Elucidation of the Mechanisms of Action of Mesenchymal Stem Cell Immunotherapy

Inzichten in het werkingsmechanisme van mesenchymale stamcel immunotherapie

Franka Luk

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

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Elucidation of the Mechanisms of Action of Mesenchymal Stem Cell Immunotherapy

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Imagination is more important than knowledge. For knowledge is limited, whereas imagination embraces the entire world.

Albert Einstein.
Cosmic Religion: With Other Opinions and Aphorisms, p. 97 (1931)]

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Chapter 1

General Introduction and Outline

Partly based on *"The Life and Fate of Mesenchymal Stem Cells"*

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Mesenchymal stem cells

Mesenchymal stem cells, alternatively named Mesenchymal stromal cells (MSC), are a heterogeneous population of adult stem cells that are virtually present throughout the whole body. MSC were first described by Alexander Friedenstein as a rare population of colony forming plastic-adherent cells within the bone marrow [1, 2]. Subsequent reports showed that these cells can also be obtained from adipose tissue, umbilical cord, dermis, spleen, muscle, dental pulp and other tissues [3-9]. The lack of a specific protein marker for MSC encouraged the International society for cellular therapy (ISCT) to set minimal criteria for the definition of MSC. These criteria state that MSC must express CD105, CD73 and CD90 markers, and lack expression of hematopoietic and endothelial markers such as CD45, CD34, CD31, CD14, CD11b and CD19 [10]. Moreover, MSC must have the capacity to differentiate into cells from the mesodermal lineages, such as osteoblasts, adipocytes and chondrocytes (figure 1). While MSC have been shown to also differentiate into other cell types, such as myocytes, tenocytes and neuron like cells, these properties are not considered requirements to define cells as MSC [11-15].

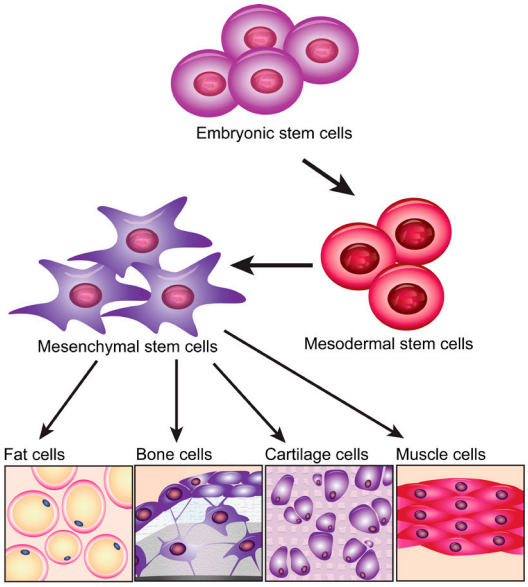

Figure 1. Multilineage differentiation capacity of Mesenchymal stem cells. MSC are adult stem cells that originate from Mesodermal stem cells. MSC have the capacity to differentiate into cells from the mesodermal lineage

Endogenous MSC

In the body, MSC are components of the stem cell niche [16]. In the occurrence of mechanical, chemical or disease-mediated tissue injury, endogenous signaling factors are released to initiate tissue- and injury-specific immune responses. Upon these signals, MSC are believed to relocate through the bloodstream to sites of injury to repair perturbed tissue as well as immunomodulate the surrounding environment. However, the migration of endogenous MSC is controversial [17]. Solid evidence for the migration of MSC via the bloodstream is sparse. One may wonder whether the recruitment of MSC from distant sites is required for the control of immune responses and initiation of repair in tissues as MSC are found locally in all tissues, from skin to brain [18]. In case of injury, local tissue-resident MSC need to travel only short distances to get to sites of injury and thereby cut the blood stream route short.

Advantages of MSC for therapeutic applications

The fact that MSC are able to differentiate into cells of the mesenchymal lineages in culture makes these cells the subject of investigation for potential use in regenerative medicine and tissue engineering. Originally it was anticipated that MSC could be used for replacement of dysfunctional cells via their capacity to differentiate into tissue cells. Over the last decades, it has become clear that MSC possess suppressive capabilities that could potentially be used to control several subsets of immune cells. For many immunological diseases, patients require lifelong treatment with immunosuppressive medication. Despite the improved quality of life of these patients on immunosuppressive medication, these drugs can lead to serious unwanted side effects, such as hypertension, development of diabetes, nephrotoxicity, infections and malignancies. Some of these side effects could be overcome by a shift from the use of generalized, nonspecific immunosuppressive drugs which inhibit both effector as well as regulatory immune cells, towards a more refined immune modulation to attain the optimal balance between effector and regulatory immune mechanisms. This led to an interest for the use of regulatory cells as cell-based therapies [19]. One of these candidates are MSC as they are relatively easy to isolate and expand in culture and have prospect as a therapeutic tool to promote immune tolerance.

Applications of MSC in regenerative medicine

The potential clinical applications of MSC for treatment of injured tissue have been abundantly tested. For example, directly injected MSC can promote tissue repair and have proven beneficial to treat heart damage and bone defects [20-23]. MSC are also studied to bioengineer functional human organs ex vivo. Macchiarini et al. successfully replaced the damaged bronchus of a patient by a bioengineered airway grew from autologous MSC and airway epithelial cells [24]. Novel 3D printing technology offers the possibility to generate custom-shaped MSC loaded hydrogels which can be transplanted at sites of injury. [25]. Although MSC have therapeutic potential for the promotion of tissue generation, more clinical trials are required to investigate the effectiveness, safety and side effects for the use of MSC in regenerative medicine.

Applications of MSC as immunomodulatory cell therapy

White blood cells or leukocytes are involved in protecting the body against infections and clean-up of aged or injured cells. Aberrant reactions of these immune cells can lead to autoimmune and inflammatory diseases. The immunomodulatory effect of MSC on immune cells in vitro is well established. MSC can suppress T cell proliferation induced by mitogens and alloantigens [26]. Along with this MSC can also alter T cell functions, such as decreasing IFNγ, IL-2, and TNFα production and increase of IL-4 secretion [27]. On the other hand, MSC promote the generation of CD4+ CD25+ CD127- Foxp3+ regulatory T cells (Tregs) [28, 29]. When co-cultured with B cells, MSC abrogate plasmablast formation and induce regulatory B cells (Bregs) [30]. Moreover, MSC can inhibit the maturation, activation and antigen presentation of Dendritic cells (DCs) and can induce DC into a distinct regulatory phenotype [31-33]. Thus, MSC are capable of both suppressing innate and adaptive immune responses and enhancing regulatory immune cells with tolerogenic properties in vitro.

Next to their immunomodulatory capacity, MSC are regarded as low immunogenic. This property could prove beneficial as it argues that MSC of allogenic origin could be used as MSC therapy without risking anti-HLA sensitization. Low expression of major histocompatibility (MHC) I and lack of MHC II and co-stimulatory molecules such as CD40, CD80 (B7-1), and CD86 (B7-2) leads to low immunogenicity, which

would prevent anti-MSC immune responses in recipients [34]. However, culture medium and plastic adherence have a major impact on the phenotype of MSC. The size of MSC dramatically increases in culture and the expression of adhesion molecules is strongly up regulated. We and others have demonstrated that activated NK cells can lyse culture-expanded MSC not only of allogeneic but also autologous origin [35, 36], suggesting that culture induces changes in MSC that makes them targets for NK cells. Therefore, further elucidation of the fate of MSC after infusion is needed to provide safe MSC therapy.

Clinical MSC immunotherapy

In 2004, MSC were first used as cellular immunomodulatory therapy in a graft versus-host disease (GVHD) patient [37]. Promising results of this study led to the initiation of several other studies in GVHD patients worldwide. Similarly, in patients with immunological diseases such as systemic lupus erythematosus (SLE) and Crohn's disease, MSC therapy showed to be feasible [38-40]. In addition, clinical studies with MSC therapy have been performed in patients suffering from aplastic anemia (AA), Type 1 diabetes mellitus, rheumatoid arthritis (RA), multiple sclerosis (MS) and Amyotrophic lateral sclerosis (ALS). Based on these many clinical trials, MSC based therapy appears safe [41]. However, the efficacy of MSC therapy is less clear as these studies mostly consist of low patient numbers, lack proper control groups and differ in MSC preparation, origin and timing and route of infusion.

MSC are short-lived after administration

The biodistribution of MSC is likely to depend on their route of administration. Most studies use the intravenous (IV) route and it has become clear that a large proportion of MSC that are injected via this route are trapped in the micro capillary network of the lungs upon first passage [42-45]. After 24 hours, the majority of MSC has disappeared and a small fraction is relocated to other organs, in particular the liver and also the spleen [42, 46]. MSC have also been reported to reappear at injured tissue sites [46]. It is however questionable whether MSC that leave the lungs are still viable. The accumulation of MSC in the lungs after IV infusion, their short survival time and limited distribution to other sites has led to the hypothesis that MSC rapidly pass on their effect to recipient cells, which may subsequently

mediate the immunomodulatory and regenerative effect induced by MSC administration. As the majority of infused MSC are around for only a short time, one might wonder how MSC modulate the host immune system during their short lifespan. By improving our understanding on the mechanistic properties of MSC immunomodulation, better tailored MSC therapy can be provided to patients.

Aim and Outline of this thesis

The aim of this thesis is to elucidate the mechanisms of action of MSC immunotherapy. Understanding how MSC based therapy works allows the design of effective MSC therapy.

In **chapter 2** the efficacy of MSC therapy is evaluated in a systematic review of 62 clinical studies which used MSC with the purpose of immunomodulation. In this chapter both clinical and immunological parameters that are associated with an immunomodulatory effect of MSC is examined to determine whether there is evidence that clinical MSC treatment leads to an immunomodulatory response and whether this is associated with an amelioration of immune disease severity. **Chapter 3** focusses on the effect of an inflammatory environment on MSC as MSC infused in patients might encounter an inflammatory environment that could influence the immunomodulatory effect of MSC. In this study we show that MSC affected B cells differently under inflammatory conditions. Contrary to preclinical studies, MSC are often cryopreserved before their use in clinical trials. In **chapter 4** we examine phenotypical differences between cryopreserved MSC and MSC from continuous culture to determine the effects of cryopreservation on MSC. Further, the effect of the lung microvasculature milieu on MSC properties is analyzed. Intravenously infused MSC do not pass the the lung barrier and have a half-life between 12 and 24 hours post infusion. This raises the question whether after IV infusion MSC live long enough to become activated by inflammatory conditions and exert their therapeutic effects via the secretome. In **chapter 5** we investigate whether infused MSC contribute to modulation of inflammatory responses by cytokine secretion and active cellular interactions or whether they merely trigger responses through recognition by host cells. Thereto inactivated MSC, that lost the capacity to respond to inflammatory stimulation and lost the ability to secrete factors are generated. Upon infusion, MSC rapidly disappear from the body. In **chapter 6** the mechanism involved in the clearance of MSC after infusion and the effects on the immune system are investigated in more detail.

In **chapter 7** we generate membrane particles from MSC and research the effect of these membrane particles on immune cells. In **chapter 8** the results obtained in the context of this thesis are summarized and appraised with respect to the elucidation of MSC cell-based therapy.

