Towards **Mesenchymal Stem Cell Therapy** in **Kidney Transplant Recipients**

MARIEKE ROEMELING – VAN RHIJN

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Mesenchymale stamcellen op weg naar klinische toepassing in niertransplantatie patiënten

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The important thing is not to stop questioning. Curiosity has its own reason for existing.

Albert Einstein

Voor Stijn, Pieter en Hendrik

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Chapter 1

Introduction
Based on: Mesenchymal Stem Cells- Application for Solid Organ Transplantation

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Body homeostasis is maintained by vital organs such as the heart, lungs, kidney and liver. Organ failure due to injury or disease will ultimately result in a life threatening situation. Heart and lung function can be supported and even temporarily replaced by artificial heart and or lung machines. In case of failure of the kidneys, kidney function can partially be replaced by renal replacement therapy in the form of dialysis. In case of failure of many other organs, no replacement therapy is available. The only curative treatment of end stage organ failure is solid organ transplantation (SOT). An organ can either be transplanted from a deceased (post mortal) donor or, in case of living kidney or liver transplantation, from a living donor. If an organ is transplanted from an identical twin, the organ will not be recognized by the recipient's immune system and will thus be accepted. In any other situation, the transplanted organ (allograft) will be recognized as foreign and will induce alloreactivity resulting in rejection of the organ. Therefore, to maintain their allograft, transplant recipients will in general depend on lifelong use of immunosuppressive medication. Improvements in the immunosuppressive regimen in the last decades have resulted in significant improvements of the short term outcome after transplantation while progress in the long term outcome lagged behind. Nevertheless, immunosuppressive drugs have several unwanted side effects such as the development of diabetes, hypertension, malignancies and nephrotoxicity. In order to optimize long term transplantation outcome, there is a need for new treatment regimens in the field of SOT.

A new therapeutic approach in the field of SOT could be provided by cell therapy. Cell therapy involves administration of a distinct population of cells with a therapeutic potential. Mesenchymal Stem or Stromal Cells (MSC) are one of the cell therapeutic options in SOT that have arisen in the last decades. These multipotent adult stem cells can be isolated from various types of tissues such as bone marrow, adipose tissue and multiple others. In absence of a specific marker, MSC are defined according to the criteria set by the International Society for Cell Therapy (ISCT) [1]. These criteria describe MSC as adherent cells with a CD73+, CD90+, CD105+, HLA-I+, CD14-, CD34-, CD45- and HLA-DR- immunophenotype and the capacity to differentiate into osteoblasts, adipocytes and chondrocytes. Interestingly, MSC have immunomodulatory and regenerative capacities.

MSC are capable of inhibiting activated T cell proliferation in vitro[2, 3] and can induce regulatory T cells [4] . They decrease the T cell stimulatory capacity of dendritic cells [5] and are reported to differentiate macrophages into a more regulatory phenotype and function [6]. Although it was previously assumed that MSC act by differentiating into functional cells [7], evidence suggests that MSC are more likely to act in a paracrine manner [8] by which they target resident cells (figure 1). MSC secrete a range of anti-inflammatory factors, including IL-10, TGF-β, hepatocyte growth factor (HGF), nitric oxide (NO), HLA-G, and PGE2, that are all indicated to play some role in their immunomodulatory effect [9-11]. In particular the expres-

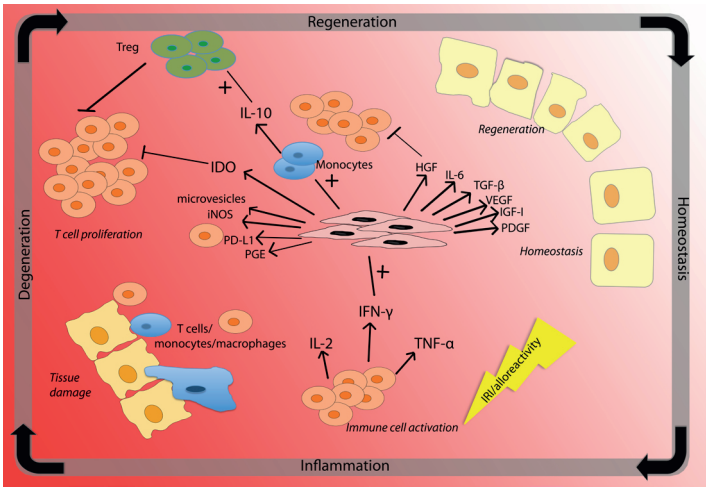

Figure 1. Proposed functionality of MSC. Immune activation as a result of ischemia-reperfusion-injury or alloreactivity results in an inflammatory environment characterized by elevated levels of the pro-inflammatory cytokines IL-2, IFNγ and TNFα produced by immune cells such as T cells and macrophages, causing tissue damage and transplant rejection. As a consequence of the inflammatory environment, MSC will be activated. Upon activation, MSC express a variety of factors such as IDO and PD-L1 which can inhibit the immune response. Finally, when the immune response is dampened, growth factors secreted by MSC such as HGF, PDGF and VEGF support regeneration, until quiescence is re-achieved.

sion of IDO by MSC is important for their inhibitory effect on T lymphocyte proliferation. In addition to their anti-inflammatory effects, paracrine mechanisms may mediate a beneficial effect of MSC on organ function in SOT by inhibition of fibrosis. HGF secreted by MSC may be one of the factors involved[11]. MSC may also exhibit a regenerative function via direct targeting of resident progenitor cells via the secretion of bFGF, BMP-7 and VEGF [11, 12].

As a result of their immunomodulatory and reparative properties, MSC have been used as an experimental cell therapeutic agent in immunological diseases such as Graft Versus Host Disease (GVHD) [13] and Crohn's disease[14]. Their properties make MSC also appealing as a cell therapeutic agent in the field of SOT (figure 2). The potential of MSC to prolong allograft survival has been investigated in a number of transplant models. These studies demonstrated that skin and heart transplant survival can be prolonged by intravenous infusion of MSC [2, 15, 16]. Administration of MSC resulted in allograft tolerance as a result of regulatory T cell generation in a mouse kidney transplant model [9]. In a rat kidney transplant model, it was shown that MSC reduce the inflammatory infiltrate, suggesting that MSC could be used to treat cellular rejection [17, 18]. Together, these data support clinical potential of MSC in SOT.

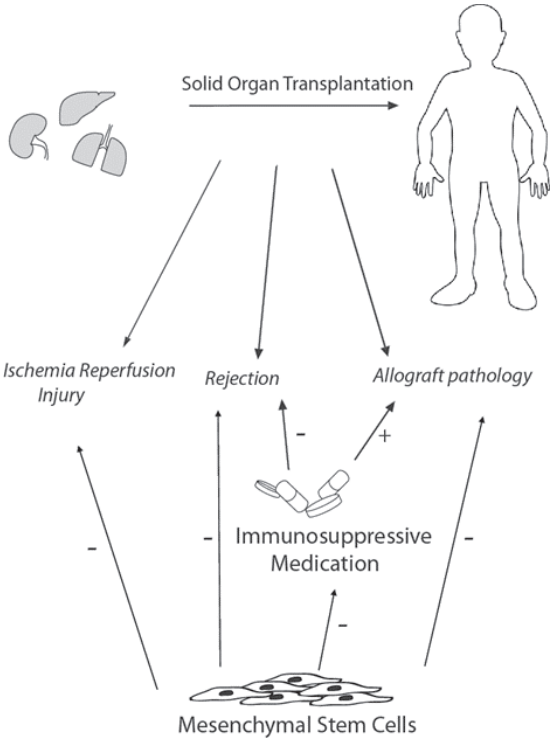

Figure 2. Challenges in SOT and possible applicability of MSC. The immunomodulatory and regenerative capacities of MSC are suggested to ameliorate transplantation related ischemia reperfusion injury, prevent or treat rejection and target allograft pathology by immunomodulation and decreasing the need for immunosuppressive medication.

In order to develop MSC into effective therapy in SOT, several issues need to be addressed involving the culture expansion process, source and origin of MSC preparation, timing, efficacy and safety of MSC therapy.

When applying MSC therapy, choices should be made considering MSC preparation. As mentioned before, MSC can be isolated from numerous, if not all, different tissues of which bone marrow is the most commonly used, especially in clinical studies. Adipose tissue is gaining interest as an alternative as it has several benefits above bone marrow. For instance, adipose tissue has a higher yield of MSC and is obtainable in a less invasive manner compared to bone marrow[19]. Yet, functional studies are required to reveal the properties of bone marrow derived MSC (BM-MSC) and adipose tissue derived MSC (ASC) in the field of transplantation.

Before clinical application, MSC are typically culture expanded. During this expansion, MSC could be at risk for genetic alterations. The fear for such alterations was fed by papers report-

ing spontaneous malignant transformation of human MSC in culture [20, 21]. However, these papers were retracted because of evidence of contamination of the MSC culture by tumor cell lines [22-24]. Nevertheless, it is evident that genetic stability deserves proper attention, that senescence of MSC cultures should be avoided and that more comprehensive stability testing than karyotyping might be necessary [25]. It has been reported that aneuploidy can occur in culture expanded MSC[26]. The implication of aneuploidy in MSC is currently not clear.

Besides a potential risk of culture expansion, the culturing period might also provide a window of opportunity to optimize the MSC product. Patient derived MSC might recover during this period before they are administered back into the patients and pre-treatment of MSC in culture with inflammatory cytokines such as IFNg might increase their immunosuppressive potential[27]. Hypoxic culture conditions are suggested to increase MSC proliferation rate which might result in a shorter laboratory-to-bedside period. However, although low-oxygen conditions are probably more physiological for MSC, whether hypoxia maintain the immunosuppressive property of MSC remains to be determined.

Next, for clinical application, MSC can of autologous or allogeneic origin. Allogeneic MSC might be most convenient as they can be isolated from healthy individuals, culture expanded, tested and cryopreserved prior to using them as a readily available cell product. The choice for allogeneic MSC therapy is supported by the paradigm that states that MSC are low immunogenic as they do not evoke immune cell responses [2, 28]. However, there is now accumulating evidence that MSC are recognized by the adaptive immune system [29-31]. Therefore, repeated allogeneic MSC therapy could potentially lead to reduced efficacy due to rapid clearance of MSC [32], or potentially, anti-HLA sensitization. Autologous MSC therapy on the other hand does not harbour any risk for sensitization but does require time to culture-expand the cells prior to usage, which makes autologous MSC therapy unsuitable for acute indications. In order to choose between the more convenient and potentially commercially interesting allogeneic MSC and non-immunogenic autologous MSC, more knowledge is desired on the potential of MSC to induce an anti-HLA response.

While autologous MSC therapy is possible desirable in order to prevent immunization, it might not be feasible as it implies using MSC derived from patients. In kidney transplantation, patients have renal or even end stage renal disease. End stage renal disease is characterized by retention of uremic toxins, an increase in oxidative stress and an inflammatory environment which affects the function of immune cells[33]. MSC might as well be affected by uremic conditions. Patient derived MSC have shown to be unaffected by Crohn's disease and multiple sclerosis [14, 34] but were functionally affected by systemic lupus erythematosus and immune thrombocytopenic purpura [35, 36]. To assess feasibility of autologous MSC

treatment in kidney transplant recipients, the effects of renal disease on the proliferation capacity and immunosuppressive function of MSC needs to be evaluated.

Taken together, the properties of MSC and the safety and preliminary efficacy data from clinical trials have initiated interest in MSC therapy in the field of SOT. In SOT, and more particularly in kidney transplantation, MSC could potentially be used to treat transplantation related ischemia reperfusion injury, acute rejection and perhaps most of all, long term allograft pathology (figure 2). Nevertheless, while current data underline the potential of MSC therapy in SOT, at the same time, it stresses the lack of understanding of the biological mechanisms that are initiated after administration of MSC. Therefore, aspects such as MSC efficacy, culture expansion, immunogenicity and source and origin of MSC need to be elucidated.

OUTLINE OF THE THESIS

MSC have immunomodulatory and regenerative properties which make them attractive as a cell therapeutic agent in the field of solid organ transplantation (**chapter 1**). To study the potential of bone marrow (BM-MSC) and adipose tissue derived MSC (ASC) in the field of transplantation, the inhibitory effect of BM-MSC and ASC in vitro was evaluated and their potential to inhibit alloreactivity in a humanized skin transplant model was studied (**chapter 2**). Before applying MSC in a clinical setting, MSC are typically culture expanded. During culture expansion, MSC may acquire genetic alterations. The occurrence and relevance of aneuploidy in culture expanded ASC was investigated (**chapter 3**). Next, as hypoxic conditions might be more physiological and potentially result in a higher yield of MSC, the effect of hypoxia on the immune modulatory capacities of ASC was evaluated (**chapter 4**). For clinical purposes, the use of allogeneic MSC is the most convenient. However, allogeneic MSC may induce alloreactivity. As this is unwanted in the field of transplantation, the current thesis describes the potential of allogeneic BM-MSC and ASC to induce anti-HLA immune responses (**chapter 5**). While allogeneic MSC therapy might provide an immunological risk, it is questionable whether autologous MSC therapy in kidney transplantation is feasible as it involves the use of cells from patients with (end stage) renal disease that may not show the same functionality as cells from healthy individuals. As renal disease can affect several other cells, the current thesis describes the effect of renal disease on ASC (**chapter 6**) and BM-MSC (**chapter 7**) and thus whether autologous MSC therapy is feasible in kidney transplant recipients. Then, after studying the efficacy, source, origin, culture expansion and immunogenicity of MSC, the mechanism of action of MSC in vivo was investigated. The immunological effect of MSC infusion in mice was assessed in search for clues on the optimal timing and mechanism of MSC therapy (**chapter 8**). Finally, we summarized and discussed our results in **chapter 9** and described future perspectives.

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Chapter 2

Human bone marrow- and adipose tissue-derived mesenchymal stromal cells are immunosuppressive in vitro and in a humanized allograft rejection model

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ABSTRACT

Background

Recent studies with bone marrow (BM)-derived Mesenchymal Stromal Cells (MSC) in transplant recipients demonstrate that treatment with MSC is safe and clinically feasible. While BM is currently the preferred source of MSC, adipose tissue is emerging as an alternative. To develop efficient therapies, there is a need for preclinical efficacy studies in transplantation. We used a unique humanized transplantation model to study the *in vivo* immunosuppressive effect of human BM-MSC and adipose tissue-derived MSC (ASC).

Methods

Gene expression of BM-MSC and ASC and their capacity to inhibit activated PBMC proliferation was evaluated. The in vivo immunosuppressive effect of BM-MSC and ASC was studied in a humanized mouse model. SCID mice were transplanted with human skin grafts and injected with human allogeneic PBMC with or without administration of BM-MSC or ASC. The effect of MSC on skin graft rejection was studied by immunohistochemistry and PCR.

Results

BM-MSC and ASC expressed TGFβ, CXCL-10 and IDO. IDO expression and acitivity increased significantly in BM-MSC and ASC upon IFN-γ stimulation. IFN-γ stimulated BM-MSC and ASC inhibited the proliferation of activated PBMC in a significant and dose dependent manner. In our humanized mouse model, alloreactivity was marked by pronounced CD45+ T-cell infiltrates consisting of CD4+ and CD8+ T cells and increased IFN-γ expression in the skin grafts which were all significantly inhibited by both BM-MSC and ASC.

Conclusion

BM-MSC and ASC are immunosuppressive in vitro and suppress alloreactivity in a preclinical humanized transplantation model.

INTRODUCTION

In the last decades, the interest in Mesenchymal Stromal Cells (MSC) as a cell therapeutic agent has grown substantially[1-5]. These fibroblast-like, plastic adherent cells have multipotent differentiation capacity and while they were originally isolated from bone marrow (BM), they can be isolated from virtually all tissues, including adipose tissue[6-10]. In absence of a specific marker, MSC are commonly defined by a panel of cell surface markers; including CD73, CD90, CD105 and CD166; and their multilineage differentiation capacity[11].

MSC are immunosuppressive cells as indicated by their capacity to inhibit activated T cell proliferation in vitro[12, 13]. This immunosuppressive potential is enhanced under inflammatory conditions in which IFNγ plays an important role[14, 15]. In animal studies, the potential of MSC has been confirmed in multiple disease models for conditions including Graft versus Host Disease (GvHD)[16, 17]; ischemia-reperfusion-induced renal injury[18, 19]; and liver failure[20, 21]. Subsequently, several clinical studies have been conducted evaluating MSC therapy in amongst others GvHD[22]; end stage liver disease[23] and Crohn's disease[24].

In the field of transplantation, MSC are of great interest due to their potential to inhibit the alloimmune response as well as to contribute to tissue repair. Animal transplant studies showed that MSC are capable to inhibit acute rejection and or to prolong allograft survival[25-28]. As a result, MSC have been applied in kidney transplant recipients[29-31] and a study with MSC in liver transplantation has been initiated[32]. Recent studies, including ours, showed the safety and feasibility of autologous MSC treatment in kidney transplant recipients when using bone marrow derived MSC (BM-MSC) in renal transplant recipients with subclinical rejection or as induction therapy [29-31, 33]. While these studies are indicative of an immunomodulatory effect of MSC in human, efficacy of MSC therapy in clinical transplantation remains to be determined. Currently, BM is the most commonly used source of MSC. However, BM aspiration is an invasive procedure accompanied with donor morbidity. In contrast, adipose tissue can be obtained in a minimal invasive way via lipectomy or liposuction and is therefore emerging as an alternative[34]. Bone marrow has a lower yield of MSC compared to adipose tissue, and adipose tissue derived MSC (ASC) have a higher proliferation capacity than BM-MSC[35-37]. Considering their basic features, BM-MSC and ASC show many resemblances. Both BM-MSC and ASC express the classical MSC markers CD73, CD90, CD105 and CD166[36, 37] although some differences in immunophenotype between BM-MSC and ASC have been reported[34, 38, 39]. BM-MSC and ASC both display the spindle shape MSC morphology and they are both capable of multilineage differentiation. Immunosuppressive capacities in vitro have been acknowledged for BM-MSC[12, 40, 41] and ASC[6, 42-44]. In animals transplant models, both BM-MSC[12, 25-27, 45, 46] and ASC[28] have shown to be beneficial in inhibiting acute rejection and or increasing allograft survival. The efficacy of BM-MSC and ASC in humanized transplant models has however never been investigated. In

the present study we focus on the immunomodulatory effects of BM-MSC and ASC in vitro and in a humanized skin allograft rejection model.

MATERIALS AND METHODS

Bone marrow and adipose tissue donors

MSC were isolated from either BM or adipose tissue from healthy controls. BM was obtained from 5 hematopoietic stem cell donors after written informed consent as approved by the Medical Ethical Committee of Leiden University Medical Centre as described before[47]. Adipose tissue was obtained from 9 live kidney donors after written informed consent as approved by the Medical Ethical Committee of the Erasmus MC (protocol no. MEC-2006- 190).

BM-MSC isolation and culture

BM was aspirated under general anesthesia. The mononucleated cell (MNC) fraction was isolated by Ficoll density gradient separation and plated in tissue culture flasks at a density of 160 x 10^3 MNC/cm^2 in low-glucose Dulbecco's modified Eagle medium DMEM (Invitrogen, Paisley, UK) supplemented with penicillin/streptomycin (Lonza, Verviers, Belgium) and 10% fetal calf serum (FCS, Thermo Scientific HyClone)). The cultures were maintained at 37^{0}C, 5% CO_2. The medium was refreshed twice a week. When the MSC cultures became confluent, cells were collected using trypsin (Lonza) and re-plated at a density of 4 x 10^3 cells/ cm^2 or frozen until further usage.

ASC isolation and culture

Abdominal subcutaneous adipose tissue was surgically removed under general anaesthesia during donor nephrectomy. Adipose tissue was mechanically disrupted and enzymatically digested with sterile 0.5mg/ml collagenase type IV (Sigma-Aldrich, St Louis, MO) in RPMI-1640 þ glutaMAX (Invitrogen) and p/s for 30min at 37 1C. Cell pellets were resuspended in MSC culture medium, consisting of MEM-α (Sigma-Aldrich(St. Louis, MO, USA) with 1% p/s and 15% fetal bovine serum (BioWhittaker, Verviers, Belgium), transferred to a 175- cm^2 culture flask (Greiner Bio-one, Essen, Germany) and kept at 37^{0}C and 5% CO_2. Medium was refreshed twice a week. When ASC cultures became 90% confluent, cells were detached using 0.05% trypsin–EDTA and frozen until further usage.

For experiments, only passage 1-5 BM-MSC and ASC were used. Prior to usage for experiments BM-MSC and ASC were cultured in parallel under the same culture conditions.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were collected from buffy coats of healthy blood bank donors (different than the MSC donors). PBMCs were isolated by density gradient centrifugation using Ficoll Isopaque (Pharmacia Amersham, Uppsala, Sweden) and used directly or frozen at -135˚C until use.

Real-time RT-PCR

For evaluation of mRNA gene-expression by BM-MSC and ASC, 100,000 MSC were seeded per well in a 6 well plate in either normal MSC culture medium or MSC culture medium supplemented with 100ng/ml IFNγ. After 24 hours, MSC cell pellets were harvested.

RNA was isolated and cDNA synthesized as described previously[48]. Quantitative expression was determined by real-time RT-PCR using universal PCR mix (Invitrogen) and Assays-on-demand for IDO (Hs00158627.m1), TGF-β (Hs00171257.m1) and CXCL-10 (Hs 00171042. m1) on an StepOnePlus real time PCR system (Applied Biosystems). Gene-expression was depicted as ratio with GAPDH.

IDO activity measurements

BM-MSC and ASC were cultured in standard MSC culture medium or culture medium supplemented with 100ng/ml IFNγ. After 1 week, medium was removed and BM-MSC and ASC were washed twice with 1X PBS. Next, BM-MSC and ASC were cultured for 24 hours in serum free MEM-α (+ 1% p/s) before medium was harvested. The tryptophan metabolic activity of IDO was determined by measurement of L-kynurenine in the conditioned medium of four unstimulated and four stimulated BM-MSC and ASC cultures. 30% trichloroacetic acid was added to the samples at a 1:3 ratio and after 30min incubation at 50°C the samples were centrifuged at 12,000 rpm for 5min. Supernatants were then diluted 1:1 in Ehrlich reagent (200mg 4-dimethylaminobenzaldehyde (Sigma, St. Louis, MO, USA) in 10ml of glacial acetic acid) in duplicate in a 96-wells flat bottom plate and absorbance was determined at 490nm using a multilabel plate reader (VersaMaxTM, Molecular Devices, Sunnyvale, CA, USA). L-kynurenine (Sigma, St. Louis, MO, USA) diluted in unconditioned medium was used as standard.

Anti-CD3/CD28 lymphocyte stimulation assay

MSC were seeded in round bottom 96-wells plates in MEMα with 10% heat inactivated human serum at 0.125×10^4; 0.25×10^4; 0.5×10^4; 1×10^4 or 2×10^4 cells per well. PBMCs were stimulated with anti-human-CD3 (0.5ug/5 x 10^5 cells), anti-human-CD28 (0.5ug/5 x 10^5 cells), and goat-anti-mouse antibody (0.5ug/5 x 10^5 cells) for cross-linking (all BD Pharmingen).

PBMCs were added to the round-bottom 96-well plates at 5 x 10^4 cells per well. Proliferation was measured by incorporation of ^{3}H- thymidine (0.5µCi) during 8h using a beta-plate reader (LKB) on day 3. All experiments were performed in triplicate and medians were used for further analysis.

Humanized SCID mouse allograft model

Animals

CB-17 SCID/ mice were obtained from Charles River Laboratories (Wilmington, Massachusetts, USA) and were used at age 8-10 weeks. The Animal Care and Use Committee of the Leiden University Medical Center approved all experiments. Animals were housed under specific pathogen free conditions in individually ventilated cages and allowed free access to food and water throughout the experiments.

Humanized SCID model

The humanized skin allograft model was approved by local medical ethical committee and local animal ethical committee. The study is based on the model described by Moulton et al. with slight adaptations[49]. In summary, human abdominal skin was obtained from healthy individuals who underwent elective plastic surgery procedures. Eight-mm punch biopsies were harvested and kept in culture medium and transplanted onto the back of CB-17 SCID mice within 12 hours. The mouse skin was shaved and skin grafts were fixed with a transparent adhesive film (Smith and Nephew, Hoofddorp, Netherlands) and allowed to heal for 4-6 weeks. For humanization, third party peripheral blood mononuclear cells (PBMCs) were isolated from fresh buffy coats, obtained from healthy controls, as described before. All animals received 100 µl of anti–asialo GM1 by intraperitoneal injection to deplete host NK cells at day-1 (Wako Chemicals USA, Richmond, VA, USA). At day 0, 24 hours after administration of anti-asialo, 3×10^8 PBMCs were transferred by intraperitoneal injection.

MSC were cultured for 1 week in the presence of 100ng/ml IFNγ (PeproTech EC Ltd, London, UK) before injection into SCID mice. BM-MSC ($0.5 * 10^6$ per infusion, n=8 mice) or ASC ($0.5 * 10^6$ per infusion, n=7 mice) were injected under the skin graft at day 0 and 4. Control mice (n=9) received 100 µl saline at the same time points. Animals were sacrificed at day 14 days and skin grafts were harvested. The humanized SCID model is depicted in figure 3a.

Immunohistochemistry

Immunohistochemical staining was performed as described previously [50]. Skin sections (4 µm) of snap-frozen skins grafts were air dried and acetone fixed. Slides were incubated overnight with mouse monoclonal IgG against human leukocytes (CD45; BD Pharminghen, Breda Netherlands) or cytotoxic T-lymphocytes (CD8; Millipore, Amsterdam Netherlands) and rabbit polyclonal anti-CD3 (NeoMarkers, Fremont, California, USA). Antibody binding was detected with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG and goat anti-rabbit IgG (both DAKO, Glostrum, Germany). After washing, sections were incubated with tyramide-fluorescein itransplantationhiocyanate in tyramide buffer (NENTM Life Science Products, Boston, MA, USA), washed and incubated with HRP-labeled rabbit anti-fluorescein itransplantationhiocyanate (DAKO, Glostrum, Germany) and developed with 3,3'-Diamino-

benzidine (DAB) (Sigma, St Louis, MO, USA). Sections were counterstained with haematoxylin (Merck, Darmstadt, Germany) and mounted with imsol (Klinipath, Duiven, the Netherlands). Quantification of immunohistochemistry was performed in a blinded manner by assessing 10 consecutive high power fields (magnification, ×200) on each section. Using Image J software, the percentage positive area in each image was determined.

Real-time RT-PCR

RNA was isolated from explanted skingrafts using TRIzol Reagent (Life technologies) and RNeasy minikit (Qiagen, Venlo, The Netherlands) according to manufacturer's recommendations. Quantitative expression was determined by real-time RT-PCR as described above using Assays- on-demand for IFNγ (Hs 00174086.m1), TNF-α (Hs99999043.m1), IL1-β (Hs00174097.m1), IL-6 (Hs 00174131.m1) and GAPDH (Hs 99999905.m1) (all Applied Biosystems, Foster City, CA). Gene-expression was depicted as ratio with GAPDH.

STATISTICAL ANALYSES

Mann Whitney statistical test was used to test for statistical significance, p value < 0.05 was considered statistical significant.

RESULTS

Bone marrow and adipose tissue donors

Study tissues were collected from healthy BM donors (n=5, mean age 15 years, range 7-31 years) and healthy adipose tissue donors (n=5, mean age 54 years, range 32-67 years). The expanded MSC from healthy BM and adipose tissue donors all expressed the common MSC markers CD73, CD90, CD105 and CD166 (data not shown).

BM-MSC and ASC express immunomodulatory genes

Both BM-MSC and ASC expressed TGF-β, CXCL-10, and IDO mRNA (figure 1A). Since MSC are activated under inflammatory conditions, we investigated the effect of the pro-inflammatory cytokine IFNγ on BM-MSC and ASC gene expression. Expression of TGFβ and CXCL-10 mRNA was not affected by 1 week of IFNγ stimulation. However, IDO mRNA expression was increased both in BM-MSC and ASC after IFNγ stimulation.

IDO activity increases after IFNγ stimulation in BM-MSC and ASC

To evaluate whether increased IDO mRNA expression was translated in enhanced IDO activity upon IFNγ stimulation of BM-MSC and ASC, we studied the concentrations of L-kynurenine

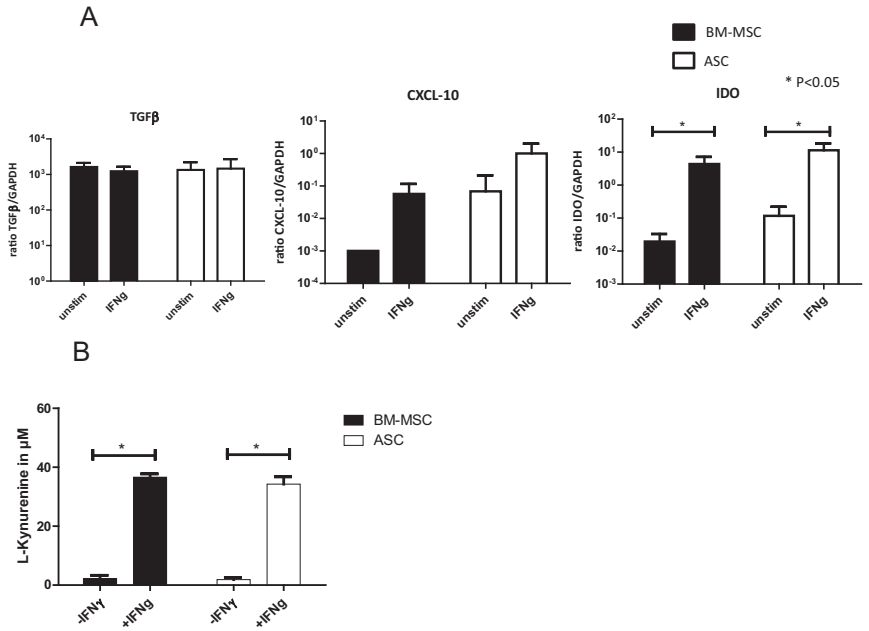

Figure 1: (A) mRNA gene expression of immunomodulatory genes of BM-MSC and ASC with or without IFNγ stimulation, n=5 for BM-MSC and n=5 for ASC. Ratio gene/GAPDH x1000 is shown. Means (SD) are depicted, *represents P<0.05. (B) Effect of IFNγ stimulation on IDO activity in BM-MSC and ASC determined by measurement of L-kynurenine in medium conditioned with unstimulated or IFNγ stimulated BM-MSC and ASC, n=4 for BM-MSC and n=4 for ASC, means (SD) are depicted, *represents P<0.05.

in BM-MSC and ASC conditioned medium. In medium conditioned with IFNγ stimulated BM-MSC and ASC, levels of L-kynurenine were significantly higher compared to medium conditioned with unstimulated BM-MSC and ASC (figure 1B). As L-kynurine is the breakdown product of tryptophan, this indicates an increase of the tryptophan depleting activity of IDO in BM-MSC and ASC after IFNγ stimulation, which is suggestive for immunosuppressive activity of BM-MSC and ASC.

BM-MSC and ASC have immunosuppressive properties in vitro

We then compared the immunosuppressive capacity of IFNγ stimulated BM-MSC and ASC. The capacity of these stimulated MSC to inhibit the proliferation of αCD3ααCD28 activated PBMCs was evaluated. Both IFNγ stimulated BM-MSC and ASC were capable of significant and dose dependent inhibition of αCD3ααCD28 activated PBMC proliferation. The BM-MSC group showed significant inhibition until a MSC:PBMC ratio of 1:10, ASC until a MSC:PBMC ratio of 1:20. No significant difference was detected between BM-MSC and ASC in the percentage inhibition (figure 2).

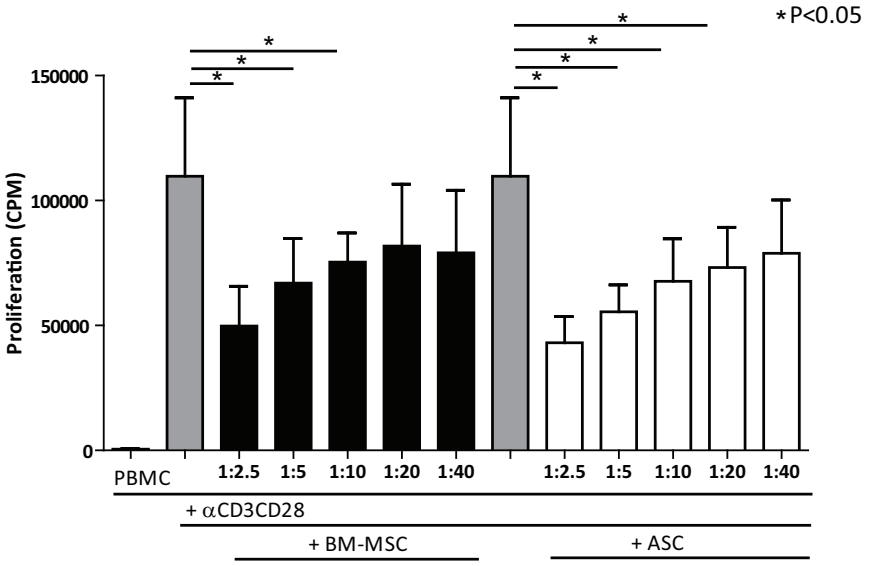

Figure 2: Inhibition of aCD3CD28 stimulated PBMC by INFγ stimulated BM-MSC (n=4) and ASC (n=4). Means (SD) are depicted, *represents P<0.05.

BM-MSC and ASC downregulate alloreactivity in vivo

After confirmation of immunosuppressive capability in vitro, we investigated the effect of IFN-γ-stimulated BM-MSC and ASC on leukocyte recruitment into human skin in a huSCID mouse model (figure 3A). Human skin was transplanted and engrafted well within 6 weeks (figure 3B). After adoptive transfer of human PBMCs into the mouse by intraperitoneal injection, there was a marked infiltration of CD45+ leukocytes in the skin graft at day 14 (figure 3C, figure 3D). Despite this marked leukocyte infiltration in the graft, skin integrity of the

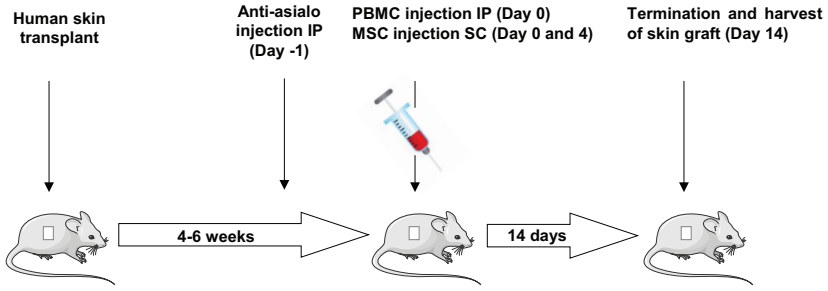

Figure 3A HuSCID mouse model: human skin is transplanted; mouse NK cells are depleted using anti-asialo GM1 injection at day-1; allogeneic PBMC are injected intraperitoneal at day 0; MSC are injected on day 0 and day 4; and after 14 days, mice are sacrificed and skin grafts are harvested.

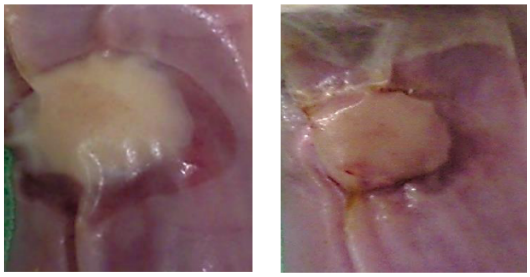

Figure 3B Representative example of engraftment process of skin graft, pictures taken at 12 and 16 days after skin graft transplantation.

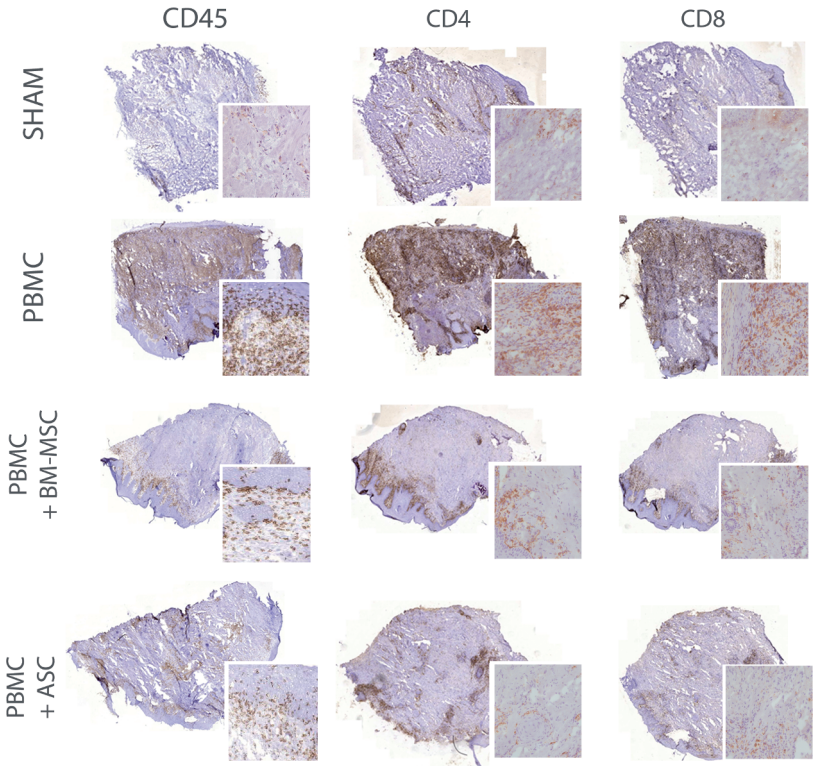

Figure 3C Immunohistological evaluation of explanted skin grafts. Staining for CD45+, CD4+ and CD8+ T cells of skin grafts explanted from mice receiving only a skin transplant (SHAM group); mice receiving skin graft and PBMC; mice receiving skin graft, PBMC and BM-MSC; and mice receiving skin graft, PBMC and ASC. Representative biopsies are shown.

