virus, more preferably HCV or HBV. In a preferred embodiment, the inhibition is the inhibition of viral replication in a host cell. In a further preferred embodiment the cells harbouring the virus are co-cultured with MSC. Alternatively, the cells harbouring the virus are contacted with exudate of MSC. In the latter case, it is preferred that the exudate is concentrated by removing small molecules, preferably smaller than 3 kD, more preferably smaller than 10 kD, even more preferably smaller than 50 kD, preferably by filtration. Also, it is preferred that the exudate is heat-treated, preferably by subjecting it to a temperature of at least 60ºC for at least 20 minutes, more preferably for at least 100ºC for at least 30 minutes.

In a further preferred embodiment, the antiviral active component produced by the MSC is a protein with an amino acid sequence of more than approximately 400 amino acids.
The invention also comprises the use of mesenchymal stem cells (MSC) for the inhibition of a viral infection, preferably a hepatitis virus, more preferably HCV or HBV. For said use, the MSC are co-cultured with cells harbouring said virus.

The invention also comprises the use of exudate of MSC for the inhibition of a viral infection, preferably a hepatitis virus, more preferably HCV or HBV. Preferably, said exudate is concentrated by removing small molecules, preferably smaller than 3 kD, more preferably smaller than 10 kD and even more preferably smaller than 50 kD, by filtration. In another embodiment, a preferred use is wherein said exudate is heat-treated, preferably by subjecting it to a temperature of at least 60ºC for at least 20 minutes, more preferably for at least 100ºC for at least 30 minutes. Further, preferably the exudate comprises a protein with an amino acid sequence of more than 80 amino acids.
Also part of the invention is a pharmaceutical composition for use in the treatment of viral infection, wherein said composition comprises MSC or exudate from MSC.

LIST OF PUBLICATIONS:

2010 Crop MJ, Korevaar SS, Kuiper, de, R, et al. Human mesenchymal stem cells are susceptible to lysis by $CD8^+$ T-cells and NK cells. (*submitted*)

2010 Hoogduijn MJ, Popp FC, Grohnert A, et al. Advancement of mesenchymal stem cell therapy in solid organ transplantation (MISOT). Transplantation 2010; 90 (2): 124-6.

2010 Crop MJ, de Rijke YB, Verhagen PC, Cransberg K, Zietse R. Diagnostic value of urinary dysmorphic erythrocytes in clinical practice. Nephron Clin Pract 2010; 115 (3): c203-12.

2010 Crop MJ, Baan CC, Korevaar SS, et al. Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. Clin Exp Immunol 2010 Sept 15 (*in press).*

2010 Crop M, Baan CC, Korevaar SS, Ijzermans JN, Weimar W, Hoogduijn MJ. Human adipose tissue-derived mesenchymal stem cells induce explosive T-cell proliferation. Stem Cells Dev 2010 Apr 5 (*in press).*

2009 Hoogduijn MJ, Crop MJ, Peeters AM, et al. Donor-derived mesenchymal stem cells remain present and functional in the transplanted human heart. Am J Transplant 2009; 9 (1): 222-30.

2009 Crop MJ, Baan CC, Korevaar SS, et al. Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients. Transplantation 2009; 87 (6): 896-906.

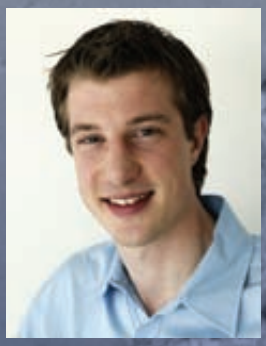
2009 Crop M, Baan C, Weimar W, Hoogduijn M. Potential of mesenchymal stem cells as immune therapy in solid-organ transplantation. Transpl Int 2009; 22 (4): 365-76.

2008 Hoogduijn MJ, Crop MJ, Korevaar SS, et al. Susceptibility of human mesenchymal stem cells to tacrolimus, mycophenolic acid, and rapamycin. Transplantation 2008; 86 (9): 1283-91.

2007 Hoogduijn MJ, Crop MJ, Peeters AM, et al. Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. Stem Cells Dev 2007; 16 (4): 597-604.

2007 Crop MJ, Hoorn EJ, Lindemans J, Zietse R. Hypokalaemia and subsequent hyperkalaemia in hospitalized patients. Nephrol Dial Transplant 2007; 22 (12): 3471-7.

CURRICULUM VITAE AUCTORIS



Meindert Johannes Crop werd samen met zijn eeneiige tweelingbroer op 31 maart 1982 geboren te Delft. Van 1994 tot 2000 zat hij op het Christelijk Gymnasium Beyers Naudé te Leeuwarden. Al sinds zijn jeugd was hij geïnteresseerd in de geneeskunde. Hoewel het genezen van dieren lange tijd zijn voorkeur had, heeft hij uiteindelijk de voorkeur gegeven aan de humane geneeskunde. Na initieel uitgeloot te zijn, kon hij in 2000 door naplaatsing toch met de studie geneeskunde beginnen aan de Erasmus Universiteit te Rotterdam.

Na het afronden van het doctoraal en co-schappen, deed hij een aanvullend keuze co-schap interne geneeskunde in het Diakonessenhuis te Paramaribo Suriname. Zijn artsexamen legde hij af in 2006. Tot zover liepen de levenspaden van hem en zijn tweelingbroer parallel, maar Meindert koos toen zijn eigen weg door met promotie onderzoek te beginnen op de afdeling niertransplantatie van het Erasmus Universitair Medisch Centrum te Rotterdam onder leiding van Dr. Carla Baan. Onder begeleiding van prof. Willem Weimar en Dr. Martin Hoogduijn deed hij onderzoek naar de mogelijkheden van mesenchymale stamcellen als immunosuppressieve behandeling in niertransplantatie.

Per 1 mei 2010 startte hij als arts-assistent in opleiding tot specialist (AIOS) interne geneeskunde in het Albert Schweitzer ziekenhuis te Dordrecht. Meindert is getrouwd met Eefje Huizer.