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Chapter 2

Efficacy of Immunotherapy with Mesenchymal Stem Cells in Man: a Systematic Review

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Abstract

Mesenchymal stem cells (MSC) are widely studied for their immunomodulatory properties. Data from in vitro and pre-clinical models demonstrate that MSC suppress activated immune cells and ameliorate the severity of experimental immune disease. In complex human studies, the immunomodulatory efficacy of MSC therapy is not well established. We conducted a systematic review of clinical studies which used MSC with the purpose of immunomodulation and included at least 10 patients to investigate the efficacy of MSC therapy. Sixty-two studies comprising 10 different immune disorders were included in the analysis, of which 18 studies represented controlled trials. Although several of the studies reported an amelioration of disease severity, other studies failed to observe a beneficial effect of MSC. The low number of randomized controlled trials, small number of studies per disease category and limited immunological readout parameters made it difficult to draw a definitive conclusion on the efficacy of MSC immune therapy.

Introduction

Mesenchymal stem cells (MSC) are characterized by their fibroblastic morphology and multilineage differentiation capacity [1]. They possess, in addition, potent immunosuppressive properties. MSC inhibit the proliferation and activity of T cells [2], modulate the differentiation of B cells [3] and induce regulatory macrophages in vitro [4]. MSC administration has been shown to be effective in ameliorating immune disease in animal models for among others colitis [5], sepsis [6], experimental autoimmune encephalitis [7] and prolong the survival of organ transplants [8]. These results have led to a vast interest in the use of MSC for clinical immunomodulatory therapy in a variety of immune disorders and organ transplantation [9,10].

In 2004, MSC were first used as an experimental immunomodulatory therapy in a graft-versus-host disease (GVHD) patient [11]. The results of this case report study were encouraging and led to the initiation of several other studies in GVHD patients worldwide. Supported by data from preclinical models, studies examining the effect of MSC in a range of immune disorders in man have been set up in recent years. Data from these trials and from trials aimed at exploiting the regenerative properties of MSC demonstrated that administration of MSC in over a 1000 patients was not associated with adverse effects, indicating that MSC therapy is safe [12].

While the safety of MSC therapy is now well established, the efficacy of MSC immunotherapy in man is under debate. There are a number of reasons for this. For instance, it has become clear that the in vivo immunomodulatory effects of MSC are not as straight forward as those seen in vitro [13]. Furthermore, a discrepancy may be expected between the immunomodulatory effects of MSC in experimental animal models and in human as inbreeding and pathogen-deprived conditions have a profound effect on the immune system. On top of that, large human studies are expensive and time consuming to set up and therefore clinical MSC studies are often limited to small numbers of patients, which makes it difficult to draw conclusions from these studies. Finally, immune therapy with MSC is examined in a wide variety of immune disorders that are caused by different immune cells and have different disease readouts.

This systematic review provides an overview of the clinical trials that have been performed with MSC in man with the purpose of immune modulation. Both clinical and immunological parameters that are associated with an immunomodulatory effect of MSC were analyzed with the aim to determine whether there is evidence that MSC treatment leads to an immunomodulatory response in man and whether this is associated with an amelioration of immune disease severity.

Methods

Eligibility criteria

All uncontrolled, non-randomized controlled and randomized controlled clinical trials examining MSC therapy in human patients of all ages with immunological disease were included in this review. Case reports and studies with less than 10 patients were excluded. Trials examining the regenerative capacity of MSC or trials using ex vivo differentiated MSC were also excluded.

Literature search

In collaboration with an information specialist from the medical library of the Erasmus MC, we performed a systemic literature search in Ovid MEDLINE, Embase.com, web-of science and the Cochrane library. Additional articles were retrieved from PubMed and Google Scholar. The final search date was 3 October 2014. Our search strategy included MSC and synonyms used for MSC in literature, along with immunological diseases or immunosuppression and related terms. When available thesaurus terms were used, MeSH terms in Medline and Emtree

terms in Embase. We limited the search results to clinical or epidemiological studies and studies in humans, and excluded conference proceedings. [supplementary material can be found online at www.informahealthcare.com/suppl/10.15861744666X.2015.1029458_Sup pl] for the complete search strategies for all databases.)

Study selection

All duplicates were removed from the search results. Two reviewers (FL, SFHW) independently screened the remaining titles and abstracts using standardized forms. Any discrepancies were resolved by discussion with a third reviewer (MJH).

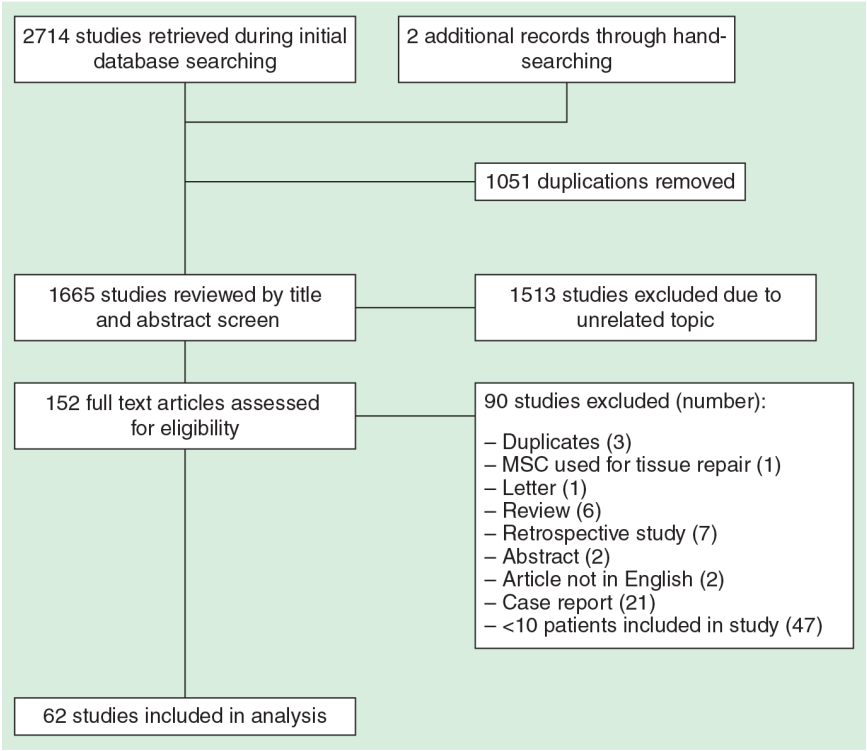

Figure 1. Flow diagram of the inclusion and exclusion of articles for this review.
MSC: Mesenchymal stem cell.

Assessment of risk of bias

The risk of biases of the included randomized controlled trials was assessed according to the Cochrane Collaboration methods [14].

Data extraction & analysis

Studies were grouped based on disease type. The type of study, patient numbers, length of follow-up after MSC transplantation and MSC characteristics were extracted from the publications. Both clinical outcomes and immunological parameters measured after MSC transplantation were extracted to judge the immunomodulatory efficacy of MSC. A narrative synthesis was performed due to the heterogeneity of the studies. The included studies differed in follow-up time, patient population, number and source of cells used, injection techniques and outcome measurements. Many of the studies lacked control groups and randomization and blinding was performed in a minority of the studies.

Results

Study characteristics

In the initial online search 2714 studies were retrieved. Duplicates were removed and 1663 studies were reviewed for eligibility criteria based on title and abstract. Of these studies, 1513 concerned an unrelated topic and were therefore excluded. Of the 150 remaining studies, 90 articles were excluded from the subsequent review process for various reasons (Figure 1). This resulted in 60 studies that met the inclusion criteria. Two studies were additionally added that did not come up in the search string but met all the criteria. Of the studies, 44 represented non-controlled trials whereas 18 concerned controlled trials (Table 1). Four of these trials made use of historic control groups [15–18], the other 14 studies were randomized controlled trials [19–23]. Two randomized controlled studies were double blinded [19,20]. The other 12 were neither blinded for the participants nor for the physicians. Twelve of the studies were multicenter studies [24–35]. The number of MSC-treated patients ranged from 6 to 105 (mean 27 ± 21). Six studies had a pediatric population [16,18,26,30,36,37] and 18 studies included a mixed adult and pediatric population [15,20,24,25,27–29,32,38–47]. The follow-up after MSC transplantation ranged from 0.6 to 89 months (Table 2). The majority of the studies used bone marrow (BM)-derived MSC, 12 studies used umbilical cord-derived MSC and one study used adipose tissue-derived MSC. In 13 studies, the

MSC were of autologous origin, in 9 studies the MSC were derived from the same donor as the hematopoietic or organ transplant and in the remaining 40 studies MSC were derived from a third party or off-the-shelf HLA matched or mismatched donor. The dose of MSC ranged from 0.03 to 10.1x106 cells/kg body weight and the frequency of infusions varied from 1- to 19-times. In most of the studies, MSC were infused intravenously (iv.). Other used routes of administration were intra-arterial [39,48–50], intraportal [38], intra-BM [32], intrasplenic [51,52], epidural [53], intrathecal [54–56] and intrafistular [47].

TABLE 1. OVERVIEW OF THE INCLUDED MESENCHYMAL STEM CELL-BASED CLINICAL TRIAL STUDIES.

Treated disease	*number of included studies (62)*	*number of controlled vs. non controlled*
Aplastic anemia	1	1; 0
COPD	1	1; 0
Crohn's disease	3	0; 3
Type 1 diabetes mellitus	2	1; 1
GVHD	32	8; 24
Liver diseases	7	5; 2
MS and ALS	6	0; 6
Kidney Tx	2	2; 0
Sjögren syndrome	1	0; 1
SLE	7	0; 7

ALS: Amyotrophic lateral sclerosis; COPD: Chronic obstructive pulmonary disease; GVHD: graft versus host disease; MS: Multiple sclerosis; SLE: Systemic lupus erythematosus; Tx: transplantation.

Quality assessment

Of the 62 trials included in this study, 44 were non-controlled trials in which the effect of MSC was compared before and after treatment. Eighteen were controlled trials, of which four compared the outcome of the MSC treatment group with historic controls. The 14 randomized controlled trials were assessed for risk of bias using the Cochrane Collaboration's tool for assessing risk of bias [9]. Twelve of the studies scored a 'high' in the risk of bias assessment for at least one of the criteria (Table 3). For one of the remaining two studies, risk of bias assessment was not possible for most of the criteria due to insufficient information in the publication. Overall, there is a strong risk of bias for the studies included in this systematic review.