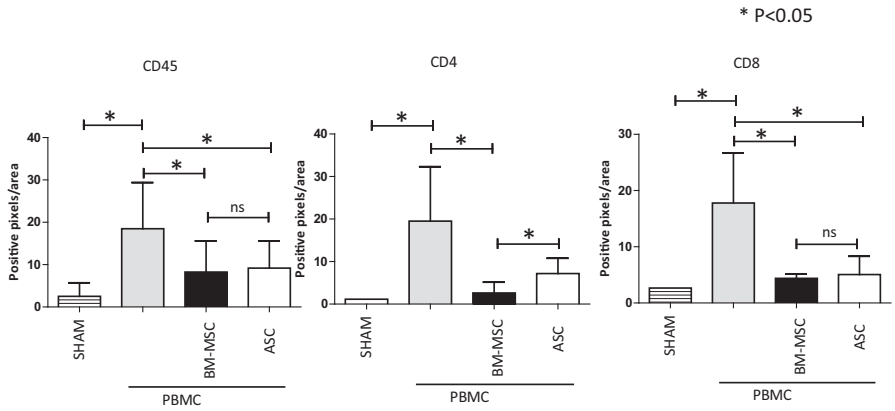

Figure 3D Quantitative evaluation of CD45+, CD4+ and CD8+ cells in biopsies of study groups. Data represent mean (SD), *indicates P<0.05. (E) mRNA gene expression in explanted skin grafts of IFNγ, TNFα, IL-6 and IL-1β of study groups. Ratio gene/GAPDH x1000 is shown. Data represent mean (SD), *indicates P<0.05.

huSCID mice remained intact and did not slough. This is probably due to the maintenance of blood flow by the murine vasculature as also described previously[49, 50]. The infiltrating CD45+ cells consisted of both CD4+ and CD8+ T cells (figure 3C, figure 3D). Mice not receiving an adoptive transfer of human PBMCs did not demonstrate leukocyte infiltration (SHAM group, figure 3C, figure 3D). Administration of BM-MSC or ASC at day 0 and day 4 after allogeneic PBMC injection resulted in a significant reduction of CD45+, CD4+ and CD8+ T cell infiltration (figure 3C, figure 3D). RT-PCR analysis for expression of various pro-inflammatory human cytokines was performed on the explanted skin grafts of the mice. The mice that had received PBMC revealed a significant increase in mRNA expression of IFNγ, TNFα, IL-6 and IL1-β indicating inflammation compared to mice with skin grafts only (figure 3E). When mice with engrafted skin grafts were treated with BM-MSC or ASC after PBMC injection, expression of IFNγ significantly decreased (figure 3E). Expression of TNFα, IL-1β and IL-6 was also reduced after BM-MSC or ASC treatment, although these differences did not reach statistical significance.

DISCUSSION

We found that MSC derived from bone marrow and adipose tissue were capable of immune suppression in vitro and inhibition of T cell mediated alloreactivity in vivo. These results are important for future clinical application of MSC. MSC are emerging as a cell therapeutic agent in the field of transplantation with now four completed trails in kidney transplant recipients

and multiple trails in preparation[29-31, 33, 51]. However, currently, there is no consensus considering the optimal source for the isolation of MSC for clinical application.

Our finding that BM-MSC and ASC are both effective indicates that the choice between BM-MSC and ASC for clinical application can be made upon practical considerations. This is important as, despite the numerous studies published on MSC and immune modulation, only a few compared their immunosuppressive capacity. Moreover, in the field of transplantation, no humanized mouse models have been used to evaluate the effect of MSC. In the present study, we first evaluated the in vitro immunosuppressive capacities of the MSC. We found that BM-MSC and ASC both express CXCL-10, TGFβ and IDO. The chemoattractive CXCL-10, the immune regulatory TGFβ and antiproliferative IDO are known factors via which MSC excert their immune modulating properties [13, 52, 53]. Thus, our results confirm the immunosuppressive potential of both types of MSC. Further, as it is known that an inflammatory environment can stimulate the immunosuppressive MSC function [54], we evaluated their response to IFNγ stimulation. Upon IFNγ stimulation, we found that IDO expression and activity was increased in both BM-MSC and ASC. This is in line with the notion that an inflammatory environment, in this study simulated with IFNγ, can potentiate MSC efficacy in both BM-MSC and ASC.

In αCD3αCD28-induced PBMC proliferation assays we found BM-MSC and ASC both capable of dose dependent inhibition. These results confirm previous data showing that BM-MSC and ASC have the capacity to inhibit proliferation of alloantigen and mitogen activated PBMC [17, 39, 55] and justifies examination of these cell populations in a preclinical transplant model.

Multiple animal studies have shown the potential of BM-MSC [12, 25-27, 45, 46] and ASC [28] to ameliorate ischemia reperfusion injury or alloreactivity. Yet, as mentioned before, human BM-MSC and human ASC have not been evaluated in a humanized transplant model. Issa et al. used such a humanized model for evaluation of regulatory T cell (Tregs) therapy. This model enabled the authors to study the effect of ex vivo-expanded Tregs and found them capable of inhibition of skin graft rejection[56]. Here, for the first time, we used such a unique humanized mouse model for evaluation of MSC treatment in allograft rejection. We found that BM-MSC and ASC were effective in inhibiting alloreactivity. Alloreactivity was marked by recruitment of CD45+ cells which consisted of CD4+ and CD8+ T cells and IFNγ, TNFα, IL-1β and Il-6 mRNA expression was increased in the explanted skin grafts. Local administration of BM-MSC as well as ASC reduced inflammation by inhibiting the recruitment of T cells and decreasing IFNγ, TNFα, IL-6 and IL-1β expression in the skin grafts. These results support the use of BM-MSC and ASC to treat transplant rejection.

In conclusion, the immunosuppressive potential of BM-MSC and ASC has been highlighted in the present study. Our experiments confirm the immunosuppressive capacities of BM-MSC and ASC in vitro. By extending our investigations to a humanised transplant model our data underscore the potential of BM-MSC and ASC therapy in clinical transplantation.

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Chapter 3

Culture expansion induces non-tumorigenic aneuploidy in adipose tissue-derived mesenchymal stromal cells

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ABSTRACT

Background aims

Adipose tissue-derived mesenchymal stromal cells (ASCs) are of interest as a cell therapeutic agent for immunologic and degenerative diseases. During in vitro expansion, ASCs may be at risk for genetic alterations, and genetic screening is a prerequisite. We examined the presence of aneuploidy in ASCs and its origin and development during culture and evaluated the implications of aneuploidy for therapeutic use of ASCs.

Methods

Adipose tissue of healthy individuals was used for isolation and expansion of ASCs. Chromosome copy numbers were studied using fluorescence in situ hybridization analysis. Aneuploidy was studied in freshly isolated ASCs, in ASCs cultured for 0–16 passages and in senescent cultures. To evaluate the plasticity of ploidy, ASCs were cloned, and the variation of ploidy in the clones was examined. Tumorigenicity was studied by subcutaneous injection of aneuploid ASCs in immunodeficient NOD/SCID mice.

Results

No aneuploidy was detected in freshly isolated ASCs. In low passages (passages 0–4), aneuploidy was detected in 3.4% of ASCs. Prolonged culture expansion of ASCs (passages 5–16) resulted in a significant increase of aneuploidy to 7.1%. With senescence, aneuploidy increased further to 19.8%. Aneuploidy was observed in clones of diploid ASCs, demonstrating the de novo development of aneuploidy. No transformation of ASCs was observed, and in contrast to cancer cell lines, aneuploid ASCs were incapable of tumor formation in immunodeficient mice.

Conclusions

ASC cultures contain a stable percentage of aneuploid cells. Aneuploidy was not a predecessor of transformation or tumor formation. This finding indicates that aneuploidy is culture-induced but unlikely to compromise clinical application of ASCs.

INTRODUCTION

Mesenchymal stromal cells (MSCs) have immunomodulatory and regenerative capacities [1-4]. They are of interest as a cell therapeutic agent for various medical conditions, including Crohn disease, graft-versus-host disease, and solid-organ transplantation [1, 5, 6]. At the present time, >300 clinical trials are registered to study the applicability and effects of MSC administration (April 2013, http://clinicaltrials.gov). In the absence of a specific marker,

MSCs are defined by the criteria set by the International Society for Cellular Therapy as plastic adherent cells with a $CD73^+$, $CD90^+$, $CD105^+$, CD14–, CD34–, CD45– and human leukocyte antigen (HLA)-DR– immunophenotype and the capacity to differentiate into osteoblasts, adipocytes and chondrocytes [7]. The primary source of MSCs is bone marrow; however, MSCs reside in virtually all tissues [8], of which adipose tissue is the most accessible and most suitable for the isolation of cells for research and therapeutic purposes. Adipose tissue-derived mesenchymal stromal cells (ASCs) are very similar to bone marrow-derived MSCs [9, 10], although un-cultured ASCs express CD34 [11, 12].

For most clinical applications, ASC are culture-expanded to obtain sufficient numbers of cells. During expansion, ASCs may be at risk for transformation. To ensure patient safety, ASCs should be subjected to strict release criteria, similar to MSCs. [13]. However, until now, genetic screening of clinical-grade MSCs has been limited to karyotyping of a small number of cells [1, 2, 13, 14].

Preclinical research evaluating the genetic stability of MSC has resulted in contradicting outcomes. Studies reporting spontaneous transformation of MSC in culture [15, 16] were retracted when cell cultures appeared to be contaminated with tumor cell lines [17, 18]. Nevertheless, there is evidence that aneuploidy occurs in cultured MSC derived from both bone marrow and adipose tissue [19-22]. These chromosomal aberrations have also been detected in MSC used in clinical trials [23]. Because aneuploidy has been suggested to be associated with cancer [24], aneuploidy in MSC cultures may be a worrisome finding and its detection in MSC cultures warrants thorough investigation.

To date, it is unknown whether aneuploidy in ASCs is already present in vivo or whether it is induced during the culture process. How aneuploidy evolves during further extensive culture expansion is also unknown. Additionally, it is unknown whether aneuploidy is associated with transformation or tumor formation and whether it compromises the safety of clinical application of ASCs. The aim of this study is to examine the occurrence and functional implications of aneuploidy in ASCs.

METHODS

Sources of adipose tissue

Subcutaneous abdominal adipose tissue was surgically removed from healthy living kidney donors during donor nephrectomy after receiving written informed consent, as approved by the Medical Ethical Committee of Erasmus Medical Center (protocol no. MEC-2006-190). Adipose tissue was collected in minimal essential medium alpha (MEM-α) (Invitrogen, Paisley, UK) supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin (Invitrogen).

ASC isolation, culture and differentiation

ASCs were isolated from adipose tissue as described previously [9, 12, 25]. Briefly, adipose tissue was mechanically disrupted and enzymatically digested with sterile 0.5 mg/mL collagenase type IV (Sigma-Aldrich, St Louis, MO, USA) in RPMI-1640 Medium with GlutaMAX (Invitrogen) and penicillin/streptomycin for 30 min at 37°C. The cell suspension was seeded in culture flasks in ASC culture medium consisting of MEM-α with 1% penicillin/streptomycin and 15% fetal bovine serum (Lonza, Verviers, Belgium). Cultures were kept at 37°C, 5% CO_2 and 95% humidity. Medium was changed every 3–4 days. Upon 90% confluency, ASC were detached using 0.05% trypsin-EDTA at 37°C. ASC were counted in order to calculate the population doublings and reseeded at a density of 1000 cells per cm2. On average, each passage represented 3.3 population doublings. ASC immunophenotype and osteogenic and adipogenic differentiation capacity were evaluated as described previously to confirm ASC identity [12, 25]

Fluorescence-activated cell sorting of un-cultured ASCs

For fluorescence-activated cell sorting (FACS) of un-cultured ASCs from the freshly isolated cell suspension, cells were washed with FACSFlow (BD Biosciences, San Jose, CA, USA) and incubated with mouse anti-human monoclonal antibodies against HLA-ABC-PE-Cy7, CD34-APC, CD45-PERCP (all BD Biosciences), CD31-APC-Cy7 (BioLegend, San Diego, CA, USA) and CD73-PE (BD Pharma, San Jose, CA, USA). To obtain ASCs, HLA-ABC$^+$CD45−CD31−CD34$^+$CD73$^+$ cells were gated and sorted (BD FACSAria II SORP; BD Biosciences). Purified ASCs were immediately fixed in 3:1 methanol acetic acid and kept at −20°C for fluorescence in situ hybridization (FISH) analysis.

FISH analysis

Dual-color FISH analysis was performed using the bacterial artificial chromosome (BAC) clones RP11-121E15 (chromosome 15q25.2) and RP11-411B10 (chromosome 18p11.21). Initially, the centromere probes Pα3.5 (chromosome 3) and D8Z2 (centromere 8) were used, but the more confined signal of the BAC clones facilitated the counting. Mbo1-digested BAC DNA or plasmid DNA was labeled with Bio-16-dUTP or Dig-11-dUTP (Roche Applied Science, Indianapolis, IN, USA) using the random prime labeling system BioPrime (Invitrogen Corporation, Carlsbad, CA, USA). Microscope chamber slides with fixed ASCs were hybridized overnight at 37°C with 10–20 ng labeled centromere probe or 40–50 ng labeled BAC probe. The following day, the slides were washed (2× saline sodium citrate [SSC] and 0.1× SSC, 0.1% Tween) at 55°C and incubated with Alexa Fluor 594 Streptavidin (Life Technologies, Bleiswijk, the Netherlands) and anti-digoxigenin-fluorescein isothiocyanate (Roche, Almere, the Netherlands) for 2 h. After washing (2× SSC, 0.1× SSC), the slides were counter-stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Zwijndrecht, Netherlands) and mounted in anti-fade solution (DABCO-Vectashield 1:1; Vector Laboratories, Burlingame, CA,

USA). For each hybridization, a minimum of 300 nuclei were scored with an Axioplan 2 imaging microscope (Carl Zeiss; Sliedrecht, the Netherlands) using a chroma-sp-100 DAPI and a chroma-sp-103v1 red filter (Chroma Technology, Bellows Falls, VT, USA), and the images were captured with ISIS software (Metasystems, Altlussheim, Germany) [26, 27].

Cloning ASCs

Sub-confluent ASC cultures were trypsinized, and single cells were seeded in 96-well flat bottom plates using FACS (BD FACSAria II SORP). After 1 day of culture, wells containing more than one cell were discarded. Other cells were cultured until colony formation occurred. Sub-confluent clones were transferred to chamber slides for FISH analysis. FISH analysis of primary cultures and clones was performed using the BAC clones RP11-121E15 (chromosome 15q25.2) and RP11-411B10 (chromosome 18p11.21).

Senescence-associated β-galactosidase staining

Two ASC cultures that were non-proliferative for a minimal period of 4 weeks despite media refreshments and passaging were seeded in six-well flat bottom plates. Staining for β-galactosidase activity at pH 6, which is a known characteristic of senescent cells [28], was performed using a Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's protocol. Sub-confluent proliferating low-passage ASCs were used as a control. Representative examples and the percentage of cells positive for β-galactosidase activity at pH 6 were depicted.

Analysis of tumorigenicity of ASCs in immunodeficient mice

As described previously [29], immunodeficient NOD/SCID mice (Erasmus MC institutional breeding) 6–8 weeks old were subcutaneously injected with $6x10^6$ ASCs (N=4) using 29-gauge needles. Control mice received $1x10^6$ cells of the human hepatoma cell line Huh7. The mice were macroscopically screened for tumor formation for 2 months. After 2 months, mice were sacrificed and the injection area was studied histologically for abnormalities. In addition, fat tissue at the injection site was removed and ASC isolated and brought into culture. ASC were then stained with antibodies against mouse CD44 and human HLA class I and analyzed by flow cytometry to confirm their ASC phenotype and to detect whether the cultures contained human ASC.

Statistical analysis

Data were analyzed using the Student t test. Statistical significance was defined as $P < 0.05$.

RESULTS

Characterization of ASCs

ASCs were successfully isolated from adipose tissue of all donors (n = 17). After culture expansion, ASCs were characterized by flow cytometry and demonstrated a $CD73^+$, $CD90^+$, $CD105^+$, $CD166^+$, HLA-ABC^+ and CD45– immunophenotype (Figure 1). Multi-lineage differentiation capacity was confirmed by adipogenic and osteogenic differentiation visualized by oil red O and von Kossa staining, respectively (data not shown).

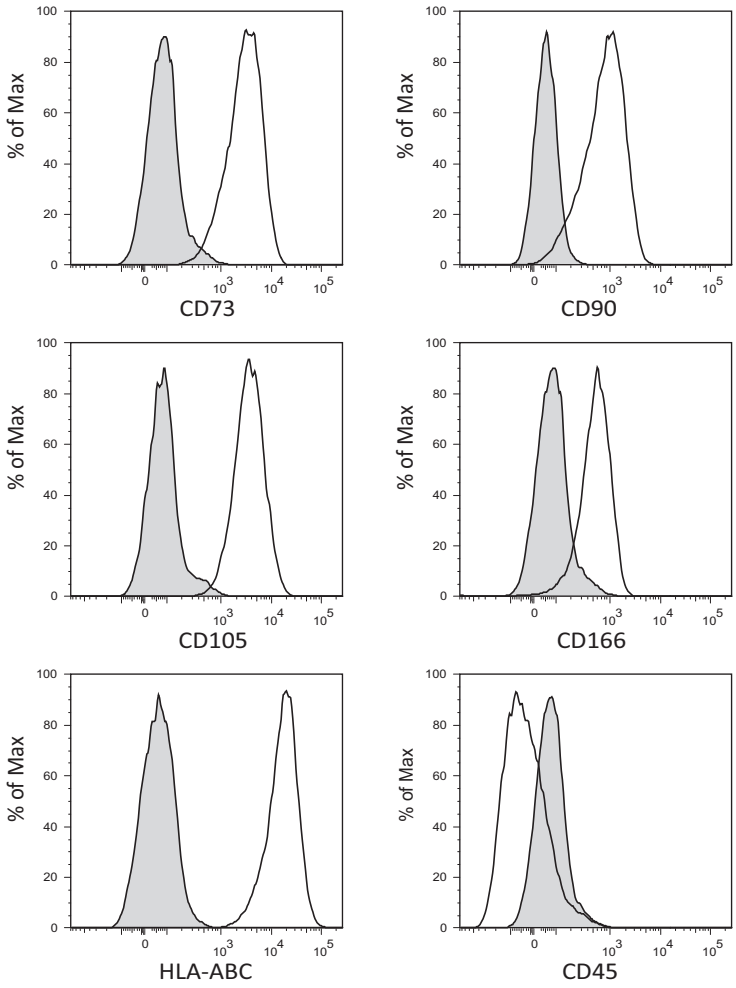

Figure 1. Immunophenotype of ASCs. Gray histograms represent unstained cells, and open histograms represent stained cells. Representative culture is shown.

Aneuploidy in ASCs

We performed FISH analysis on cultured ASCs to detect the presence of aneuploid cells (Figure 2). Because there is a general consensus to use low-passage ASCs for clinical studies, we performed FISH analysis on 12 different ASC cultures of low passage (0–4 passages, approximately 2–17 population doublings) to detect the presence of aneuploid cells in cultures of therapeutic potential. On average, 96.6% of ASCs were diploid, and 3.4% (range, 1.3–7; standard error of the mean [SEM] 0.4) were aneuploid (Table I, Figure 3). The occurrence of aneuploidy was not related to the age of the ASC donor and even occurred in ASCs that

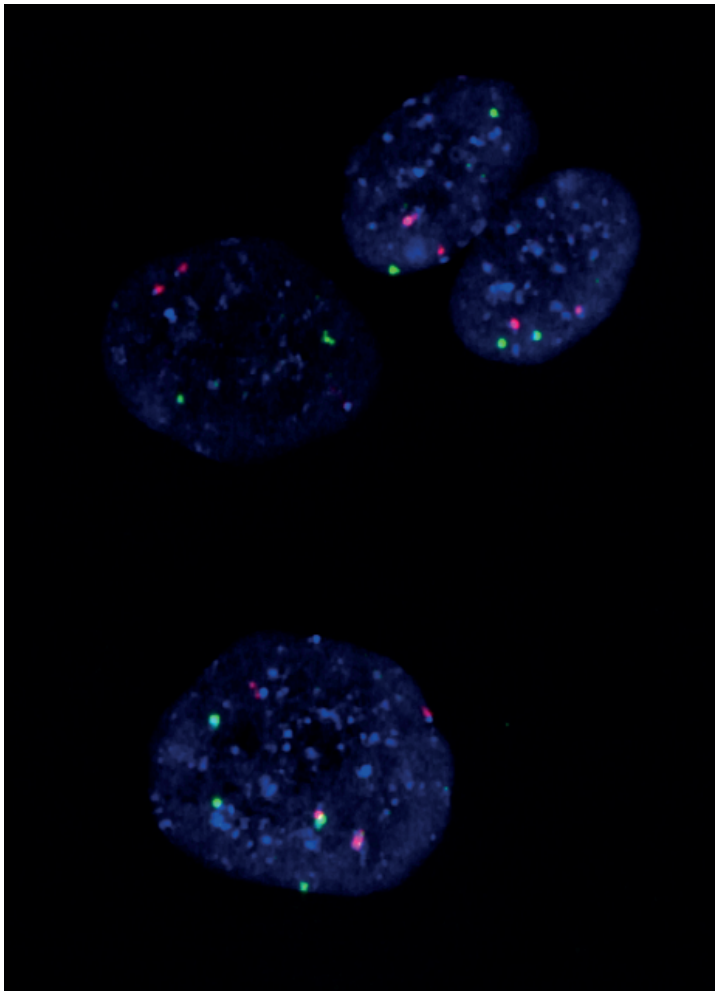

Figure 2. FISH analysis of ASCs. Representative example of FISH staining of three diploid ASCs and one tetraploid ASC (bottom cell) is shown. Colors indicate hybridized probes on chromosome 15q25.2 and chromosome 18p11.21.

Table I. Overview of 12 ASC cultures between passages 0 and 4 analyzed for aneuploidy.

Culture number	Sex	Age	Passage analysed	Approx. population doublings	Aneuploidy (%)
1	f	64	0	2-4	3.9
2	m	44	0	2-4	4.8
3	f	30	1	6-7	2.5
4	f	42	2	9-10	3.9
5	m	44	2	9-10	1.3
6	m	77	2	9-10	3.3
7	f	56	2	9-10	4.1
8	m	73	3	13-14	7.0
9	m	39	3	13-14	2.3
10	m	42	3	13-14	2.3
11	m	47	4	16-17	3.1
12	f	32	4	16-17	2.6

Approximate population doublings are indicated. F, female; M, male.

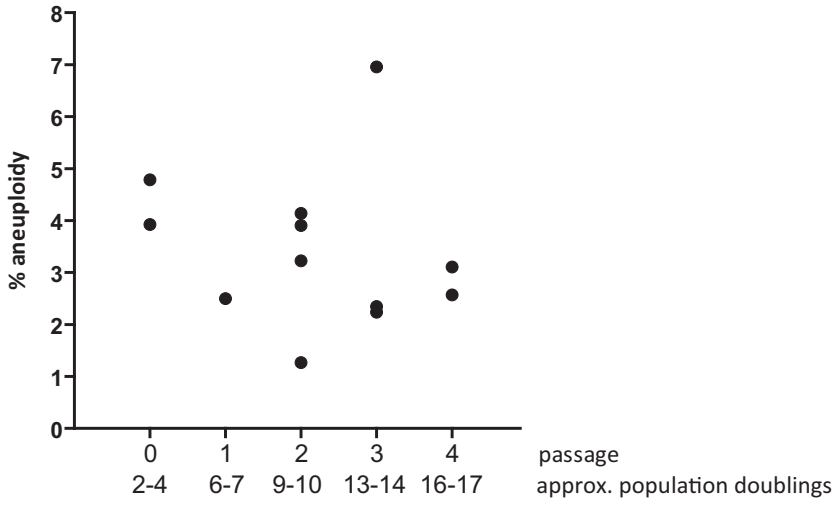

Figure 3. Aneuploidy in ASC cultures of clinically relevant passages. Percentages of aneuploid ASCs in 12 cultures of passages 0-4 (approximately 2-17 population doublings) (see Table II for details) were determined by FISH analysis.

were cultured for only a few days (passage 0, approximately 2–4 population doublings). Most aneuploid ASCs were tetraploid (86.7% of aneuploid cells). Octaploidy and other forms of poliploidy were detected at low frequencies.

Aneuploidy in ASC clones

To determine whether aneuploidy can arise from diploid ancestor cells, ASC clones were generated from three cultures. The ancestor ASC populations consisted of 2.4% (passage 3), 1.2% (passage 2) and 3.1% (passage 4) aneuploid cells; if diploid cells would give rise only to diploid cells, the large majority of clones would be expected to be fully diploid. On average, the possibility of cloning a diploid cell was 97.8%. FISH analysis on the generated clones showed that most clones contained a percentage of aneuploid cells, ranging from 0.3–6% (Figure 4). These results indicate that aneuploid ASCs can arise from diploid ancestor

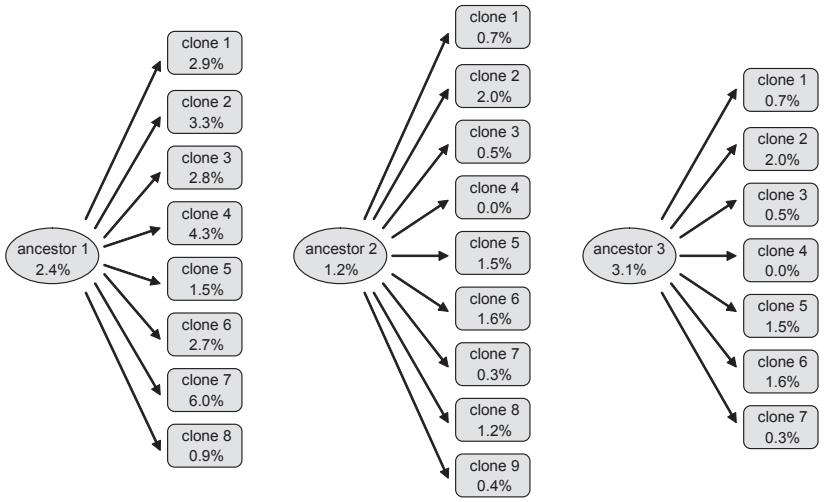

Figure 4. Schedule of percentages of aneuploidy in ASC clones. Three ancestor cultures contained 2.4%, 1.2% and 3.1% of aneuploid cells. Clones from ASCs of these cultures derive from diploid ancestor cells in 97.6%, 98.8% and 96.9% of the cases. Of 24 analyzed clones, 22 contained aneuploid cells, indicating that aneuploidy arises de novo from diploid cells.

Aneuploidy in non-cultured ASCs

To evaluate whether aneuploidy in ASCs is a culture-induced phenomenon, the occurrence of aneuploidy in non-cultured ASCs was evaluated. Using FACS, HLA-ABC$^+$CD31−CD45−CD34$^+$CD73$^+$ cells were isolated from the stromal vascular fraction. From this cell fraction, a population of cells with spindle-shaped morphology develops in culture within a few days (data not shown), demonstrating that these cells represent ASCs. The percentage of

aneuploidy in non-cultured ASCs of three donors was examined by direct fixation of the freshly isolated HLA-ABC$^+$CD31−CD45−CD34$^+$CD73$^+$ cells and subsequent FISH analysis. In contrast to cultured ASCs, freshly isolated ASCs were 100% diploid. These results indicate that aneuploidy in ASCs is culture-induced.

Aneuploidy in high-passage and senescent ASCs

To study whether aneuploidy increased after prolonged culture expansion of ASCs, we analyzed the percentage of aneuploidy in five ASC cultures of passages ranging from 5–16 (approximately 19–57 population doublings). These high-passage ASCs displayed normal ASC morphology and proliferation. The incidence of aneuploidy in these cultures was on average 7.1% (SEM 1.5), which was significantly higher than the 3.4% aneuploidy in passages 0–4 ASCs (Table II, Figure 5A).

Table II. Overview of five ASC cultures between passages 5 and 16 analyzed for aneuploidy.

Culture number	Sex	Age	Passage analysed	Approx. population doublings	Aneuploidy (%)
13	f	62	5	19-20	2.1
13	f	62	10	36-37	11.1
13	f	62	12	42-43	3.5
14	f	56	8	29-30	10.9
14	f	56	9	33-34	6.4
15	m	48	11	39-40	5
16	f	39	14	49-50	13.3
17	m	36	16	56-57	4.5
13	**f**	**62**	**15**	**49-50**	**13.7**
14	**f**	**56**	**15**	**49-50**	**25.8**

Table 2. Approximate population doublings are indicated. **Bold** items indicate senescent cultures.
F, female; M, male.

We also studied aneuploidy in two senescent passage 15 ASC cultures (approximately 49–50 population doublings). These cultures were non-proliferative for >4 weeks and displayed an altered morphology and increase in cell size (Figure 5B lower panel). Their state of senescence was confirmed by staining for β-galactosidase activity at pH 6, which is specific for senescent cells and indicated a mean percentage of senescent cells of 94% (SEM 0.6) (Figure 5B lower panel, C) compared with low-passage proliferating ASCs (Figure 5B upper panel, C). In these senescent cultures, the percentage of aneuploidy increased dramatically to 19.8% (SEM 6.1) (Table II, Figure 5D).

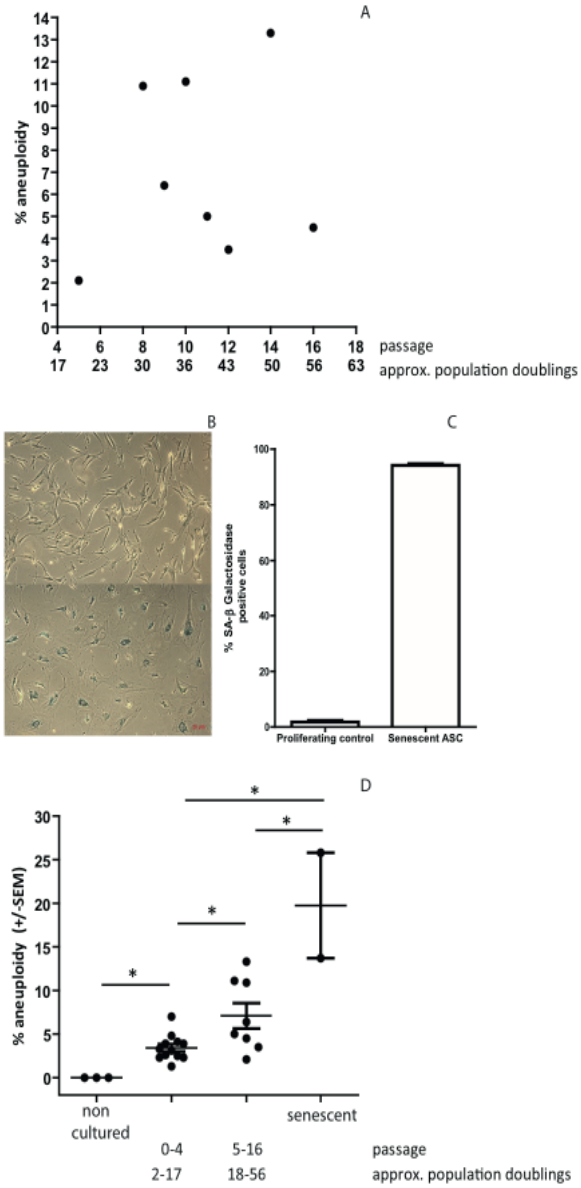

Figure 5. Aneuploidy in ASC cultures of high passages. (A) Percentages of aneuploid ASCs in eight cultures of passages 5-16 (see Table II for details) determined by FISH analsis. (B) Qualitative determination of senescence by staining for senescence-associated (SA) – β-galactosidase of low-passage proliferating ASCs (upper panel) and senescent ASCs (lower panel). Blue cells indicate cells positive for β-galactosidase activity at pH 6, a known marker for senescence. Representative examples are shown. (C) Quantitative measurement of senescence—percentage of cells positive for β -galactosidase at pH 6 in senescent cultures and controls. (D) Aneuploidy in non-cultured ASCs, ASCs of passages 0-4, ASCs of passages 5-16 and senescent ASCs determined by FISH analysis. *P < 0.05.

Tumorigenicity of aneuploid ASCs

Because aneuploidy is considered to be associated with tumor formation, we examined whether aneuploid ASCs had tumorigenic potential. In culture, no transformation of ASCs was observed in any of the cultures, including cells that were senescent and cultured for >6 months.

The tumorigenicity of aneuploid ASCs was evaluated by subcutaneous injection of $6x10^6$ ASCs containing a known proportion of aneuploid cells in immunodeficient NOD/SCID mice. Four mice were injected with ASCs in the flank just above the hind limb. The first three mice received ASCs of three different ASC cultures of passages 2–4 (approximately 9–17 population doublings), and the fourth received a mixture of the ASCs. Before injection, a sample of the ASCs was collected for FISH analysis. The ASC cultures consisted of 2.3%, 2.6% and 3.3% aneuploid cells; this implies that 140,000–200,000 aneuploid cells were injected in the mice. Mice receiving ASCs remained in good condition, and no tumor formation was macroscopically observed 2 months after injection. No cutaneous or subcutaneous tumor formation had occurred in any of the mice (Figure 6A–C). Mice injected with the hepatoma cell line Huh7 at

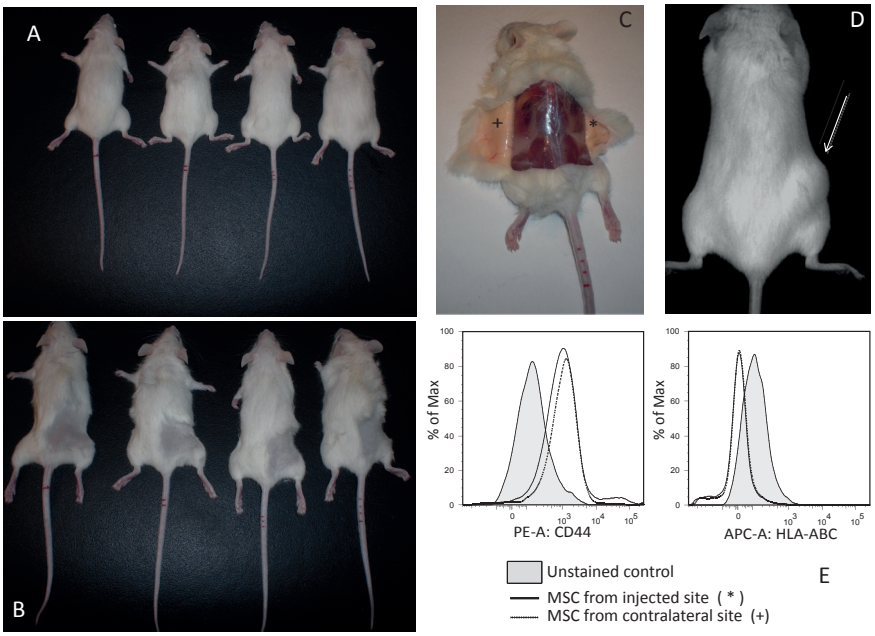

Figure 6. Tumorigenicity of ASCs. Immunodeficient NOD/SCID mice received subcutaneous injections of 6×10^6 ASCs including 2.3-3.3% aneuploid cells. After 2 months, the mice were macroscopically examined for tumor formation. No cutaneous or subcutaneous tumor formation was found (A-C). Injection of 1×10^6 Huh7 human hepatoma cells induced tumor formation (D). ASCs were isolated from the adipose tissue of the injection and contralateral site and characterized by flow cytometry (E). The cells stained positive for anti-mouse CD44 but were negative for anti-human HLA-ABC, indicating the mouse origin of the cells.

these same locations formed tumors, confirming that the subcutaneous environment supports tumorigenesis (Figure 6D). The subcutaneous adipose tissue located at the site of injection and at the contralateral side was collected for ASC isolation and culture. After 1 week, spindle-shaped ASCs appeared in the cultures. Flow cytometry analysis of the ASCs revealed their mouse origin (Figure 6E). No human cells were detected in cultures from adipose tissue of the injection side, indicating that even in immunodeficient mice ASC injection harbors no threat despite the presence of aneuploid cells.

DISCUSSION

In this study, we show that ASC cultures contain a fraction of aneuploid cells. The finding of aneuploid cells in ASC cultures is in accordance with earlier studies that reported aneuploidy in bone marrow-derived MSC [20] and ASC [19, 21] cultures. This phenomenon has also been described in clinical-grade MSCs [23].

Aneuploidy refers to a state in which cells possess an abnormal number of chromosomes and is suggested to be associated with cancer[24]. Because safety is crucial for MSC therapy, it is surprising that little is known about the clinical implications of aneuploidy in culture-expanded ASCs. In the present study, we examined the occurrence of aneuploidy during short-term and long-term culture of ASCs. The absence of aneuploidy in freshly isolated ASCs indicates that aneuploidy in ASCs is induced by culture expansion. To avoid the risk of injecting aneuploid ASCs, the possibility of using freshly isolated ASCs, as studied by others [30], could be considered. However, efficacy studies are needed to determine whether the therapeutic potential of fresh ASCs is comparable to culture-expanded ASCs. The percentage of aneuploidy remained stable in clinically relevant passage numbers (passages 0–4). In higher passage numbers, the percentage of aneuploidy significantly increased. Aneuploid ASCs did not transform in vitro and did not form tumors in vivo. Senescent ASC cultures had a high percentage of aneuploid cells, suggesting aneuploidy may occur in particular in old ASCs.