TABLE 2. CHARACTERISTICS OF THE INCLUDED TRIALS

Disease, study (year)	*Country*	*Control groups*	*n patients (% male)*		*Age yrs (range)*		*Immunosuppressive co-medication*	*Follow up m (range)*	*Source; route of administration*	*Dosages x10 ^{6} /kg (range)*	*Frequency of infusion*	*Ref.*
			T	*C*	*T*	*C*						
Aplastic Anemia												
Xiao et al. (2013)	CHN	Historical	18 (78%)	18 (67%)	33 (16-58)	41.5 (13-79)	*not mentioned*	12	BM; 3rd; IV	0.6 (0.5 - 0.71)	1-6x	[15]
COPD												
Weiss et al. (2013)	USA	RCT: Placebo	30 (60%)	32 (56%)	68.1	64.1	*not mentioned*	24	BM; 3rd; IV	total: 400x10^6	4x	[19]
Crohn's disease												
Forbes et al. (2014)	AUS	-	16 (38%)	-	35.8 (21-55)	-	AZA, PR, MMF	1.4	BM; 3rd; IV	8.9 (7.1-10.1)	4x	[35]
Mayer et al. (2013)	USA	-	12 (75%)	-	38.3 (18-75)	-	PR, MESA,	24	PL; 3rd; IV	2 or 8	2x	[61]
Ciccocioppo et al. (2011)	ITA	-	12 (67%)	-	33.3 (16-59)	-	AZA, PR, MESA, MTX	12	BM; A; intrafistular	20 (15-30)	4x (range 2-5)	[47]
Type 1 Diabetes mellitus												
Hu et al. (2013)	CHN	RCT: Placebo	15 (60%)	14 (57%)	17.6	18.2	*not mentioned*	21	UC; IV	total: 2.6x10^7 (1.5 - 3.2x10^7)	2x	[20]
Vanikar et al. (2010)	IND	-	11 (64%)	-	21.1 (13-43)	-	-	7.3 (2.2-12)	AT; 3rd; intraportal	total 11.56x10^7	1x	[62]
GVHD												
Acute												
Kurtzberg et al (2014)	USA	-	75 (59%)	-	8.6 (0.2-17.5)	-	CST	3.28	BM, 3rd; IV	2	8x	[36]
Yin et al. (2014)	USA	-	10 (60%)	-	39 (20-71)	-	MEP, PR, Tac, BUD, INX, BX, SRL, MMF, CSA,	9.9 (7.3-13.2)	BM, 3rd; IV	2	3x	[67]
Calkoen et al. (2013)	NLD	-	22 (54%)	-	6.3 (0.7-18.1)	-	CSA, MTX, PR, MMF or TAC	12.5 (8-89)	UC; IV	1-2	2x (range 1-3)	[37]
Jitschin et al. (2013)	SWE	RCT: Placebo	6 (83%)	5 (80%)	48.2 (27-66)	53.8 (44-65)	CST CSA or Tac, SRL, MTX	6	BM, 3rd; IV	2 (1.5–2.2)	1x	[21]
Muroi et al. (2013)	JPN	-	14 (29%)	-	52 (4–62)	-	CSA, MTX, TAC, PR, MEP	24	BM, 3rd; IV	2	8x (range 3-12)	[29]
Resnick et al. (2013)	ISR	-	50 (56%)	-	19 (1-69)	-	MEP, CSA, Tac, SRL, MMF, ATG, anti CD25 AB	43	BM, 3rd; IV or IA	1.14	1-4x	[39]
Prasad et al. (2011)	USA	-	12 (83%)	-	6 (0.4-15)	-	MEP, Tac, INX, DZB, CSA, ETN	20 (14-36.5)	BM, 3rd; IV	2 or 8	2-21x	[30]

3rd: 3rd third party; A: autologous; AB: antibody; ALS: Amyotrophic lateral sclerosis; AT: adipose tissue; ATG: antithymocyte globulin; AZA: Azathioprine; BM: bone marrow; BUD: budesonide, BX: Basiliximab; CNI: calcineurin inhibitors; C: Control group; COPD: Chronic obstructive pulmonary disease; CSA: Cyclosporine; CST: corticosteroids; CTX: cyclophosphamide; D: donor derived; DZB: daclizumab; ETN: etanercept; GVHD: graft versus host disease; IA: intra-arterial; INX: infliximab, IV: intravenous; LEF: Leflunomide; MEP: Methylprednisolone; MESA: Mesalamine; MMF: Mycophenolate mofetil; MS: Multiple sclerosis; MTX: methotrexate; PL: placenta; PR: prednisone/ prednisolone; RCT: randomized controlled trial; SLE: Systemic lupus erythematosus; SRL: sirolimus, T: Treatment group; Tac: Tacrolimus; Tx: transplantation; UC: umbilical cord.

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Disease, study (year)	*Country*	*Control groups*	*n patients (% male)*		*Age yrs (range)*		*Immunosuppressive co-medication*	*Follow up m (range)*	*Source; route of administration*	*Dosages x10 ^{6} /kg (range)*	*Frequency of infusion*	*Ref.*
			T	*C*	*T*	*C*						
GVHD												
Acute												
Kebriaei et al. (2009)	USA	-	31 (68%)	-	52 (34-67)	-	CST, MMF, Tac, CSA, MTX, INX,	2.9	BM, 3rd; IV	2 or 8	2x	[31]
Von Bonin et al. (2009)	DEU	-	13 (54%)	-	58 (21–69)	-	FLU, ATG, INX, PR, MTX	8.4	BM, 3rd; IV	0.9 (0.6-1.1)	2x (range 1–5)	[68]
Le Blanc et al. (2008)	SWE	-	55 (62%)	-	22 (0.5-64)	-	CST, CNI, MMF	16 (1.5-64)	BM, 3rd; IV	1.4	1-5x	[24]
Chronic												
Peng et al. (2014)	CHN	-	23 (78%)	-	31 (14–51)	-	CNI, MMF	12	BM, 3rd; IV	1	3x	[40]
Peng et al. (2014)	CHN	-	38 (76%)	-	29.5 (18-51)	-	PR, MMF, Tac, CSA, SRL	12	BM, 3rd; IV	1	2-4 x	[69]
Weng et al. (2012)	CHN	-	22 (77%)	-	27.7 (16-39)	-	CST, MTX or CSA	3	BM, 3rd; IV	0.95 (0.23–2)	1x (range 1-6)	[41]
Weng et al. (2010)	CHN	-	19 (74%)	-	29.4 (18-39)	-	CSA, Tac, MTX,	22 (7.4-36.5)	BM, 3rd; IV	0.6 (0.23–1.42)	2x (range 1–5).	[70]
Acute and chronic												
Introna et al. (2014)	ITA	-	40 (68%)	-	27.8 (1-65)	-	CSA, MTX, ATG, ETN, MMF	33.6	BM, 3rd; IV	1.5 (0.8-3.1)	3x (range 2-11)	[27]
Herrmann et al. (2012)	AUS	-	19 (68%)	-	43 (21-58)	-	CNI, ETN	30	BM, 3rd; IV	1.7–2.3	2x (range 2-19)	[71]
Pérez-Simon et al. (2011)	ESP	-	18 (50%)	-	40 (21-66)	-	MEP	12	BM, 3rd; IV	1-2	1x (range 1-4)	[72]
Lucchini et al. (2010)	ITA	-	11 (72%)	-	9.3 (4-15)	-	MMF, ECP, CSA, AZA, ETN, Tac	8 (4-18)	BM, 3rd; IV	1.2 (0.7-3.7)	1-5x	[26]
Ringdén et al. (2006)	SWE	RCT: Standard therapy	9 (89%)	16 (56%)	56 (8–61)	40 (3–60)	ATG, MTX, CSA, MMF, PR, DZB, INX	36	BM, 3rd; IV	1.0 (0.7 to 9)	1-2x	[42]
Profylaxis for GVHD development												
Liu et al. (2014)	CHN	-	20 (70%)	-	26 (14-46)	-	CTX, ATG, CSA, MTX, MEP	16.7 (5.5 - 30)	BM, 3rd; IV	1	1-3x	[28]
Wu et al. (2014)	CHN	-	21 (52%)	-	18 (4-31)	-	CSA, MMF, ATG, CTX, anti-CD25 AB	2.5-78	UC; IV	5	1x	[43]
Xiong et al. (2014)	CHN		26 (27%)		40.5 (14-55)		*not mentioned*	11.3 (4.2-25.8)	BM, 3rd; IV	2.73 - 4.3 (2 - 5.47)	2-4x	[25]

3rd: 3rd third party; A: autologous; AB: antibody; ALS: Amyotrophic lateral sclerosis; AT: adipose tissue; ATG: antithymocyte globulin; AZA: Azathioprine; BM: bone marrow; BUD: budesonide, BX: Basiliximab; CNI: calcineurin inhibitors; C: Control group; COPD: Chronic obstructive pulmonary disease; CSA: Cyclosporine; CST: corticosteroids; CTX: cyclophosphamide; D: donor derived; DZB: daclizumab; ETN: etanercept; GVHD: graft versus host disease; IA: intra-arterial; INX: infliximab, IV: intravenous; LEF: Leflunomide; MEP: Methylprednisolone; MESA: Mesalamine; MMF: Mycophenolate mofetil; MS: Multiple sclerosis; MTX: methotrexate; PL: placenta; PR: prednisone/ prednisolone; RCT: randomized controlled trial; SLE: Systemic lupus erythematosus; SRL: sirolimus, T: Treatment group; Tac: Tacrolimus; Tx: transplantation; UC: umbilical cord.

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Disease, study (year)	*Country*	*Control groups*	*n patients (% male)*		*Age yrs (range)*		*Immunosuppressive co-medication*	*Follow up m (range)*	*Source; route of administration*	*Dosages x10 ^{6} /kg (range)*	*Frequency of infusion*	*Ref.*
			T	*C*	*T*	*C*						
Profylaxis for GVHD development												
Wu et al. (2013)	CHN	-	50 (48%)	-	26 (9-58)	-	FLU, CTX, ATG, MMF, BX, CSA	1-58	UC; IV	5	1x	[44]
Kuzmina et al. (2012)	RUS	RCT: Standard therapy	18 (39%)	19 (42%)	29 (19–60)	34 (20–63)	CTX, ATG, CSA, MTX, MMF, PR	2.5 - 32	BM, D; IV	0.9-1.3	1x	[57]
Bernardo et al. (2011)	NLD/ITA	Historical	13 (54%)	39 (61%)	2 (0.8–14)	4 (0.8–17)	CST, MTX	28 (19–38);	BM, 3rd; IV	1.9 (1–3.9)	1x	[16]
Liu et al. (2011)	CHN	RCT: Standard therapy	27 (74%)	28 (68%)	30 (14–46)	31.5 (12–48)	CTX, ATG, CSA	23.7 (0.7–33.5)	BM, D or 3rd; IV	0.3-0.5	1x	[45]
Baron et al. (2010)	BEL	Historical	20 (70%)	16 (81%)	58 (21-69)	55 (10-69)	Tac, PR, MEP, SRL	18.4 (13.1-29.8)	BM, 3rd; IV	*not mentioned*	1x	[17]
Zhang et al. (2010)	CHN	-	12 (67%)	-	38.2 (21-53)	-	CTX, CSA, MEP	29–57	BM, D; IV	1.78	1x	[73]
Guo et al. (2009)	CHN	-	33 (73%)	-	23 (7-43)	-	CSA, MMF, anti-CD25 AB, CTX, ATG,	1.5 to 60	BM, D; intra-BM	0.37 (0.05 - 1.7)	1x	[32]
Ning et al. (2008)	CHN	RCT: Standard therapy	15 (73%)	15 (87%)	38 (17–52)	37 (16–61)	MTC, CSA, CTX,	36.6 (0.6–44)	BM, D; IV	0.5 (0.03-1.53)	1x	[46]
Ball et al. (2007)	NLD	Historical	14 (61%)	47 (60%)	8 (1-16)	7.1 (1-17)	*not mentioned*	MSC group: (3-28) control group: (32-110)	BM, D; IV	1.6 (1-3.3)	1x	[18]
Lazarus et al. (2005)	USA	-	46 (52%)	-	44.5 (19-61)	-	CSA, MTX, CTX	12-24	BM, D; IV	1.0, 2.5 or 5.0	1x	[33]
Liver diseases												
Cirrhosis												
Xu et al. (2014)	CHN	RCT: Standard therapy	27 (58%)	29 (65%)	45	44	*not mentioned*	24	BM; A; intrahepatic	*not mentioned*	1x	[50]
Amin et al. (2013)	EGY	-	20 (70%)	-	51.3 (42-60)	-	*not mentioned*	6	BM; A; intrasplenic	total 1x10^7	1x	[51]
El-Ansary et al. (2012)	EGY	RCT: Standard therapy	9	10 (80%)	48 (32-60)	51.6 (39-60)	*not mentioned*	6	BM; A; IV	1	1x	[58]

3rd: 3rd third party; A: autologous; AB: antibody; ALS: Amyotrophic lateral sclerosis; AT: adipose tissue; ATG: antithymocyte globulin; AZA: Azathioprine; BM: bone marrow; BUD: budesonide, BX: Basiliximab; CNI: calcineurin inhibitors; C: Control group; COPD: Chronic obstructive pulmonary disease; CSA: Cyclosporine; CST: corticosteroids; CTX: cyclophosphamide; D: donor derived; DZB: daclizumab; ETN: etanercept; GVHD: graft versus host disease; IA: intra-arterial; INX: infliximab, IV: intravenous; LEF: Leflunomide; MEP: Methylprednisolone; MESA: Mesalamine; MMF: Mycophenolate mofetil; MS: Multiple sclerosis; MTX: methotrexate; PL: placenta; PR: prednisone/ prednisolone; RCT: randomized controlled trial; SLE: Systemic lupus erythematosus; SRL: sirolimus, T: Treatment group; Tac: Tacrolimus; Tx: transplantation; UC: umbilical cord.