At the present time, MSC therapy is the subject of extensive basic research and is under clinical evaluation in many medical fields [31-34]. Safety is a main theme of these studies. Results from first clinical trials using human MSCs have not provided evidence for tumor formation, although longer follow-up is needed. A few articles were published in recent years reporting the alarming finding of transformation of MSCs [15, 35, 36]. Although some of these reports were retracted when cultures appeared to be contaminated with tumor cell lines 17 and 18,[17, 18], transformation has been proven to occur in murine MSCs and to cause sarcoma formation [37, 38]. Similar results were obtained with macaque MSCs [39]. These findings suggest that murine and macaque MSCs are susceptible to transformation and capable of tumor formation, whereas human MSCs are not [40, 41]. This suggestion indicates an essential difference between MSCs of different species. However, there is still debate

concerning the genetic stability of MSCs and the implication for clinical safety [42, 43]. We recently carried out a single nucleotide polymorphism array-based whole-genome analysis of expanded MSCs and found no evidence for culture-obtained genomic translocations or deletions in MSCs [12]. Our analysis suggests that the occurrence of aneuploidy in MSCs is not associated with the development of other genetic abnormalities.

Nevertheless, the occurrence of aneuploidy in ASC cultures is an interesting biologic phenomenon. Cloning of low-passage ASCs resulted in clones that were predominantly diploid but contained low percentages of aneuploid ASCs. These results indicate that there is plasticity in the ploidy of ASCs. The fact that the percentage of aneuploidy remained stable in these clones for several passages suggests that some form of autoregulation of ploidy in ASCs exists. It is possible that this autoregulatory capacity is lost when ASC cultures age and become senescent and show elevated frequencies of aneuploidy.

In conclusion, we found aneuploidy in ASCs to be a culture-induced phenomenon, which is stable during culture and increased in senescent cultures. We demonstrated plasticity of ploidy in ASCs and found no transformation or tumor formation by aneuploid ASCs. Aneuploidy in low-passage ASCs is unlikely to compromise clinical application of ASCs.

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Chapter 4

Effects of Hypoxia on the Immunomodulatory properties of Adipose tissue-derived Mesenchymal Stem Cells

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ABSTRACT

Adipose tissue-derived mesenchymal stem cells (ASC) are of great interest as a cellular therapeutic agent for regenerative and immunomodulatory purposes. The function of ASC adapts to environmental conditions, such as oxygen tension. Oxygen levels within tissues are typically much lower than under standard culture conditions and ASC used for therapy therefore encounter a change from normoxic to hypoxic conditions. The effect of hypoxia on the regenerative potential of ASC has been investigated in a number of studies. The effect of hypoxia on the immunomodulatory function of ASC, however, remains to be determined. In the present study the effect of hypoxic (1% oxygen) culture conditions on human ASC was examined. ASC showed no signs of toxicity under low oxygen levels and no major immunophenotypical changes were observed, apart from a down regulation of the marker CD105. Oxygen tension had no effect on the proliferation of ASC and colony forming unit (CFU) efficiency remained the same under 1% and 20% oxygen. Under both oxygen levels ASC were capable of strong upregulation of the immunomodulatory molecules indolamine 2,3-dioxygenase (IDO) and programmed death ligand-1 (PD-L1) upon stimulation with IFN-γ and TNF-α, and, in addition, IDO activity as measured by the accumulation of L-kynurenine was not affected under hypoxia. The ability of ASC to inhibit anti-CD3/CD28 stimulated $CD4^+$ and $CD8^+$ T cell proliferation was not hampered by hypoxia. The results of the present study demonstrate that the immunosuppressive capacity of ASC is maintained under hypoxic conditions. These findings are important for the therapeutic use of ASC and may be applied for the *in vitro* generation of ASC with improved functionality for therapeutic use.

INTRODUCTION

Mesenchymal stem cells (MSC) have emerged as cells with great clinical potential. *In vitro* studies have demonstrated the immunosuppressive and regenerative capacities of MSC. Currently, MSC have been evaluated as a cell therapeutic agent in many medical fields including graft versus host disease, solid organ transplantation and Crohn's disease [1-3]. MSC can be isolated from a wide range of tissues [4, 5], of which bone marrow is the classical, and most frequently used source. However, bone marrow aspiration is invasive and is accompanied with donor morbidity [6]. In contrast, adipose tissue is more accessible, has a higher yield of MSC, and adipose tissue-derived MSC (ASC) share many properties with their bone marrow derived counterparts [7, 8]. Adipose tissue is therefore the favored source of MSC in an increasing number of studies [9-11]. To obtain sufficient numbers of MSC for research and certainly clinical application the cells are culture expanded. Culture conditions, however, have striking effects on the phenotype and function of MSC. Advantage of this can be taken by modulating culture conditions in such a way that cells with superior functionality are obtained.

Oxygen concentration is an important environmental factor that affects MSC. While MSC are normally cultured under 20% oxygen tension, tissue-resident MSC face much lower oxygen concentrations. Oxygen tension in adipose tissue, for instance, fluctuates depending on blood flow, but varies typically between 3 and 11% [12]. Lower oxygen concentrations can occur in the incidence of injury. It has been demonstrated that hypoxic conditions affect the function of bone marrow derived MSC. Culturing under 1% oxygen reduces MSC senescence while it increases proliferation and maintains the differentiation properties of the cells [13]. Similar results have been obtained for MSC derived from adipose tissue and Wharton's jelly [14-16]. This suggests that MSC are triggered by injury-induced hypoxic conditions to expand and eventually differentiate. MSC have also been shown to enhance their angiogenic potential under hypoxia by increasing their secretion of vascular endothelial growth factor (VEGF) and bFGF [17].

Tissue trauma is almost without exception followed by inflammation. Inflammation in its turn is a major activator of the immunosuppressive capacity of MSC [18], which thereby allows regeneration by inhibiting immune activity. It is however unknown whether hypoxia, occurring before the initiation of inflammation, alters the immunosuppressive capacities of MSC. As these capacities are essential for MSC therapy, it is important to evaluate the effect of hypoxia on MSC. Therefore, in the present study we examined the effect of low oxygen concentrations on the phenotype and immunomodulatory properties of human ASC.

MATERIALS AND METHODS

Adipose tissue

Subcutaneous adipose tissue was surgically removed from healthy live kidney donors during the kidney donation procedure after written informed consent, as approved by the Medical Ethical Committee of the Erasmus MC (protocol no. MEC-2006-190). Adipose tissue was collected in essential medium alpha (MEM-α) (Life Technologies, Paisley, UK) supplemented with 100U/ml penicillin and 10.000U/ml streptomycin (p/s) and 2mM L-glutamine (Lonza, Verviers, Belgium).

ASC isolation

Adipose tissue-derived mesenchymal stem cells were isolated from adipose tissue of five donors as described previously [5, 19]. In brief, adipose tissue was mechanically disrupted, enzymatically digested with sterile 0.5mg/mL collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) in RPMI-1640 + glutaMAX (Life Technologies) and p/s for 30 min at 37°C. Cells were resuspended in ASC culture medium, consisting of MEM-α with 15% fetal bovine serum (FBS) (Lonza), transferred to a 175cm^2 culture flask (Greiner Bio-one, Essen, Germany) and kept at 37°C, 5% CO_2, 20% O_2 and 95% humidity. Medium was changed every 3-4 days. When >90% confluent, ASC were detached using 0.05% trypsin-EDTA at 37°C and either directly used for experiments or frozen until usage. ASC were used for experiments between passages 1 and 5.

Immunophenotyping by flow cytometry

Adipose tissue-derived mesenchymal stem cells were immunophenotyped by flow cytometry after standard culture expansion and after one week exposure to hypoxic culture conditions. Subconfluent ASC were trypsinized and washed with FACSFlow (BD Biosciences, San Jose, CA, USA). Cell suspensions were incubated with mouse-anti-human monocolonal antibodies against CD45-PerCP, CD73-PE, CD166-PE, HLA-ABC-APC, HLA-DR-APC-Cy7 (all BD Biosciences) and CD105-FITC (R&D Systems, Abingdon, UK) at room temperature in the absence of light for 15 min. After two washes with FACSFlow, flow cytometric analysis was performed using an eight color FACSCANTO-II with FACSDIVA Software (BD Biosciences) and FlowJo Software (Tree Star, Palo Alto, CA).

Hypoxic culture conditions

Hypoxic conditions were induced by culture of ASC in 1% O_2, 5% CO_2 and 94% N_2 in a 95% humidified atmosphere. Control cells were kept under normoxic conditions (20% O_2).

Lactate measurements

Adipose tissue-derived mesenchymal stem cells were seeded in six-well plates at a concentration of 200,000 cells per well in ASC culture medium and kept under either normoxic or

hypoxic conditions. After 3 days, the conditioned medium was collected and frozen at -80°C until usage. A lactate assay kit (BioVision, Milpitas, CA, USA) was used for measurement of lactate levels. In brief, samples were incubated with 50µl of the Reaction Mix for 30 min at room temperature in the dark. The formed product was measured spectrophotometrically at 570nm on a Victor^2 1420 multilabel plate reader (PerkinElmer, Santa Clara, MA, USA) and corrected for background.

Measurement of metabolic activity by MTT assay

Adipose tissue-derived mesenchymal stem cells were seeded in 96-well flat bottom plates at a concentration of 3000 cells per well in 200µl ASC culture medium and kept for 24h, 48h or 72h under normoxic or hypoxic conditions. Five hours prior to the end of the incubation, 20µl sterile MTT (Sigma-Aldrich) (5mg MTT/ml disolved in 1x PBS) was added to the wells. Culture medium was then removed and 100µl DMSO added to the cells to dissolve formed crystals. Mitochondrial conversion of MTT was determined by absorbance measurements at 550nm wavelength on a Victor^2 1420 multilabel plate reader.

Colony forming unit (CFU) assay

Adipose tissue-derived mesenchymal stem cells were seeded at 50 cells per 6 cm diameter culture dishes in quintuple (3.0 cells/cm^2). After 2 weeks of culture under normoxic or hypoxic conditions, medium was removed, the dishes washed with PBS and fixed in 70% ethanol. Colonies were stained with 2.3% crystal violet solution (Sigma-Aldrich) for 30 min. Dishes were then washed with tap water and colonies with a diameter of more than 1mm counted. Colony forming unit (CFU) efficiency was expressed as the percentage of cells capable of forming colonies.

Proliferation assay

Adipose tissue-derived mesenchymal stem cells were seeded at a density of 1000 cells/cm^2 in 75cm^2 culture flasks and kept under normoxic or hypoxic conditions for 1 week without medium changes. Cells were then detached by trypsinization, counted, and re-seeded. Population doublings were calculated using the formula ^{2}LOG(number of cells at t_{d7}/number of cells at t_{d0}). Cumulative population doublings during 5 consecutive weeks of culture were plotted.

Quantitative mRNA expression

Adipose tissue-derived mesenchymal stem cells were seeded in six-well plates at a concentration of 1×10^5 per well in the presence or absence of 20ng/ml TNFα and 50ng/ml IFNγ and kept under normoxic or hypoxic conditions. After 6h, 24h or 72h, RNA*later* (Life Technologies) was added to the cells. RNA was isolated and 500ng used for cDNA synthesis as described previously [20]. Gene expression was determined by real-time RT-PCR using universal PCR

master mix (Life Technologies) and Assays-on-demand for hypoxia induced factor 1α (HIF1-α) (Hs00153153.m1), HIF2-α (Hs1026149.m1), VEGF (Hs00173626.m1), IDO (Hs00158627.m1), PDL-1 (Hs00204257.m1), TGFβ1 (Hs00171257.m1), IL10 (Hs00174086.m1), IL6 (Hs00174131.m1) and CXCL10 (Hs00171042.m1) (all Applied Biosciences, Foster City, CA, USA) on an ABI PRISM 7700 sequence detector (Applied Biosystems). As housekeeping gene expression fluctuates under hypoxia, data was expressed as relative copy number of the PCR products per 500ng RNA. Relative copy number was calculated using the formula $2^{(40\text{-Ct value})}$.

IDO activity measurements

Adipose tissue-derived mesenchymal stem cells were cultured for 24h or 72h with or without 20ng/ml TNF-α and 50ng/ml IFN-γ under normoxic or hypoxic conditions. The tryptophan metabolic activity of IDO was determined by measurement of L-kynurenine in the conditioned medium of five ASC cultures. Thirty percent of trichloroacetic acid was added to the samples at a 1:3 ratio and after 30 min incubation at 50°C the samples were centrifuged at 12,000 rpm for 5 min. Supernatants were then diluted 1:1 in Ehrlich reagent (200mg 4-dimethylaminobenzaldehyde (Sigma, St. Louis, MO, USA) in 10ml of glacial acetic acid) in duplicate in a 96-wells flat bottom plate and absorbance was determined at 490nm using a multilabel plate reader (VersaMaxTM, Molecular Devices, Sunnyvale, CA, USA). L-kynurenine (Sigma, St. Louis, MO, USA) diluted in unconditioned medium was used as standard.

PBMC isolation

Peripheral Blood Mononuclear Cells (PBMC) were collected from buffy coats of healthy blood bank donors. PBMC were isolated by density gradient centrifugation using Ficoll Isopaque (δ=1.077, Amersham, Uppsala, Sweden) and frozen -135°C until use .

Anti-CD3/CD28 lymphocyte stimulation assay

Peripheral blood mononuclear cells (PBMC) were labeled using the PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich) and stimulated with anti-CD3 antibody (0.5µl/5×10^5 cells), anti-CD28 antibody (0.5µl/5×10^5 cells) and a goat-anti-mouse antibody (1µl/5×10^5 cells) for cross-linking (all BD Biosciences). PBMC were seeded in round-bottom 96-well plates at 5×10^4 cells per well and ASC added at 1:2.5, 1:5, 1:10 and 1:20 ratios in MEM-α with p/s and 10% heat inactivated (HI)-human serum. After 3 days of incubation under normoxic or hypoxic conditions, PBMC were collected, washed twice with FACSFlow (BD Biosciences) and incubated with monoclonal antibodies against TCR-FITC (Serotec, Oxford, UK), CD3-AmCyan, CD4-Pacific Blue, CD8-PerCP (all BD Biosciences) for 15 minutes at room temperature in the absence of light. After two washes with FACSFlow, flow cytometric analysis was performed using an 8 color FACSCANTO-II with FACSDIVA Software (BD Biosciences).

Statistical analysis

Data were statistically analyzed using Mann Whitney, and Kruskal-Wallis statistical tests. $P < 0.05$ was considered significant.

RESULTS

ASC identity

In several previous studies we have demonstrated that the adherent cells of the stromal vascular fraction of human adipose tissue are ASC with properties similar to bone marrow MSC [5, 19, 21]. Thus ASC have a spindle-shaped fibroblastic morphology in culture, have a $CD45^-$ $CD73^+CD105^+CD166^+HLAclass\ I^+$ immunophenotype and can differentiate in adipogenic and osteogenic lineages [19] (data not shown).

Culture of ASC under hypoxic conditions

Under hypoxic conditions, cells are forced to switch to anaerobic metabolism and will produce lactate. In our model, ASC significantly increased lactate concentrations in the culture medium after 3 days of culture under hypoxic conditions (28.3mM, range 25-31, versus 12mM, range 10-14, under normoxic conditions) (Figure 1A).Culture of ASC under hypoxic conditions (1% O_2) for 24 h had no effect on mRNA expression levels of hypoxia-inducible factor 1-α (HIF1-α) and HIF2-α (Figure 1B). As hypoxia stabilizes HIF protein, we examined whether gene expression downstream of HIF was affected by hypoxia in ASC. Expression of (VEGF) downstream of HIF1-α was significantly increased under hypoxia by nearly a factor two (Figure 1C).

Effect of hypoxia on ASC characteristics

To study the effect of hypoxia on ASC characteristics; metabolic activity, survival, growth, and proliferation of ASC was examined. Culture of ASC under 1% O_2 for 24 h up to 72h had no effect on ASC metabolic activity and there was no evidence for loss of cell viability, as measured by the mitochondrial reduction of MTT to formazan (Figure 2A). There was a similar increase in the conversion of MTT under normoxic and hypoxic conditions at 24 h up to 72 h, which reflects the similar increase in cellular metabolic activity under both conditions. Parallel culture of ASC under normoxic and hypoxic conditions for five consecutive weeks demonstrated no difference in population doubling times, which were 2.6 days (range 1.9-3.1), under normoxia and 2.5 days (range 1.9-5.0) under hypoxia. Therefore, cumulative population doublings were the same under both oxygen tensions (Figure 2B). Furthermore, there was no difference in the colony forming unit (CFU) capacity of ASC cultured under 20 and 1% O_2 (Figure 2C), suggesting oxygen tension does not affect the stemness of ASC. Finally, the immunophenotype of ASC was determined after 10 days of culture under normoxic or hypoxic conditions. Under

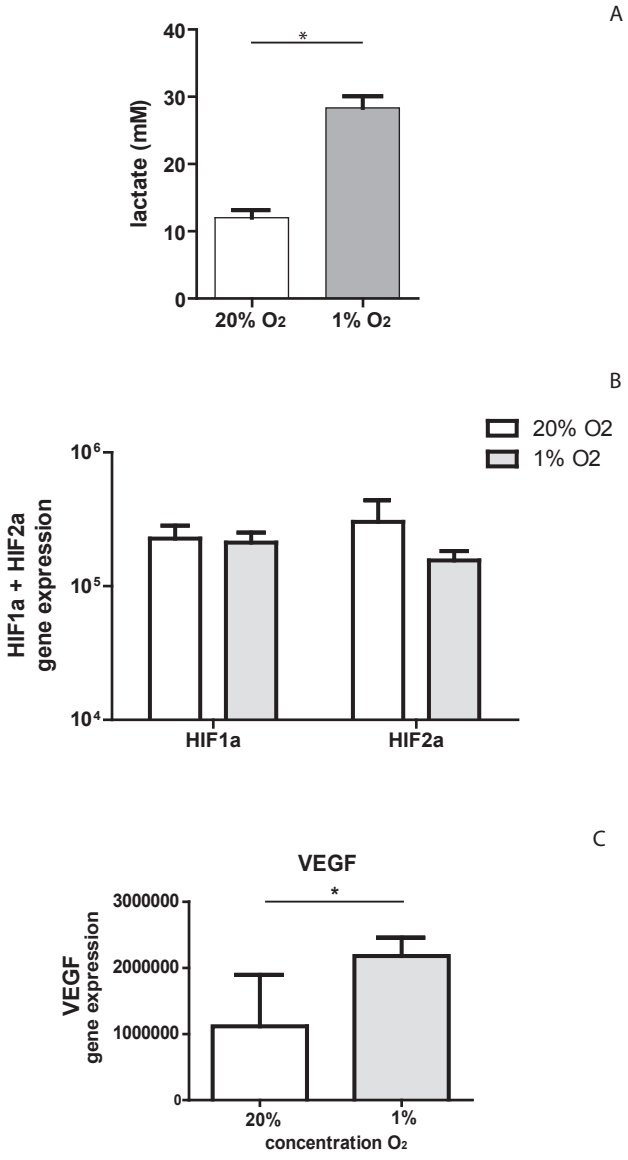

Figure 1. (A) Lactate production by ASC. ASC were cultured under 20 or 1% O2 for 3 days and medium collected for lactate measurements. Mean with SEM of three experiments is shown. (B) Effect of hypoxia on gene expression of hypoxia-inducible genes; mRNA expression of HIF1-α and HIF2-α by ASC after 24h culture under normoxic (20% O2) and hypoxic (1% O2) conditions. Mean with SD of four different experiments is shown. (C) mRNA expression of VEGF, downstream of HIF1-α under normoxia and hypoxia. Mean with SD of four different ASC cultures, *Indicates $p < 0.05$.

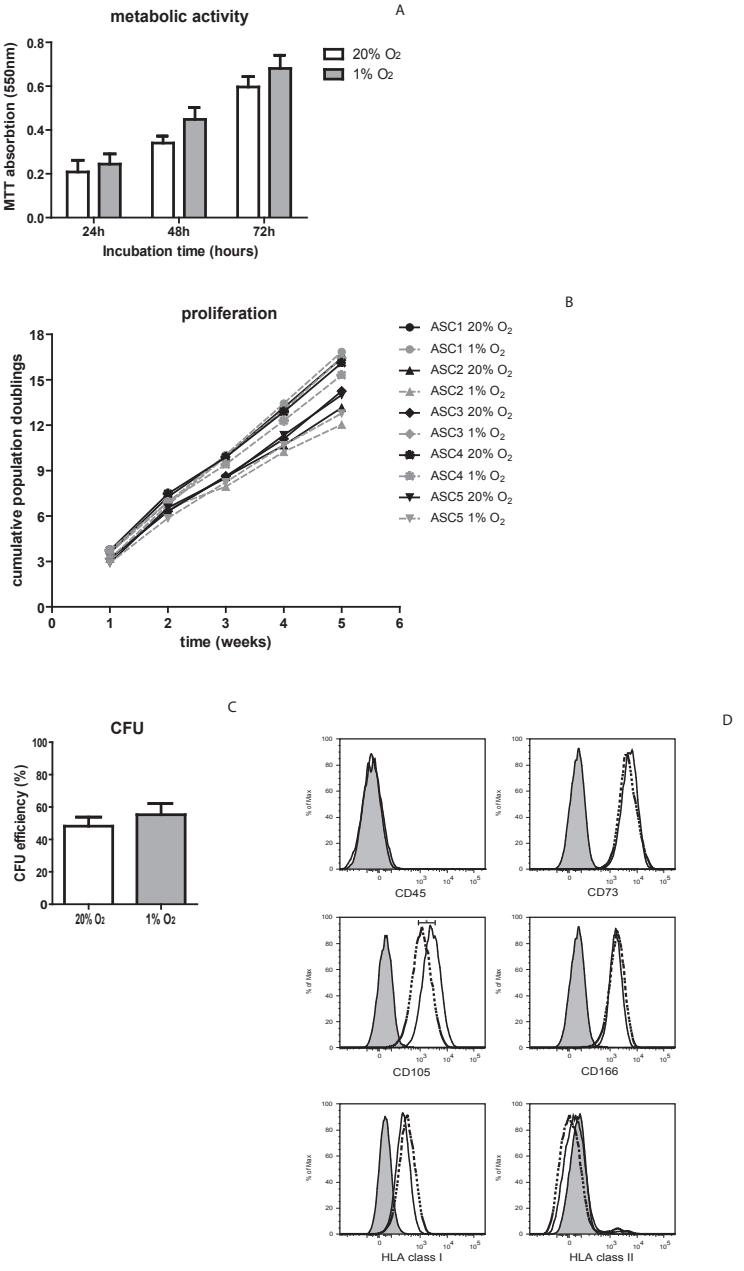

Figure 2. Effect of hypoxia on ASC characteristics. (A) ASC metabolic activity measured by MTT assay. Mean with SEM of three different ASC cultures shown. (B) Cumulative population doublings of ASC cultured under normoxic and hypoxic conditions. (C) CFU efficiency of ASC. Mean with SEM of four different ASC cultures shown. (D) Immunophenotype of ASC after culturing for 10 days under normoxic and hypoxic conditions. Filled histogram; unstained ASC, solid line; ASC cultured under 20% O2, dotted line; ASC cultured under 1% O2. *Indicates $p < 0.05$.

both conditions, the ASC immunophenotype was $CD45^{-}CD73^{+}CD105^{+}CD166^{+}HLA\text{-}I^{+}HLA\text{-}II^{-}$ (Figure 2D). The expression of CD105 was, however, significantly lower in ASC cultured under hypoxic conditions (MFI 2530) than in ASC cultured under normoxia (MFI 4215).

Effect of hypoxia on the expression of immunomodulatory genes by ASC and IDO activity

It is known that the immunomodulatory capacity of ASC is induced under inflammatory conditions. This was confirmed in the present study by showing that treatment of ASC with IFN-γ and TNF-α for 6, 24 and 72 h induced a strong increase in the mRNA expression of immunomodulatory programmed death ligand 1 (PD-L1) (10^{2}-fold), CXCL10 (10^{6}-fold) and IDO (10^{5}-fold). Under hypoxic conditions, ASC maintained the capacity to induce the expression of IDO, PD-L1 and CXCL10 in response to IFN-γ and TNF-α to a similar extent as under normoxic concentrations (Figure 3A-C).

To determine whether hypoxia affected the tryptophan depleting activity of IDO in ASC we measured concentrations of L-kynurenine, the breakdown product of tryptophan, in conditioned medium of ASC cultured for 24h or 72h with or without TNF-α and IFN-γ under 20 or 1% oxygen. Culture with TNF-α and IFN-γ strongly increased L-kynurenine levels at 24 and 72 h (Figure 3D and Figure 3E). Hypoxia did not affect L-kynurenine levels indicating preserved IDO activity under hypoxic conditions.

Furthermore, TGF-β, associated with the immunomodulatory capacity of ASC, was expressed by ASC under resting conditions and at similar levels upon stimulation with IFN-γ and TNF-α. Hypoxia did not affect TGF-β expression (data not shown). IL10 was not expressed by ASC under any of the conditions tested (data not shown). Pro-inflammatory IL6 was expressed by ASC, and its expression did not change under low oxygen conditions (data not shown). These data suggest that ASC maintain their capacity to express immunomodulatory factors and respond to inflammatory conditions by activating their immunomodulatory apparatus under hypoxic conditions.

Immunosuppressive capacities of ASC under hypoxia

To study whether hypoxia influences the *in vitro* immunosuppressive capacities of ASC, ASC were added to anti-CD3 and anti-CD28 stimulated PKH-labeled allogeneic PBMC. The proliferation of $CD4^{+}$ and $CD8^{+}$ T cells tended to be lower under hypoxia than under normoxia, although the difference was not significant. Under normoxia, there was a significant inhibition of $CD4^{+}$ and $CD8^{+}$ T cell proliferation at ASC:PBMC ratios of 1:2.5 (Figure 4A-B). Under hypoxia, inhibition of T cell proliferation was more profound as T cell proliferation was significantly inhibited till ASC:PBMC ratios up to 1:5 (Figure 4A-B). Direct comparison of the T cell inhibition by ASC under normoxia and hypoxia demonstrated that inhibition of $CD4^{+}$ and $CD8^{+}$T cell proliferation was significantly higher at a 1:5 under 1% oxygen level (Figure 4C-D).

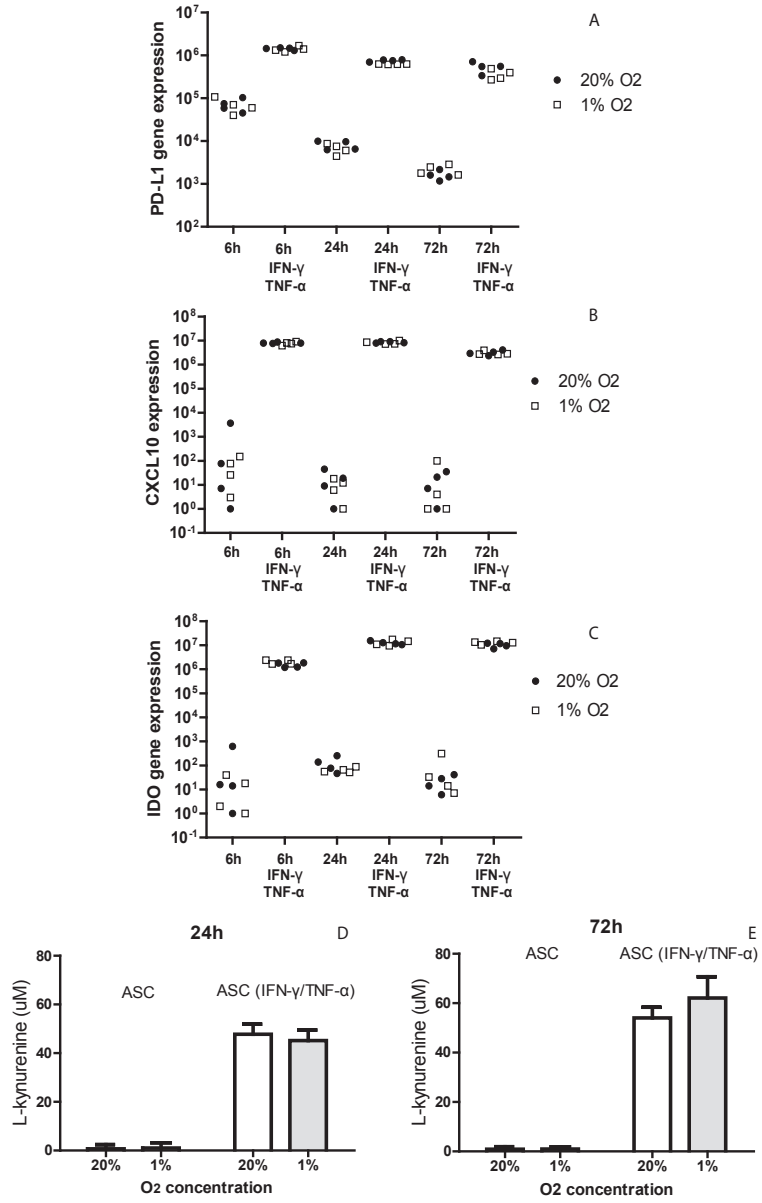

Figure 3. Effect of hypoxia on the induction of PD-L1 (A), CXCL-10 (B), and IDO (C); mRNA expression in ASC after IFN-γ/TNF-α stimulation. ASC were cultured under 20 or 1% O2 for 6, 24, or 72h with or without 50ng/ml IFN-γ and 20ng/mlTNF-α. Every data point represents a distinct ASC culture (n =4 different ASC cultures). (D,E) IDO activity determined by accumulation of L-kynurenine in ASC conditioned medium in the absence and presence of TNF-α and IFN-γ [24h (D) and 72h (E)]. Means of five ASC cultures with SEM shown.

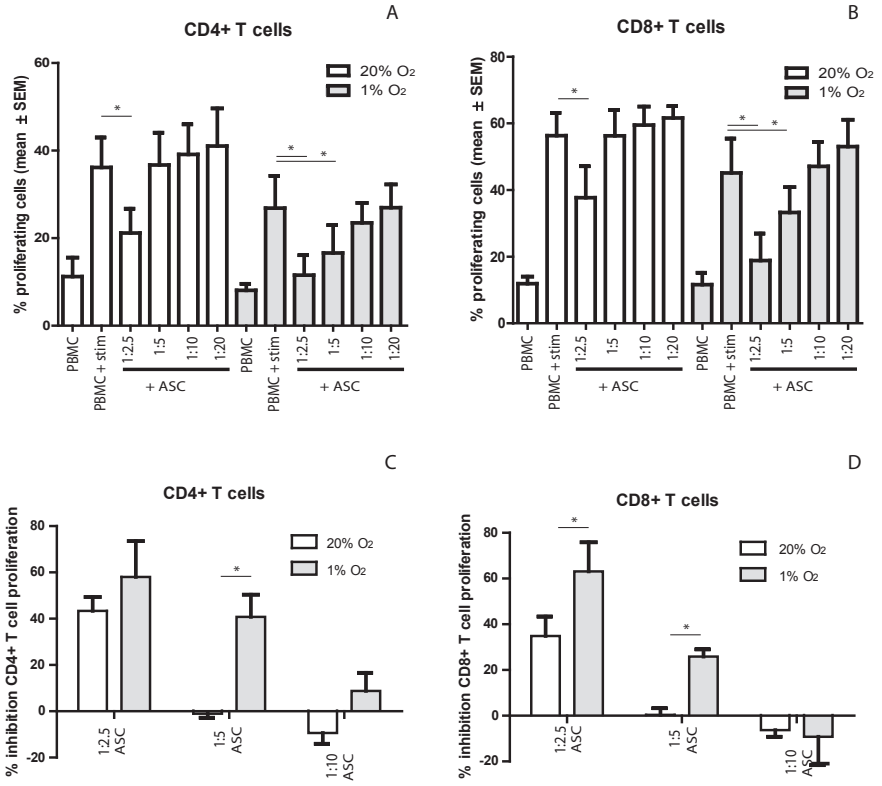

Figure 4. (A–D) PBMC were labeled with PKH and stimulated with anti-CD3/CD28 antibodies. On day 3, the proliferation of CD4+ and CD8+ T cells was analyzed by flow cytometry. (A,B) percentage proliferating cells shown. (C,D) percentage inhibition of proliferation shown, compared to the condition without ASC. Mean with SEM of three experiments with different ASC cultures shown, *p-value<0.05.

DISCUSSION

Adipose tissue-derived mesenchymal stem cells possess an assortment of properties that make them suitable for regenerative and immunomodulatory applications. The properties of ASC are affected by changing environmental conditions that induce ASC to adapt a particular function. Oxygen tension is one of these environmental factors. Oxygen tension in tissue fluctuates between 3% and 11% [22] and may be lower in case of trauma, and such changes may affect the function of ASC. In the present study, we cultured ASC under 20% and 1% oxygen to examine whether oxygen tension would affect the *in vitro* properties of ASC.

In the present study we found that ASC were resistant to hypoxic conditions and detected no signs of toxicity or decreased CFU efficiency under low oxygen concentrations. There is some controversy on the effect of hypoxia on the proliferation of bone marrow and adipose

tissue-derived MSC, with some studies reporting an inhibitory effect of low oxygen levels on proliferation [23-25], others reporting increased proliferation [26], while we found that ASC proliferation was not affected over at least five passages. There are a number of factors that may influence the outcome of studies, such as the percentage of oxygen used to generate hypoxic conditions with some studies using 1% oxygen and others up to 5%. Furthermore, the composition of the cell culture medium, in particular the use of serum, and the species of ASC used will affect outcomes. In the present study, the immunophenotype of ASC was only moderately affected by hypoxia, which is in line with earlier studies [24, 25]. In our hands, only the expression of CD105 (endoglin) was consistently down regulated under low oxygen tension. This is quite surprisingly, as CD105 is known to be up regulated under hypoxia in endothelial cells [27]. The consequence of the down regulation of CD105, which is an adhesive molecule and part of the TGFβ receptor complex remains to be determined.

In the current study, we further found that a hypoxic pre-conditioning regimen does not hamper the immunosuppressive properties of ASC. The immunosuppressive function of ASC is induced by inflammatory cytokines such as IFN-γ and TNF-α [18, 28]. These cytokines stimulate the expression of anti-proliferative IDO and of the inhibitory co-stimulatory molecule PD-L1. The induction of these proteins and the activity of IDO were not affected by hypoxic conditions, indicating that the immunosuppressive machinery of ASC is maintained at low oxygen concentrations. In addition, the strongly enhanced expression of the chemokine CXCL10 under inflammatory conditions was not affected by hypoxia, suggesting that the chemoattractive properties of ASC for immune cells are preserved under low oxygen concentrations.

Subsequently, we found that culture under 1% O_2 did not hinder the suppressive effects of ASC on T cell proliferation. The percentage of inhibition of t cells by ASC was even increased under hypoxia. Thus although ASC under hypoxia were phenotypically indifferent from ASC under normoxic conditions, they had a more profound effect on T cell proliferation. One explanation could be that hypoxic conditions shift the balance between T cell proliferation and the inhibitory effect of ASC. Thus while T cells are affected in their proliferation by hypoxia, as demonstrated earlier [29] and shown in the present study, ASC maintain their suppressive capacity. It is possible that under hypoxic conditions, T cells are more sensitive to low tryptophan concentrations induced by IDO expression in ASC or by PD-L1 induced inhibition of proliferation. ASC therefore have a relatively larger impact on inflammatory conditions and are more effective in inhibiting immune responses under hypoxic conditions, such as in case of trauma, than under normoxic conditions. Alternatively, the enhanced efficacy of ASC to inhibit T cell proliferation under hypoxia may be explained by the fact that ASC employ additional mechanisms of immunomodulation under hypoxic conditions that were not analyzed in the present study.

In summary, our data indicates that a reduction of oxygen tension up to at least 1% does not hamper the immunomodulatory therapeutic efficiency of ASC. This is of relevance as for

most clinical purposes, the immunosuppressive capacities of ASC are essential. Assertion of these capacities under hypoxia is crucial as ASC will encounter hypoxic conditions when administered for most, if not all, immunomodulatory applications. Subsequently, as there is evidence that hypoxic pre-conditioning enhances the regenerative potential of ASC [30, 31], maintenance of immunosuppressive capacities under hypoxia is needed when hypoxia pre-conditioning will be used for regenerative application. The present study shows that in these situations, the immunomodulatory capacity of ASC is preserved.

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Chapter 5

Human allogeneic bone marrow and adipose tissue derived mesenchymal stromal cells induce CD8$^+$ cytotoxic T cell reactivity

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ABSTRACT

Introduction

For clinical applications, Mesenchymal Stromal Cells (MSC) can be isolated from bone marrow and adipose tissue of autologous or allogeneic origin. Allogeneic cell usage has advantages but may harbor the risk of sensitization against foreign HLA. Therefore, we evaluated whether bone marrow and adipose tissue-derived MSC are capable of inducing HLA-specific alloreactivity.