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			T	*C*	*T*	*C*						
Cirrhosis												
Zhang et al. (2012)	CHN	RCT: Placebo	30 (86.7%)	15 (93%)	48 (25–64)	47 (29–64)	*not mentioned*	11	UC; IV	0.5	3x	[22]
liver failure												
Shi et al. (2012)	CHN	RCT: Placebo	24 (83%)	19 (79%)	40 (24–59)	45 (26–62)	*not mentioned*	16.6	UC; IV	0.5	3x	[23]
Peng et al. (2011)	CHN	RCT: Standard therapy	53 (94%)	105 (94%)	42.2	42.22	*not mentioned*	11	BM; A; IA	total 1x10^7	1x	[48]
El-Ansary et al. (2010)	EGY	-	12 (75%)	-	(32-69)	-	*not mentioned*	6	BM; A; intrasplenic or IV	total 1x10^7	1x	[52]
MS and ALS												
Karussis et al. (2010)	ISR	-	34 (50%)	-	35.3-53	-	*not mentioned*	6-25	BM; A; intrathecal and IV	total 63.2x10^6	1x	[54]
ALS												
Mazzini et al. (2010)	ITA	-	10 (70%)	-	41 (20–61)	-	*not mentioned*	30.2 (12-44)	BM; A; epidural	total: 7.46x10^7 (1.14-10.9)	1x	[53]
MS												
Bonab et al. (2012)	IRN	-	25 (24%)	-	34.7 (24-50)	-	*not mentioned*	12	BM; A; intrathecal	total: 2.95x10^7	1x	[55]
Connick et al. (2012)	UK	-	10 (70%)	-	48.8 (40–53)	-	*not mentioned*	10	BM; A; IV	1.6 (1.1-2)	1x	[59]
Yamout et al. (2010)	LBN	-	10 (40%)	-	42.8 (34-56)	-	MTX	12	BM; A; IV	total: 4.7x10^7 (3.2-10)	1x	[60]
Bonab et al. (2007)	IRN	-	10 (30%)	-	33 (22-40)	-	*not mentioned (attacks*	19 (13-26)	BM; A; intrathecal	total: 8.73x10^6 (2.26-18)	1x	[56]
Kidney Tx												
Peng et al. (2013)	CHN	RCT: Standard therapy	6 (100%)	6 (67%)	33.67	30.67	CTX, MEP, CNI, MMF	12	BM; D; IA and IV	IA total: 5x10^6; IV: 2	2x	[49]
Tan et al. (2012)	CHN	RCT: Standard therapy	105 (63%)	51 (67%)	37-39 (34-42)	37 (34-39.9)	MEP, MMF, CNI	12	BM; A; IV	1-2	2x	[63]

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			T	*C*	*T*	*C*						
Sjögren syndrome												
Xu et al. (2012)	CHN	-	24 (4%)	-	45 (27-68)	-	*not mentioned*	12	UC; IV	1	1x	[74]
SLE												
Gu et al. (2014)	CHN	-	81 (15%)	-	31.6 (12–55)	-	prednisone, CTX, MMF	12	BM; 3rd or UC; IV	1	1x	[75]
Wang et al. (2014)	CHN	-	40 (5%)	-	34 (16-54)	-	CSA, CTX, MMF, PR, LEF	12	UC; IV	1	2x	[34]
Wang et al. (2013)	CHN	-	87 (8%)	-	31.5 (12–56)	-	PR, CTX, MMF, LEF	27 (12–48)	BM; 3rd or UC; IV	1	1-2x	[76]
El-Ansary et al. (2012)	EGY	-	30 (57%)	-	48.2 (22-68)	-	*not mentioned*	6	BM; A or D; IV	0.7–1.0	2x	[77]
Wang et al. (2012)	CHN	-	58 (12%)	-	30-33 (12-54)	-	PR, MMF, LEF, CTX	27 (12-48)	BM; 3rd or UC; IV	1	1-2x	[78]
Liang et al. (2010)	CHN	-	14 (7%)	-	28.3 (12–44)	-	PR, MMF	17.2 (3-36)	BM; 3rd, IV	1	1x	[64]
Sun et al. (2010)	CHN	-	16 (13%)	-	31.8 (17-55)	-	PR, CTX	8.3 (3-28)	UC; IV	1	1x	[65]

3rd: 3rd third party; A: autologous; AB: antibody; ALS: Amyotrophic lateral sclerosis; AT: adipose tissue; ATG: antithymocyte globulin; AZA: Azathioprine; BM: bone marrow; BUD: budesonide, BX: Basiliximab; CNI: calcineurin inhibitors; C: Control group; COPD: Chronic obstructive pulmonary disease; CSA: Cyclosporine; CST: corticosteroids; CTX: cyclophosphamide; D: donor derived; DZB: daclizumab; ETN: etanercept; GVHD: graft versus host disease; IA: intra-arterial; INX: infliximab, IV: intravenous; LEF: Leflunomide; MEP: Methylprednisolone; MESA: Mesalamine; MMF: Mycophenolate mofetil; MS: Multiple sclerosis; MTX: methotrexate; PL: placenta; PR: prednisone/ prednisolone; RCT: randomized controlled trial; SLE: Systemic lupus erythematosus; SRL: sirolimus, T: Treatment group; Tac: Tacrolimus; Tx: transplantation; UC: umbilical cord.

Clinical outcomes of the included studies

All studies were grouped based on disease and treatment outcome was extracted and described in table 4. The majority of the included studies investigated the effect of MSC treatment on established GVHD or on the prevention of the development of GVHD (n = 19 and 13, respectively). The studies on established GVHD were sub grouped into acute GVHD (n = 10), chronic GVHD (n = 4) or combined acute and chronic GVHD (n = 5). Four of the studies were randomized controlled trials [21,45,46,57], whereas the remainder compared clinical parameters pre- and post-infusion. All except for one study used the iv. route of administration of MSC. In one study MSC were injected in the BM [32]. The main readout parameter for the outcome of the GVHD studies was defined as response to treatment, where a complete response (CR) is described as a complete resolution of all signs of GVHD and partial response (PR) as a reduction of GVHD to a less severe grading. The mean CR and PR in acute GVHD patients after MSC treatment was 51% (± 21%) and 15% (± 9%), respectively. The single randomized controlled trial in acute GVHD showed no significant difference in CR and PR rates between the MSC and placebo groups (Table 4). In chronic GVHD, two studies reported a response rate of on average 71% and two studies showed CR and PR in on average 17 and 57% of the patients, but in none of the cases–control groups was included. The mixed acute and chronic GVHD trials showed a CR of 32% (± 17%) and PR of 49% (± 16%).

TABLE 3. RISK OF BIAS ASSESSMENT OF RANDOMIZED CONTROLLED TRIALS ACCORDING TO THE COCHRANE COLLABORATION'S TOOL FOR ASSESSING RISK OF BIAS

Source	*Random sequence generation*	*Allocation concealment*	*Blinding of personnel*	*Blinding of outcome assessment*	*Incomplete outcome data*	*Selective reporting*	*Ref.*
Kuzmina et al. (2012)	U	U	H	H	L	L	[57]
Liu et al. (2011)	U	U	H	H	L	L	[45]
Ning et al. (2008)	H	U	H	H	H	U	[46]
Jitschin et al. (2013)	U	U	H	H	L	L	[21]
Tan et al. (2012)	L	L	H	H	L	L	[63]
Peng et al. (2013)	U	U	H	H	L	L	[49]
Zhang et al. (2012)	U	U	H	H	L	L	[22]
El-Ansary et al. (2012)	U	U	U	U	L	U	[58]
Xu et al. (2014)	L	L	H	H	H	U	[50]
Shi et al. (2012)	U	U	H	H	L	L	[23]
Peng et al. (2011)	H	H	H	H	U	U	[48]
Hu et al. (2013)	L	L	L	L	L	L	[20]
Weiss et al. (2013)	L	L	L	L	H	L	[19]
Ringdén et al. (2006)	H	H	H	H	L	U	[42]

H: High; L: Low; U: Unclear

The studies that investigated the prevention of GVHD by MSC determined the outcome of treatment by the incidence of the development of acute or chronic GVHD. The controlled studies within this group all show less incidence of GVHD in the MSC group compared with the control group [16–18,46,57]. One controlled study failed to show a reduction in the incidence of acute GVHD in the MSC versus control group (51.8 vs 38.9%), but was able to show a lower incidence of chronic GVHD (51.4 vs 74.1%) [45]. In summary, the results of the studies on GVHD hint toward a clinical immunomodulatory effect of MSC, but the lack of randomized controlled groups in the majority of the studies make it difficult to draw decisive conclusions at this point.

In seven studies, the effect of MSC on liver cirrhosis (n = 4) or liver failure (n = 3) was examined. These studies included two randomized [22,50] and three non-randomized controlled trials [23,48,58]. These studies differed in follow-up time and route of MSC administration (Table 2) and are therefore difficult to compare. All studies except for one used the Model for End-stage Liver Disease (MELD) score as a readout for liver function. The MELD score was significantly lower in MSC-treated groups compared with the control groups or before treatment (Table 4). Although there is a considerable risk for bias of the included studies, there is a careful indication that MSC therapy may be beneficial to improve the MELD score of liver disease patients. However, larger randomized controlled trials are needed to confirm the preliminary data.

The seven studies on systemic lupus erythematosus (SLE) were all non-controlled trials. The studies were similar in follow-up time and the route and dose of MSC administration. All studies except for one used the Systemic Lupus Erythematosus Disease Activity Index score to determine the clinical improvement. In all studies, the Systemic Lupus Erythematosus Disease Activity Index score improved significantly after MSC treatment. The lack of control groups make these outcomes difficult to interpret.