Methods

MSC were isolated from healthy human Bone Marrow (BM-MSC) and adipose tissue (ASC) donors. Peripheral Blood Mononuclear Cells (PBMC) were co-cultured with HLA-AB mismatched BM-MSC or ASC pre-cultured with or without IFNy. After isolation via FACS sorting, the educated CD8+ T effector populations were exposed for 4 hours to Europium labeled MSC of the same HLA make up as in the co-cultures or with different HLA. Lysis of MSC was determined by spectrophotometric measurement of Europium release.

Result

CD8+ T cells educated with BM-MSC were capable of HLA specific lysis of BM-MSC. The maximum lysis was 24% in an effector: target (E:T) ratio of 40:1. Exposure to IFNγ increased HLA-I expression on BM-MSC and increased lysis to 48%. Co-culturing of PBMC with IFNγ-stimulated BM-MSC further increased lysis to 76%. Surprisingly, lysis induced by ASC was significantly lower. CD8+ T cells educated with ASC induced a maximum lysis of 13% and CD8+ T cells educated with IFNγ-stimulated ASC of only 31%.

Conclusion

Allogeneic BM-MSC, and to a lesser extend ASC, are capable of inducing HLA specific reactivity. Our results suggest that clinical therapy with allogeneic MSC should be carefully considered.

INTRODUCTION

Mesenchymal stromal cells (MSC) are adult stem or progenitor cells which can be isolated from virtually all postnatal tissues including bone marrow and adipose tissue[1-3]. MSC are defined by their capacity to adhere to plastic, their multilineage differentiation capacity and a panel of cell surface markers including CD13, CD73, CD90 , CD105, CD166 and HLA-class I [1, 4]. HLA-class-II expression on MSC is low or absent. MSC have immune modulatory and reparative properties which makes them appealing as a cell therapeutic agent for degenerative disease and immune disorders. When exposed to inflammatory conditions such as IFNγ stimulation, MSC increase their immunosuppressive properties but also their expression of HLA-class I and II[5, 6].

In the last decades, multiple clinical trials evaluating the potential of MSC for a wide spectrum of medical conditions have been conducted. Next to safety and feasibility of MSC therapy, preliminary efficacy results were obtained in some of these studies evaluating MSC amongst others in rheumatoid arthritis[7, 8]; Crohn's disease[9, 10]; liver cirrhosis[11] and solid organ transplantation[12-15].

For clinical application, the choice of MSC is likely to affect the outcome of the therapy.

MSC are typically isolated from bone marrow and culture expanded. As donor age might be of influence for bone marrow derived MSC (BM-MSC) composition and function[16-18], use of MSC derived from young bone marrow donors might be preferable. Yet, this choice has some drawbacks as bone marrow aspiration is an invasive procedure and the use of BM from young individuals involves ethical dilemmas. Adipose tissue derived MSC (ASC) might serve as an alternative with several advantages: adipose tissue can be obtained in a minimal invasive manner via lipectomy or mini liposuction; adipose tissue has a higher yield of MSC[19]; and finally, ASC are at least as immunosuppressive as BM-MSC in vitro[20, 21].

Next, there is a choice of using MSC of autologous or allogeneic origin. Allogeneic cells provide a practical 'off the shelf' cell therapeutic agent as they can be isolated and cultured in advance. Recent studies suggest that allogeneic MSC have comparable efficacy compared to autologous MSC [22, 23]. However, allogeneic MSC might possibly elicit an anti-HLA immune response [24]. In contrast, autologous MSC avoid potential immunogenic responses, but need to be prepared per individual and are unsuitable for acute indications. Furthermore, although suitable in some situations[25, 26], patient MSC can be affected by the disease rendering them unfit for clinical application[27, 28]. In contrast, allogeneic cells provide a practical 'off the shelf' cell therapeutic agent as they can be isolated from healthy donors and cultured in advance. Recent studies further suggest efficacy of allogeneic MSC therapy [22, 23]. However, allogeneic MSC might elicit a HLA-specific immune response [24]. The risk of anti-HLA sensitization by allogeneic MSC has been considered insignificant in the past as MSC were suggested to be low-immunogeneic[29-32]. However, we have shown that allogeneic MSC are susceptible for lysis by pre-activated CD8+ T cells[33] in an HLA-specific fashion,

which challenges this old paradigm. It is however unknown whether MSC themselves are capable of inducing HLA-specific CD8+ cytotoxicity. This would signify a risk of sensitization by allogeneic MSC therapy and could potentially affect the efficacy of repeated MSC administrations or even future organs transplantations. The development of anti-MSC donor immune responses should therefore be studied before new studies are planned with allogeneic MSC. Thus, in the present study we evaluated whether repeated exposure to BM-MSC and/or ASC induced HLA-class-I specific lysis by CD8+ T cells.

METHODS

Isolation of ASC

During live donor kidney transplantation, abdominal subcutaneous adipose tissue was surgically removed from 5 healthy kidney donors after written informed consent as approved by the medical ethical committee of the Erasmus Medical Center Rotterdam (protocol no. MEC-2006-190). Adipose tissue was collected in MEM-α medium (Sigma-Aldrich(St. Louis, MO, USA) supplemented with 100IU/ml penicillin and 100µg/ml streptomycin (p/s, Gibco BRL, Paisley UK) and 2mM L-glutamine (Lonza, Verviers, Belgium),. ASC were isolated as described previously[1]: adipose tissue was mechanically disrupted with a scalpel knife, and then enzymatically digested with 0.5mg/ml collagenase type IV (Sigma-Aldrich, St Louis, MO) in RPMI-1640 glutaMAX (Invitrogen) and p/s for 30 minutes at 37°C under continuous shaking. The obtained cell suspension was transferred to a T175 cm^2 culture flask (Greiner Bio-one, Essen, Germany) and cultures were kept at 37°C, 5% CO_2 and 95% humidity. MSC culture medium which consisted of MEM-α (Sigma-Aldrich, St. Louis, MO, USA) with 1% p/s, 2nM L-glutamine and 15% fetal bovine serum (BioWhittaker, Verviers, Belgium) was refreshed twice a week. When ASC cultures reached >90% confluency, MSC were detached with 0.5% trypsin-EDTA (Lonza, Verviers, Belgium) and used for experiments or frozen at -150°C until usage

Isolation of BM-MSC

BM was obtained from 5 hematopoietic stem cell donors after written informed consent as approved by the Medical Ethical Committee of Leiden University Medical Centre as described before[34]. In brief, BM was aspirated under general anesthesia. The mononucleated cell (MNC) fraction was isolated by Ficoll density gradient separation (Ficoll Isopaque, δ=1.077, Amersham, Uppsala, Sweden) and plated in tissue culture flasks at a density of 160 x 10^3 MNC/cm^2 in low-glucose Dulbecco's modified Eagle medium DMEM (Invitrogen, Paisley, UK) supplemented with 1% p/s (Lonza) and 10% fetal calf serum (FCS, Thermo Scientific HyClone,). The cultures were maintained at 37°C, 5% CO_2. The medium was refreshed twice a week. When the MSC cultures became confluent, cells were collected using trypsin (Lonza) and re-plated at a density of 4 x 10^3 cells/ cm^2 or frozen until further usage.

BM-MSC and ASC donor characteristics

MSC isolated from 5 healthy HLA-AB mismatched bone marrow (mean age 15.4 years, range 7-31) and adipose tissue donors (mean age 50.7 years, range 27-67) (table 1) were used for experiments. Prior to using them in experiments, BM-MSC and ASC were cultured in parallel in MEM- with 1% p/s and 15% fetal bovine serum under standard culture conditions. When indicated, MSC were then stimulated with 100 ng/ml IFNγ for 1 week. Passage 2-6 MSC were used.

Table 1: HLA typing of BM-MSC, ASC and PBMC used in the 5 experiments.

No	BM-MSC/ASC	HLA-I		HLA-I	
		A	A	B	B
1	BM-MSC 1	1	3	7	35
	ASC 1	24		15	51
	PBMC 1	32(19)		44(12)	18
2	BM-MSC 2	3	24(9)	35	38(16)
	ASC 2	1	30(19)	8	41
	PBMC 2	68(28)		51 (5)	53
3	BM-MSC 3	24(9)		62(15)	55(22)
	ASC 3	11	30(19)	52(5)	35
	PBMC 3	1	3	8	64(40)
4	BM-MSC 4	2	29(19)	7	44(12)
	ASC 4	1	26(10)	38(16)	51(5)
	PBMC 4	3	23	49	61
5	BM-MSC 5	2	24(9)	35	58(17)
	ASC 5	3		7	13
	PBMC 2	68(28)		51 (5)	53

Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected from healthy blood bank donors. Donors with 4 mismatches for HLA-A and HLA-B with the BM-MSC and ASC were selected (table 1). PBMC were isolated by density gradient centrifugation using Ficoll Isopaque and frozen at -150°C until usage.

Immunophenotypic characterization of BM-MSC and ASC

Unstimulated and 1 week 100ng/ml IFNγ-stimulated BM-MSC and ASC were trypsinized and washed with FACSFlow (BD Biosciences, San Jose, CA). Cell suspensions were incubated with mouse-anti-human monoclonal antibodies against CD13-PECy7; CD45-PERCP; HLA-DR-FITC; HLA-ABC-PE-Cy7; CD31-FITC; CD73-PE; CD166-PE (all BD Biosciences); CD90-APC and CD105-FITC (R&D Systems, Abingdon, UK) at room temperature in the absence of light for 15

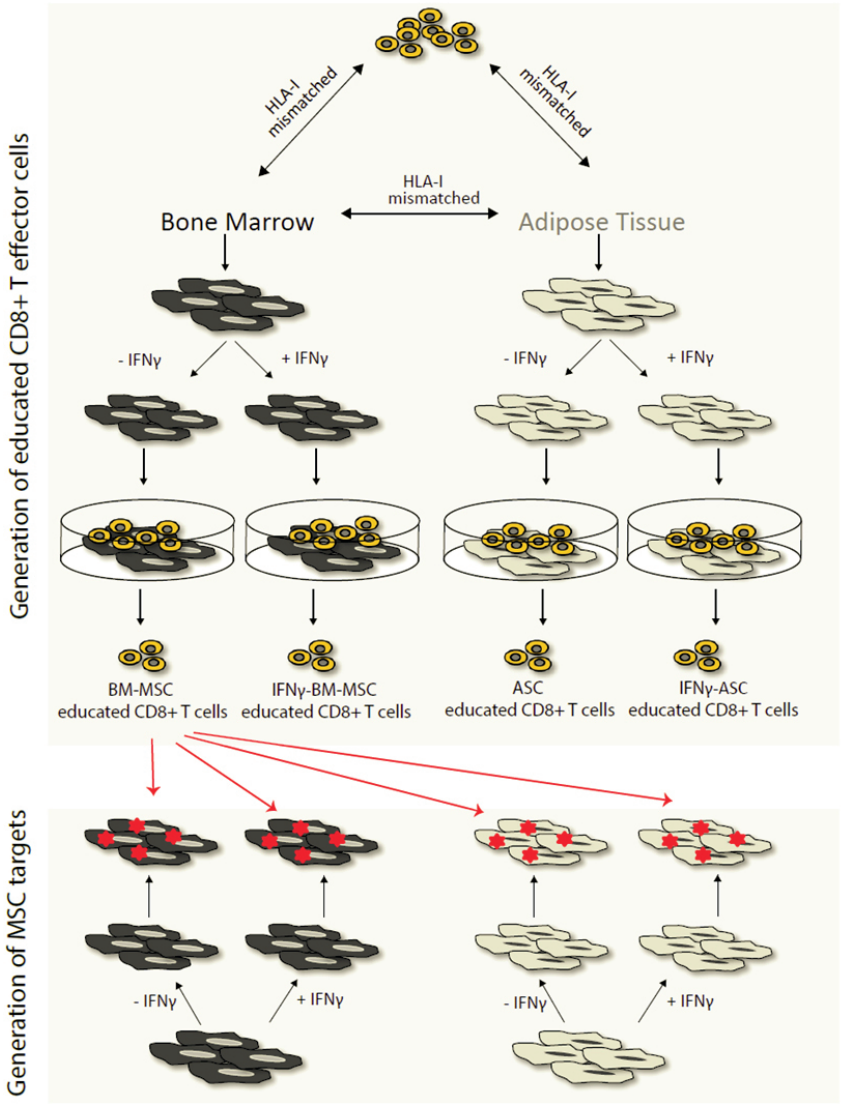

Figure 1: Experimental design of study. Five HLA-class I mismatched BM- MSC and ASC pairs were used. BM-MSC and ASC were either unstimulated or pre-stimulated for 1 week with IFNγ. To obtain educated CD8+ T cells, a co-culture was established with BM-MSC or ASC and HLA-class I mismatched PBMC in the presence of 200 U/ml IL-2. CD3+CD8+ effectors (BM-MSC educated CD8+ T cells; IFNγ-BM-MSC educated CD8+ T cells; ASC educated CD8+ T cells and IFNγ-ASC educated CD8+ T cells) were selected via FACS sorting. CD3+CD8- cells were isolated from all co-cultures as a negative control. After Europium labeling, target cells, either IFNγ-stimulated or unstimulated BM-MSC and ASC, were exposed to the effector cells as depicted here for BM-MSC educated CD8+ T cells. Europium release was assessed as a measure of CD8+ T cell mediated lysis.

minutes. After two washes with FACSFlow, flow cytometric analysis was performed using an 8 color FACSCANTO-II with FACSDIVA Software (BD Biosciences) and FlowJo Software (Tree Star Inc. Palo Alto, CA). For analysis, background MFI was substracted and the mean MFI of 4 experiments were calculated.

Generation of BM-MSC and ASC educated CD8+ effector populations

Five BM-MSC and ASC cultures with different HLA-A and HLA-B subtypes were selected (table 1). These BM-MSC and ASC were cultured in parallel under the same conditions for 1 week with or without 100ng/ml IFNγ before they were seeded in 24-well flat bottom plates. PBMC with a 2-2 mismatch for HLA-A and HLA-B with both the BM-MSC and the ASC were then selected. To generate effector cells, 5 x 10^5 PBMC were co-cultured with 1,2 x 10^6 BM-MSC or, in parallel, with the ASC in MEMα containing 10% of human heat inactivated serum and 200IU/mL IL-2 (Chiron, Amsterdam, The Netherlands). After 1 week of co-culture, the PBMC were removed from the co-cultures, washed with 1X PBS and incubated with mononuclear antibodies against CD3-Amcyan, CD8-PE-Cy7 and 7AAD-viaprobe (all BD Biosciences) for 15 minutes in the dark. The cells were washed and the BM-MSC and ASC educated effector CD3+CD8+ cell populations were isolated by FACS sorting (FACS-ARIA Cell-sorter, BD Biosciences).

This procedure resulted in the isolation of the following effector populations: CD3+CD8+ cells educated by BM-MSC (BM-MSC educated CD8+ T cells); CD3+CD8+ cells educated by IFNγ-stimulated BM-MSC (IFNγ-BM-MSC educated CD8+ T cells); CD3+CD8+ cells educated by ASC (ASC educated CD8+ T cells) and CD3+CD8+ cells educated by IFNγ-stimulated ASC (IFNγ-ASC educated CD8+ T cells); as a control, the CD3+CD8- populations were isolated from all four co-cultures. Our experimental design is depicted in figure 1.

Evaluation of cytotoxicity mediated lysis of BM-MSC and ASC by Europium release assay

To examine the cytotoxic capacity of the CD3+CD8+ effector T cells, BM-MSC and ASC identical to those used in the co-cultures were cultured for 1 week in the presence or absence of 100ng/ml IFNγ and labeled with Europium–diethylenetriaminepentaacetate (DTPA) (Sigma-Aldrich, St. Louis, MO) (figure 1). These target MSC were used in the Europium release cytotoxicity assay as described previously[33]. In brief, the different effector populations were exposed for 4 hours to each of the different Europium labeled targets cells; unstimulated BM-MSC; IFNγ-stimulated BM-MSC; unstimulated ASC and IFNγ-stimulated ASC. The effectors were incubated with 2500 target cells at effector: target (E:T) ratios of 40:1 to 0.3:1 in round-bottom 96-well plates (Nunc, Roskilde, Denmark) at 37°C. The plates were then centrifuged and 20µl of the supernatant was transferred to 96-well plates with low background fluorescence (fluoroimmunoplates [FluoroNunc plates]; Nunc). Subsequently, 100µl of enhancement solution (PerkinElmer, Groningen, the Netherlands) was added to each well and release of Europium was measured in a time-resolved fluorometer (Victor

1420 multilabel counter; LKB-Wallac, Turku, Finland). Maximal release of Europium by target cells was measured by incubation of 2500 labeled target cells with 1% Triton (Sigma-Aldrich, Zwijndrecht, the Netherlands) for 4hr. Spontaneous release of Europium was measured by incubation of labeled target cells without effector cells for 4hr; the percentage leakage was calculated as (spontaneous release/maximal release)x100%. The percentage cytotoxicity mediated lysis was calculated as %lysis = (measured lysis-spontaneous release)/(maximal release- spontaneous release)x100%.

Statistical analysis

Paired t-test was used to test for statistical significance in MFI of the staining for cell surface markers on BM-MSC and ASC. Two-way ANOVA was used to evaluate statistical significance of differences in lysis of different target populations and by the CD8+ T effector cells and CD8- T cell control populations. Significant lysis was defined as lysis of a target cell by a certain CD8+ T effector population which was significantly higher than the background lysis of the CD8- negative control T cell population.

RESULTS

BM-MSC and ASC immunophenotype

Cell surface marker expression was analyzed by flow cytometry using a characterization panel for MSC. Both BM-MSC and ASC expressed CD13; CD73; CD90; CD105; CD166 and HLA-ABC and were negative for HLA-DR; CD31 and CD45, confirming their MSC immunophenotype (figure 2A).

Expression of HLA-class I was upregulated in BM-MSC and ASC after IFNγ stimulation (figure 2B). IFNγ stimulation did not affect MSC cell surface marker expression on ASC. In BM-MSC, only CD13 was slightly upregulated after IFNγ stimulation (data not shown).

BM-MSC induce HLA-class I specific lysis by CD8+ T cells

To evaluate the capacity of BM-MSC to induce CD8+ T cell mediated lysis, CD8+ T effector cells were isolated via FACS sorting after one week of co-culture of PBMC with HLA-AB mismatched BM-MSC. BM-MSC educated CD8+ T effector cells were capable of lysing Europium labeled BM-MSC identical to the one used in the co-culture (figure 3A). Lysis was dose dependent and reached a maximum of 24% (mean, range 13-37%) at a 40:1 effector: target (E:T) ratio, which was significantly higher than lysis by CD8- T cells (figure 3B). IFNγ stimulation of BM-MSC targets further increased lysis to 48% (mean, range 25-80%, figure 3A). In contrast, when BM-MSC-educated CD8+ T cells were exposed to IFNγ-stimulated or unstimulated ASC with a different HLA make-up, no lysis was detected (figure 3A) indicating that the BM-MSC induced lysis was HLA-class I specific.

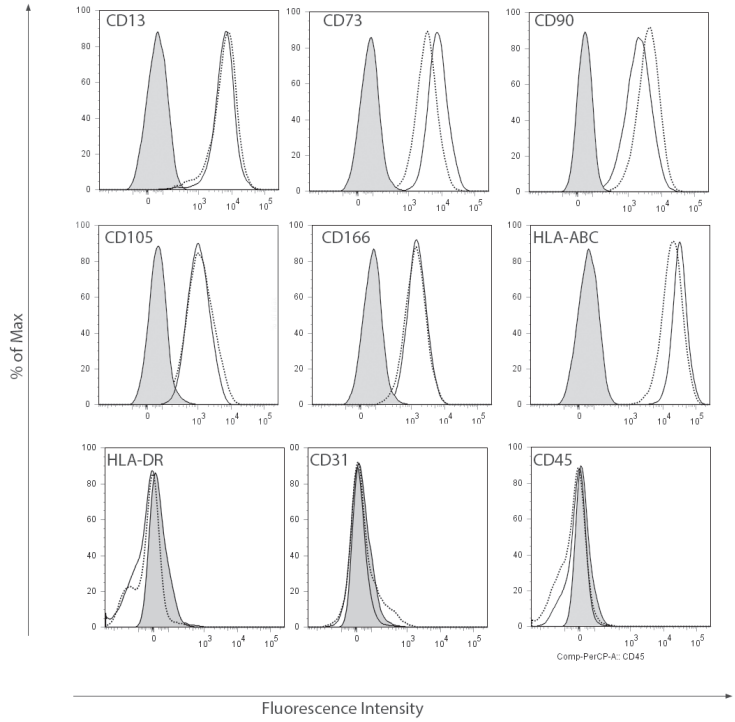

Figure 2A: Flow cytometric immunophenotyping of BM-MSC and ASC. BM-MSC (black solid lines) and ASC (black dotted lines) expressed MSC markers CD13, CD73, CD90, CD105, CD166 and HLA-ABC and were negative for HLA-DR, CD31 and CD45. No significant differences were detected. Grey solid histograms represent unstained control, n=4, representative examples are shown.

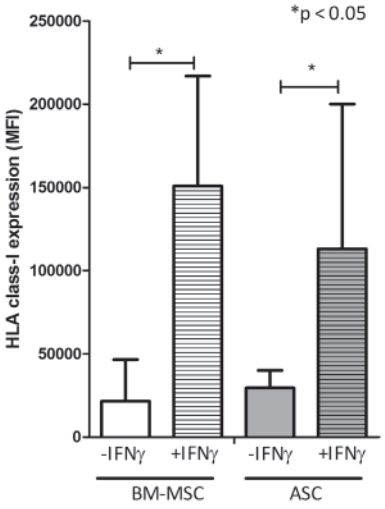

Figure 2B: IFNγ stimulation resulted in upregulation of HLA-class I expression on BM-MSC and ASC, mean MFI of n=5, * indicates p<0.05.

Figure 3

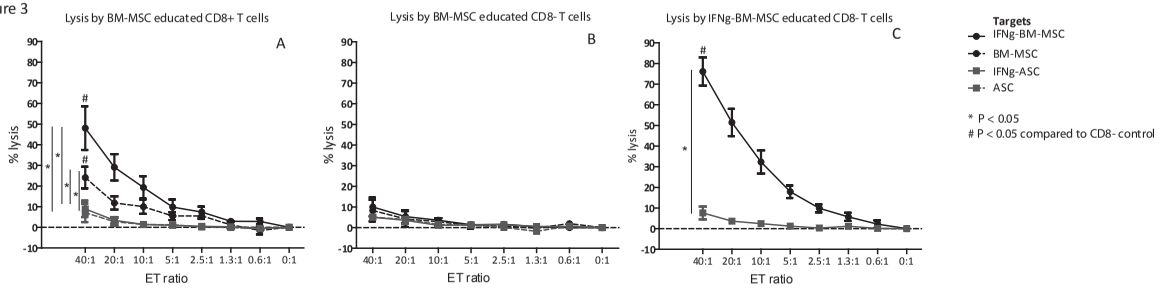

Figure 3ABC: A: Dose dependent lysis of BM-MSC by BM-MSC educated CD8+ T cells (black dashed line). Lysis increased when BM-MSC targets were IFNγ-stimulated (black solid lines) and there was no lysis of the mismatched ASC (grey lines) indicating HLA-class I specific lysis. Mean ± SEM, n = 5. B: The BM-MSC educated CD8- T cells were not capable of lysing any of the target populations. Mean ± SEM, n = 5. C: When CD8+ T cells were educated with IFNγ-stimulated BM-MSC, lysis of IFNγ-stimulated BM-MSC targets increased (black solid line). Lysis was HLA-class I specific as IFNγ-BM-MSC educated CD8+ T cells did not lyse the mismatched ASC (grey lines). Mean ± SEM, n = 5.

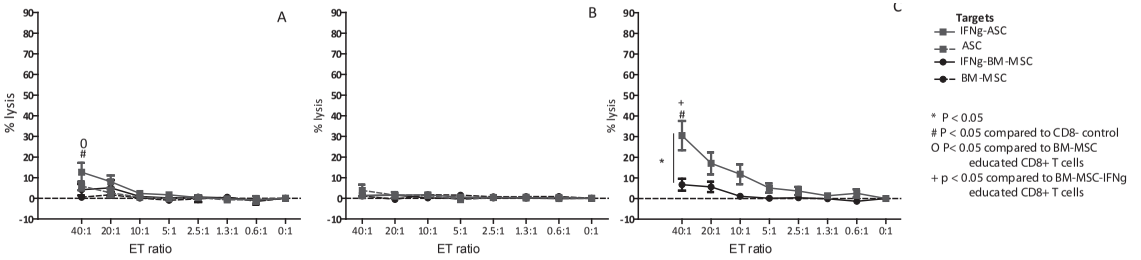

Figure 4ABC: A: CD8+ T cells educated with ASC were only capable of lysis of IFNγ-stimulated ASC (grey solid line) and not of the of the unstimulated ASC (grey dashed lines) nor the mismatched BM-MSC (black lines). Mean ± SEM, n = 5. B: The ASC educated CD8- T cells did not lyse any of the target populations. Mean ± SEM, n = 5. C: IFNγ-ASC educated CD8+ T cells were capable of significant and dose dependent lysis of IFNγ-stimulated ASC (grey solid line) and not of the mismatched BM-MSC (black line). This lysis was markedly lower than the lysis induced by IFNγ-stimulated BM-MSC (figure 3C, black solid line). Mean ± SEM, n=5.

IFNγ stimulation increases BM-MSC induced lysis

CD8+ T effector cells were educated with IFNγ-stimulated BM-MSC to study the effect of inflammatory conditions on the induction of CD8+ T cell mediated lysis. IFNγ-BM-MSC educated CD8+ T cells induced 76% lysis of IFNγ-stimulated target BM-MSC (mean, range 58-91%) (figure 3C3c), which was significantly higher than the lysis induced by unstimulated BM-MSC (figure 3a). No lysis of ASC with different HLA was observed (figure 3C) confirming the HLA-class I specific character of the lysis.

ASC induce less lysis compared to BM-MSC

Next, we studied the capacity of ASC to induce CD8+ T cell mediated lysis. Educated CD8+ T effector cells were generated by co-culturing ASC and mismatched PBMC. In contrast to BM-MSC educated CD8+ T cells, which significantly lysed BM-MSC, ASC- educated CD8+ T cells were only capable of lysing ASC when target ASC were pre-stimulated with IFNγ (mean 13%, range 4-21, figure 4A). This lysis of IFNγ-stimulated ASC target cells was significantly lower than the lysis of IFNγ-stimulated BM-MSC targets (fig 3Aby BM-MSC-educated CD8+ T cells (figure 3a).

IFNγ stimulation increases ASC induced lysis

Finally, to evaluate the influence of inflammatory conditions on ASC on their capacity to induce CD8+ cytotoxicity, PBMC were co-cultured with IFNγ-stimulated mismatched ASC. The IFNγ-ASC educated CD8+ T cells were capable of significant lysis of IFNγ-stimulated ASC targets (mean lysis of 31%, range 19-51%, figure 4C) and not of the mismatched IFNγ-stimulated BM-MSC (figure 4C). These data confirm the HLA-class I specificity of ASC induced lysis and further denote an effect of IFNγ stimulation on the ASC induced CD8+ mediated lysis, as also seen in the experiments performed with BM-MSC. However, the maximum lysis of 31% IFNγ stimulated ASC by IFNγ-ASC educated CD8+ T cells was substantially lower than the maximum lysis of 76% of IFNγ-stimulated BM-MSC by IFNγ-BM-MSC educated CD8+ T cells. Therefore, these results indicate a lower capacity of IFNγ-stimulated ASC to induce CD8+ cytotoxity compared to IFNγ-stimulated BM-MSC.

DISCUSSION

Currently, MSC have been applied in several clinical studies. Some of these studies indicated a clinical effect of MSC while others could not confirm MSC efficacy [12, 35-37]. These contradicting outcomes might be explained by a great variety in MSC preparations. MSC of bone marrow or adipose tissue and of autologous as well as allogeneic origin have been used. The effect of these differences in MSC preparation on study outcome is currently unknown. To decide on the use of allogeneic or autologous MSC, more knowledge on immunogenicity

of allogeneic MSC is required and potential differences between bone marrow and adipose tissue derived MSC need to be evaluated.

The use of allogeneic MSC has several benefits. It can be used as an 'off the shelf' therapy and recent studies suggest that allogeneic MSC can provide an effective treatment[22, 23] And, as several preclinical studies found MSC to be low immunogeneic [29-32], it has been suggested that allogeneic MSC can be used without risk for sensitization. However, other studies showed that allogeneic MSC are susceptible for lysis by CD8+ T cells[38] and NK cells [38, 39] and can induce memory T cells [40-42] and the production of IgG antibodies[43], which argues against a low-immunogeneic profile of MSC. In the current study we investigated whether MSC are capable of inducing HLA-class I specific CD8+ T cell cytotoxicity. We tested this for the most commonly used bone marrow derived MSC as well as adipose derived MSC, which are an important alternative.

In this study, we generated educated CD8+ T cells by co-culturing BM-MSC with HLA-AB mismatched PBMC, simulating the first exposure of allogeneic MSC to the recipient immune system. Next, we challenged those educated CD8+ effector T cells with MSC from the same donor that were used in the co-culture, to mimic a second exposure to MSC, or with MSC from an allogeneic HLA-AB mismatched donor. In this system, we found BM-MSC capable of inducing of HLA-class I specific lysis of allogeneic BM-MSC. This indicates that though allogeneic BM-MSC can be immunosuppressive in vitro [29], activation of the adaptive immune system by BM-MSC is not prevented by this anti-proliferative effect.

When used for treatment of inflammatory disease, MSC might face an inflammatory environment. Inflammatory conditions, which can be simulated with IFNγ, increase the immunosuppressive potential of MSC [6, 44]. However, in accordance with others [5], we found that BM-MSC and ASC also increase their expression of HLA-class I upon IFNγ stimulation. This upregulation could influence the induction of CD8 cytotoxicity as this is a HLA-class I restricted process. Thus, we used CD8+ T cells educated with IFNγ-stimulated BM-MSC and found that the lysis of IFNγ-stimulated BM-MSC was increased. This implies that inflammatory conditions in vivo might increase the immunogenicity of BM-MSC.

Next, we evaluated the immunogenicity of ASC. ASC are arising as an alternative for BM-MSC as they have several favorable characteristics: adipose tissue is easy to obtain and has a high yield of MSC; further, ASC are at least as immunosuppressive in vitro as BM-MSC[20, 21]. Surprisingly, we found that ASC were not capable of inducing CD8+ T cell mediated lysis. Significant lysis was only detected when ASC targets were IFNγ-stimulated. This indicates a less immunogeneic profile of ASC compared to BM-MSC as BM-MSC did induce significant CD8+ T cell mediated lysis. When using IFNγ-stimulated ASC to educate the CD8+ T cells, an increase in lysis of ASC was observed. However, this lysis was again markedly lower than the lysis induced by IFNγ-stimulated BM-MSC, confirming the less immunogeneic profile of ASC. We hypothesized that this difference in immunogenicity could be the result of levels of HLA-class I expression. Yet, although BM-MSC showed a higher fold increase in HLA-class

I expression upon IFNγ stimulation, HLA-class I levels were lower in unstimulated BM-MSC compared to ASC. Another potential explanation for the difference in lysis induced by BM-MSC and ASC might be found in the differences in age of BM-MSC and ASC donors used in this study. Age has been suggested to be of relevance for the differentiation capacity and composition of BM-MSC [16, 17]. However the proliferation rate of ASC was found to be unaffected by age [45] and the effect of age on other properties of BM-MSC and ASC such as the immunosuppressive capacity as well as their immunogeneic potential remains to be determined. Finally, the difference in immunogenicity between BM-MSC and ASC can be explained by for instance a difference in the expression of lysis inhibiting proteins such as serpins remains to be determined.

Taken together, our results indicate that BM-MSC and ASC can induce anti HLA sensitization. Whether this property will translate into sensitization when MSC are infused in vivo is unknown. It can be questioned whether MSC reside long enough after infusion to initiate this immune response [46]. Yet, the potential of MSC to induce anti HLA sensitization as described in this study plea for the use of autologous MSC treatment if possible and a careful consideration when applying allogeneic MSC. In particular situations, the use of allogeneic MSC is preferable such as in situations of acute organ failure or in cases when the patients' disease affect MSC functionality. In those situations, the risk of allogeneic MSC therapy can possibly be reduced by choosing a low risk study design e.g. using allogeneic MSC which are mismatched with the organ donor and screening of MSC recipients for absence of anti-donor reactivity prior to MSC treatment and monitoring for the development of anti-MSC donor immune responses after MSC treatment.

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Chapter 6

Adipose tissue derived mesenchymal stem cells are not affected by renal disease

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ABSTRACT

Mesenchymal stem cells are a potential therapeutic agent in renal disease and kidney transplantation. Autologous cell use in kidney transplantation is preferred to avoid anti-HLA reactivity; however, the influence of renal disease on mesenchymal stem cells is unknown. To investigate the feasibility of autologous cell therapy in patients with renal disease, we isolated these cells from subcutaneous adipose tissue of healthy controls and patients with renal disease and compared them phenotypically and functionally. The mesenchymal stem cells from both groups showed similar morphology and differentiation capacity, and were both over 90% positive for CD73, CD105, and CD166, and negative for CD31 and CD45. They demonstrated comparable population doubling times, rates of apoptosis, and were both capable of inhibiting allo-antigen- and anti-CD3/CD28-activated peripheral blood mononuclear cell proliferation. In response to immune activation they both increased the expression of pro-inflammatory and anti-inflammatory factors. These mesenchymal stem cells were genetically stable after extensive expansion and, importantly, were not affected by uremic serum. Thus, mesenchymal stem cells of patients with renal disease have similar characteristics and functionality as those from healthy controls. Hence, our results indicate the feasibility of their use in autologous cell therapy in patients with renal disease.

INTRODUCTION

Mesenchymal stem or stromal cells (MSC) are multipotent fibroblastic cells that can be derived from bone marrow and many other tissues including adipose tissue [1-3]. In culture, MSC are rapidly proliferating, adherent cells, showing a spindle-shaped morphology. They have colony forming capacity and the ability to differentiate into osteoblasts, chondrocytes and adipocytes [4]. There is no specific MSC marker and according to the criteria of the International Society of Cellular Therapy (ISCT), cultured MSC are characterized by a $CD14^-$, $CD45^-$, $HLA\text{-}DR^-$, $CD73^+$, $CD90^+$, $CD105^+$ phenotype[5]. In addition, they express CD13, CD166, HLA class I and lack the endothelial marker CD31. Recently, it is reported that freshly isolated adipose tissue derived MSC are initially CD34+ and increasingly express ISCT MSC markers after culture expansion [6].

MSC can modulate the immune system by inhibition of mitogen and allo-activated lymphocyte proliferation [7-9]. This effect is to a large extent dependent on soluble factors. MSC inhibit lymphocyte proliferation via the secretion of TGF-β, IL-10, nitric oxide (NO), HLA-G and hepatocyte growth factor (HGF) [7, 10-12] and the expression of indoleamine 2,3-dioxygenase (IDO) [13]. MSC furthermore secrete trophic factors that stimulate vasculogenesis and angiogenesis and aid tissue regeneration [14].

As a result, MSC receive a lot of interest in many medical fields as a cellular therapy. Currently, MSC therapy is subject of 216 registered clinical trials (http://clinicaltrials.gov, February, 2012).

In renal disease, patients may profit from the repair capacity and immunomodulatory potential of MSC [15]. In kidney transplantation, MSC therapy could find use as an induction/immunosuppressive therapy [16-19] and for repair of ischemia reperfusion injury.

There is a choice of MSC tissue source for therapeutic applications. While most studies have used MSC from bone marrow origin, adipose tissue has an advantage above bone marrow as it is obtainable in a less invasive manner and large numbers of cells can be cultured in less time. Another critical choice is whether to use MSC of autologous or allogeneic origin. In a (pre-) transplant setting it is crucial to avoid induction of HLA reactivity against a potential future donor and therefore exposure to allogeneic MSC is to be avoided. Autologous adipose tissue-derived MSC are thus the preferred choice of cells for use in renal disease and (pre-) transplant patients.

Although RD is known to affect the immune system [20], it is reported that functional regulatory T cells can be obtained from uremic patients [21]. However, it is unknown whether renal impairment affects the functionality of MSC. In patients with immune thrombocytopenic purpura, systemic lupus erythematosus, rheumatoid arthritis and aplastic anemia, numbers and/or functionality of MSC were reported to be impaired [22-25], whereas no influence of disease on MSC was found in multiple sclerosis and Crohn's disease [26-28].

There is a need to clarify whether MSC derived from RD patients are of the same quality as those obtained from healthy controls in order to determine whether it is safe and feasible to

use autologous MSC for RD and kidney transplant patients. Besides the influence of RD on the intrinsic properties of MSC, uremic conditions found in these patients may have an impact on MSC functionality as well. There is evidence that uremia affects endothelial progenitor cell differentiation and number [29, 30], but it is unknown whether uremia affects MSC functionality. Analysis of the effect of uremic conditions on MSC may predict the efficacy of MSC after administration to renal disease patients.

This is the first study reporting the effect of uremic conditions on human MSC functionality. Furthermore, we isolated adipose tissue-derived MSC from RD patients and healthy controls and compared their expansion potential and differentiation and immunomodulatory capacity. In addition, the genetic stability of MSC of renal disease patients in culture was investigated.

RESULTS

MSC isolation

Cultures of MSC were established by plating adipose tissue derived cell suspension in culture flasks. After 3 days, non-adherent cells were removed. MSC were successfully isolated from all 16 healthy controls (MSC-HC) and 16 renal disease patients (MSC-RD). MSC donor characteristics are depicted in table 1. Renal disease patients received standard of care medication for renal insufficiency. Additionally used medications are listed in the table.