Five studies examined the effect of MSC on multiple sclerosis (MS) and two on amyotrophic lateral sclerosis (ALS). None of the studies was controlled. The effect of MSC was determined by the Expanded Disability Status Scale (EDSS) score for MS patients and by the ALS Functional Rating Scale and MRI assessment for ALS patients and compared between pre- and post-infusion. In two MS studies, MSC were infused iv., which resulted in a lower EDSS score [59,60]. In the studies where MSC were administered via the intrathecal route, no differences or an increase in EDSS score was measured [55,56]. MSC induced no differences in ALS Functional Rating Scale or MRI assessment in ALS patients [53,54].

TABLE 4. THERAPEUTIC EFFECTS OF MSC THERAPY

Disease	*Comparison*	*Main readout parameter*	*Outcome*	*Ref.*
Aplastic Anemia				
Xiao et al. (2013)	MSC infusion/historical control	Response to treatment (complete and partial)	MSC: 33.3%; control: 5.56%	[15]
COPD				
Weiss et al. (2013)	MSC infusion/ placebo infusion	improvement in pumonary function (FEV_1 , FVC, FEV_1/FVC)	No statistically significant differences	[19]
Crohn's disease				
Forbes et al. (2014)	pre/post infusion	disease activity (CDAI score); response to treatment	↓ post MSC; clinical response: 80%	[35]
Mayer et al. (2013)	pre/post infusion	disease activity (CDAI score); IBD questionnaire	↓ post MSC (low dose group); ↑ IBD questionnaire score	[61]
Ciccocioppo et al. (2011)	pre/post infusion	disease activity (CDAI score; PDAI score)	↑ post MSC; ↓ post MSC	[47]
Type 1 Diabetes mellitus				
Hu et al. (2013)	MSC infusion/ placebo infusion	serum C-peptide; exogenous insulin requirement	↑ in MSC group; ↓ in MSC group	[20]
Vanikar et al. (2010)	pre/post infusion	serum C-peptide; exogenous insulin requirement	↑ post MSC; ↓ post MSC	[62]
GVHD				
acute				
Kurtzberg et al. (2014)	pre/post infusion	clinical response (responders/ no responders)	57.5% response	[36]
Yin et al. (2014)	pre/post infusion	Response to treatment (complete, partial)	CR: 50%; PR: 20%	[67]
Calkoen et al. (2013)	pre/post infusion	Response to treatment (complete, partial)	CR: 50%; PR: 27%	[37]
Jitschin et al. (2013)	MSC infusion/ placebo infusion	Response to treatment (complete, partial)	↑ in MSC group: CR: 66,7%; PR: 16.7% vs. Placebo: CR: 60%; PR: 20%	[21]
Muroi et al. (2013)	pre/post infusion	Response to treatment (complete, partial)	CR: 57.1%; PR: 0%	[29]
Resnick et al. (2013)	pre/post infusion	Response to treatment (complete)	34%	[39]
Prasad et al. (2011)	pre/post infusion	Response to treatment (complete, partial)	CR: 58.3%; PR: 17%	[30]
Kebriaei et al. (2009)	pre/post infusion	Response to treatment (complete, partial)	CR: 77.4%; PR: 16.1%	[31]
Von Bonin et al. (2009)	pre/post infusion	Response to treatment (complete, partial)	CR: 8%; PR: 8%;	[68]
Le Blanc et al. (2008)	pre/post infusion	Response to treatment (complete, partial)	CR: 54.5%; PR: 16.4%	[24]
chronic				
Peng et al. (2014)	pre/post infusion	Response to treatment	87%	[40]
Peng et al. (2014)	pre/post infusion	Response to treatment (complete, partial)	CR: 13%; PR: 61%;	[69]
Weng et al. (2012)	pre/post infusion	Response to treatment	55%	[41]
Weng et al. (2010)	pre/post infusion	Response to treatment (complete, partial)	CR: 21%; PR: 52,6%	[70]
Acute and chronic				
Introna et al (2014)	pre/post infusion	Response to treatment (complete, partial)	CR: 27.5%; PR: 40%	[27]
Herrmann et al. (2012)	pre/post infusion	Response to treatment (complete, partial)	CR: 47.4%; PR: 31.6%	[71]
Pérez-Simon et al. (2011)	pre/post infusion	Response to treatment (complete, partial)	CR: 11%; PR: 50%	[72]
Lucchini et al. (2010)	pre/post infusion	Response to treatment (complete, partial)	CR: 23.8%; PR: 47.6%	[26]
Ringdén et al. (2006)	MSC infusion/ standard therapy	Response to treatment (complete, partial)	CR: 50%; PR: 75%	[42]
profylaxis for GVHD development				
Liu et al. (2014)	pre/post infusion	Response to treatment (complete, partial)	CR: 25%; PR: 60%	[28]
Wu et al. (2014)	pre/post infusion	Development of acute or chronic GVHD	aGVHD: 57.1%; cGVHD: 50%	[43]
Xiong et al. (2014)	pre/post infusion	Response to treatment (complete, partial)	CR: 77.3%; PR: 13.6%	[25]
Wu et al. (2013)	pre/post infusion	Development of acute or chronic GVHD	aGVHD: 42%; cGVHD: 37.7%	[44]
Kuzmina et al. (2012)	MSC infusion/ standard therapy	Development of acute or chronic GVHD	MSC group: aGVHD: 5.3%; cGVHD: 27,8% control group: aGVHD: 33.3%; cGVHD: 35.3%	[57]
Bernardo et al. (2011)	MSC infusion/historical control	Development of acute or chronic GVHD	MSC group: aGVHD: 31%; cGVHD: 0% control group: aGVHD: 41%; cGVHD: 10%	[16]
Liu et al. (2011)	MSC infusion/ standard therapy	Development of acute or chronic GVHD	MSC group: aGVHD: 51.8%; cGVHD: 51.4% control group: aGVHD: 38.9%; cGVHD: 74.1%	[45]

ALS: Amyotrophic lateral sclerosis; ALSFRS: Amyotrophic Lateral Sclerosis Functional Rating Scale; CDAI: Crohn's Disease Activity Index; COPD: Chronic obstructive pulmonary disease; CR: compete responders; EDSS: Expanded disability status scale; FEV: Forced expiratory volume; FVC: Forced vital capacity; GVHD: graft versus host disease; IBD: Inflammatory Bowel Disease; MELD: Model for End-stage Liver Disease; MS: Multiple sclerosis; PDAI: perianal disease activity index; PR: partial responders; SLE: Systemic lupus erythematosus; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; SSDAI: Sjögren Syndrome Disease Activity Index; Tx: Transplantation.

TABLE 4. THERAPEUTIC EFFECTS OF MSC THERAPY

Disease	*Comparison*	*Main readout parameter*	*Outcome*	*Ref.*
GVHD				
acute				
Baron et al. (2010)	MSC infusion/historical control	Development of acute GVHD	MSC group: 55%; control group: 75%	[17]
Zhang et al. (2010)	pre/post infusion	Development of acute or chronic GVHD	aGVHD : 16.7%; cGVHD : 16.7%	[73]
Guo et al. (2009)	pre/post infusion	Development of acute or chronic GVHD	aGVHD : 45.5%; cGVHD : 31%	[32]
Ning et al. (2008)	MSC infusion/ standard therapy	Development of acute or chronic GVHD	MSC group: aGVHD: 11.1%; cGVHD: 14.3% control group: aGVHD: 53,3%; cGVHD: 28,6%	[46]
Ball et al. (2007)	MSC infusion/historical control	Development of acute or chronic GVHD	MSC group: aGVHD: 14%; cGVHD: 7% control group: aGVHD: 30%; cGVHD: 13%	[18]
Lazarus et al. (2005)	pre/post infusion	Development of acute or chronic GVHD	aGVHD : 28%; cGVHD : 61%	[53]
Liver diseases				
Cirrhosis				
Xu et al. (2014)	MSC infusion/ standard therapy	MELD score	↓ in MSC group	[50]
Amin et al. (2013)	pre/post infusion	serum albumin and bilirubin	albumin: ↑ post MSC; bilirubin ↓post MSC	[51]
El-Ansary et al. (2012)	MSC infusion/ standard therapy	MELD score	↓ in MSC group	[58]
Zhang et al. (2012)	MSC infusion/ placebo infusion	MELD Na score	↓ in MSC group	[22]
Liver failure				
Shi et al. (2012)	MSC infusion/ placebo infusion	MELD score	↓ in MSC group	[23]
Peng et al. (2011)	MSC infusion/ standard therapy	MELD score	↓ in MSC group	[48]
El-Ansary et al. (2010)	pre/post infusion	MELD score	↓ post MSC	[52]
MS and ALS				
Karussis et al. (2010)	pre/post infusion	EDSS score (MS patients) and ALSFRS scores (ALS patients)	EDSS score ↓ post MSC; ALSFRS score: no significant differences	[54]
ALS				
Mazzini et al. (2010)	pre/post infusion	MRI assessments	no changes pre and post infusion	[53]
MS				
Bonab et al. (2012)	pre/post infusion	EDSS score	no significant differences pre and post infusion	[55]
Connick et al. (2012)	pre/post infusion	EDSS score	↓ post MSC	[59]
Yamout et al. (2010)	pre/post infusion	EDSS score	↓ post MSC	[60]
Bonab et al. (2007)	pre/post infusion	EDSS score	↑ post MSC	[56]
Kidney Tx				
Peng et al. (2013)	MSC infusion/ standard therapy	biopsy proven acute rejection	MSC group: 0%; control group: 16.7%	[49]
Tan et al. (2012)	MSC infusion/ standard therapy	biopsy proven acute rejection	MSC group: 16.2%; control group: 25.5%	[63]
Sjögren syndrome				
Xu et al. (2012)	pre/post infusion	SSDAI score	↓ post MSC	[74]
SLE				
Gu et al. (2014)	pre/post infusion	SLEDAI score	↓ post MSC	[75]
Wang et al. (2014)	pre/post infusion	SLEDAI score	↓ post MSC	[34]
Wang et al. (2013)	pre/post infusion	SLEDAI score	↓ post MSC	[76]
El-Ansary et al. (2012)	pre/post infusion	Serum creatinine and haemoglobin levels	Creatinine: ↓ post MSC; Hb: no significant differences	[77]
Wang et al. (2012)	pre/post infusion	SLEDAI score	↓ post MSC	[78]
Liang et al. (2010)	pre/post infusion	SLEDAI score	↓ post MSC	[64]
Sun et al. (2010)	pre/post infusion	SLEDAI score	↓ post MSC	[65]

ALS: Amyotrophic lateral sclerosis; ALSFRS: Amyotrophic Lateral Sclerosis Functional Rating Scale; CDAI: Crohn's Disease Activity Index; COPD: Chronic obstructive pulmonary disease; CR: compete responders; EDSS: Expanded disability status scale; FEV: Forced expiratory volume; FVC: Forced vital capacity; GVHD: graft versus host disease; IBD: Inflammatory Bowel Disease; MELD: Model for End-stage Liver Disease; MS: Multiple sclerosis; PDAI: perianal disease activity index; PR: partial responders; SLE: Systemic lupus erythematosus; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; SSDAI: Sjögren Syndrome Disease Activity Index; Tx: Transplantation.

A single study was included in which refractory aplastic anemia patients were treated with MSC [15]. This study showed an improvement in blood count recovery after MSC treatment compared with the historical control group (Table 4).

Weiss et al. [19] conducted a randomized double-blinded controlled trial in chronic obstructive pulmonary disease (COPD) and found no significant differences in pulmonary function between the MSC and control group. In this study, a large number of patients in the MSC group terminated the study prematurely (37 vs 16% in the control group), which could possibly lead to attrition bias. Nevertheless, this well-executed study does not support the idea that MSC have a clinical immunomodulatory effect in COPD.

Three non-controlled trials conducted on therapy refractory luminal Crohn's disease patients are included in this review [35,47,61]. These studies showed improvement in disease activity score.

Two studies on type 1 diabetes mellitus were included, one of which was a randomized controlled double-blind trial [20]. This study showed a significant increase in serum C-peptide levels, a measure for insulin production, in the treatment group. The dose of exogenous insulin was abated in MSC-treated patients. Vanikar and collaborators [62] showed in a non-controlled trial an increase in serum C-peptide levels and a decrease in exogenous insulin requirement post-MSC infusion.

Two trials examining the effect of MSC treatment in kidney transplant (Kidney Tx) recipients were included in this review [49,63]. Both studies showed less biopsy-proven acute rejection after MSC therapy compared with the conventional therapy control groups. In the study by Tan et al. [63], MSC therapy was compared with IL-2 receptor blocking therapy in the control group.

One study on Sjögren syndrome was included in this review [64]. This non-controlled study showed an improved Sjögren Syndrome Disease Activity Index score post-MSC infusion.

Immunological outcomes of the included studies

Of the 62 included studies, 16 studies measured immunological parameters in blood of patients treated with MSC. These immunological parameters are grouped in Table 5.

Immune cell subset analysis

The percentage of Tregs in patient blood was measured in 10 studies. Jitschin et al. and Xu et al. [21,50] found a significant increase in Treg percentages compared with the placebo or conventional therapy control group, respectively. Interestingly, both studies found a higher percentage of Tregs in the treatment group up until 6 months after MSC infusion. Of note, Jitschin et al. also measured the percentages of anti-inflammatory IL-10 producing type 1 regulatory cells but no large differences were found between the treatment and control group. Six studies investigated the Treg percentages pre- and post-MSC infusion. Xiao et al. and Guo et al. [15,32] found no significant differences in Treg percentages at any time point after MSC treatment. In contrast, four studies found a significant increase in Treg percentages post-MSC infusion [47,54,65,66]. Xu et al. [50] measured a significant decrease in the percentage of Th17 cells after MSC treatment. In contrast, in the study by Jitschin et al. [21], Th17 cell numbers were indifferent between the treatment and placebo groups. Guo et al. And Weng et al. [32,41] showed a significant increase in CD8+ T-cell numbers post-MSC infusion. However, Peng et al. [49] found no significant differences in CD8+ T cells between MSC and standard treatment groups, whereas they observed an increase in the number of B cells in the MSC-treated patients. In addition, another study showed that regulatory CD5+ B cells and IL-10 producing regulatory CD5+ cells were significantly higher at 3 months post-MSC [40].

Serum cytokine levels

In eight studies, levels of various cytokines were measured in the blood of MSC-treated patients. In the study by Peng et al. [49], percentages cytokine producing cells were determined by intracellular staining and no differences between MSC-treated patients and the control group were found. Anti-inflammatory IL-10 levels were found to be reduced in a study comparing chronic GVHD patients before MSC infusion with patients responding to MSC post-infusion [41]. A study comparing SLE patients pre- and post-MSC infusion did not show a significant difference in IL-10 levels [66]. Levels of TGF-β were increased in liver cirrhosis patients treated with MSC compared with patients receiving standard therapy and in SLE patients post-MSC treatment [50,66]. IL-2 was increased in the MSC group compared with placebo control group and in responders post-infusion compared with chronic

GVHD patients pre-MSC infusion [21,41]. TNF-α and IFN-γ levels were measured in four studies. Weng et al. [41] showed an increase in IFN-γ in responders to MSC treatment. In contrast, Lucchini et al. [26] showed a decrease in IFN-γ and TNF-α following MSC treatment. Decreased TNF-α levels after MSC treatment were confirmed by Xu et al. [50]. Sun et al. [66] demonstrated no significant difference in IFN-γ levels post-treatment. IL-4 was measured in two studies and was found to be decreased post-MSC infusion in both studies [41,66]. Xu et al. [50] measured the levels of IL-6 and IL-17 in MSC-treated and conventional therapy-treated liver cirrhosis patients and found a decrease of both cytokines in the MSC-treated patients. Although these results indicate that MSC infusion leads to immunological changes, the precise response to MSC treatment remains obscured. The variation in study setup, administered cell dose, immunosuppressive co-medication and follow-up time is too large to draw balanced conclusions on the immunological impact of MSC treatment.

TABLE 5. IMMUNOLOGICAL EFFECTS OF MSC THERAPY

Parameters	*Disease + study*	*Comparison*	*Time of measurement*	*Outcome*	*Ref.*
Anti inflammatory immune cell subsets					
	aplastic anemia				
Regulatory T cells	Xiao et al. (2013)	pre/post infusion	0.5, 1, 3, 6 and 12 months	No significant differences	[14]
	Crohn's disease				
	Ciccocioppo et al. (2011)	pre/post infusion	0 and 12 months	mucosal ↑ post MSC (p= <.0001); circulating ↑ post MSC (p= <.001)	[47]
	acute GVHD				
	Yin et al. (2014)	responders/ non responders	1, 4, 7, 14, 21 and 28 days	No significant differences	[64]
	Jitschin et al. (2013)	MSC infusion/placebo	30, 90 and 180 days	↑ in MSC group (p= .003 at d30; p=.037 at d90; n.s. at d180)	
	chronic GVHD				
	Weng et al. (2012)	pre/ responders post infusion	3 months	No significant differences	[40]
	profylaxis for GVHD development				
	Guo et al. (2009)	pre/post infusion	1, 3, 6, 12 and 18 months	No significant differences	[31]
	Liver cirrhosis				
	Xu et al. (2014)	MSC infusion/standard therapy	2, 4, 12 and 24 weeks	↑ in MSC group (p=<.05 at d14; p=<.05 at d28; p=<.05 at d84; n.s. at d168)	[48]
	MS and ALS				
	Karussis et al. (2010)	pre/post infusion	4 and 24 hours	↑ post MSC (p= <.05)	[52]
	SLE				
	Liang et al. (2010)	pre/post infusion	1 week, 3 and 6 months	↑ post MSC (p= <.05)	[61]
	Sun et al. (2010)	pre/post infusion	3 and 6 months	↑ post MSC (p= <.05)	[62]
	acute GVHD				
IL-10+ Tr1 cells	Jitschin et al. (2013)	MSC infusion/placebo	30, 90 and 180 days	similiar in both groups (n.s. at d30; p=.036 at d90 ↑; n.s. at d180)	[20]
	chronic GVHD				
Regulatory CD5+ B cells	Peng et al. (2014)	pre/post infusion	3 months	↑ post MSC (p= <.05)	[39]
IL-10 producing regulatory CD5+ B cells	Peng et al. (2014)	pre/post infusion	3 months	↑ post MSC (p= <.01)	[39]

ALS: Amyotrophic lateral sclerosis; GVHD: graft versus host disease; IL: interleukin; MS: Multiple sclerosis; NK: Natural killer; SLE: Systemic lupus erythematosus; SS: Systemic Sclerosis; Th: T helper; Tr1: Type 1 regulatory; Tx: transplantation.

TABLE 5. IMMUNOLOGICAL EFFECTS OF MSC THERAPY

Parameters	*Disease + study*	*Comparison*	*Time of measurement*	*Outcome*	*Ref.*
Immune cell subsets					
	acute GVHD				
Th17 cells	Jitschin et al. (2013)	MSC infusion/placebo	30, 90 and 180 days	similar in both groups (p= .032 at d30 ↓; n.s. at d90; n.s. at d180)	[20]
	Liver cirrhosis				
	Xu et al. (2014)	MSC infusion/standard therapy	2, 4, 12 and 24 weeks	↓ in MSC group (n.s. at d14; p=<.05 at d28; p=<.05 at d84; n.s. at d168)	[48]
	MS and ALS				
CD40+, CD83+, CD86+ and HLA-DR+ myeloid dendritic cells	Karussis et al. (2010)	pre/post infusion (24hrs)	4 and 24 hours	↓ post MSC (p= <.05)	[52]
	chronic GVHD				
CD8+ CD28- T cells	Weng et al. (2012)	pre/ responders post infusion	3 months	↑ post MSC (p= .008)	[40]
	profylaxis for GVHD development				
CD8+ T cells, NK cells and NKT cells	Guo et al. (2009)	pre/post infusion	1, 3, 6, 12 and 18 months	↑ post MSC (p= not mentioned)	[31]
CD4+ T cells and CD3+CD19+ cells	Guo et al. (2009)		1, 3, 6, 12 and 18 months	No significant differences	[31]
T cells and CD4+ T cells	Liu et al. (2014)	pre/post infusion	56 days	↑ post MSC (p= .015 and p=.012)	[27]
	MS				
CD3, CD4, CD8, CD19, and CD56 subsets	Connick et al. (2012)	pre/post infusion	1, 2, 3 and 4 weeks	No significant differences	[57]
	Kindey Tx				
T cells, CD4+ and CD8+ T cells and NK cells	Peng et al. (2013)	MSC infusion/standard therapy	0, 3, 6 and 12 months	No significant differences between groups	[47]
B cells and CD27+ memory B cells	Peng et al. (2013)	MSC infusion/standard therapy	0, 3, 6 and 12 months	B cells: ↑ in MSC group at d90 (p= <.05); memory B cells n.s	[47]
	Sjögren syndrome				
SS-related auto-antibodies	Xu et al. (2012)	pre/post infusion		↓ post MSC	[71]
Cytokine levels					
	Crohn's disease				
IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IFNγ, TNFα	Ciccocioppo et al. (2011)	pre/post infusion	0, 1,2 and 12 months	No significant differences	[47]
	Acute GVHD				
TNFR1, Elafin, IL2RA, CK18, IL-8, MIG, IP10, and Reg3α	Yin et al. (2014)	responders/ non responders	1, 4, 7, 14, 21 and 28 days	↓ in responders (p<.05)	[64]
IL-2	Jitschin et al. (2013)	MSC infusion/placebo	30, 90 and 180 days	↑ in MSC group (p= <.05 at d30; p=<.01 at d90; p= <.05 at d180)	[20]
	Chronic GVHD				
IL-2, IL-4, IL-10, IFNγ	Weng et al. (2012)	pre/ responders post infusion	3 months	IL-2: ↑ post MSC (p= .001); IFNγ↑ post MSC (p= .013); IL-10: ↓ post MSC (p= .004); IL-4: ↓ post MSC (p= .04)	[40]
	Acute and chronic GVHD				
TNFα and IFNγ	Lucchini et al. (2010)	pre/post infusion	24 hrs	↓ post MSC	[25]
SDF-1α, TPO, and IL-11	Liu et al. (2011)	pre/post infusion	8, 16 and 28 days	SDF-1α: n.s; TPO and IL-11 ↑ post MSC (p= <.05)	[44]
	Liver cirrhosis				
TGFβ, IL-6, IL-17 and TNFα	Xu et al. (2014)	MSC infusion/standard therapy	1, 2, 4, 8, 12 and 24 weeks	TGFβ ↑ in MSC group (d7, d14 and d28 p=<.05); IL-6, IL-17 and TNFα ↓ in MSC group (p=<.05)	[48]
	Kidney Tx				
Intracellular staining of TNFα, IL-4, IFNy and IL-10	Peng et al. (2013)	MSC infusion/standard therapy	0, 3, 6 and 12 months	No significant differences between groups	[47]
	SLE				
TGFβ, IL-4, IFNy and IL-10	Sun et al. (2010)	pre/post infusion	3 and 6 months	TGFβ ↑ post MSC (p=<.05 at d90; n.s. at d180); IL-4 ↓ post MSC (p= <.05); IFNy and IL-10 n.s.	[62]

ALS: Amyotrophic lateral sclerosis; GVHD: graft versus host disease; IL: interleukin; MS: Multiple sclerosis; NK: Natural killer; SLE: Systemic lupus erythematosus; SS: Systemic Sclerosis; Th: T helper; Tr1: Type 1 regulatory; Tx: transplantation.