Phenotypical characteristics of MSC

Morphology

MSC-HC and MSC-RD cultured in standard culture medium showed a similar spindle shaped morphology (Figure 1a).

Immunophenotype

The immunophenotype of MSC-HC and MSC-RD was characterized by flow cytometric analysis. In the freshly isolated cell fraction, we selected the HLA-ABC+CD45-CD31- fraction to obtain the non-hematopoietic, non-endothelial cell fraction and the CD34+CD73+ population to study the MSC. These fresh MSC further showed CD90 expression and CD105 and CD166 were expressed at low levels. There was no difference between the immunophenotype of freshly isolated MSC-HC and MSC-RD (figure 1b).

After culture expansion, MSC cell surface markers CD13, CD73, CD105, CD90 and CD166 were expressed in over 95% of MSC-HC and MSC-RD (figure 1c). CD31, CD34 and CD45 were absent, while HLA-DR was expressed at a low level. No differences between the immunophenotypes of cultured MSC-HC and MSC-RD were detected.

Table 1 Characteristics of mesenchymal stem cell donors (healthy controls and renal disease patients) at time of sampling the adipose tissue

	Healthy controls n=16	**Renal Disease patients n=16**
Gender (f/m)	10/6	6/10
Age, year (mean, range)	54.5 (25-73)	56.2 (26-72)
BMI (mean, range)	29.8 (22-38)	27.1 (20-31)
Creatinine, µmol/L (mean, range)	76 (65-95)	757 (240-1536)
GFR, mL/min/1.73 m^2 (mean, range)[a]	76.8 (63->90)	10.3 (8-17)[b]
Renal replacement therapy (RRT) (ND/PD/HD)	(16/0/0)	(6/8/2)
Duration of RRT (months, **range)**[c]	NA	16.2 (8.4-30.3)
Diabetes Mellitus[d]	0/16	4/16
Hypertension[e]	3/16	16/16
Medication use[f]		
anti-hypertentive drugs	3/16	16/16
erythropoiesis-stimulating agents	0/16	9/16
antiplatelet drugs	1/16	5/16
cholesterol lowering drugs	0/16	4/16
prednisone	0/16	2/16
insuline	0/16	2/16
KTx indication	NA	
Nephrosclerosis		6
Focal segmental glomerulosclerosis		2
Medullary cystic kidney disease		1
Adult-onset polycystic kidney disease		1
Rapidly progressive glomerulonephritis		1
Henoch-Schonlein purpura		1
IgA nephropathy		1
Membranous glomerulopathy		1
Diabetic nephropathy		1
Renal insufficiency e.c.i.		1

Abbreviations: BMI, body mass index; GFR, glomerular filtration rate; HD, hemodialysis; KTx, kidney transplantation indication; NA, not applicable; ND, no dialysis; PD, peritoneal dialysis; RRT, renal replacement therapy. [a]GFR was calculated with the Modification of Diet in Renal Disease Study (MDRD) calculation. [b]In the renal disease patient group, only pre-emptive patients are included. [c]Duration of RRT: NA for healthy controls. [d]Number of subjects with diabetes mellitus.[e]Number of subject with therapy requiring hypertension. [f]Most commonly used drugs (besides standard of care medication for renal insufficiency).

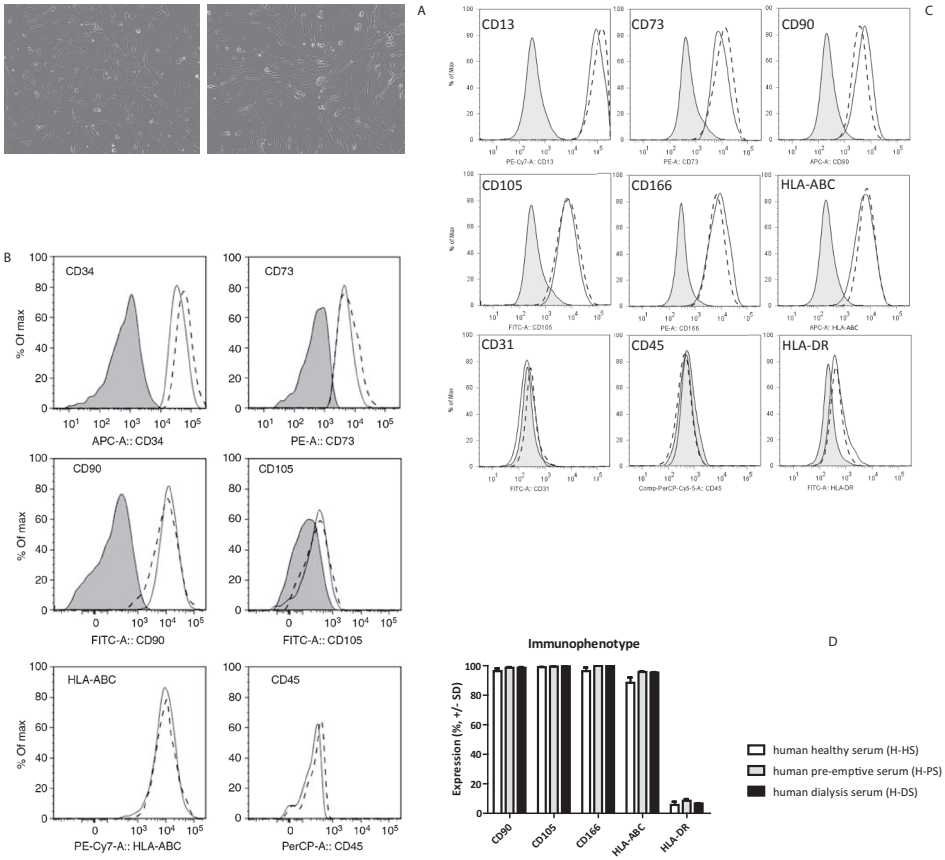

Figure 1. Characteristics of mesenchymal stem or stromal cell healthy control (MSC-HC) and mesenchymal stem or stromal cell renal disease (MSC-RD). (a) Microscopic image of representative example of MSC-HC (left) and MSC-RD (right; original magnification x 100; n=16 for MSC-HC and MSC-RD). (b) Representative example of flow cytometric analysis of cell surface marker expression on freshly isolated MSC-HC (solid lines) and MSC-RD (dashed lines; n=3 for patients and controls). Isotype IgG controls are represented by the solid histograms. For human leukocyte antigen (HLA) class I and CD45 no isotypes could be used, as these markers were used for gating of MSC from the cell suspension. (c) Representative example of flow cytometric analysis of cell surface marker expression on cultured MSC-HC (solid lines) and MSC-RD (dashed lines). Solid histograms represent unstained controls (n=4 for patients and controls). (d) Comparison of cell surface marker expression in MSC-RD cultured for 1 week in serum of healthy individuals (H-HS, white bars), pre-emptive renal disease patients (H-PS, gray bars), and dialysis patients (H-DS, black bars; n=4).

Serum analysis

To investigate the influence of uremia, MSC were studied after exposure to media containing 10% pooled human serum derived from healthy controls (human healthy serum H-HS) or pre-emptive (GFR 15-25) (human pre-emptive serum, H-PS) or dialysis patients (human dialysis serum, H-DS). The sera were tested for levels of creatinine, urea, C-Reactive Protein

(CRP), IL-1β and IL-6. Creatinine, urea and CRP levels were significantly increased in serum of pre-emptive renal disease patients compared to healthy individuals (respectively 86.7 versus 424.7 µmol/L, 6.25 versus 24.1 mmol/L and 1.55 versus 10.9 mg/L). Creatinine was further increased in serum of dialysis patients (920.8 µmol/L) while urea was still elevated compared to healthy serum but lower than in pre-emptive serum (15.9 mg/L). IL-1β and IL-6 were increased in dialysis serum only (Figure 2).

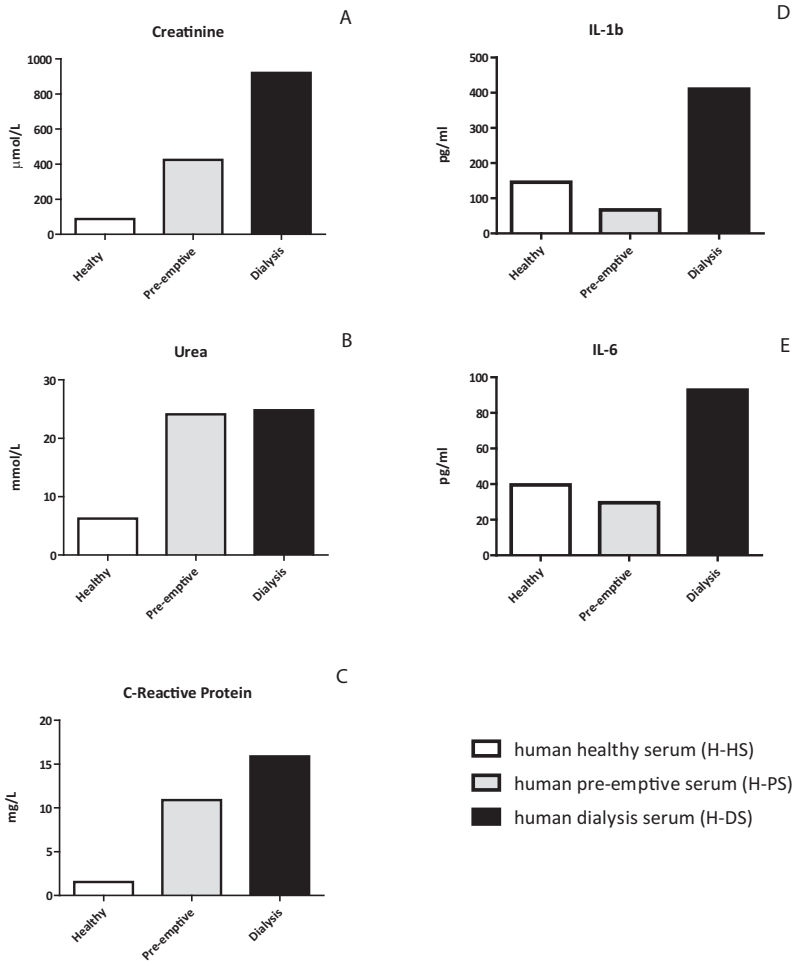

Figure 2. Characteristics of the different pooled human sera used to evaluate the influence of uremia on mesenchymal stem or stromal cell (MSC). Levels of (a) creatinine, (b) urea, (c) C-reactive protein, (d) interleukin (IL)-1β, and (e) IL-6 in pooled heat-inactivated human serum of healthy male individuals (H-HS, white bars), pre-emptive male RD patients (H-PS, gray bars), and male dialysis patients (H-DS, black bars). For the experiments, minimum essential medium-a (MEM-a) with 10% serum was used.

Phenotypical characteristics of MSC exposed to uremic conditions

Morphology

MSC-RD were cultured for 1 week in medium containing 10% H-HS, H-PS or H-DS. No morphological changes were detected.

Immunophenotype

Immunophenotypical analysis of MSC-RD was performed by flow cytometry after exposure for 1 week to medium containing 10% H-HS, H-PS or H-DS. No effects of the various sera on the immunophenotype of MSC-RD were detected (Figure 1d).

Proliferation potential of MSC

Population doubling time

The proliferation potential of MSC of healthy controls and renal disease patients was evaluated over 4 passages. MSC-RD demonstrated a mean population doubling time of 2.7 days (range 2.2-4.0), not significantly different from MSC-HC (2.8 days, range 2.2-4.3) (figure 3a). The population doubling times of MSC-HC and MSC-RD furthermore remained stable for at least 70 days of culture (figure 3b). Exposure of MSC-RD to human serum led to a decrease in population doubling time to 1.9 days (range 1.5-2.9). Culture in medium containing serum of pre-emptive and ESRD patients had no effect on the proliferation rate of MSC-RD (population doubling time 1.7, range 1.5-2.0 and 1.9, range 1.7-2.3 respectively)(Figure 3a).

Colony Forming Unit Efficiency

For comparison of the colony forming unit (CFU) efficiency of MSC-HC and MSC-RD, MSC were seeded at 3.0 cells/cm^2 and cultured for two weeks before colonies were counted. 28% of MSC-HC showed CFU capacity (range 19.2-48%) versus 21% of MSC-RD (range 14.0-30.4%), which was not significantly different (Figure 3c).

Apoptosis analysis

Apoptosis detection was performed using flow cytometric analysis of Annexin V staining in combination with 7-AAD viability staining. The experiment was performed with 3 MSC-HC and 3 MSC-RD cultures. A representative example is shown in Figure 3d. On average, viable cells represented 94.6% and apoptotic cells 4.1% of the expanded MSC-HC compared to 95% and 3.7% respectively of the MSC-RD, indicating there was no difference in apoptosis rate between MSC-HC and MSC-RD.

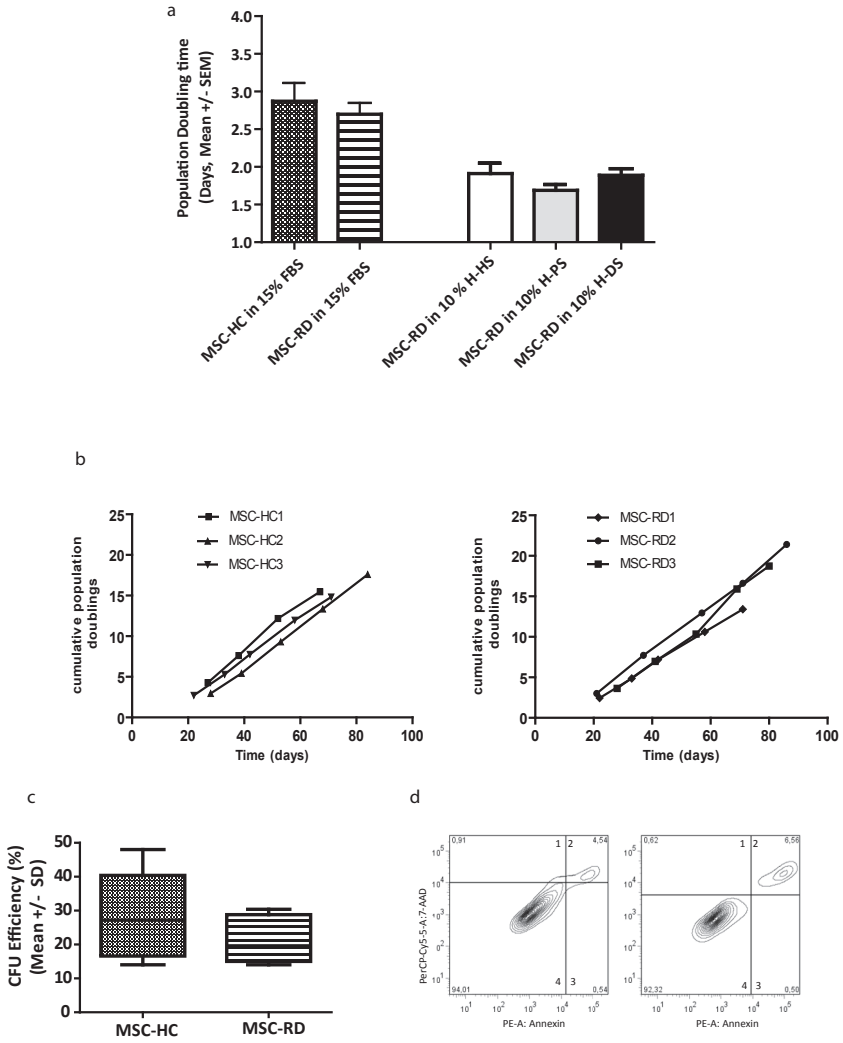

Figure 3. Proliferation, colony-forming unit (CFU) capacity, and apoptosis of mesenchymal stem or stromal cell healthy control (MSC-HC) and mesenchymal stem or stromal cell renal disease (MSC-RD). (a) Comparison of population doubling times of MSC-HC and MSC-RD cultured in 15% fetal bovine serum (FBS), of MSC-RD in 10% healthy human serum (H-HS), pre-emptive RD patients (H-PS), and of dialysis patients (H-DS; n=6). (b) Population doubling times of MSC-HC (left) and MSC-RD (right) remained stable for at least 70 days of culture (n=3). (c) CFU efficiency of MSC-HC and MSC-RD (n=5). (d) Representative example of apoptosis analysis in MSC-HC (left) and MSC-RD (right) under standard culture conditions (n=3).

Functional characteristics of MSC

Differentiation

When exposed to differentiation medium, both MSC-HC and MSC-RD showed osteogenic and adipogenic differentiation capacity as demonstrated by histological detection of calcium depositions with von Kossa staining for osteogenic differentiation and staining of lipid droplets with oil red O for adipogenic differentiation (Figure 4). Differentiation capacity was not affected by exposure to uremic conditions (data not shown).

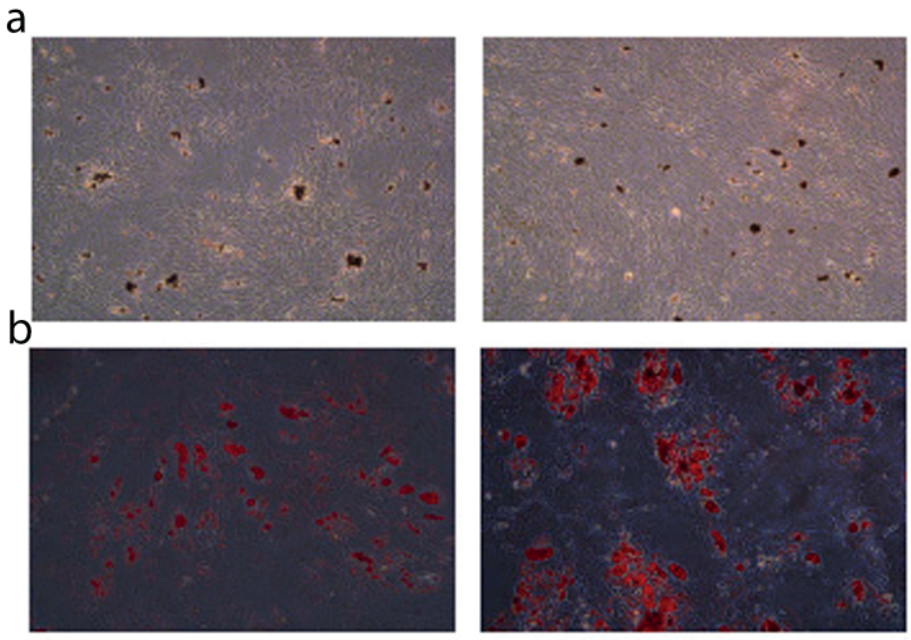

Figure 4. Differentiation capacity of mesenchymal stem or stromal cell healthy control (MSC-HC) and mesenchymal stem or stromal cell renal disease (MSC-RD). (a) Osteogenic differentiation of MSC-HC (left) and MSC-RD (right) was determined after culturing for 3 weeks under osteogenic conditions by von Kossa staining (original magnification x100; n=3). (b) Adipogenic differentiation of MSC-HC (left) and MSC-RD (right) was determined after culturing for 2 weeks under adipogenic conditions by oil red O staining (original magnification x100; n=3).

Immunosuppressive capacities

The immunomodulatory capacities of MSC-HC and MSC-RD were examined by analysis of their ability to inhibit the proliferation of allo-antigen and anti-CD3/CD28 activated peripheral blood mononuclear cells (PBMC). The effect of MSC on allo-antigen activated PBMC was evaluated in Mixed Lymphocyte Reaction (MLR) assays (n=16) using 5 different MSC-HC, 5

different MSC-RD and minimal 1-2-2 HLA-A, -B, and -DR mismatched PBMC. In all experiments both MSC-HC and MSC-RD were capable of a significant and dose-dependent suppression of PBMC proliferation up to a 1:20 dilution. No significant difference was detected between the suppressive function of MSC-HC and MSC-RD (Figure 5a).

The ability of MSC-HC and MSC-RD to inhibit the proliferation of activated PBMC was confirmed by adding MSC to anti-CD3/CD28 activated PBMC. MSC-HC and MSC-RD showed a significant and similar inhibition of anti-CD3/CD28 activated PBMC at ratios of 1:2.5 to 1:40 (Figure 5b).

We next examined whether uremic serum containing medium affected the immunosuppressive effect of MSC. While MSC survival and proliferation was unaffected by uremia, PBMC showed a severely reduced proliferation capacity after stimulation with allogeneic PBMC in medium containing serum from dialysis patients. Therefore no inhibitory effect of MSC could be determined (Figure 5c). Proliferation of PBMC was preserved after stimulation with anti-CD3/CD28 in medium containing serum of pre-emptive RD patients, while proliferation was reduced but detectable when exposed to 10% dialysis serum. MSC were capable of further inhibiting the proliferation of anti-CD3/CD28 stimulated PBMC proliferation in 10% pre-emptive and dialysis serum, demonstrating that PBMC functionality was, but MSC functionality was not affected by uremic conditions. To summarize, MSC maintained their inhibitory effect on PBMC proliferation under uremic conditions (Figure 5d).

Expression of pro-inflammatory and anti-inflammatory gene products

MSC respond to inflammatory conditions with an increase in the expression of both pro- and anti-inflammatory factors. To compare the capacity of MSC-HC and MCS-RD to respond to inflammatory stimuli, MSC were cultured with allo-antigen activated PBMC (MLR) in a transwell system. MSC-RD were furthermore cultured in serum of pre-emptive renal disease and dialysis patients. After 1 and 3 days MSC were harvested and mRNA isolated. MSC-HC and MSC-RD cultured in the different sera showed a similar response to the pro-inflammatory conditions. They increased expression of anti-inflammatory indoleamine 2,3-dioxygenase (IDO) and programmed death ligand 1 (PD-L1), while the expression of transforming growth factor beta TGF-β was mildly reduced (Figure 5e). The expression of the pro-inflammatory cytokines IL-1β, IL-6 and IL-8 was increased in all MSC tested, demonstrating that MSC-HC and MSC-RD follow the same trend in gene expression in response to inflammatory stimuli (figure 5e). MSC-RD exposed to 10% uremic sera showed the same gene expression profile as MSC-RD in healthy serum, except for IL-1β, which was induced at lower levels after 1 day of culture with MLR (figure 5f).

Cytogenetic and molecular genetic analysis of expanded MSC-RD

As genomic stability of MSC is crucial for their use in future clinical trials, SNP array based whole genome analysis of 3 expanded MSC-RD was performed. As a control the non-cultured

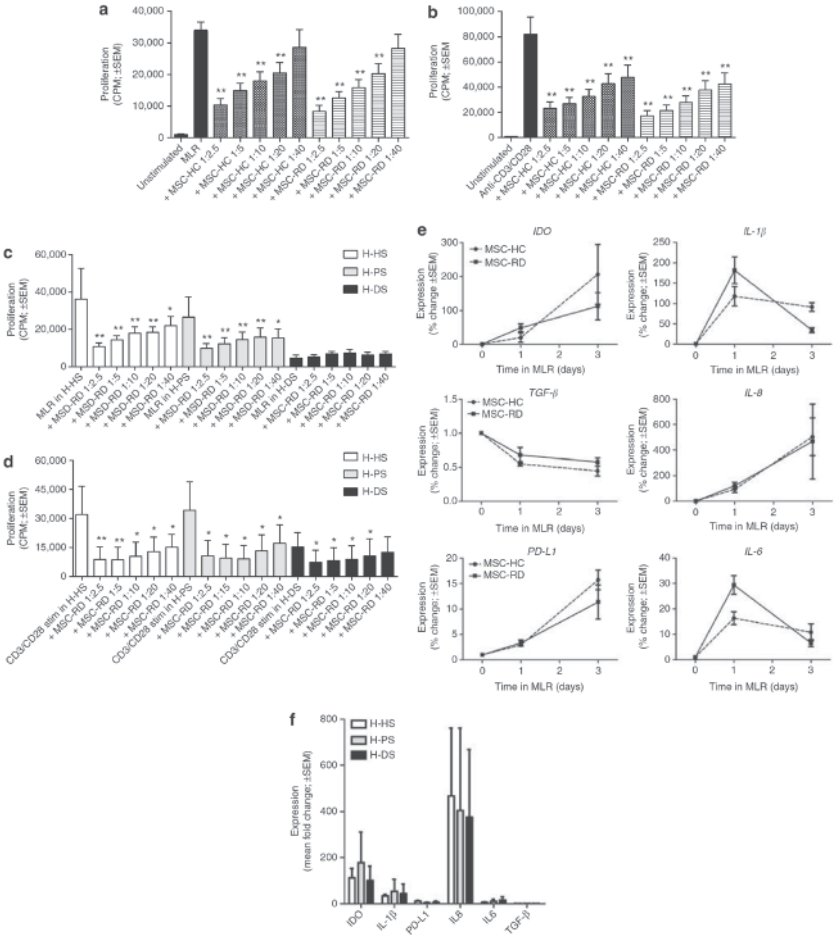

Figure 5. Immunomodulatory effect of mesenchymal stem or stromal cell healthy control (MSC-HC; n=5) and mesenchymal stem or stromal cell renal disease (MSC-RD; n=5). (a) Inhibition of the proliferation of allo-activated PBMCs (mixed lymphocyte reaction (MLR), minimal 1-2-2 human leukocyte antigen (HLA)-A, -B, and -DR mismatched) by MSC-HC and MSC-RD (n=16). (b) Inhibition of the proliferation of anti-CD3/CD28 stimulated PBMCs by MSC-HC and MSC-RD (n=12). (c) Inhibition of the proliferation of allo-activated PBMCs (MLR, minimal 1-2-2 HLA-A, -B, and -DR mismatched) by MSC-RD in human serum of healthy individuals, pre-emptive RD patients and of patients on dialysis (n=6). (d) Inhibition of the proliferation of anti-CD3/CD28-stimulated PBMCs by MSC-RD in the three sera. *P<0.05, **P<0.01, compared with no MSCs in the corresponding serum (n=3). (e) mRNA expression of anti-inflammatory (indoleamine 2,3-dioxygenase (IDO), transforming growth factor-β (TGF-β), and programmed death ligand 1 (PD-L1)) and pro-inflammatory (interleukin (IL)-1β, IL- 6, and IL-8) genes by MSCs after stimulation with allo-activated PBMCs (MLR). Data shown represent the fold change in the expression compared with control MSCs cultured without MLR (MSC-HC n=5, MSC-RD n=4). (f) mRNA expression of IDO, TGF-β, PD-L1, IL-1β, IL-6, and IL-8 genes by MSC-RD after 3-day stimulation with allo-activated PBMCs (MLR; mean fold change in expression compared with control MSCs cultured without MLR) in medium containing 10% human healthy serum (H-HS), human pre-emptive serum (H-PS), or human dialysis serum (H-DS; n=3). CPM, counts per minute.

PBMC of the MSC donor were used. Using a window of 3 SNP or 50KB, we did not detect any Copy Number Variations (CNV) among the PBMC/MSC pairs. Even on the single SNP level we did not observed a significant change in SNP calling (less than 2 out of 317000)

Karyotyping of cultured MSC-HC and MSC-RD revealed only normal karyotypes.

In addition, fluorescence in situ hybridisation (FISH) was performed on 5 MSC cultures (3 healthy controls (age 62, 64 and 56) controls and 3 renal disease patients (age 26, 37 and 77)) which were all cultured for 10 population doublings. The results indicated karyotypic normal diploid cells in >95% of MSC of both healthy controls and RD patients (representative example of diploid cell in figure 6). A small number of tetraploidic MSC was detected. This has been described previously [31].

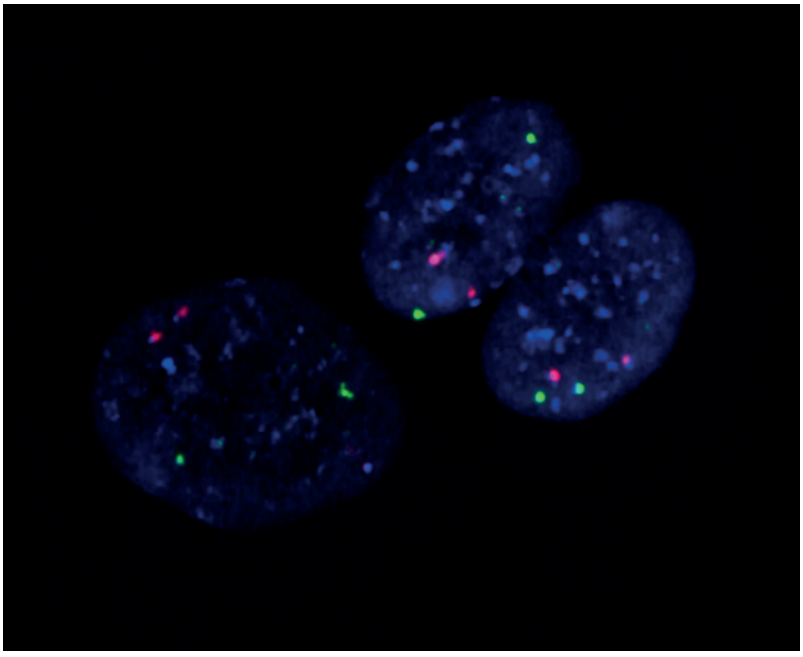

Figure 6. Fluorescence in situ hybridization (FISH) analysis: representative example of three diploid cells. The 4,6–diamidino-2–phenylindole (DAPI) counterstained nuclei are visible. In each nucleus two green spots (centromere 3 signal) and two red spots (centromere 8) can be detected.

DISCUSSION

In this study we investigated the effects of kidney disease on adipose tissue derived MSC. Kidney disease is characterised not only by a systemic accumulation of creatinine and urea but also by associated chronic inflammation, shown by high levels of C-reactive protein in patients with RD [32]. Uremic conditions and systemic inflammation have a clear impact on human health. On the cellular level, kidney disease impairs the functioning of immune cells, as we confirmed in the present study by showing a decreased proliferative response to allo-activation of PBMC in uremic serum. In contrast, *in vitro* exposure of MSC to uremic conditions did not compromise the proliferation rate, differentiation or immunomodulatory capacity of MSC. This is the first study that indicates that MSC are resistant to high levels of uremia. Earlier we demonstrated that adipose tissue derived MSC increased their immunosuppressive capacity when stimulated with the proinflammatory cytokines IFN-γ, TNF-α and IL-6 [33]. In the present study we found no effect of serum of RD patients on the immunosuppressive capacity of MSC. This suggests that the uremic and inflammatory conditions in RD patients do not compromise the immunosuppressive function of MSC.

In addition, MSC of renal disease patients did not differ phenotypically and functionally from MSC of healthy individuals. Immediately after isolation from adipose tissue, the immunophenotype of renal disease MSC was indistinguishable from MSC of healthy individuals. After culture expansion both MSC-HC and MSC-RD adapted to the ISCT immunophenotypes which remained stable in culture. Their proliferation rate was rapid and non-declining, which would guarantee that sufficient numbers of MSC of RD patients can be generated in short amount of time. Our results therefore indicate that renal disease has no intrinsic and extrinsic effect MSC. This is important for the feasibility of clinical application of autologous MSC in renal disease patients.

An important matter of concern for cellular therapy is the genetic stability of the cells in culture. Studies reporting malignant transformation of MSC in culture [34, 35] have recently been retracted as the cultures investigated were contaminated with tumor cell lines [36]. This strengthens the hypothesis that MSC are genetically stable[37]. Nevertheless, screening for genetic stability remains important to ensure that healthy cells are put back into patients, particularly when the cells have been derived from a diseased milieu. The copy number variation analysis as carried out in the present study is very suitable for this purpose.

Whether MSC used for cell therapy should be of allogeneic or autologous origin is a delicate question. It has been suggested that autologous cell usage harbours the risk for homing of malignant cells. However, we recently found that autologous MSC are susceptible to lysis by NK cells [38] and are therefore unlikely to be of greater risk for tumor formation than allogeneic cells. Although allogeneic MSC are more practical and cost effective, as they can be cultured in advance and used as an off-the-shelf product, their use may have some hazards. It has been demonstrated that allogeneic MSC can be immunogeneic [39]. Whether potential

immunogenicity of MSC would hamper their clinical effects is unknown. In clinical organ transplantation, however, the introduction of foreign HLA might trigger sensitisation against a potential future donor organ. At this point in time, therefore, MSC therapy with autologous cells is recommended in RD patients. When there is more known about the development of sensitisation against allogeneic MSC, therapy with allogeneic MSC may become preferable.

We have demonstrated that MSC can be successfully isolated and expanded from adipose tissue of RD patients and that these MSC are fully functional, even under uremic conditions. These results remove one of the barriers for developing therapies with autologous MSC in RD.

METHODS

Sources of adipose tissue

Pre kidney transplantation patients (renal disease patients) (n=16) and healthy live kidney donors (healthy controls) (n=16) were included after written informed consent as approved by the Medical Ethical Committee of the Erasmus MC (protocol no. MEC-2006-190). Their characteristics are shown in table 1. (GFR was calculated using the MDRD Study Equation). During the kidney donation and transplantation procedure, subcutaneous adipose tissue was surgically removed from the abdominal incision and collected in minimum essential medium alpha (MEM-α) (Invitrogen, Paisley, Scotland) with 100IU/ml penicillin and 100µg/ml streptomycin (p/s) (Invitrogen).

MSC isolation, culture and differentiation

MSC from renal disease patients (MSC-RD) and healthy controls (MSC-HC) were isolated from the adipose tissue as described previously [3, 40]. In brief, adipose tissue was mechanically disrupted, enzymatically digested with sterile 0.5mg/mL collagenase type IV (Sigma-Aldrich, St. Louis, MO) in RPMI-1640 + glutaMAX (Invitrogen) and p/s for 30 min at 37°C. For characterization of freshly isolated MSC, erythrocytes were lysed with RBC Lysis Buffer (eBioscience) according to protocol. For culture expansion, cells were resuspended in MSC-culture medium, consisting of MEM-α with 1% p/s and 15% fetal bovine serum (FBS) (Biowithaker), transferred to a 175 cm^2 culture flask (Greiner Bio-one, Essen, Germany) and kept at 37°C, 5% CO_2, 95% humidity. Medium was changed every 3-4 days. When >90% confluent, MSC were detached using 0.05% trypsin-EDTA at 37°C. Different MSC cultures were used for experiments between passages 1 and 5.

To confirm the differentiation capacity of MSC, osteogenic and adipogenic differentiation was induced as described previously [40].

Proliferation

For calculation of population doubling time, MSC were seeded at a density of 1000 cells/cm^2 and cultured until 90% confluency was reached, after which the cells were detached by trypsinisation, counted and re-seeded.

Colony forming unit (CFU) assay

MSC were seeded at 50 cells per 6 cm diameter culture dishes in quintuple (3.0 cells/cm^2). After 2 weeks of culture, medium was removed, the dishes washed once with 1x PBS and fixed in 70% ethanol for 5 minutes. Colonies were stained with 2.3% crystal violet solution (Sigma-Aldrich) for 30 minutes. Dishes were then washed with tap water and colonies (>1mm) counted. CFU efficiency was expressed as the percentage of cells capable of forming colonies.

Flow Cytometric Characterization of MSC

MSC were immunophenotyped immediately after isolation or after culture expansion. Cultured cells were trypsinised and washed with FACSFlow (BD Biosciences, San Jose, CA). Cell suspensions were incubated with mouse-anti-human monocolonal antibodies against CD13-PECy7, CD45-PERCP, HLA-DR-FITC, HLA-ABC-APC (all BD Biosciences), CD14-PE (Serotec), CD31-FITC, CD73-PE, CD166-PE (BD Pharma) CD90-APC, CD105-FITC (R&D Systems) at room temperature in the absence of light for 15 minutes. After two washes with FACSFlow, flow cytometric analysis was performed using an 8 color FACSCANTO-II with FACSDIVA Software (BD Biosciences) and FlowJo Software (Tree Star Inc. Palo Alto, CA). Appropriate IgG antibodies were used (BD Pharmingen) for isotype control. For apoptosis analysis, an Annexin V-PE apoptosis Detection Kit I (BD Pharmingen) was used according to the manufacturer's description.

PBMC isolation

Peripheral blood mononuclear cells (PBMC) were collected from buffy coats of healthy blood bank donors (different than the MSC donors). PBMC were isolated by density gradient centrifugation using Ficoll Isopaque (δ=1.077, Amersham, Uppsala, Sweden) and frozen -135°C until use [41] .

Serum collection

Serum of pre-emptive male renal disease patients listed for kidney transplantation was collected and pooled from 10 individuals (Human Pre-emptive Serum H-PS). Furthermore, serum of male patients with terminal renal insufficiency who received renal replacement therapy by hemodialyis was collected just pre dialysis and pooled from 10 individuals (Human Dialysis Serum, H-DS). Control serum was collected from male healthy individuals (Blood Bank donors) and pooled from 7-10 individuals (Human Healthy Serum (H-HS).

All sera were heat inactivated and were tested for creatinine, CRP, urea and Il-1 β and IL-6 levels.

Mixed Lymphocyte Reactions

In mixed lymphocyte reactions (MLR), 5×10^4 PBMC were seeded in round-bottom 96-well plates and stimulated with 5×10^4 γ-irradiated (40 Gy) HLA-A, -B and -DR mismatched PBMC in MEM-α with p/s and 10% heat-inactivated (HI)-human serum (pre-emptive, dialysis or healthy). MSC were added to the MLR at a 1:2.5, 1:5, 1:10, 1:20 and 1:40 ratio. On day 7, proliferation was measured by incorporation of ^{3}H-thymidine (0.5 µCi/well, 8-hour incubation) using a β-plate reader (LKB, Bromma, Sweden).