Discussion

In the present systematic review, we aimed to determine whether there is evidence for efficacy of MSC immune therapy for the treatment of immunological diseases in human. In addition, the included manuscripts were screened for measurements of immunological parameters that would support the clinical effect of MSC on immunological disease outcome. Sixty-two studies met the inclusion criteria. Several of these studies were set up as safety studies in the first place with immunomodulatory efficacy as a secondary objective. Only 18 of the studies included control groups, and in four of them controls were historic. The lack of control groups in the majority of the studies made it difficult to draw decisive conclusions on the outcome of the studies as there is no correction for placebo effects. This systematic review is, therefore, limited by the lack of randomized controlled trials with large patients numbers. Another aim of the review was to determine whether there are immunological disorders in which MSC therapy is effective, and disorders in which this is not the case. For many disorders, there were however few studies included, which increases the risk of facing a publication bias. For instance, the results of a Phase III trial in GVHD that failed to demonstrate a beneficial effect of MSC are not available in the public domain [67] and a Phase III trial in Crohn's disease was put on hold when the placebo effect turned out to be higher than expected. It seems clear that there is an underreporting of clinical trials with neutral/negative outcome in peer-reviewed journals [68]. In addition, there were differences between the small number of studies within the same disease category when looking at the number of cells administered, the route of administration, immunosuppressive co-medication and follow-up time, which further split up the studies. Seventeen studies described measurements of immunological parameters in blood of patients after MSC treatment. The main reason for measuring these parameters was to find a biomarker for the efficacy of MSC therapy. Outcomes of different cell and cytokine levels were inconsistent between studies, mainly because the studies differed excessively in study design, patient population and outcome parameters. The result of this systematic review is that there are indications that MSC therapy is capable of modulating the immune system in GVHD, liver disease, Crohn's disease, diabetes mellitus and kidney transplantation, and that there are weaker indications for aplastic anemia, Sjögren syndrome and SLE. There is so far no convincing evidence for an immunomodulatory effect of MSC in COPD, ALS and MS. However, for none of the disease categories a decisive conclusion can be drawn. Larger studies are needed to study the effect of MSC in more controlled manners.

The immunosuppressive effect of MSC is well studied in in vitro cultures and experimental animal models and thus, multiple clinical studies on MSC therapy have been initiated in recent years. Whereas it has become clear that MSC therapy is safe, the efficacy of the therapy is more obscure. The majority of the trials that have been performed on MSC therapy lacked sufficient sample size or the studies were not randomized, making it difficult to determine if there is a positive effect of MSC immune therapy. Whereas some of the controlled trials included in this review showed a positive effect of MSC treatment compared with the control group [20,50,63], other randomized studies did not show amelioration of disease symptoms after treatment with MSC therapy [19]. These results underline that it is still not known if MSC therapy is efficient and that MSC might be beneficial for some immunological diseases but not for all immunological diseases. The small amount of evidence for a clinical immunomodulatory effect of MSC that is available today suggests that the outcomes of future controlled studies can confirm as well as disapprove the effect of MSC in any of the analyzed disease categories. There is, however, an encouraging prospect: several larger placebo-controlled trials are currently ongoing and reports can be expected in the coming years (e.g., clinicaltrials.gov registered randomized placebo-controlled trials on ALS patients [69,70], heart failure patients [71,72], type 1 diabetes mellitus patients [73] and MS patients [74] are currently recruiting). With this large number of clinical trial reports forecasted to be published in the years to come, a systematic review performed in a few years from today is likely to be able to generate more conclusive results. The results of these randomized trials will help to establish whether MSC have a clinically relevant immunomodulatory effect and for which disorders this can be exploited most efficiently. Moreover, if the right immunological parameters are measured in the patients, these studies might give more insight in the immunological mechanism of action of MSC therapy.

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Chapter 3

Inflammatory Conditions Dictate the Effect of Mesenchymal Stem or Stromal Cells on B cell Function

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Abstract

The immunomodulatory capacity of mesenchymal stem or stromal cells (MSC) makes them a promising tool for treatment of immune disease and organ transplantation. The effects of MSC on B cells are characterized by an abrogation of plasmablast formation and induction of regulatory B cells (Bregs). It is, however, unknown how MSC interact with B cells under inflammatory conditions. In this study, adipose tissue-derived MSC were pretreated with 50 ng/ml IFN-γ for 96 h (MSC–IFN-γ) to simulate inflammatory conditions. Mature B cells were obtained from spleens by CD43− selection. B cells were co-cultured with MSC and stimulated with anti-IgM, anti-CD40, and IL-2; and after 7 days, B cell proliferation, phenotype, Immunoglobulin-G (IgG), and IL-10 production were analyzed. MSC did not inhibit B cell proliferation but increased the percentage of CD38high CD24high B cells (Bregs) and IL-10 production, while MSC–IFN-γ significantly reduced B cell proliferation and inhibited IgG production by B cells in a more potent fashion but did not induce Bregs or IL-10 production. Both MSC and MSC–IFN-γ required proximity to target cells and being metabolically active to exert their effects. Indoleamine 2,3 dioxygenase expression was highly induced in MSC–IFN-γ and was responsible of the anti-proliferative and Breg reduction since addition of tryptophan (TRP) restored MSC properties. Immunological conditions dictate the effect of MSC on B cell function. Under immunological quiescent conditions, MSC stimulate Breg induction; whereas, under inflammatory conditions, MSC inhibit B cell proliferation and maturation through depletion of TRP. This knowledge is useful for customizing MSC therapy for specific purposes by appropriate pretreatment of MSC.

Introduction

B cells contribute to immunological diseases in various ways by production of auto-antibodies, presentation of auto-antigen, and secretion of inflammatory cytokines. In the context of post solid organ transplantation, B cells mediate humoral rejection by the production of donor-specific human leukocyte antigen (HLA) antibodies (DSAs) and provide co-stimulatory signals to T cells [1, 2]. On the other hand, a population of regulatory B cells (Bregs) has been described that can regulate immune responses mainly via the secretion of IL-10 [3, 4]. Bregs have been shown to be involved in suppressing autoimmune reactions as

well as in maintaining transplant tolerance [5, 6]. Current treatments for B cell-mediated disease are mainly based on global B cell depletion, thereby eliminating pathogenic B cells as well as Breg subsets. A more refined modulation of B cell activity could prove beneficial for patient treatment. Mesenchymal stem or stromal cells have potent immunomodulatory properties and target the proliferation and differentiation of a variety of immune cells [7]. The effect of MSC on T cells has been extensively studied but also regulation of natural killer cells [8], macrophages [9], dendritic cells [10], and more recently B cells by MSC has drawn attention. Previously, we have shown that MSC can abrogate plasmablast formation and induce IL-10+ and CD19+ CD38high CD24high B cells [11], which are the two main signatures to define Bregs [12]. However, it appears that the nature of the immunosuppressive and anti-proliferative effects of MSC on lymphocytes is dependent on the inflammatory microenvironment [13–16]. In particular, IFN-γ has a prominent role in potentiating the anti-proliferative capacity of MSC via the induction of indoleamine 2,3-dioxygenase (IDO) activity [17] and contact dependent mechanisms of action [18, 19]. Priming of MSC with inflammatory factors is likely to occur in vivo as MSC-treated patients often suffer from acute or chronic inflammatory diseases. MSC infused in patients might encounter an inflammatory environment that could influence the immunomodulatory effect of MSC. We previously showed that B cell proliferation is increased when B cells are stimulated by an anti-CD40 + anti-IgM + IL-2 cocktail as well as with activated T cells. MSC reduced B cell proliferation induced by stimulated T cells but not by the cocktail in the absence of T cells [11]. In our previous work, we hypothesized that the anti-proliferative effect of MSC on B cells in the presence of activated T cells was due to the secretion of IFN-γ by activated T cells and the subsequent activation of MSC. In this study, we examined how IFN-γ affected the immunomodulatory role of MSC on B cells by comparing the effects of MSC and IFN-γ treated MSC on B cell proliferation and differentiation into plasmablasts or IL-10 producing Bregs.

Methods

Isolation and culture of human subcutaneous adipose tissue MSC

Subcutaneous adipose tissue from healthy human donors that became available as a waste product during kidney donation procedures was collected after obtaining written informed consent as approved by the Medical Ethical Committee of the Erasmus University Medical Centre Rotterdam (protocol no. MEC-2006-190). The

tissue was collected in minimum essential medium-α (MEM-α) (Sigma Aldrich, St. Louis, MO, USA) supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml) (1% P/S; Lonza, Verviers, Belgium), and 2 mM L-glutamine (Lonza) and stored at 4°C for 3–16 h. MSC were isolated as described previously [20]. Briefly, adipose tissue was mechanically disrupted and digested enzymatically with 0.5 mg/mL collagenase type IV (Life Technologies, Paisley, UK) in RPMI 1640 Medium with glutaMAX (Life Technologies) for 30 min at 37°C under continuous shaking. Cultures were kept at 37°C, 5% CO2, and 95% humidity and refreshed weekly with MEM-α with 1% P/S, and 15% heat-inactivated fetal bovine serum (FBS; Lonza).

At 90% confluence, adherent cells were removed from culture flasks by incubation in 0.05% trypsin-EDTA (Life Technologies, Bleiswijk, The Netherlands) at 37°C and cells used for experiments or frozen at −150°C until further use. MSC were used for experiments between passages 2 and 5 and their phenotypic markers and osteogenic and adipogenic potential were tested as described before [21]. MSC from 19 different donors were used in the experiments.

Stimulation of MSC

Mesenchymal stem or stromal cells were pretreated for 4 days with IFN-γ (50 ng/ml; Life technologies). For co-culture experiments, MSC were washed with phosphate buffered saline (PBS) and detached by incubation with 0.05% trypsin-EDTA before seeding them in 96 well plates in Iscove's Modified Dulbecco's Medium (IMDM, Lonza) with 10% heat inactivated FBS. Phenotypical characteristics of MSC before and after IFN-γ were assessed measuring several markers on their surface: CD13-PeCy7 (clone L138), CD31-V450 (clone WM59), CD45-APC-H7 (clone 2D1), HLA-ABC-APC (clone G46-2.6), HLA-DR PerCP (clone L243) and CD73-PE (clone AD2; all BD Biosciences), CD90-APC (clone Thy-1A1), and CD105-FITC (clone 166707; all R&D Systems, Minneapolis, MN, USA) and PD-L1 PE (clone B7-H1; Biolegend, San Diego, CA, USA) by Flow Cytometry and optical microscopy morphology (Figure S1 in Supplementary Material).