To determine the proliferation capacity of the PBMC, 5×10^4 cells were stimulated with 1 µg/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. Experiments were performed in triplicate and medians were used for further analysis.

Anti-CD3 /CD28 lymphocyte stimulation assay

PBMC were stimulated with anti-human-CD3 (0.5 µl/5×10^5 cells), anti-human-CD28 (0.5 µl/5×10^5 cells) and goat-anti-mouse antibody (1 µl/5×10^5 cells) for cross-linking (all BD Pharmingen). PBMC were seeded in round-bottom 96-well plates at 5×10^4 cells per well and MSC were added at 1:2.5, 1:5, 1:10, 1:20 and 1:40 ratios in MEM-α with p/s and 10% heat inactivated (HI)-human serum (pre-emptive, dialysis or healthy).

On day 3, proliferation was measured by incorporation of ^{3}H-thymidine using a β-plate reader (LKB, Bromma, Sweden). Only results of PBMC with sufficient proliferation (>10,000 cpm) were included. All experiments were performed in triplicate and medians were used for further analysis.

Real-time RT-PCR

MSC were seeded in 6 well plates at 2×10^5 per well in the presence or absence of an MLR separated from the MSC by a transwell system (1×10^6 responder x 1×10^6 stimulator PBMC, HLA-A, -B and -DR mismatched). At day 0, day 1 and day 3, MSC cell pellets were harvested. RNA was isolated and cDNA synthesized as described previously [42]. Quantitative expression was determined by real-time RT-PCR using universal PCR mix (Invitrogen) and Assays-on-demand for IDO (Hs00158627.m1), PDL-1 (H200204257.m1), TGF-β (Hs00171257.m1), IL-1β (Hs01555410.m1), IL-6 (Hs00174131.m1), and IL-8 (Hs00174114.m1) (all Applied Biosystems, CA) on an ABI PRISM 7700 sequence detector (Applied Biosystems). Expression levels were calculated as ratio per 18S RNA or as copies mRNA per 500 ng of total RNA.

Screening genetic stability

Cytogenetic and molecular analysis

GTG banded chromosomes were prepared following standard procedures and karyotypes were described following the recommendations for cytogenetic nomenclature [43].

FISH analysis

From 5 control and 3 renal disease patients, part of the cultured MSC cells were used for dual-color FISH using centromeric chromosome 3 and 8 probes. DNA of the plasmids probes Pα3.5(centromere 3) and D8Z2 centromere 8) were labelled with the Random Prime labelling system (Invitrogen, Corporation, Carlsbad, California, USA) using Bio-16-dUTP or Dig-11-dUTP (Roche Applied Science, Indianapolis, USA). Of each probe 5 ng labelled product was mixed denatured and hybridised overnight at 37ºC Next day the slides were washed (2x SSC and 0.1x SSC, 0.1% Tween) at 55ºC and incubated with Streptadivine Alexa 594 and anti-Digoxiginine FITC for 2 hours. After washing (2xSSC, 0.1xSSC, 55C) the slides were counter-stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Zwijndrecht, Netherlands) and mounted in anti-fade solution (Dabco-Vectashield 1:1(Vector Laboratories, Burlingame, California, USA).

For each hybridization, a minimum of 300 nuclei were scored with an Axioplan 2 Imaging microscope (Carl Zeiss,Sliedrecht the Netherlands) using a chroma-sp-100 DAPI and a chroma-sp- 103v1 red filter(Chroma Technology, Bellows Falls, VT, USA) and the images were captured with the ISIS software (Metasystems, Altlussheim, Germany).

[44, 45].

DNA isolation

DNA was isolated from frozen PBMC ($10x10^6$) and MSC ($10x10^6$) using the QIAamp DNA mini-kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA quantity was measured using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA).

High-density SNP-based oligonucleotide array analysis

Whole genome analysis using Illumina cyto-SNP bead chip version 12.2 (Illumina San Diego, CA, USA) array was performed on DNA derived from MSC-RD expanded roughly 1000-fold in culture. Genomic DNA (200ng) from the PBMC and MSC sample was processed using reagents and protocols as described by the supplier. The data were analyzed using Beadstudio software (Illumina), which allows for the visualization of several different variables relevant to the detection of copy number alterations (CNA), including the B-allele frequency (BAF) and log R ratio (LRR) [46]. The normalized intensity ratio for each SNP in the samples was compared with a 40 CEU HapMap reference set. The BAF and LRR output data were also analyzed and visualized for CNA's by using the software program Nexus Copy Number (Nexus

BioDiscovery, El Segundo, CA, USA). Results were compared with an in-house database of known copy number variations, and the public available copy number variations dataset containing ~3500 healthy controls (DGV Dataset of Genomic Variants and CHOPS data base).

In addition to the paired analysis for deletions and duplications in MSC versus PBMC DNA, we also looked for non concordance for each SNP marker present on the array using the Genomestudio GT® paired analysis settings.

STATISTICAL ANALYSIS

Data were analyzed using the (two-tailed) Wilcoxon signed-rank test. Statistical significance was defined as P less than 0.05.

DISCLOSURE

None of the authors have a relationship with companies that have a financial interest in the information contained in this manuscript.

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Chapter 7

Bone marrow-derived mesenchymal stromal cells from patients with end-stage renal disease are suitable for autologous therapy

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ABSTRACT

Background aims. Mesenchymal stromal cells (MSCs) are pluripotent cells that have immunosuppressive and reparative properties *in vitro* and *in vivo*. Although autologous bone marrow (BM)-derived MSCs are already clinically tested in transplant recipients, it is unclear whether these BM cells are affected by renal disease. We assessed whether renal failure affected the function and therapeutic potential of BM-MSCs. Methods. MSCs from 10 adults with end-stage renal disease (ESRD) and 10 age-matched healthy controls were expanded from BM aspirates and tested for phenotype and functionality *in vitro*. Results. MSCs from ESRD patients were >90% positive for CD73, CD90 and CD105 and negative for CD34 and CD45 and showed a similar morphology and differentiation capacity as MSCs from healthy controls. Of importance for their clinical utility, growth characteristics were similar in both groups, and sufficient numbers of MSCs were obtained within 4 weeks. Messenger RNA expression levels of self-renewal genes and factors involved in repair and inflammation were also comparable between both groups. Likewise, microRNA expression profiling showed a broad overlap between ESRD and healthy donor MSCs. ESRD MSCs displayed the same immunosuppressive capacities as healthy control MSCs, demonstrated by a similar dose-dependent inhibition of peripheral blood mononuclear cell proliferation, similar inhibition of proinflammatory cytokines tumor necrosis factor-a and interferon-g production and a concomitant increase in the production of interleukin-10. Conclusions. Expanded BM-MSCs procured from ESRD patients and healthy controls are both phenotypically and functionally similar. These findings are important for the potential autologous clinical application of BM-MSCs in transplant recipients.

INTRODUCTION

It has been postulated in recent years that mesenchymal stromal cells (MSCs) may be useful in modifying and potentially reversing loss of function in kidney disease and in preventing allograft rejection in transplant recipients [1-6]. MSCs were first discovered in bone marrow (BM) aspirates but have also been isolated and expanded from various other adult tissues [7]. At the present time, no unique phenotype has been identified that allows the prospective isolation of MSCs. Their isolation and characterization relies primarily on their ability to adhere to plastic, their multi-lineage differentiation and their membrane antigen profile [8].

Pre-clinical studies indicate that administration of MSCs ameliorates renal injury and accelerates tissue repair, and numerous experimental studies have demonstrated beneficial effects after solid organ transplantation [2, 4, 5, 9-13]. The potential mechanism of MSC-induced kidney repair has been addressed in numerous studies [10, 13-18]. There is increasing evidence that the process of trans-differentiation is rare and of limited biologic relevance [15]. It is currently believed that paracrine factors are responsible for the effects of MSCs, including their mitogenic, anti-apoptotic, angiogenic and various immune-modulating properties [19-25].

In human kidney transplantation, there is great interest in the therapeutic application of MSCs, and the first clinical trials using autologous BM-MSCs in kidney transplantation with the initial focus on their potential as induction therapy and to treat allograft rejection have started [20, 26, 27]. A concern for the use of allogeneic MSCs is the potential induction of an allo-response with negative consequences for MSC efficacy and potentially for graft survival in the case of transplantation. A concern for the use of autologous MSCs includes their potential dysfunction secondary to the underlying disease, as reported in systemic lupus erythematosus, immune thrombocytopenic purpura, rheumatoid arthritis, multiple myeloma and aplastic anemia [28-32], and a potential safety risk related to genetic stability. A few pre- clinical studies have reported on the impact of renal disease on the phenotype and function of MSCs. In mice, functional incompetence of MSCs was reported after exposure to uremic conditions [33]. In human BM-MSCs, uremic serum induced an osteoblast-like phenotype accompanied by matrix remodeling and calcification [34], whereas human adipose tissue- derived MSCs are not affected by renal disease [35]. None of these studies has studied human BM-MSCs isolated from patients with end-stage renal disease (ESRD); so far, BM-MSCs are the only cell product used in clinical trials with MSCs in nephrology.

We performed a head-to-head comparison between BM-MSCs from patients with ESRD and from healthy controls regarding their phenotypic and functional characteristics. More recently, micro- RNAs (miRNAs) have been shown to play a critical role in differentiation and MSC functionality[36], and their dysregulation has been shown in various diseases including kidney diseases [37, 38].Because miRNA expression in MSCs from patients with ESRD is completely unknown, we have included in this study comparison of miRNA expression in

MSCs from patients with ESRD and healthy controls. We believe our results to be important for clinical application in transplant recipients.

METHODS

Patients and controls

BM samples were collected from patients with ESRD and from healthy controls that were either BM donors in a clinical renal transplantation setting or patients undergoing orthopedic surgery. Ethics committee approval was obtained for the aspiration

protocol, and individual written consents were obtained from all individuals. Two age-matched groups of 10 individuals each were compiled for the analyses.

Isolation and culture of MSCs

Heparinized BM was aspirated under local or general anesthesia. The mononucleated cell fraction was isolated by Ficoll density gradient separation and plated in tissue culture flasks at a density of 16 x 10^3mononucleated cells/cm^2in low-glucose Dulbecco's modified Eagle medium (DMEM; Invitrogen, Breda, the Netherlands) supplemented with penicillin/ streptomycin (Lonza) and 10% fetal calf serum (Thermo Scientific HyClone). The cultures were maintained at 37°C 5% carbon dioxide. The medium was refreshed twice a week. When the MSC colonies or cultures reached confluence, the cells were collected using trypsin (Lonza) and re-plated at 4 x 10^3 cells/cm^2.

Characterization of MSCs

The expanded MSC populations were characterized by morphology (spindle-shaped cells) and by fluorescence-activated cell sorter analysis for expression of the following membrane markers: HLA class I (ABC), HLA class II (DR), CD31, CD34, CD 45, CD73, CD80, CD90 and CD105. All specific fluorochrome-labeled antibodies and isotype controls were purchased from BD Biosciences (BD Bioscience, Franklin Lakes, NJ, USA) except for CD105 (Ancell Corporation, Bayport, MN, USA).

Differentiation of MSCs

For adipogenic and osteogenic differentiation, 50 x 10^3 MSCs were plated in a 24-well plate in duplicate. Adipogenic and osteogenic differentiation medium consisted of basic medium 1 (a-MEM/ 4 mmol/L L-glutamine/penicillin/streptomycin, 10% fetal calf serum) supplemented with insulin (10 µg/mL), 5 µmol/L 3-isobutyl-1-methylxanthine, 50 µmol/L indomethacin, 100 nmol/L dexamethasone and 50 µg/mL vitamin C. The medium was refreshed weekly with adipogenic and osteogenic differentiation medium supplemented with 5 mmol/L β-glycerophosphate. After a culture time of 3 weeks, the cells were stained for alkaline

phosphatase expression using fast blue staining, for calcium deposition using alizarin red and for lipids using oil red O. For chondrogenic differentiation, 2 x 10^5 cells were pelleted by centrifugation (7 min at 350g) and maintained in basic medium 2 (high-glucose DMEM/4 mmol/L L-gluta- mine/penicillin/streptomycin), supplemented with proline(40µg/mL), ITS(50mg/mL), sodiumpyruvate (100 µg/mL), ascorbate-2-phosphate (50 µg/mL), transforming growth factor (TGF)-β3 (10 ng/mL) and 100 nmol/L dexamethasone. The medium was refreshed weekly. After a total culture time of 3 weeks, the pellets were formalin-fixed (o/n 4% formalin) and embedded in paraffin. Subsequently, 5-µm sections

were de-paraffinized, re-hydrated and stained (20 min) with 1% toluidine blue.

The stained adipogenic and osteogenic differentiation cultures were subjected to semi-quantitative visual scoring resulting in a number from 0 (no staining) to 4 (maximal staining of positive control). The stained paraffin sections from the chondrogenic differentiation were scored from 0 (no staining) to 2 (maximal staining of positive control).

Karyotype analysis

Karyotype analysis was performed by routine G-banding on sub-confluent, expanding MSC cultures. At least 20 metaphases were analyzed.

RNA isolation and real-time polymerase chain reaction

Total RNA from MSCs was extracted using TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized from 1 µg total RNA, using an oligo dT primer, RNase out, M-MLV reverse transcriptase, M-DTT and buffers in a volume of 20 µL (Invitrogen). Quantitative real-time polymerase chain reaction (PCR) was performed in duplicate by using iQ SYBR Green Supermix on iCycler real- time detection system (BioRad, Veenendaal, the Netherlands). The amplification reaction volume was 12.5 µL, consisting of 6.25 µL iQ SYBR Green PCR master mix, 0.5 µL primers, 2.5 µL cDNA and 3.25 µL water. The messenger RNA (mRNA) level was normalized by the housekeeping gene glycerin aldehyde-3-phosphate dehydrogenase (GAPDH).

Profiling miRNAs by TaqMan Array MicroRNA Cards

Total RNA from selected MSC samples was isolated using TRIzol reagent (Invitrogen) and 350 ng of total RNA was reverse transcribed using the miRNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) in combination with the stemloop Megaplex Primer Pools, Human Pools A v2.1 (Applied Biosystems) according to the manufacturer's instructions. For each cDNA sample, 384 microRNAs including 6 controls (RNU44, RNU48, 4*U6), were profiled using TaqMan Array Human MicroRNA Card A v2.0 (Applied Biosystems) according to the manufacturer's instructions. All arrays were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems) and default thermal-cycling conditions. For each array, the obtained CT-values were converted to relative quantities normalized to RNU48.

Peripheral blood mononuclear cell isolation and fibroblast culture

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy blood donors (different from the MSC donors) by density gradient centrifugation using Ficoll-isopaque and frozen until use (-80°C). The SV40-transformed human renal fibroblast cell line TK173 (from normal kidney) has been previously characterized [39] and was cultured as described [40].

In vitro MSC and PBMC proliferation assay

Cultured MSCs from patients with ESRD and healthy controls were plated in flat-bottom 96-well plates (Costar; Sigma-Aldrich, Zwijndrecht, the Netherlands) and allowed to attach overnight. PBMCs were stimulated with anti-CD3/anti-CD28 Dynabeads (1 bead/5 cells; Invitrogen) and were seeded in DMEM with 5% human serum (Sanquin, Amsterdam, the Netherlands) and 10% fetal bovine serum. PBMCs were seeded in triplicate at a concentration of 1 x 10^5/well. MSCs were added to the PBMC proliferation assay at 1:1250, 1:250, 1:50; 1:10 and 1:2 ratios. After 5 days, supernatants were assayed for cytokine production, and cells were cultured for an additional 16 h in presence of ^{3}H-thymidine (0.5 μCi). ^{3}H-Thymidine incorporation was determined as a measure for proliferation.

Determination of cytokine production

Production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10 and interferon (IFN)-γ in supernatants from the PBMC proliferation assay was measured by immunoassays specific for human cytokines. Cytokine production was also measured in expanding MSC cultures without stimulation. At 70% confluence, the medium was refreshed and supernatants were collected 2 and 3 days later. Cytokines were simultaneously tested with the Bio-Plex Human Cytokine 17-Plex Panel following the manufacturer's instructions (Bio-Rad Laboratories) [41]. Samples were analyzed using a Bio-Plex Array Reader with Bio-Plex software (Bio-Rad Laboratories) [41].

Statistical analysis

Continuous normally distributed data are presented as mean ± SD. Differences between two groups were analyzed using the unpaired two-sample t test. When criteria for parametric testing were not met, the Mann-Whitney test was used. Differences were considered statistically significant with $P < 0.05$. Data analysis was performed using SPSS version 17.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism, version 5.0 (GraphPad Prism Software Inc, San Diego, CA, USA).

RESULTS

Isolation, expansion and characterization of MSCs

Because differentiation potential and proliferation rate are reported to decline in aged human MSCs [39, 40], we selected two age-matched study populations and used expanded MSC populations at similar low passage numbers (P3-4). Healthy controls (five women and five men) had a median age of 64 years (±7.98), which was comparable to the patients with ESRD. Patient demographics are presented in Table I. The MSC cultures of patients with ESRD and healthy controls showed similar expansion kinetics and population doubling times (Figure1A,B) and were morphologically identical, showing the frequently described spindle shape (data not shown). In addition, the membrane marker profiles of the expanded MSCs from both groups were indistinguishable (Figure 1C).

All MSC cultures showed the capacity to differentiate along at least one but typically two of the three lineages tested (Figure 1D). There are no significant differences in *in vitro* dif-

Table 1 Characteristics of ESRD patients

Patients characteristics	**ESRD patients (N=10)**
Mean age (years)	66 ± 4.78
Gender (male/female)	7/3
Mean Body Mass Index (kg/m^2)	22.16 ± 4.06
Initial Kidney Disease, N (%)	
- ADPKD	1 (10%)
- Hypertension	2 (20%)
- Hypertension and Diabetes	1 (10%)
- ATN	1 (10%)
- IgA -nephropathy	2 (20%)
- Granulomatosis with polyangiitis	1 (10%)
- ECI	2 (20%)
Dialysis, N(%)	
Non	4 (40%)
Hemodialysis	2 (20%)
Peritoneal ialysis	4 (40%)
Mean time on dialysis (months)	10.1± 3.65
Medication, N (%)	
- EPO	7 (70%)
- Insulin	1 (10%)
- Oral anti-diabetic agent	1 (10%)
Anti-hypertensive agents, N (%)	
- 0	1 (10%)
- 1	3 (30%)
- 2	3 (30%)
>2	3 (30%)

All values are mean± SD. ADPKD, autosomal dominant polycystic kidney disease. ATN, acute tubular necrosis. EPO, Erythropoïetine. ESRD, end stage renal disease.

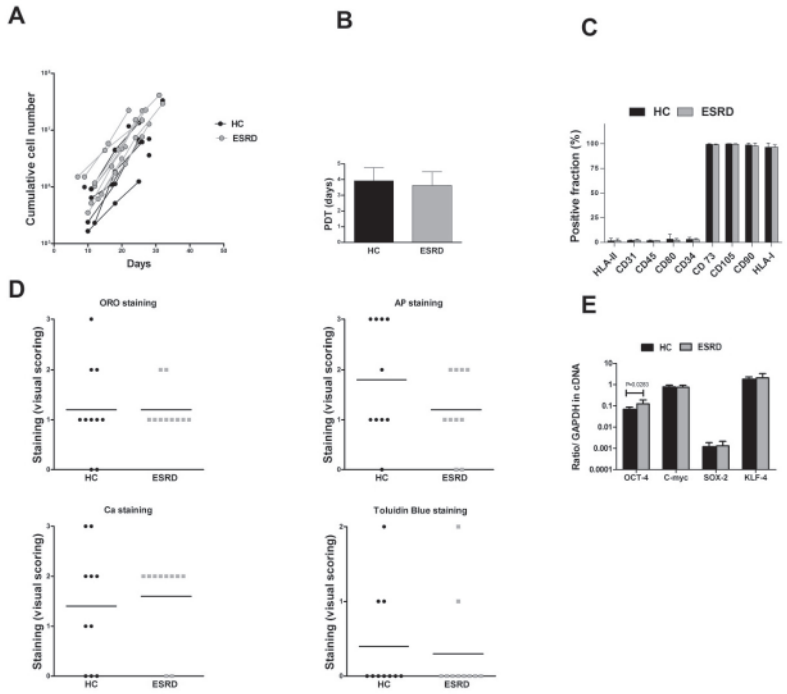

Figure 1. Isolation, expansion and characterization of MSCs. Expansion characteristics of MSC cultures from healthy controls (HC) and patients with ESRD for individual cultures (A) and expressed in population doubling time (PDT) per group (B). Immunophenotype of HC and ESRD patients (C). Differentiation capacity of BM-MSCs from HC and ESRD patients semi-quantitatively assessed by oil red O (ORO), toluidine blue, alkaline phosphatase (AP) and calcium (Ca) staining (D). Expression of mRNA for self-renewal genes Oct-4, C-myc, Sox2 and KLF-4 was assessed by quantitative real-time PCR in BM-MSCs of HC and ESRD patients (E). GAPDH expression was used as a control.

ferentiation capacities between the MSCs from both groups, although the differentiation capacities of the healthy controls seemed more variable. Quantitative PCR analysis showed that mRNA levels of the self-renewal genes c-myc, Sox-2 and Klf-4 were highly similar in both groups, with the exception of OCT4 mRNA levels, which were significantly higher in patients with ESRD (Figure 1E).

MSC products expanded in parallel for potential clinical use were subjected to karyotype analysis. All four tested MSC cultures from the healthy donors showed a normal karyotype. Of the eight MSC cultures from patients with ESRD, one displayed a clonal karyotypic abnormality, 46,XY,del(3) (q11q21) (data not shown). This abnormality was not detected in a repeated karyotype analysis of the MSC culture or in the karyotype of the patient's peripheral blood.

mRNA expression of cytokines and angiogenic factors and cytokine expression in supernatants of MSC cultures

We next focused our analysis on mRNA encoding cytokines and angiogenic factors in both groups. mRNA levels of indoleamine 2,3-dioxygenase and the cytokines IL-10, TNF-α and IFN-γ and of the angiogenic factor basic fibroblast growth factor were of very low abundance (data not shown). mRNA levels of Angiopoietin-1 (Ang-1), Tie-2, vascular endothelial growth factor (VEGF), IL-6 and TGF-β were highly similar in both groups (Figure 2A,B). Ang-2 was significantly higher and IL-8 significantly lower in patients with ESRD.

Cytokine production was measured in expanding, non-stimulated MSC cultures. From the 17 cytokines analyzed, only monocyte chemotactic protein-1 and IL-6 could be detected in the supernatants of MSC cultures. There was no difference in expression levels of both cytokines between BM-MSCs of patients with ESRD and healthy controls (Figure 2C,D).

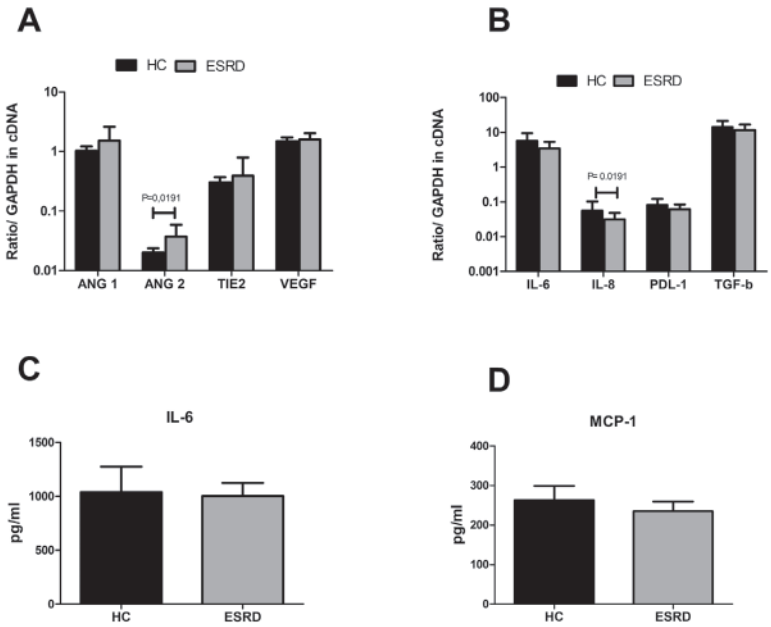

Figure 2. Similar expression levels of cytokines and angiogenic factors between BM-MSCs of patients with ESRD and healthy controls (HC). Expression of mRNA of angiogenic factors Ang-1, Ang-2, Tie-2 and VEGF (A) and IL-6, IL-8, PDL-1 and TGF-β (B) in BM-MSCs of HC and ESRD patients. GAPDH expression was used as a control. Concentration of IL-6 (pg/mL) (C) and monocyte chemotactic protein-1 (MCP-1) (D) in supernatants of BM-MSCs of HC and ESRD patients after 2 days of culture.

BM-MSC miRNA expression profiles of ESRD patients and healthy controls

Differential expression of miRNA is increasingly acknowledged as a determining factor in lineage differentiation[36].We performed miRNA profiling as a broad, non-biased measure of the differentiation status of our MSC cultures. When the normalized data are subjected to un-biased cluster analyses, the BM-MSCs from both patients with ESRD and controls grouped together and separated from the hematopoietic phenotypes CD4, CD8 and CD14 (Figure3). As predicted, the most related expression profile was from the human kidney fibroblast cell line TK173, confirming the phenotypic similarity between MSCs and fibroblasts. Based on the miRNA profiles, we conclude that the impact of ESRD on the BM-MSC phenotype is minimal. Only three miRNAs were differentially expressed between the two groups (hsa-miR-876-5p, hsa-miR-146a and hsa- miR-517b; Mann Whitney $P < 0.02$, $P < 0.04$, $P < 0.04$). However, these were low abundant miRNAs, and the value of these differences is limited. A few more

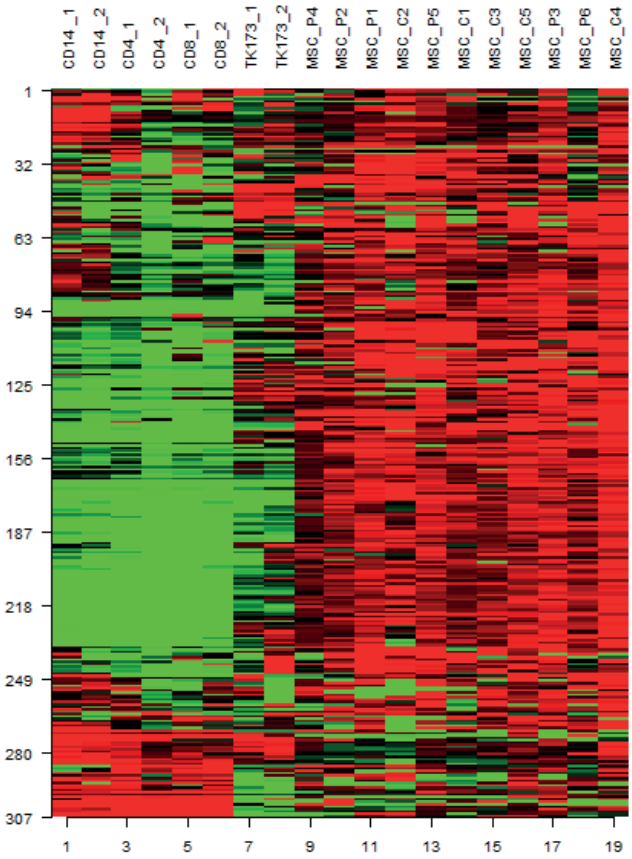

Figure 3. BM-MSCs miRNA expression profiles of patients with ESRD and healthy controls. miRNA signature of BM-MSCs of ESRD (P1-P6) compared with BM-MSCs from HC (C1-5) and compared with fibroblasts (TK173) and immune cells (CD4, CD8 and CD14; all two samples) as indicated.

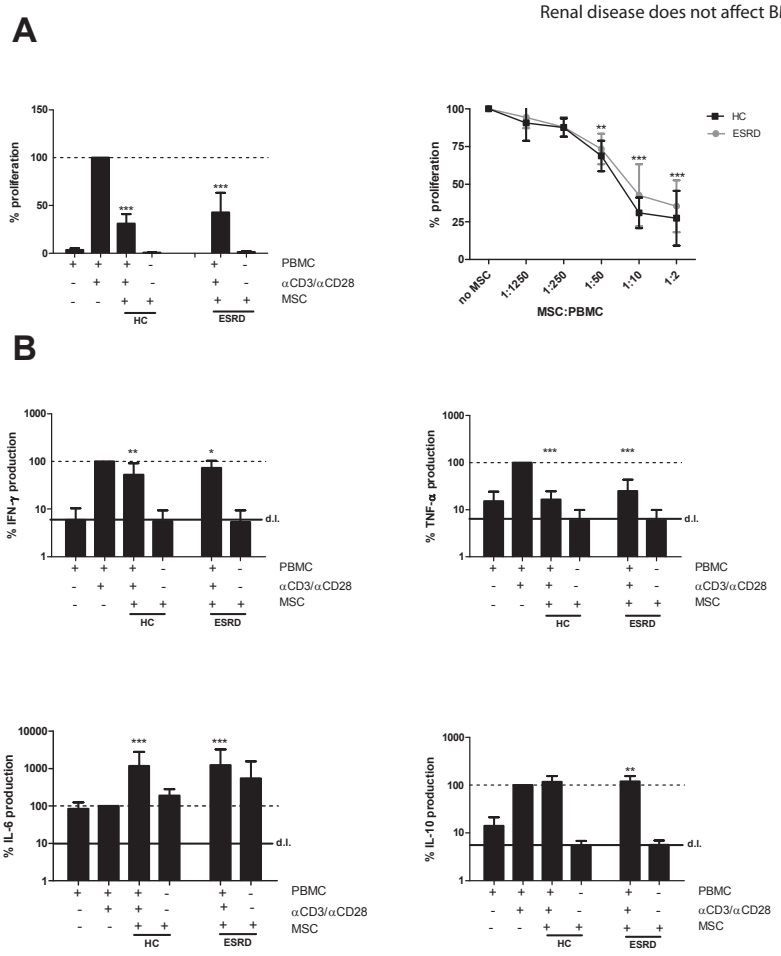

Figure 4. BM-MSCs from patients with ESRD suppress immune responses *in vitro*. BM-MSCs from ESRD patients (at different ratios of MSCs to PBMCs) have the same immunosuppressive capacities as BM-MSCs from HC in the mixed lymphocyte reaction. (A) MSCs from HC and ESRD patients were added at a ratio of 1:10 to stimulated PBMCs (left panel) and proliferation of 100,000 stimulated PBMCs with anti-CD3/CD28 beads and different amounts of MSCs (right panel) (as indicated). Proliferation measured by 3H-thymidine incorporation in counts is expressed as a percentage (%). Stimulated PBMCs with anti-CD3/CD28 are depicted as 100%. (B) Cytokine production of IFN-γ, TNF-α, IL-6 and IL-10 in the supernatants of PBMC cultures, anti-CD3/CD28 stimulated PBMCs and 10,000 MSC/100,000 PBMC co- cultures. Cytokine production is depicted as a percentage compared with the cytokine production of anti-CD3/CD28 stimulated PBMCs (depicted as 100%). Mean production of cytokines in the anti-CD3/CD28 stimulated PBMCs group are as follows: IFN-γ, 23.8 ng/mL (range, 10.7e40.4 ng/mL), TNF-α, 1.4 ng/mL (range, 0.4e2.8 ng/mL), IL-10, 0.4 ng/mL (range, 0.2e0.6 ng/mL) and IL-6, 11.2 ng/mL (range, 0.1e17.4 ng/mL). Mean production of IFN-γ, TNF-α and IL-10 in the MSCs of the HC and ESRD groups were below the detection limit (see lines 1 ng/mL, 62.5 pg/mL and 18.8 pg/mL), and the mean IL-6 production in the MSCs of HC was 29.5 ng/mL. Bars represent the mean and SD of data from 10 patients in triplo. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with anti-CD3/CD28 stimulated PBMCs. (d.l., detection limit.)

recent studies have reported miRNA expression profiles in differentiating MSCs [36]. These miRNAs include miR-143, miR-103 and miR-107 for adipocyte differentiation, miR-140 for cartilage development, miR-683, 663 in chondrocyte and miR-24, let7a, let7b, let7c, miR-138 and miR-320 as osteocyte-specific miRNAs [44]. Although we did not test the expression of these miRNAs in differentiating cultures, at baseline none of these miRNAs were differentially expressed between the two groups. miR-143, miR-140-5b, miR-24, let7b, miR-138 and miRNA 320 were included in the 30 most abundant miRNAs present in the MSCs.

BM-MSCs from ESRD patients suppress immune responses *in vitro*

An important characteristic of MSCs, especially in the context of potential use in renal transplantation, is the anti-inflammatory and immunosuppressive function. Autologous PBMCs were activated by polyclonal CD3/CD28 activation in the absence or presence of MSCs. MSCs from both healthy controls and patients with ESRD inhibited this proliferation in a dose-dependent manner, reaching near to optimal inhibition at a 1:10 MSC-to-PBMC ratio (Figure 4A). The same cultures were analyzed for cytokine production by these polyclonal PBMCs. MSCs from both healthy controls and patients with ESRD showed a similar effect- no inhibition of IL-10 production but an inhibition for IFN-γ and TNF-α (Figure4B). MSC cultures alone did not show production of any of these cytokines. In contrast, MSCs produced IL-6 (as also described in Figure 2C), explaining the increased production in co-cultures (Figure 4B).

DISCUSSION

The great potential for MSCs as a therapeutic tool for allograft rejection after renal transplantation is supported by positive results in different experimental models [5, 9, 11, 13, 41]. In these studies, recipient and donor-derived MSCs were shown to have better immunomodulatory properties than third-party MSCs [41-44]. However, donor- derived MSCs were also reported to cause sensitization, and application with autologous MSCs seems preferable [45-47].The first clinical trials using autologous BM-MSCs in kidney transplantation have started [26, 27, 48]. However, a premise in these studies is that BM-MSCs from patients with renal failure are not affected by the disease background. So far, no formal assessment of this assumption has been made. This assumption is very relevant because BM-MSCs from patients with rheumatoid arthritis, systemic lupus erythematosus and systemic sclerosis were shown to be deficient in their ability to support hematopoiesis and exhibited features of early senescence [28, 29, 49].

In our study, expanded MSCs of patients with ESRD complied with the phenotypic criteria for MSCs as defined by the International Society for Cellular Therapy (ISCT) [8], indicating that ESRD did not affect MSC phenotype. Also, the growth and differentiation characteristics were not impaired in expanded MSCs from patients with ESRD. These data are important

for our clinical protocols because they validated our ability to obtain sufficient numbers of autologous MSCs at a low passage within 4 weeks.

We observed a clonal karyotypic aberration in the MSC product from one of the patients with ESRD. Because subsequent karyotype analysis on the patient's peripheral blood did not show this aberration, we could not attribute these findings to patient chimerism. Theoretically, it would be possible that the generation of this aberration was caused by the MSC expansion process. However, we argue this to be very unlikely because clonal karyotypic aberrations were never detected in expanded MSC populations from healthy individuals and never found in any of 37 MSC preparations from healthy donors that we have manufactured for clinical applications under the same culture conditions. At the present time, we consider chimerism in the patient's MSC compartment, possibly related to medication over a long period, the most plausible explanation for this observation, which warrants further investigations.

mRNA expression of self-renewal genes was highly similar between ESRD patient groups and healthy controls with the exception of increased mRNA levels of Oct4 in the patient-derived MSCs. The potential clinical significance of this difference is unclear. More recent studies have demonstrated increased Oct4 mRNA expression in MSCs after hypoxia [50]; however, the effect of uremia on the expression of self-renewal genes is unknown. We also focused on the mRNA profiles of inflammatory cytokines and angiogenic factors that have been associated with immune modulation and regenerative functions in acute kidney injury (AKI) and renal transplantation. mRNA levels of cytokines of cultured MSCs were highly similar between ESRD patient groups and healthy controls. In addition, we found similar expression levels of VEGF, Tie-2 and Ang-1 in both groups. The last-mentioned is of great relevance because Ang-1 is recognized as a crucial stromal cell derived factor that is indispensable for stabilization of the microcirculation, can preserve peritubular perfusion and can prevent renal failure in AKI [51]. Uremic BM-derived murine MSCs showed decreased expression of VEGF and stromal cell derived factor, decreased proliferative capacity, decreased *in vitro* tube formation and decreased angiogenesis *in vivo* [33]. This discrepancy could be attributed to species or culture conditions during MSC expansion.

More recent studies have focused on the importance of miRNAs in MSC differentiation and function [36]. It was demonstrated that micro-vesicles released from MSCs may account for their paracrine mechanism of action by a horizontal transfer of mRNA and miRNA. In a rat model, micro-vesicles released from MSCs protected from AKI induced by ischemia-reperfusion injury and from subsequent chronic renal damage [52]. miRNA expression and clearance may also be altered in renal failure. A more recent study demonstrated reduced circulating miRNA levels in chronic kidney disease [37]. Another study compared miRNA profiles of human proximal tubular cells (HK-2) cultured in control versus uremic dialysate and indicated that the uremic environment can alter miRNA expression [53]. In contrast, we found that miRNA expression signatures in MSCs from patients with ESRD were highly similar to miRNA expression signatures from healthy controls, contributing to their extensive phenotypic and

functional overlaps. Cell type and culture methods could be important factors accounting for these differences.

In conclusion, MSCs are an interesting candidate for the induction of tolerance or the treatment of allograft rejection because of their immunosuppressive properties. In our experiments, we found that MSCs from patients with ESRD and healthy controls have similar immunosuppressive capacities. Functionally, MSCs were shown to have a similar dose-dependent inhibition of PBMC proliferation, similar decrease in the production of pro-inflammatory cytokines TNF-α and IFN-γ and similarly increased production of tolerogenic cytokine IL-10. Taken together, our study demonstrates that ex vivo expanded BM-MSCs from patients with ESRD are both phenotypically and functionally similar to BM-MSCs from healthy controls. These findings are important for the potential clinical use of autologous MSCs in transplant recipients.

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Chapter 8

Mesenchymal stem cells induce an inflammatory response after intravenous infusion

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ABSTRACT

Mesenchymal stem cells (MSCs) have potent immunosuppressive effects *in vitro* and are considered as a therapeutic option for autoimmune disease and organ transplantation. While MSCs show beneficial effects on immune disease progression and transplant survival in animal models, the immunomodulatory mechanisms involved are largely unknown. In the present study, we show that intravenously infused C57BL/6- green fluorescent protein (GFP) MSCs home to the lungs in C57BL/6 recipient mice and induce an inflammatory response. This response was characterized by increased mRNA expression of monocyte chemoattractant protein-1 (MCP1), IL1-β, and TNF-α and an increase in macrophages in lung tissue 2 h after MSC infusion. Simultaneously, serum levels of proinflammatory IL6, CXCL1, and MCP1 protein increased, demonstrating systemic immune activation after MSC infusion. In liver tissue, no C57BL/6-GFP MSCs were detected, but MCP1 and TNF-α mRNA levels peaked 4 h after MSC infusion. The expression of the anti-inflammatory cytokines TGF-β, IL4, and IL10 was only marginally affected. Nevertheless, 3 days after MSC infusion, animals developed a milder inflammatory response to lipopolysaccharides. Our results suggest that the in vivo immunomodulatory effects of MSCs originate from an inflammatory response that is induced by the infusion of MSCs, which is followed by a phase of reduced immune reactivity.

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent cells that reside in most, if not all, tissues [1]. They are capable of osteogenic, adipogenic and chondrogenic differentiation [2] and have particularly potent immunosuppressive properties [3, 4]. These characteristics have raised interest in the use of MSCs for the treatment of degenerative and immunological diseases such as graft versus host disease [5], multiple sclerosis [6], Crohn's disease [7] and rejection of organ transplants [8].

The immunosuppressive capacity of MSCs has been a subject of extensive research over the last decade. In vitro experiments have demonstrated that MSCs inhibit the proliferation and cytokine secretion of T cells [9], B cells [10] and NK cells [11]. MSCs target lymphocytes via a variety of mechanisms, which depend on both the secretion of soluble anti-inflammatory factors, such as TGF-β, hepatocyte growth factor (HGF) [3], HLA-G [12], and on cell contact interactions via the inhibitory co-stimulatory programmed death ligand 1 (PD-L1) pathway [13]. The expression by human MSC of the tryptophan-depleting enzyme indolamine 2,3-dioxygenase (IDO) is crucial for the inhibition of lymphocyte proliferation [14]. The expression of IDO and other anti-inflammatory factors by MSCs is strongly induced under inflammatory conditions [15-17], suggesting that the immunomodulatory effects of MSC act as a feedback mechanism to control ongoing immune responses.

Studies in animal models and early clinical trials have attempted to translate the potent in vitro immunosuppressive effects of MSCs in effective immune therapy. The most widely used route of administration of MSCs in these studies is via intravenous (IV) infusion. In contrast to the in vitro immunomodulatory effects of MSCs, however, little is known about the effects of MSCs after IV infusion. IV-infused MSCs home to the lungs [18-21] and it is believed that they migrate from there to sites of injury and inflammation [22, 23] and exert their immunosuppressive function. However, recent evidence suggests that this is not the case. It has been shown that living MSCs are only detectable in the lungs after IV infusion, and not in any other tissue or at sites of injury [24, 25]. Furthermore, the presence of living MSCs in the lungs is temporary and within 24 h, the majority of MSCs in the lungs die and their cell debris is distributed to other sites, in particular, the liver.

Even though MSCs appear to be short-lived after IV infusion, they induce potent immunomodulatory effects in various disease models. For instance, infusion of MSCs has been shown to alleviate experimental colitis [26], ameliorate experimental encephalomyelitis [27] and inhibit allograft rejection [28]. In these models, infused MSCs are unlikely to be present at the site of inflammation or live long enough to become activated by inflammatory conditions and exert their immunosuppressive effect. Therefore, it is unlikely that the in vitro established mechanisms of immunomodulation by MSCs are operational after infusion of MSCs. Other mechanisms are likely to mediate the effect of MSCs, probably involving other cell types. The

purpose of the present study was to map the type, origin, and duration of the immunomodulatory response that is induced upon IV infusion of the MSC.

MATERIALS AND METHODS

Isolation and culture of MSCs

MSCs were isolated from the adipose tissue of C57BL/6 mice expressing enhanced green fluorescent protein (GFP) under the human ubiquitin C promoter (The Jackson Laboratory). The adipose tissue was a kind gift from E. Koning en Dr. R. Mebius of the Free University Amsterdam. Animals were kept in accordance with the institutional guidelines. The adipose tissue of five animals was pooled, minced with a scalpel knife, and enzymatically digested with 0.5 mg/mL collagenase type IV (Life Technologies) in a minimal essential medium eagle-alpha (MEM-α) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (1% p/s) (all Life Technologies) for 30 min at 37°C under continuous shaking. After centrifugation at 1,200 g for 10 min, the cell pellet was resuspended in a red blood cell lysis buffer (eBioscience) and incubated for 10 min at room temperature. The cells were then washed, resuspended in the MEM-α supplemented with 2 mM L-glutamine, 1% p/s, and 15% fetal bovine serum (FBS; Lonza), and filtered through a 70-µm cell strainer (BD Biosciences). The cells were then transferred to a 175-cm2 cell culture flask (Greiner Bio-One) and expanded in a humidified atmosphere with 5% CO_2 at 37°C. Nonadherent cells were removed after 3–4 days. The culture medium was refreshed twice weekly. Cells were removed from culture flasks at subconfluency using 0.05% trypsin-EDTA (Life Technologies) and reseeded at 1,000 cells/cm^2 to ensure optimal proliferation. MSCs were used for experiments between passages 4 to 10.

Characterization of MSCs

MSCs were examined for GFP expression by fluorescence microscopy. For immunophenotyping, MSCs were harvested, washed in FACSFlow (BD Biosciences), and stained with Sca-1-PE-Cy7, CD11b-APC, CD44-PE, and CD45-PERCP (all BD Biosciences) and measured on a FACSCanto II flow cytometer (BD Biosciences) using FACSDiva software.

Adipogenic differentiation was induced by culturing confluent MSCs in the MEM-α supplemented with 2 mM L-glutamine, 1% p/s, 15% heat-inactivated FBS (FBS-HI), 50 µg/mL L-ascorbic acid (Sigma-Aldrich), 500 µM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), 60 µM indomethacin, 2 µM insulin (Sigma-Aldrich), and 10 nM dexamethasone (Sigma-Aldrich) for 14 days. Lipid-filled vesicles were detected by Oil Red O staining. Cells were washed with phosphate-buffered saline (PBS), fixed with 60% isopropanol for 1 min, and incubated with filtered 0.3% Oil Red O (Sigma-Aldrich; in 60% isopropanol) for 30 min. Following three washes with PBS, the cells were photographed.

Osteogenic differentiation was induced by culturing confluent MSCs in the MEM-α supplemented with 2 mM L-glutamine, 1% p/s, 15% FBS-HI, 5 mM β-glycerophosphate (Sigma-Aldrich), 50 µg/mL L-ascorbic acid, and 10 nM dexamethasone for 21 days. The deposition of calcified nodules was identified using von Kossa staining. Cells were washed with PBS and fixed with cold 4% paraformaldehyde for 5 min. Following an additional wash step with PBS and two wash steps with distilled water, cells were incubated with 1% silver nitrate (in water) on a light box until blackening occurred. Cells were washed three times with water, incubated with 2.5% sodium thiosulfate (in water) for 5 min, washed again twice with water, and photographed.

For examination of the immunosuppressive capacity of MSCs, Swiss mouse splenocytes were stimulated with 5 µg/mL Concanavalin A (ConA; Sigma Aldrich) and MSCs added at ratios of 1:5 to 1:640 in 96-well plates. After 3 days, proliferation was measured by incorporation of ^{3}H-thymidine (0.25 µCi/well; PerkinElmer) during 8 h of incubation using a Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer (PerkinElmer).

Infusion of MSCs

Male C57BL/6 mice, 8–10 weeks old and weighing ~25 g, were obtained from Harlan. The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act and complied with the 1986 directive 86/609/EC of the Council of Europe. MSCs were trypsinized, washed, and resuspended in PBS. Three hundred thousand MSCs in 200 µL PBS were infused via tail vein injections. Control mice received 200 µL PBS only. After 2, 4, 20, 68, and 216 h, mice were sacrificed and blood collected in Minicollect serum separation tubes (Greiner Bio-One). Two experiments were carried out, totaling five animals per time point. Lungs, livers, and kidneys were removed and frozen at −80°C. Spleens were removed and cell suspensions prepared. After red blood cell lysis (RBC lysis buffer; eBioscience), splenocytes were frozen in 10% DMSO at −150°C.

Treatment with lipopolysaccharides

One day or 3 days after infusion of MSCs as described above, animals were injected with 5 mg/kg body weight lipopolysaccharides (LPS; Sigma-Aldrich) dissolved in PBS via the tail vein. Animals were sacrificed 4 h later and blood collected in Minicollect serum separation tubes.

PCR analysis

Frozen lung, liver, and kidney tissue were sectioned in 20-µm slices and RNA isolated using the Trizol reagent (Life Technologies). cDNA was synthesized from 500 ng RNA with random primers (Promega). Quantitative gene expression was determined using TaqMan Gene Expression Master Mix (Life Technologies) and assays-on-demand for IL1-β (mm01336189.m1), IL10 (mm00439614.m1), CCL2 [monocyte chemoattractant protein-1 (MCP1)] (mm00441242.m1),

CCL3 (MIP1-α) (mm00441258.m1), TNF-α (mm00443258.m1), IFN-γ (mm01168134.m1), TGF-β (mm01178820.m1), IL4 (mm00445259.m1), CD68 (mm03047340.m1), F4/80 (mm008002529.m1), Ly6G6c (mm00458275.m1), and housekeeping gene HPRT (mm01545399.m1) (all Applied Biosystems) on a StepOnePlus (Applied Biosystems). GFP expression was determined by SYBR green-based (Applied Biosystems) RT-PCR. Primer sequences used for the detection of GFP were 5′-CCA CAT GAA GCA GCA GGA CTT-3′ and 5′-GGT GCG CTC CTG GAC GTA-3′ (BaseClear). Results were expressed as a ratio to HPRT.

Analysis of serum proteins by Milliplex

Serum concentrations of a broad panel of chemokines, cytokines, and acute-phase proteins were measured by Milliplex cytokine/chemokine panel I (Millipore) according to the manufacturer's instructions. The panel existed of the following proteins: CRP, CXCL1, CXCL5 (LIX), CCL11 (Eotaxin), G-CSF, GM-CSF, IFN-γ, IL1-α, IL1-β, IL2, IL3, IL4, IL5, IL6, IL7, IL9, IL10, IL12p40, IL12p70, IL13, IL15, IL17, IP10, LIF, CCL2 (MCP1), M-CSF, CCL3 (MIP1-α), MIP1-β, MIP2, MIG, Rantes, SAP, TNF-α, and VEGF.

Splenocyte analysis

Splenocytes were thawed and stimulated with 5 ng/mL PMA (Sigma-Aldrich) and 500 ng/mL ionomycin (Sigma-Aldrich) for 4 h at 37°C in the presence of 1 µl Golgiplug (BD Biosciences) per $1\times1x10^6$ cells. Controls were not stimulated with PMA/ionomycin. The cells were then washed and stained with CD3-PERCP, CD4-FITC, and CD25-PE-Cy7 antibodies (all BD Biosciences). Subsequently, cells were washed and incubated in a Fixation/Permeabilization solution (BD Biosciences) for 30 min at 4°C, followed by a wash in the Perm/Wash buffer (BD Biosciences), and stained with the IFN-γ-APC antibody (BD Biosciences). After washing, the cells were analyzed on a FACSCanto II flow cytometer using FACSDiva software.

To determine the effect of the MSC on the proliferative capacity of splenocytes after LPS treatment, splenocytes were collected 4 h after LPS treatment and seeded at 150,000 cells per well in a 96-well round-bottom plate and stimulated with 5 µg/mL ConA for 3 days. They were then incubated with 3H-thymidine (0.25 µCi/well) for 8 h. 3H-thymidine incorporation was measured using the Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer.

RESULTS

Characterization of adipose tissue-derived MSCs

MSCs were isolated from the adipose tissue of C57BL/6 mice expressing GFP under the ubiquitin promoter. In culture, MSCs displayed a characteristic fibroblast-like morphology (Figure 1A) and showed strong GFP expression (Figure 1B). Flow cytometric analysis confirmed

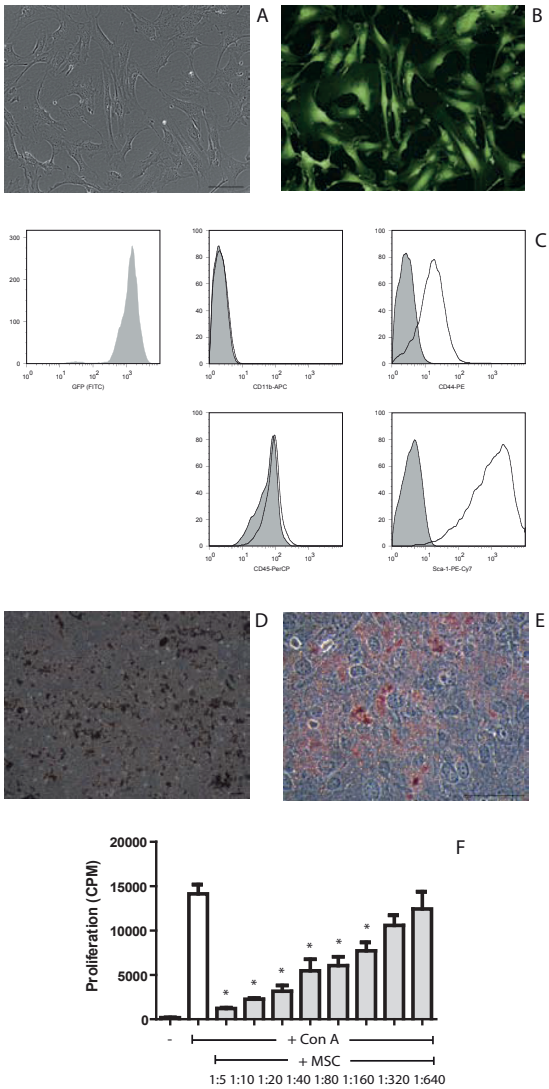

Figure 1. Characterization of MSCs isolated from adipose tissue of C57BL/6 mice expressing GFP under the ubiquitin promoter. (A) Bright-field image of cultured MSCs showing characteristic MSC morphology. (B) Fluorescence (FITC) image of cultured MSCs showing GFP expression. Scale bars represent 100mm. (C) Expression of GFP, CD11b, CD44, CD45, and Sca-1 by MSCs. Representative experiment shown. Closed histograms: unstained cells, open histograms: stained cells. (D) MSCs cultured under control (upper panel) or osteogenic conditions (lower panel) for 21 days stained with von Kossa staining. Calcified nodules appear in brown/black. (E) MSCs cultured under control (upper panel) or adipogenic conditions (lower panel) for 14 days stained with Oil Red O. Lipid-filled vesicles appear in red. Scale bars represent 100mm. (F) Dose-dependent inhibition of ConA stimulated splenocyte proliferation, measured by 3H-thymidine in- corporation assay (n=3). *P<0.01 compared to white bar. MSC, mesenchymal stem cells; GFP, green fluorescent protein; ConA, concanavalin A.

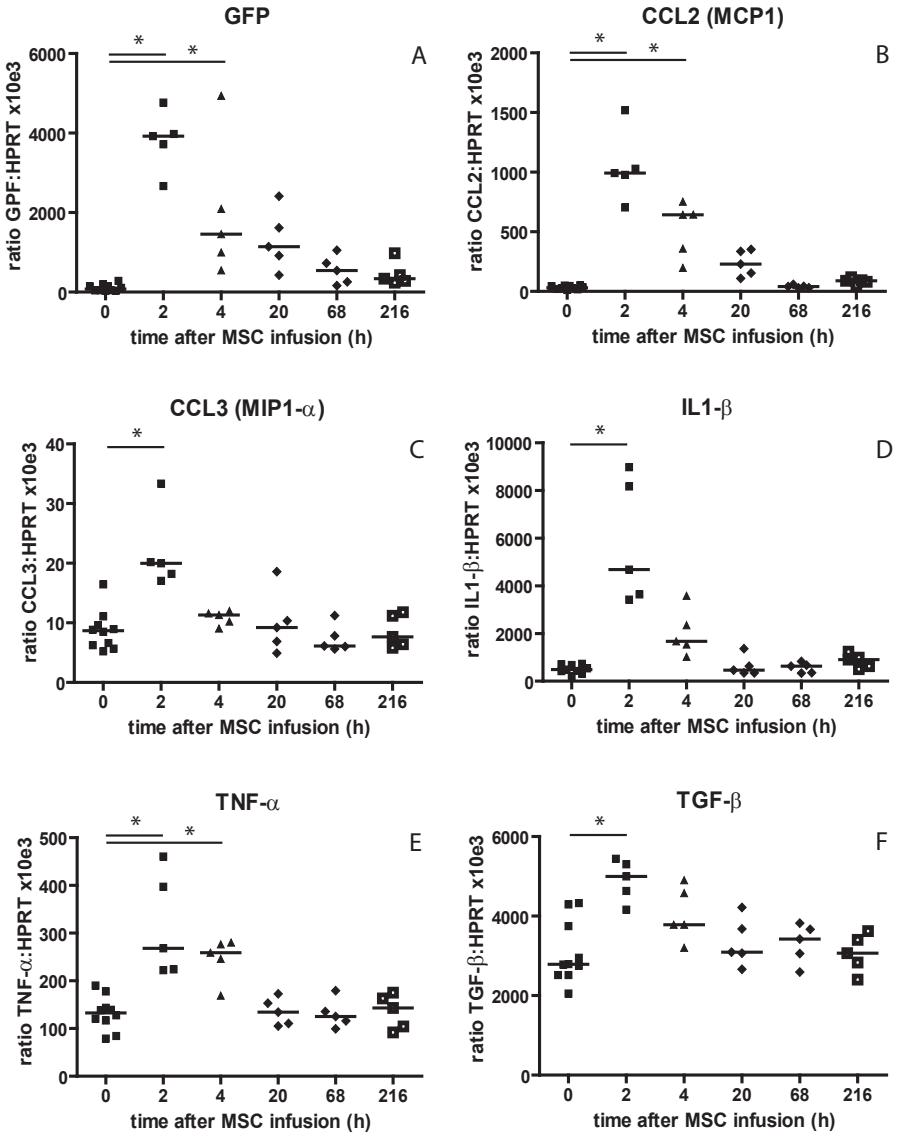

Figure 2. Gene expression changes in lung tissue in response to IV infusion of MSCs. Lungs were removed at various time points after administration of MSCs and mRNA isolated. Expression of GFP, CCL2 (MCP1), CCL3 (MIP1-a), IL1-β, TNF-α and TGF- β is depicted as a ratio to HPRT. (A–F) show single measurements of five animals per time point (control group contains 10 animals) with medians. *P<0.01.

GFP expression in close to 100% of MSCs and demonstrated positive expression of the MSC markers CD44 and Sca-1 (Figure 1C). None of the cells expressed the hematopoietic markers, CD11b and CD45, indicating the absence of macrophages and dendritic cells in the cultures. Culture under osteogenic and adipogenic differentiation-inducing conditions stimulated the production of calcified nodules (Figure 1D) and lipid-filled vesicles (Figure 1E) by MSCs. Finally, MSCs were capable of inhibiting the proliferation of ConA-stimulated splenocytes in a dose-dependent manner (Figure 1F). These results demonstrate that the MSCs used in this study were fully functional.

Effects of IV infusion of MSCs on lung tissue

Three hundred thousand GFP-MSCs were infused via the tail vein in syngeneic C57BL/6 mice. At 2, 4, 20, 68, and 216 h after infusion, five mice were sacrificed and lungs removed for analysis. MSCs could be detected in the lungs 2 h after infusion by means of GFP mRNA expression (Figure 2A). GFP expression in the lungs subsequently decreased. The presence of MSCs in the lungs was accompanied by an inflammatory response, which peaked at 2 h2h after infusion of MSCs and was characterized by increased mRNA levels of the proinflammatory chemokines and cytokines CCL2 (MCP1; 32-fold), CCL3 (MIP1-α; 2-fold), IL1-β (5-fold), and TNF-α (2-fold) (Figure 2B-E). Simultaneously, there was a twofold increased expression of anti-inflammatory TGF-β (Figure 2F). All gene expression levels returned to basal levels at 4 to 20 h after MSC infusion. The expression of IL4, IL10, and IFN-γ did not significantly change upon administration of MSCs (data not shown).

MSCs increase the number of macrophages in the lungs

To investigate whether the inflammatory response in the lungs induced by the infusion of MSCs resulted in increased numbers of macrophages and neutrophils in the lungs, PCR analysis for the macrophage markers CD68 and F4/80 and for the neutrophil marker Ly6G6c was performed. There was an increase in CD68 and F4/80 expression in the lungs 2 h after infusion of MSCs (Figure 3A,B). At 20 h, expression levels had returned to basal levels. There was no significant change in Ly6G6c, although at 2 h, there was a trend for increased expression (Figure 3C).

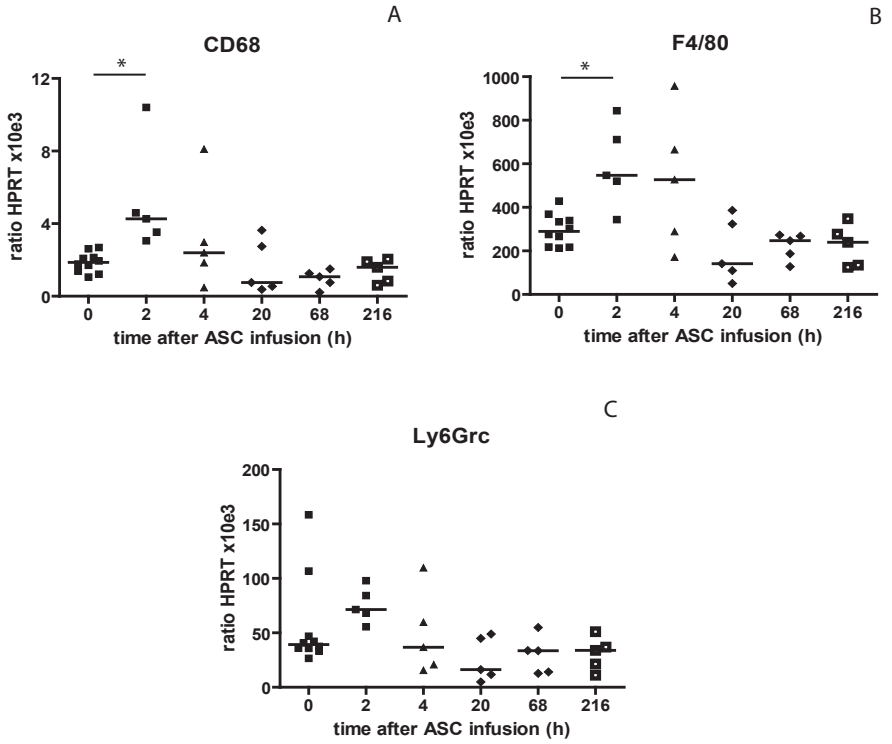

Figure 3. Expression of macrophage and neutrophil markers in lung tissue in response to IV infusion of MSCs. (A–C) Expression of CD68 and F4/80 for macro- phages and Ly6G6c for neutrophils is shown as a ratio to HPRT. Single measurements of 5 animals per time point (control group 10 animals) with medians shown. *P<0.01.

Systemic effects of IV infusion of MSCs

To analyze whether the inflammatory response in the lungs evoked by MSC infusion was also detectable systemically, levels of 34 cytokines, chemokines, and acute-phase proteins were measured in serum at the various time points after administration of MSCs. Two hours after infusion of MSCs, there were increases in the concentrations of the neutrophil stimulator G-CSF (4-fold), proinflammatory IL6 (18-fold), the neutrophil chemoattractant CXCL1 (10-fold) and CCL2 (MCP1, 4-fold) (Figure 4A-D). Concentrations of the proteins returned to basal levels at 20 h after administration of MSCs. In contrast, levels of the neutrophil chemoattractant, CXCL5, dropped significantly 2 h up to 20 h after infusion of MSCs, while the eosinophil chemoattractant, CCL11, was decreased at 20h (Figure 4E-F). No significant changes in serum concentrations of CRP, GM-CSF, IFN-γ, IL1-α, IL1-β, IL2, IL3, IL4, IL5, IL7, IL9, IL10, IL12p40, IL12p70, IL13, IL15, IL17, IP10, LIF, M-CSF, CCL3 (MIP1-α), MIP1-β, MIP2, MIG, Rantes, SAP, TNF-α, and VEGF were detected (data not shown).

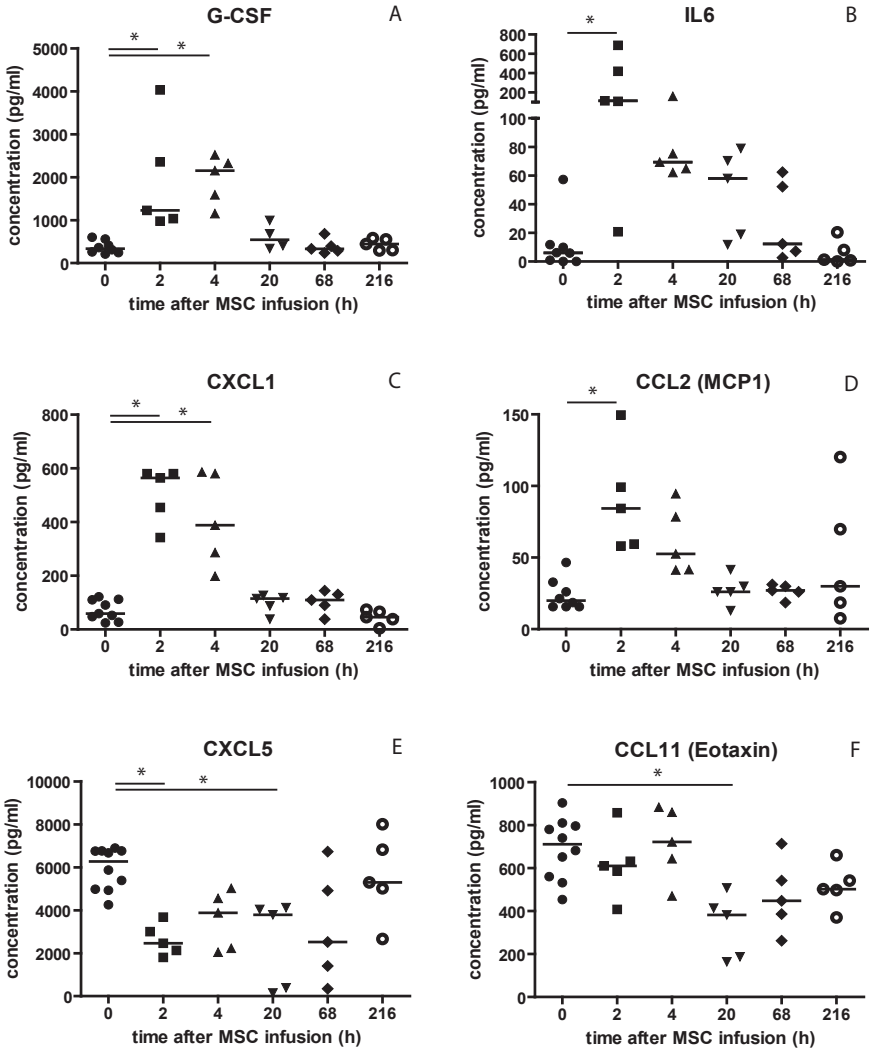

Figure 4. Serum concentrations of cytokines/chemokines after IV infusion of MSCs. Serum was collected at various time points after administration of MSCs. Protein concentrations of G-CSF, IL6, CXCL1, CCL2 (MCP1), CXCL5, and CCL11 (Eotaxin) were determined by Milliplex assay. (A–F) show single measurements of 5 animals per time point (control group 10 animals) with medians. *P<0.01.

Effects of IV infusion of MSCs on liver and kidney tissue

Earlier, we found that the MSC label, but no viable MSCs, accumulated in liver tissue 1 day after infusion of MSC [29]. To examine whether this would leave an immunological footprint in liver tissue, livers were removed for analysis 2, 4, 20, 68, and 216 h after MSC infusion and mRNA expression of a number of cytokines and chemokines analyzed. No expression of GFP was detected in the liver at any time after MSC infusion, confirming that no viable MSCs

localized to the liver (data not shown). Expression of TNF-α, CCL2 (MCP1), and CCL3 (MIP1-α) increased 2 h and peaked 4 h after infusion of MSCs, showing twofold, fourfold, and twofold increases, respectively (Figure 5A-C). IFN-γ and IL1-β levels also peaked, but at 68 and 216 h after MSC infusion, respectively (Figure 5D-E). TGF-β showed a 1.5-fold increase after 4h (Figure 5F), while no changes in IL10 expression were observed (data not shown). In kidney

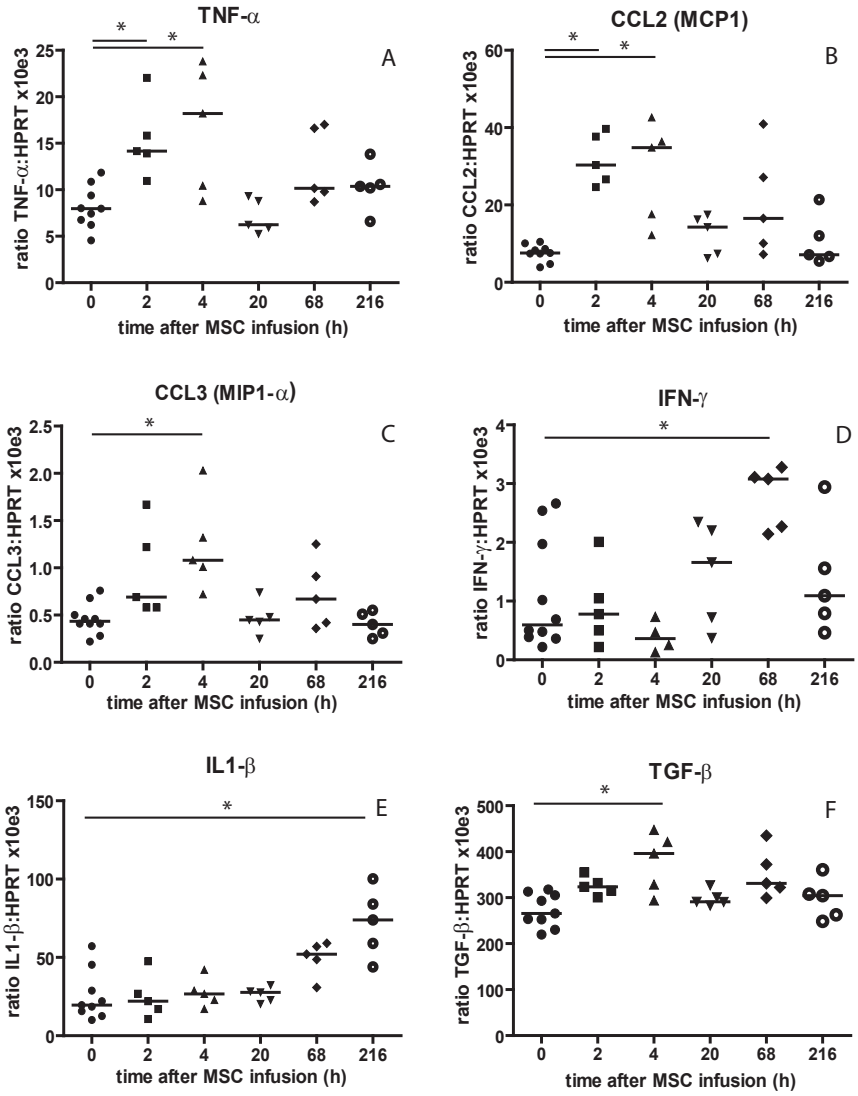

Figure 5. Gene expression changes in liver tissue in response to IV infusion of MSCs. Livers were removed at various time points after administration of MSCs and mRNA isolated. Expression of IFN-γ, CCL2 (MCP1), CCL3 (MIP1-a), IL1- β, TNF-α, and TGF- β is depicted as a ratio to HPRT. (A–F) show single measurements of 5 animals per time point (con- trol group 10 animals) with medians. *P<0.01.

tissue, no significant changes in cytokine and chemokine gene expression were detected at any time point after infusion of MSCs and no expression of GFP was found (data not shown).

Effect of IV infusion of MSCs on splenocytes

The effect of MSC infusion on the responsiveness of splenocytes was determined by *in vitro* stimulation of splenocytes with PMA/ionomycin and measurement of their IFN-γ production. PMA/ionomycin induced an IFN-γ response in ~6% of $CD3^+$ and CD3– splenocytes of control animals. The IFN-γ response in $CD3^+$ splenocytes was not significantly different in animals that received MSCs, although there was a trend for a reduced IFN-γ response at 2, 4, 20, and 68 h after administration of MSCs (Figure 6A). The IFN-γ response in CD3– splenocytes was not affected by MSC treatment (Figure 6B).Infusion of MSCs dampens the immune response to LPS

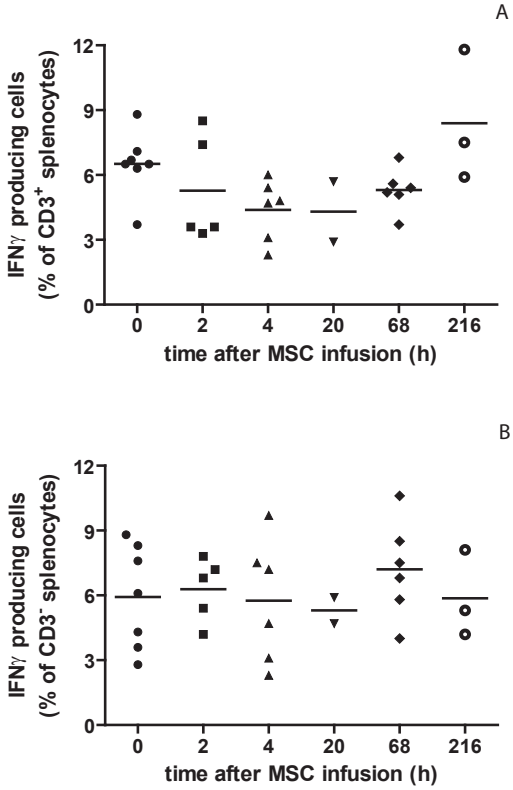

Figure 6. In vitro IFN- γ response of splenocytes isolated at various time points after IV infusion of MSCs. Splenocytes were stimulated for 4h with PMA/ionomycin and intracellular IFN- γ production measured by flow cytometry. The percentage of IFN- γ producing cells of CD3+ (A) and CD3- (B) splenocytes is depicted.

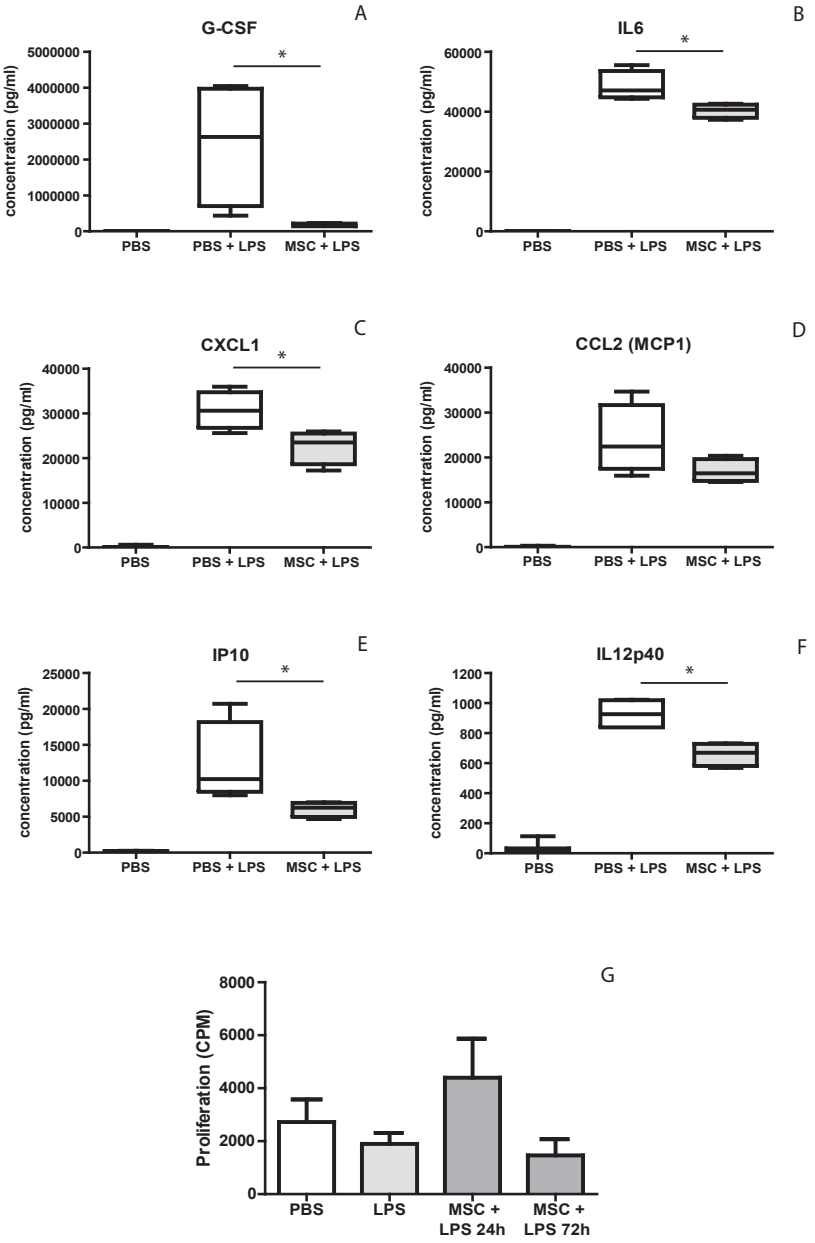

Figure 7. Effect of MSC infusion on the response to LPS. Mice received 5mg/kg LPS 3 days after IV infusion of 300,000 MSCs or phosphate- buffered saline. Four hours later, mice were sacrificed and serum and splenocytes collected. (A-F) Protein concentrations of G-CSF, IL6, CXCL1, CCL2 (MCP1), IP10, and IL12p40 in serum were determined by Milliplex assay. Means with 95% percentiles of 4 measurements with SD shown. (G) Proliferative response to ConA of splenocytes from animals injected with LPS with and without prior treatment with MSCs (24 or 72h). *P<0.01.

To examine whether the early immune activation induced by the infusion of MSCs would lead to an immunomodulatory effect in vivo later on, animals received 5 mg/kg LPS at day 1 or 3 after administration of MSCs or PBS and were sacrificed after 4 h. LPS induced major increases in serum concentrations of multiple cytokines and chemokines, including G-CSF, IL6, CXCL1, MCP1, IP10, and IL12p40. Infusion of MSCs 1 day before administration of LPS had no effect on the serum levels of these factors (data not shown). However, infusion of MSCs 3 days before LPS significantly reduced the increases in G-CSF, IL6, CXCL1, IP10, and IL12p40, suggesting a dampened immune response to LPS (Figure 7A–F).

To examine their proliferative response, splenocytes were stimulated with ConA. Splenocytes from animals that were treated with MSCs 24 h before LPS tended to show an increased proliferative capacity, whereas splenocytes from animals that were treated with LPS 72 h earlier tended to show reduced proliferation, although the differences were not significant (Figure 7G).

DISCUSSION

There is increasing interest in the use of MSC therapy for immunomodulatory purposes. In contrast to pharmacological immunosuppressants, which need to be taken lifelong and which suppress specific molecular pathways, MSC therapy is thought to be able to achieve long-term effects by modulating the function of various cells of the immune system. Although beneficial effects of MSCs have been demonstrated in inflammatory disease and transplant models, the mechanisms of immune modulation by MSCs after *in vivo* administration remain obscured. Understanding of these mechanisms is essential for the development of efficient MSC therapy.

Surprisingly, in the present study, we found that infusion of syngeneic MSCs induced an inflammatory response, which was detectable systemically and in lung and liver tissue. No inflammatory response was evident in the kidneys. The response in lung tissue was characterized by increased expression of monocyte and neutrophil chemoattractants and proinflammatory cytokines. In addition, we observed an increase in the expression of the macrophage markers, CD68 and F4/80, suggesting that intravenous infusion of MSCs causes an influx of macrophages in the lungs. We and others have described that MSCs interact with macrophages and induce macrophages with an immunomodulatory phenotype [30]. Whether macrophages that are recruited to the lungs upon infusion of MSCs are adapting a regulatory function will need further investigation.

The question rises whether the administered MSCs themselves were the source of the proinflammatory cytokine response. Gene expression changes in lung tissue coincided with the presence of MSCs and the genes that showed significant upregulation in the lung encode proteins that are expressed by MSCs. Nevertheless, a more likely possibility is that MSCs trig-

ger the expression of proinflammatory factors in resident cells of the lungs. MSCs are shown to accumulate in the microvasculature after infusion due to space restriction [31], where they mechanically interact with endothelial cells and leukocytes. The encounter of MSCs with lung endothelial cells and cells of the immune system may evoke the inflammatory response.

Our results strongly suggest that the increased expression of proinflammatory factors in liver tissue did not directly derive from MSCs. At the time the increases were observed, 4 h up to 216 h after MSC infusion, no MSCs were detected in the liver, indicating that liver resident cells are responsible for the gene expression changes. It is possible that the initial inflammatory response in the lungs is transferred via circulating chemokines and cytokines, which trigger a response in the liver. Another possibility is that mobile cell types like macrophages are activated by MSCs and transfer the signal from the lung to the liver via the blood stream. Some of the systemic changes in the levels of chemokines and cytokines are also unlikely to be a direct effect of MSCs. We observed a significant increase in G-CSF levels in serum after MSC infusion, but MSCs do not produce G-CSF [32]. Furthermore, MSCs decreased serum levels of CXCL5 and CCL11, showing that MSCs inhibit the production of these chemokines by other cells.

Although it seems that MSCs elicit a response of the immune system after infusion, at the moment, we can only speculate about the trigger that induces this response. As we used syngeneic MSCs, an alloresponse can be excluded. The infused MSCs, however, expressed GFP, whereas the recipients did not, but as GFP is an intracellular protein, it is unlikely to evoke an immune response as rapidly as was observed. It is possible that the immune system responds to infused MSCs as the cells are culture generated and differ in size and protein expression pattern from non-cultured MSCs [33]. In addition, under normal conditions, MSCs are present in the circulation at extreme low numbers [34, 35] and the high numbers present after MSC administration may activate a clearance response. It is thus likely that the immune reaction induced after infusion of MSCs is a response to cells that are seen as foreign and/or are present at an unusual location, rather than a MSC-specific response. Other cell types may well induce a similar response. We have not set out to test this in the present study.

How the immunoactivating effects of MSC administration lead to beneficial immunomodulatory effects in disease models remain unclear. The immune responses, which are evoked by MSCs, may result in an immunosuppressed status. In line with this hypothesis, we observed that the systemic inflammatory response to LPS was reduced 3 days after infusion of MSCs. We were not able to demonstrate reduced in vitro responsiveness of splenocytes from animals that received MSCs 3 days before LPS. In fact, splenocytes from animals that received LPS did not respond more potently to in vitro ConA stimulation than splenocytes from PBS control animals. This observation could relate to the fact that the ConA stimulation assay takes 3 days, after which the effect of LPS treatment of the animals may have been washed out.

In line with our data, organ transplant models have demonstrated that infusion of MSCs 7 to 4 days before transplantation was effective in prolonging transplant survival, whereas administration at later time points was not [28, 36]. In contrast, in models for arthritis [37] and colitis [38], administration of MSCs after inflammatory disease onset was shown to be effective. There may be differences in the effect of MSCs under inflammatory and homeostatic conditions. First, cytokine footprints are different under these conditions and MSCs will have a different modulating effect, and, second, MSCs, which are exposed to inflammatory conditions, become more immunosuppressive in vitro [15, 16] and in vivo [39] and may induce a different response upon infusion. Further research should clarify which conditions are optimal for effective MSC therapy for various applications.

In summary, intravenous infusion of syngeneic MSCs in mice triggers a systemic inflammatory response, which originates in the lungs and proceeds systemically. This response may lead to an immunosuppressed status in a few days via feedback mechanisms. Even though MSCs are present for only a few hours, their effects last for much longer and this offers possibilities to use MSCs for immunomodulatory therapy.

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Chapter 9

Summary and discussion

SUMMARY

Mesenchymal Stem or Stromal Cells (MSC) are multipotent cells with the ability to modulate immune responses *in vitro* and *in vivo*. These properties make them appealing for application as a cell therapeutic agent in the field of solid organ transplantation (SOT) (**chapter 1**). However, there are many queries considering the clinical application of MSC in SOT. These queries include MSC efficacy and mechanism of action, the optimal MSC preparation and safety and immunogenicity of MSC. The current thesis aimed to provide answers to these questions.

In **chapter 2**, the immunomodulatory effect of bone marrow-derived MSC (BM-MSC) and adipose tissue derived MSC (ASC) was evaluated *in vitro* and *in vivo*. BM-MSC and ASC were immunosuppressive *in vitro* and inhibited alloreactivity, as marked by decreased CD45+ leukocyte infiltration and IFNγ expression, in a humanized skin transplant model in the mouse. These results underline the potential of BM-MSC as well as ASC in the field of transplantation.

For MSC therapy, safety is crucial. Genetic stability of culture expanded ASC is an important safety aspect and was studied in **chapter 3**. It was found that ASC cultures contain on average 3.4% of aneuploid cells. This percentage increased with extended culture expansion and was highest in senescent ASC. Freshly isolated ASC did not contain aneuploid cells indicating that aneuploidy in ASC is a culture induced phenomenon. Importantly, aneuploid ASC did not transform *in vitro* nor did they cause tumour formation when injected in immunocompromised mice, indicating that aneuploidy in low passage ASC does not endanger safety of ASC therapy.

Culture conditions may provide opportunities to improve the cell therapeutic agent. There are indications that oxygen tension affects the differentiation capacity of MSC. The effect of oxygen tension on the immunosuppressive capacities of ASC was studied in **chapter 4**. It was found that the immunosuppressive potential of ASC is preserved under hypoxic conditions *in vitro* indicating that ASC can be used under conditions involving low oxygen tension such as in ischemic tissues.

In **chapter 5**, the allogenicity of BM-MSC and ASC was studied. When exposed to allogeneic PBMC, BM-MSC induced CD8+ T cells capable of HLA-class I specific cytotoxicity. ASC were also capable of inducing HLA-class I specific lysis, yet this lysis was significantly lower than the lysis induced by BM-MSC. IFNγ stimulation caused up-regulation of HLA-class I expression on BM-MSC and ASC and increased the lysis induced by BM-MSC and ASC. These results indicate that allogeneic BM-MSC and ASC can be immunogenic and advocate the use of autologous MSC in clinical organ transplantation to avoid sensitization.

It is questionable whether autologous MSC therapy in kidney transplantation is feasible as it implies using cells from patients with (end stage) renal disease. In this thesis, the effect of (end stage) renal disease on ASC (**chapter 6**) and BM-MSC (**chapter 7**) was investigated. ASC and BM-MSC derived from patients with (end stage) renal disease displayed a normal MSC immunophenotype and proliferation capacity. The immunosuppressive effects of

ASC and BM-MSC derived from renal disease patients were comparable to those from ASC and BM-MSC derived from healthy controls. Further, when ASC derived from renal disease patients were exposed to uremic conditions *in vitro*, ASC maintained their characteristics, including their anti-proliferative effect on immune cells. Together, these results indicate that it is feasible to isolate and use ASC and BM-MSC from renal disease patients and suggest that ASC remain functional when infused in a uremic patient.

Finally, while MSC are currently entering the clinical arena, little is known about how MSC exert their immunomodulatory potential after administration. In **chapter 8** it was aimed to untwine the effect of MSC infusion in mice. Infusion of syngeneic MSC resulted in a rapid accumulation of MSC in the lungs 2 hours after infusion. This was accompanied with a local and systemic inflammatory response which vanished within a day. When mice were challenged with LPS 3 days after infusion of MSC, the LPS induced inflammation was significantly decreased. Together, these results show that infusion of MSC induces a phase of immunosuppression after an initial phase of immune activation.

DISCUSSION

The current thesis describes the results of research aimed to get insight in the applicability of MSC therapy in clinical SOT, and in particular in kidney transplantation. In accordance with other studies [1-3], the potential of MSC as an immunosuppressive agent in transplantation was underlined in this thesis as it was demonstrated that MSC of both adipose and bone marrow origin are capable of inhibition of alloreactivity in vitro and in vivo. As a result of these immunomodulating capacities, MSC have already made their entrance in the clinical arena. While initial clinical studies were performed in osteogeneis imperfecta [4] and graft versus host disease (GvHD) patients [5], the indications for clinical MSC therapy expanded and include now a wide range of mainly immunological conditions including Crohn's disease [6], rheumatoid arthritis [7] and clinical organ transplantation. Recently, the first clinical trials using MSC in clinical kidney transplantation have been effectuated [8-13]. These studies mainly focussed on safety and feasibility of MSC therapy in kidney transplantation (table 1). The main conclusion of these studies is that MSC therapy in kidney transplantation is feasible and safe, and in addition, indications of immunosuppression were reported [8, 11].

These results are in accordance with studies performed in other medical fields showing safety and feasibility of clinical MSC therapy [6, 14]. However, substantial efficacy data is still lacking and an industry supported phase III trial in GvHD did not live up to expectations [15]. The absence of efficacy data from MSC trials might be explained by the great diversity in study design and the mainly small study sizes. Thus, to obtain reproducible and consistent efficacy data on the therapeutic effect of MSC, profound studies should be initiated including appropriate endpoints and standardized immune monitoring assays [16]. Yet, when designing

Table 1. Overview of published clinical trials with MSC in the field of kidney transplantation up till December 2013.

Reference	Primary end points	MSC preparation	MSC dose (MSC/kg BW)	Study population	Study design	Main conclusions
Perico et al.2011 (9)	Safety and feasibility of MSC therapy post KTx	Autologous BM-MSC	1.7- 2x10^6	Living KTx recipients (n=2)	MSC at day 7 post KTx + Basiliximab and low dose rATG induction therapy	-Feasibility of post KTx MSC therapy -After MSC infusion: - ↑ serum creatinine - ↑ peripheral Tregs - ↓ CD8+ memory cells
Tan et al. 2012 (11)	Efficacy of MSC therapy post KTx to reduce CNI levels	Autologous BM-MSC	1-2x10^6	Living KTx recipients (n=106)	MSC at day 0 and 14 + standard or low dose CNI versus IL2-R Ab + standard dose CNI	-After MSC therapy: -↓ acute rejections at 6 months -↓ risk of opportunistic infections -↑eGFR at 1 year
Perico et al. 2013 (10)	Safety and feasibility of MSC therapy pre KTx	Autologous BM-MSC	2x10^6	Living KTx recipients (n=2)	MSC infusion at day -1 KTx +low-dose ATG induction therapy	-Safety and feasibility of pre KTx MSC therapy -After MSC therapy: -↑ peripheral Tregs -↓memory CD8+T cells
Reinders et al., 2013 (8)	Safety and feasibility of MSC therapy for SCR and/or IF/TA post KTx	Autologous BM-MSC	2 X 1-2x10^6	Living KTx recipients with SCR and/or IF/TA 4 weeks or 6 months post KTx (n=6)	2 x MSC infusion, 7 days apart	-Safety and feasibility of post KTx MSC therapy -After MSC therapy: -opportunistic infections in 3/6 patients -↓donor specific lymphocyte proliferation
Peng et al., 2013 (12)	Safety and efficacy of donor derived MSC therapy post KTx	Allogeneic (kidney donor derived) BM-MSC	5x10^6 * (day 0) and 2x10^6 * (day 30)	Living KTx recipients (n=6)	MSC infusion intra (renal)-arterially at day 0 and intravenous at day 30 plus low dose CNI versus normal dose CNI	-Safety and feasibility of donor derived MSC therapy -After MSC therapy: -stable renal function with low dose CNI -↑peripheral B cells
Lee et al., 2013 (13)	Safety and feasibility of intra osseous injected MSC post KTx	Allogeneic (kidney donor derived) BM-MSC	1x10^6	Living KTx recipients (n=7)	Intra osseous injection of MSC at time of KTx	-Safety and feasibility of intra osseous-donor derived MSC therapy -After MSC therapy: -no peripheral chimerism

MSC dosages represent the number of infused MSC per kg body weight (BW) if not otherwise indicated with * as for the study of Peng et al. in which total numbers of infused MSC are depicted. MSC, Mesenchymal Stromal Cells; BM-MSC, bone marrow derived MSC; KTx, kidney transplantation; CNI, Calcineurin Inhibibitor; eGFR, estimated Glomerular Filtration Rate; SCR, subclinical rejection; IF/TA, interstitial fibrosis/tubular atrophy.

such studies, researchers will face multiple queries. These queries involve the optimal MSC preparation for clinical application and the mechanism of action and safety of MSC therapy.

Considering the optimal MSC preparation, culture expansion of MSC is an important topic. Culture expansion might involve a risk for contamination or transformation. Therefore, to ensure safety of MSC therapy, the MSC therapeutic product is subject to strict release criteria. There is a consensus on comprehensive testing of MSC for microbiological contamination, yet the genetic stability of MSC [17-22], and subsequently, the way to test MSC for genetic stability [23, 24] is under debate. When using FISH analysis, as described in this thesis, aneuploidy in culture expanded ASC was detected. Importantly, aneuploidy amongst ASC remained stable in low passage MSC and did not result in transformation *in vitro* or tumour formation *in vivo*. These results indicate that aneuploidy in MSC does not comprise a risk when low passage MSC are used.

Interestingly, it is unknown how aneuploidy in ASC develops and evolves. Aneuploidy in ASC might reflect aging and subsequently senescence of ASC in culture. This theory is supported by the increased percentage of aneuploidy in ASC with extended culture expansion as discussed in this thesis. However, while aneuploidy in ASC appears to be induced by culture expansion, aneuploidy ASC in vivo might also be undetectable as they are immunological cleared. Nonetheless, genetic stability of MSC for clinical application deserves continued attention; MSC cultures should be monitored for morphological changes and conventional karyotyping should be combined with other techniques such as CGH/SNP array or FISH[23, 24]. Additionally, though there are currently no indications for a clinical risk for MSC induced malignancies [25], the occurrence of de novo malignancies should be closely monitored after clinical MSC treatment.

Next to a potential risk, culture expansion may also provide a window of opportunity to improve the MSC preparation. For survival, MSC require growth factors and nutrients which are typically provided by supplemented fetal bovine serum (FBS) in the media. Adding IFNγ to the media has shown to increase the immunosuppressive potential of MSC [26] and the use of platelet lysate containing media increases the proliferation rate of MSC [27, 28]. Moreover, the use of platelet lysate (or human serum) containing medium provides an animal-free culture expansion protocol which is desirable to avoid potential xeno-reactivity.

Next to the culture medium, the environment in which MSC are expanded might affect MSC functionality. Culturing under hypoxic conditions has shown to affect the differentiation capacity of MSC [29]. As oxygen levels in tissues are substantially lower than the oxygen levels in incubators [30], hypoxic culture conditions might be more physiological for MSC. In the current thesis, it was described that hypoxia did not abolish the immunomodulatory effect of MSC *in vitro*. This implies that MSC can maintain their immunosuppressive effect when they are applied in ischemic conditions.

An alternative to the use of culture expanded MSC is the use of freshly isolated MSC. However, as described in the current thesis and by others [31, 32], freshly isolated MSC have

different characteristics compared to their cultured counterparts. The immunosuppressive potential of freshly isolated MSC is unknown and needs to be evaluated before these freshly isolated cells might be applicable in clinic.

Next to the question how and if MSC should be expanded, the choice of MSC preparation includes two other important questions: is there a favourable tissue to isolate MSC from? And should MSC from autologous or allogeneic origin be used? While MSC are originally isolated from bone marrow [33] and bone marrow derived MSC (BM-MSC) are still the most commonly used MSC, MSC can be isolated from multiple tissues[34]. Currently, based on MSC functionality, no superior source of MSC has been identified. In this thesis, it was demonstrated that BM-MSC and adipose tissue derived MSC (ASC) were equally effective in inhibiting PBMC proliferation *in vitro* and alloreactivity in a humanized mouse model. This indicates that, in accordance with others [35, 36], ASC provide a good alternative for BM-MSC.

Next, the choice arises whether to use ASC or BM-MSC from autologous or allogeneic tissues. Allogeneic MSC have the benefit that they can be isolated from healthy individuals, culture expanded and tested and stored in advance. Yet, as described in the current thesis, BM-MSC and ASC can induce HLA-class I specific cytotoxicity when exposed to allogeneic PBMC. Surprisingly, BM-MSC induced more lysis than ASC. The underlying mechanism explaining this difference between BM-MSC and ASC is unknown and it is up to now undetermined whether the induction of HLA-class I cytotoxicity will also take place *in vivo*. Nonetheless, the induction of HLA-class I specific cytotoxic T cells by allogeneic BM-MSC and ASC *in vitro* contradicts the old paradigm that MSC are low immunogenic. Furthermore, although contradicting data is published, MSC induced antibody production seems possible[37]. Hence, allogeneic MSC administration might induce T and B immune responses. Such immune activation could impair the effect of repeated MSC administration and even future organ transplantations if the transplanted organ shares HLA typing with MSC. Studies using allogeneic MSC for indications such as Crohn's disease and rheumatoid arthritis might be able to provide valuable data on the risk of sensitization by allogeneic MSC therapy. At this stage however, usage of autologous MSC in transplant patients seems the safest option to avoid any risk of sensitization. However, in situations when allogeneic MSC therapy is preferential, or the only feasible option, mismatching of MSC donor and organ donor can provide in a low-risk regimen to exclude the possibility of T and B cell activation against the allograft. Finally, screening for signs of sensitization after MSC therapy, such as MSC-HLA specific antibodies, should be part of studies using allogeneic MSC.

While the use of autologous MSC might be preferential in the field of SOT, feasibility of this strategy was unknown as several diseases [38, 39] have been shown to affect MSC functionality. In this thesis, it was documented that BM-MSC and ASC were not affected by renal disease. Their proliferation rate was unaffected which enables expansion of sufficient numbers of MSC, and importantly, the *in vitro* immunosuppressive potential of BM-MSC and ASC was not affected by the renal disease. So as renal disease is known to affect several other

cell types [40], the current thesis shows that it does not affect MSC functionality and that autologous MSC therapy in kidney transplantation is thus feasible.

Taken together, the results presented in the current thesis support the use of culture expanded autologous BM-MSC or ASC for clinical application in kidney transplantation. Still, besides MSC preparation, multiple other aspects of clinical MSC therapy need further elucidation. An important topic is the timing of MSC therapy. To date, MSC have been applied, pre-[9], shortly after [10] and late after [8] kidney transplantation. MSC infusion shortly after transplantation in the present of (ATG) induction therapy caused increases in serum creatinine in two patients, which was not the case when MSCs were administrated pre transplantation. These data indicate, in accordance with findings in animal studies [2, 10] that pre-transplantation infusion might be the preferred time of administration of MSC. Yet, the optimal timing is closely related to the mechanism of action of MSC therapy and this requires further evaluation.

It has been suggested that MSC migrate *in vivo* to sites of injury and or inflammation after infusion [41, 42]. However, multiple studies, including a study described in this thesis, indicate that MSC do not migrate beyond the lungs after infusion [43, 44]. The contradiction of these results with other tracing studies can be explained by the fact that most studies trace MSC label and cannot discriminate between label and MSC. It furthermore raises the question how MSC therapy works if MSC are only shortly present after infusion.

The last aim of this thesis was therefore to study the effect of MSC infusion in mice. It was found that after intravenous administration, MSC accumulated in the lungs. This was accompanied with a rapid pulmonary, and slightly later, a systemic inflammatory response. Within hours, the MSC in the lungs as well as the inflammatory response vanished. After 3 days, MSC infusion resulted in immune suppression. Hence, as MSC are presumably not present anymore at this time, it can be hypothesized that MSC might act via an initial phase of immune activation. During this phase of inflammation, MSC might be able to interact with immune cells such as macrophages resulting in immunosuppression at a later stage. Nevertheless, this hypothesized mechanism of action can probably only account for part of the effect of MSC as locally administrated MSC can also be effective as described in this thesis. Therefore, the importance of the MSC induced immune activation needs further study. Additionally, the optimal dosing of MSC therapy needs investigation. Currently, most studies used a treatment regimen including 1 or 2 dosages of approximately 1 – 2 X 10^6 MSCs/kg body weight. However, dose escalating studies, such as designed for liver transplant recipients[45], and studies evaluating frequency and interval of MSC infusion will be informative of the best treatment regimen. Evaluation of repeated MSC infusion is of particular importance in studies using allogeneic MSC as they might evoke alloreactivity which could affect study outcome.

CONCLUSION

Taken together, in this thesis it was aimed to eliminate some of the preclinical issues which come across when applying MSC in clinical (kidney) transplantation. It was concluded that both MSC derived from bone marrow and adipose tissue are immunosuppressive in a preclinical transplant model; that low passage culture expanded MSC do not transform and form no oncogenic risk; but that the use of allogeneic MSC might involve the risk for anti-HLA sensitization which argues for the use of autologous MSC for clinical application. The feasibility of using autologous MSC treatment in kidney transplantation was confirmed by showing that bone marrow and adipose tissue derived MSC are not affected by renal disease. Finally, in search for the mechanism of action of MSC therapy, MSC were found capable of immune suppression after an initial phase of immune activation.

The results discussed in this thesis are of relevance for the clinical application of MSC in solid organ transplantation and in particular for the application of MSC in kidney transplantation.

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Chapter 10

Dutch summary
(samenvatting)

Vitale organen zoals het hart, de longen, lever en nieren zijn essentieel voor overleving. Wanneer organen falen als gevolg van ziekte of schade is orgaantransplantatie de enige genezende behandeling. Ieder individu heeft unieke 'transplantatie moleculen' (HLA-moleculen) op zijn of haar organen. Lichaamsvreemde (allogene) HLA-moleculen op getransplanteerde organen zullen herkend worden door het immuunsysteem van de ontvanger van dit orgaan. Deze herkenning initieert een afstotingsreactie (alloreactiviteit). Deze afstotingsreactie dient geremd te worden met afweer-onderdrukkende medicijnen om het orgaan te behouden. Ontwikkelingen op het gebied van deze afweer-onderdrukkende medicijnen hebben de afgelopen decennia gezorgd voor indrukwekkende verbetering van de korte termijn uitkomst van orgaantransplantatie. Deze medicijnen hebben echter ook bijwerkingen die vooral de lange termijn uitkomst na transplantatie beïnvloeden. Om de lange termijn uitkomst na transplantatie te verbeteren is er behoefte aan nieuwe behandelmogelijkheden. Celtherapie, waarbij specifieke therapeutische cellen worden toegediend, kan mogelijk voorzien in deze behoefte.

Mesenchymale Stam- of Stromale Cellen (MSC) zijn voorloper cellen die kunnen differentiëren in onder andere bot- en vetcellen. Verder zijn deze MSC in staat om een afweerreactie te remmen. Deze eigenschappen maken MSC interessant voor toepassing als celtherapie in orgaantransplantatie patiënten (**hoofdstuk 1**).In transplantatie patiënten zouden MSC gebruikt kunnen worden om afstotingsreacties te remmen en beschadigde organen te repareren. Er zijn echter nog veel onzekerheden omtrent de klinische toepassing van MSC. Deze vragen betreffen de effectiviteit, werkingsmechanismen en veiligheid van MSC en de optimale MSC preparatie. Het doel van dit proefschrift was het beantwoorden van deze vragen.

In **hoofdstuk 2** werd het immuun modulerend effect van beenmerg MSC (BM-MSC) en vetweefsel MSC (ASC) bestudeerd. BM-MSC en ASC waren allebei in staat om de proliferatie van imuuncellen te remmen. Vervolgens werd het effect van BM-MSC en ASC bestudeerd in een diermodel. In dit model werd menselijke huid getransplanteerd op de rug van een muis. Toediening van menselijke allogene imuuncellen veroorzaakte vervolgens afstoting van de huid wat gekenmerkt werd door toename van de imuuncellen en het ontstekingseiwit IFNγ in de huid. Deze afstotingsreactie werd geremd door therapie met BM-MSC en ASC. Deze resultaten ondersteunen het toedienen van BM-MSC en ASC als celtherapie in orgaantransplantatie patiënten.

Veiligheid is cruciaal voor MSC therapie. De genetische stabiliteit van de cellen is daarbij van groot belang en werd bestudeerd in **hoofdstuk 3.** Er werd aangetoond dat een percentage van de gekweekte ASC een abnormaal aantal kopieën van de chromosomen had (aneuploidie). Dit percentage nam toe wanneer ASC langer gekweekt werden. Ongekweekte ASC bevatten geen aneuploide cellen wat impliceert dat aneuploidie wordt geïnduceerd door het kweken van ASC. Van belang is dat geen van ASC kweken kwaadaardig transformeerden of resulteerden in tumor groei wanneer de ASC werden geïnjecteerd in muizen zonder afweer.

Het kweken van MSC biedt de mogelijkheid tot optimalisatie van het MSC celproduct. Het gebruik van lage zuurstof condities (hypoxia) bevorderde de differentiatie capaciteit van MSC. Het effect van hypoxia op de immuunmodulerende eigenschappen van MSC is echter niet bekend en werd bestudeerd in **hoofdstuk 4**. Wanneer ASC werden blootgesteld aan hypoxia behielden ze hun immuun onderdrukkende eigenschappen. Dit betekent dat ASC kunnen worden toegediend in situaties met lage zuurstof spanning zoals getransplanteerde organen.

In **hoofdstuk 5** werd onderzocht of HLA-moleculen op allogene BM-MSC en ASC zelf een immuunreactie kunnen opwekken. Blootstelling van BM-MSC aan allogene imuuncellen resulteerde in de inductie van cytotoxische immuuncellen die in staat waren tot HLA specifieke lysis. ASC waren ook in staat tot het induceren van HLA specifieke lysis door cytotoxische immuuncellen. Echter, deze lysis was significant lager dan de lysis geïnduceerd door BM-MSC. Stimulatie van BM-MSC en ASC met het ontstekingseiwit IFNγ zorgde voor verhoging van het HLA op BM-MSC en ASC en de geïnduceerde lysis. Deze resultaten tonen aan dat allogene BM-MSC en ASC immunogeen kunnen zijn. Dus, om alloreactiviteit te voorkomen heeft het gebruik van patiënt eigen (autologe) MSC in orgaantransplantatie patiënten de voorkeur boven het gebruik van allogene MSC.

Het is echter de vraag of autologe MSC therapie in niertransplantatie patiënten mogelijk is omdat dit het gebruik betekent van cellen van nierziekte patiënten. In dit proefschrift werd het effect van nierziekte op BM-MSC (**hoofdstuk 6**) en ASC (**hoofdstuk 7**) onderzocht. ASC en BM-MSC afkomstig van patiënten met nierziekte vertoonden een normaal MSC uiterlijk en deling capaciteit. De anti-proliferatieve effecten van ASC en BM-MSC afkomstig van nierziekte patiënten waren vergelijkbaar met die van gezonde donoren. Ten slotte waren de karakteristieken van ASC, inclusief het immuun onderdrukkende effect, niet aangedaan wanneer ASC werden blootgesteld aan bloedserum van nierziekte patiënten. Deze resultaten tonen aan dat het mogelijk is om functionele BM-MSC en ASC te isoleren van patiënten met nierziekte en suggereren bovendien dat ASC hun functionaliteit behouden wanneer ze toegediend zouden worden in een patiënt met nierziekte.

Ten slotte, terwijl MSC momenteel al in de kliniek gebruikt worden is er nog weinig bekend over hoe MSC werken. In **hoofdstuk 8** werd het effect van MSC infusie in muizen bestudeerd. Infusie van MSC in muizen resulteerde in een snelle ophoping van de MSC in de longen. Tegelijkertijd was er in de longen een lokaal en in het bloedserum een systemische ontstekingsreactie te detecteren. Deze reactie verdween binnen een dag. Drie dagen na het toedienen van MSC was het immuunsysteem van de muizen echter onderdrukt.

Samenvattend tonen de resultaten besproken in dit proefschrift aan dat BM-MSC en ASC in staat zijn om een afweerreactie te onderdrukken, dat het kweken van MSC veilig is maar dat allogene MSC zelf wel een alloreactie kunnen veroorzaken. Om deze alloreactie te voorko-

men heeft het gebruik van autologe therapie de voorkeur. Dit is ook mogelijk aangezien de BM-MSC en ASC van patiënten met nierziekte niet aangetast zijn in hun functie. Ten slotte werd geconcludeerd dat infusie van MSC in muizen een ontstekingsreactie veroorzaakte waarna een fase van onderdrukking van het immuunsysteem volgde.

Appendix

ACKNOWLEDGEMENTS / DANKWOORD

Het gaat om het scheppen van een orde waarin ruimte is voor chaos (Ida Gerhardt).

Promotieonderzoek doen, een proefschrift schrijven; dat doe je niet alleen.

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Enne, binnenkort weer samen naar het werk fietsen?

Marieke

LIST OF PUBLICATIONS

Advancement of Mesenchymal Stem Cell Therapy in Solid Organ Transplantation (Misot).

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PHD PORTFOLIO

Name PhD student: Marieke Roemeling-van Rhijn
Erasmus MC department: Internal Medicine, Section Nephrology and Transplantation
PhD period: January 2010-January 2014
Research school: Postgraduate School Molecular Medicine
Promotor: Prof.dr. Willem Weimar
Co-promotor: dr. Martin J. Hoogduijn

1.PhD Training

Courses and workshops:

2010 Good Clinical Practice (Basiscursus Regelgeving Klinisch Onderzoek, BROK)*
2010 Short Introductory Course on Statistics & Survival Analysis for MD's*
2011 Laboratory animal competence (Article 9)*
2011 Photoshop and Illustrator CS5 workshop, Molmed*
2011 European Transplant Fellow Workshop, European Society of Organ Transplantation
2011 Biomedical English Writing and Communication, Molmed*
2012 Molecular Immunology for PhD students*

*Erasmus MC, Rotterdam

Participation and presentations at (inter)national conferences

2010	Science days, Dept. of Internal Medicine, Antwerp, Belgium	participation
2010	Misot, Rotterdam	participation
2010	Annual Meeting NTV (Bootcongres), Rotterdam	participation
2010	TTS, Vancouver	poster
2010	Dutch Stem Cell meeting, Utrecht	participation
2010	Regional Meeting ISCT-Europe, Belgirate, Italy	poster
2011	Annual Meeting NTV (Bootcongres), Amsterdam	presentation
2011	Misot, Leuven, Belgium	presentation
2011	American Transplantation Congres(ATC), Philedelphia, USA	poster*
2011	Annual ISCT Meeting, Rotterdam	poster
2011	ESOT, Glasgow, UK	mini-oral
2012	Science days, Deptof Internal Medicine, Antwerp, Belgium	poster
2012	Annual Meeting NTV (Bootcongres), Maastricht	presentation
2012	Molmed day, Rotterdam	poster
2012	American Transplantation Congres (ATC), Boston, USA	poster*
2013	Annual Meeting NTV (Bootcongres), Duiven	presentation
2013	ESOT, Vienna, Austria	presentation

2013	ISN, Florence, Italy	poster*
2013	ATC, Seattle, Washington, USA	poster*
2013	Basic Science Meeting TTS/ESOT, Paris, France	poster
2013	PLAN day, Dutch Federation of Nephrology), Rotterdam	presentation

* presented by dr. M.J. Hoogduijn

2. Teaching activities

Lecturing

2011 Lectures for Transplantation minor, Erasmus University (medical students)

2011 Seminars on stem cells for HOVO(Hoger Onderwijs Voor Ouderen), Erasmus University

Supervising

Supervision of HLO (Hoger Laboratorium Onderwijs) bachelor students:

Fane Mensah (November 2010- June 2011)

Esther Jongste (November 2011-June 2012)

3. Other

Memberships

2010-present	Dutch Transplant Society (Nederlandse Transplantatie Vereniging, NTV)
2010-present	Mesenchymal Stem Cells in Solid Organ Transplantation (MiSOT) study group
2010-2012	International Society for Cellular Therapy (ISCT)
2010-2013	European Society for Organ Transplantation (ESOT)
2013-present	Dutch Society of Hepatology (Nederlandse Vereniging van Hepatologie, NVH)

ABOUT THE AUTHOR

Marieke was born as Marieke van Rhijn on November 8th 1982, in Schiedam, The Netherlands. She was raised by her beloved parents Hans van Rhijn and Anne Mieke Groeneveld in Schiedam. In 2002, she completed secondary school and started her medical school at the Erasmus University in Rotterdam, the Netherlands. During her study, Marieke became interested in the field of (liver) transplantation and research. In 2007, she performed her graduation research under supervision of prof.dr. H.J. Metselaar entitled 'The value of ultrasonography in the long term follow after liver transplantation'. Marieke finished her internships with a final internship 'Transplantation surgery' in the Queens Elisabeth Health and Sciences Centre in Halifax, Nova Scotia, Canada under supervision of dr. Ian P. Alwayn. In 2010, she graduated from medical school and started the work for the PhD project presented in this thesis. At the internal medicine transplant laboratory at the Erasmus Medical Center Rotterdam, she studied Mesenchymal Stem Cells under the enthusiastic supervision of dr. Martin J. Hoogduijn and prof.dr. W. Weimar. Marieke married her love Stijn in 2012, together they have 2 children: Pieter (2011) and Hendrik (2012). In 2014 they started their new live together in Groningen. Marieke started her training to become a gastro-enterologist and hepatologist with her training residencies in Internal medicine at the University Medical Center Groningen (UMCG) under supervision of prof.dr. R.O. Gans which will be continued under supervision of dr. M.A. Meijssen at the Isala clinic Zwolle and under supervision of prof.dr. J.H. Kleibeuker in the UMCG.