IDO activity measurement

The activity of IDO was determined by the measurement of L-kynurenine in the supernatant of four MSC cultures as described previously [22]. Briefly, MSC were seeded at a density of 100,000 cells/well in a 6 wells plate and cultured for 4 days with or without 50 ng/mL IFN-γ. 30% trichloroacetic acid was added to the supernatant in a 1:3 ratio. Samples were incubated for 30 min at 50°C and spun

down at 12,000 rpm for 5 min. Samples were plated in a 96 wells flat bottom plate and diluted 1:1 in Ehrlich reagent [200 mg 4-dimethylaminobenzaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in 10 ml of glacial acetic acid]. Absorbance was read at 490 nm using a Wallac Victor2 1420 multilabel plate reader (Perkin Elmer, Waltham, MA, USA).

Isolation of B cells from spleens

Spleens were obtained from post-mortal kidney donors (Erasmus MC Hospital, Rotterdam) and anonymously used for research purposes as described in article 13 of The Netherlands law of organ donation (Wet op Orgaandonatie, WOD). All samples and data were analyzed anonymously. Spleens were mechanically disrupted and filtered through a 70-µm cell strainer (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) to obtain a single-cell suspension. Mononuclear cells, isolated using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient, were stored at −150°C until use. Upon thawing, quiescent B cells were isolated by negative selection using anti-CD43- magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) [23]. Purity was determined by flow cytometry (FACS Canto II). Typically, cell suspensions consisted of >98% pure CD19+ B cells. B cells from spleens from 12 different donors were used it the experiments.

B cell stimulation

B cells were co-cultured in IMDM-10%FBS with a cocktail to mimic antigen and T cell help: 10 mg/ml F(ab)2 anti-IgM (Jackson, ImmunoResearch laboratories, Inc., West Grove, PA, USA), 103 IU IL-2 (Proleukin, Prometheus laboratories Inc., San Diego, CA, USA), and 5 mg/ml anti-CD40 agonistic monoclonal antibody (Bioceros, Utrecht, The Netherlands). In some of the experiments, 200 µM tryptophan (TRP, l-tryptophan, SigmaAldrich) was added to the stimulation cocktail to counteract the activity of IDO.

Transwell (TW) cultures

24-wells plates with 0.4 µm pore polycarbonate membrane inserts (Costar, Corning, Kennebunk, ME, USA) were used for the TW cultures. MSC were seeded on the membrane of the inserts and B cells were added to the lower chamber at a ratio MSC:B cells 1:5. After 7 days, inserts were removed; and B cells from the lower chamber were collected for further analysis and B cell subsets characterization.

Heat inactivated MSC

To study the effect of cell surface molecules but not the secreted factors, MSC were inactivated as previously described [24]. Shortly, MSC were heated in suspension in PBS in parafilm-sealed tubes by 30 min incubation at 50°C in a temperature-regulated waterbath. The inactivated cells were then washed and counted and used for further experiments.

B cell subset characterization

B cells were labeled by incubation with 5,6-carboxysuccinimidyl-fluoresceine-ester (CFSE) (Molecular Probes Invitrogen, Karlsruhe, Germany) for 10 min at 37°C. After 7 days, B cells were collected and processed for flow cytometric analysis (FACS Canto II, Diva Software, BD Biosciences, San Jose, CA, USA), and supernatants were stored at −80°C for cytokine and Immunoglobulin-G (IgG) determination. The antibodies used for flow cytometry phenotyping were as follows: CD27-PE-Cy7 (clone 0323), CD38-PE (clone HB7), CD19-BV512 (clone HIB19) and CD24-APC (clone SN3 A5-2H1D) (eBioscience, San Diego, CA, USA), IL-10-Bv421 (Clone Jes3-9D7, Biolegend), and Via Probe for determination of cell viability (BD Biosciences, San Jose, CA, USA). After 7 days, proliferation of B cells was assessed by measuring CFSE dye dilution on a FACSCanto II flow cytometer (BD Biosciences). 12 h before harvesting the cells, Monensin (Golgi Stop, BD Biosciences) was added to the wells and the intracellular staining was performed without restimulation using Intrastain kit (Dako, Denmark).

Measurement of cytokine secretion

Supernatants from MSC-B cell co-cultures kept at −80°C were thawed and used for measurement of cytokine levels. IL-10 was quantified using a Milliplex kit (Merck Millipore, Amsterdam, the Netherlands) according to manufacturer's instructions. Human cytokine standards were provided by the kit and a standard curve was prepared from 10,000 to 3.2 pg/ml. Samples and standards mixed with antibody-coated magnetic beads were incubated overnight in a 96-well plate at 4°C under continuous agitation. Plates were washed and incubated with detection antibodies for 1 h. Finally, plates were washed and incubated with streptavidin-phycoerythrin for 30 min. The samples were measured on a Luminex 100/200 cytometer (Luminex, Austin, TX, USA) using Xponent software.

IgG ELISA

Plates were coated with goat anti human Ig-UNLB (Southern Biotechnology Associates; Birmingham, AL, USA). Plates were washed with PBS 0.05% Tween and blocked with PBS 5% FBS for 2 h. Diluted samples and standard IgG (Sigma-Aldrich) were added to the plate and incubated for 90 min. IgG-HRP (My Biosource; San Diego, CA, USA) was used as a conjugate and 3,3,5,5-tetramethylbenzidine (TMB) was used to visualize bound IgG. Absorbance was read at 595 nm using a Wallac Victor2 1420 multilabel plate reader (Perkin Elmer, Waltham, MA, USA).

RNA expression quantification

After 7 days of co-culture, B cells were recovered, pelleted in PBS–DEPC and snap frozen. RNA was isolated and 500 ng was used for cDNA synthesis as described previously [25]. Gene expression was determined by real-time RT-PCR using universal PCR master mix (Life Technologies) and an assay-on-demand for IL-10 (Hs00174086.m1) (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI PRISM 7700 sequence detector (Applied Biosystems). Data are expressed as relative copy number of the PCR products with respect to the housekeeping gene GAPDH.

Statistical analysis

Data are expressed as means ± SEM. Significant differences within groups were calculated using repeated measures non-parametric analysis of variance (ANOVA: Friedman test) with Dunnett's posttest performed by GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). P values were indicated as * for $P < 0.05$; ** for $P < 0.01$; and *** for $P < 0.001$.

Results

IFN-γ-pretreated MSC inhibit B cell proliferation

Previously, we showed that the inhibition of B cell proliferation by MSC was dependent on the presence of T cells [11]. We hypothesized that MSC needed to be activated by IFN-γ secreted by T cells to mediate their anti-proliferative effects on B cells. Here, we analyzed the anti-proliferative capacity of MSC and IFN-γ-pretreated MSC (MSC–IFN-γ) on anti-CD40, anti-IgM, and IL-2 stimulated B cells using flow cytometry. After 7 days of co-culturing with MSC or MSC–IFN-γ, viable naïve and memory B cells were distinguished based on intensity of CD27

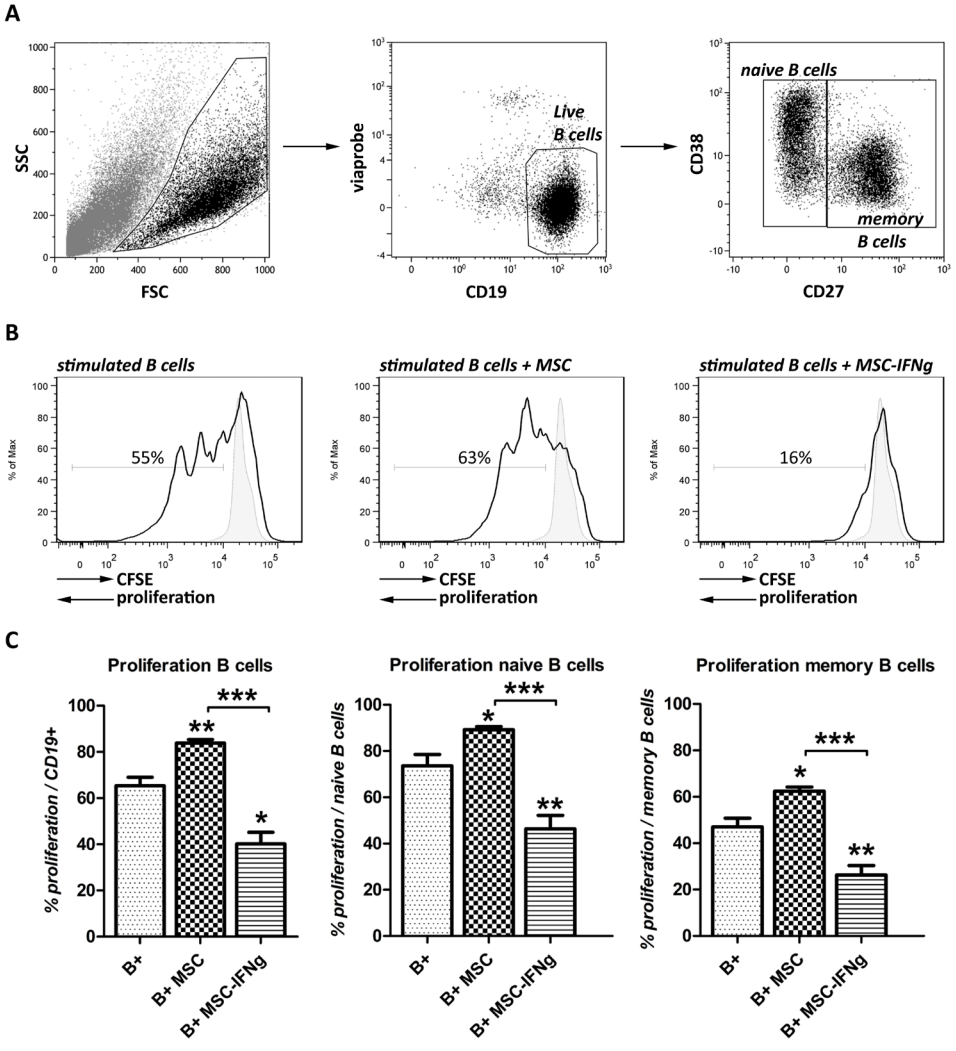


Figure 1. IFN-γ stimulated mesenchymal stem or stromal cells (MSC) reduce B cell proliferation. B cells from human splenocytes were stimulated for 7 days with anti-CD40, anti-IgM and IL-2 in the presence of adipose tissue-derived MSC or IFN-γ-pretreated MSC (MSC–IFN-γ). (A) Representative FACS plots of the gating strategy of live, naïve, and memory B cells based on intensity of CD27 expression. (B) Proliferation of B cells in the presence or absence of MSC or MSC–IFN-γ at a 5:1 (B cell:MSC) ratio was assessed through measurement of 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE) label dilution. Representative histograms were shown. Gray, solid histograms represent unstimulated B cells. (C) Percentage of proliferation of B cells (left graph), naïve B cells (middle graph), and memory B cells (right graph). Bars indicate mean ± SEM of three experiments with three different MSC cultures and three different B cell donors.

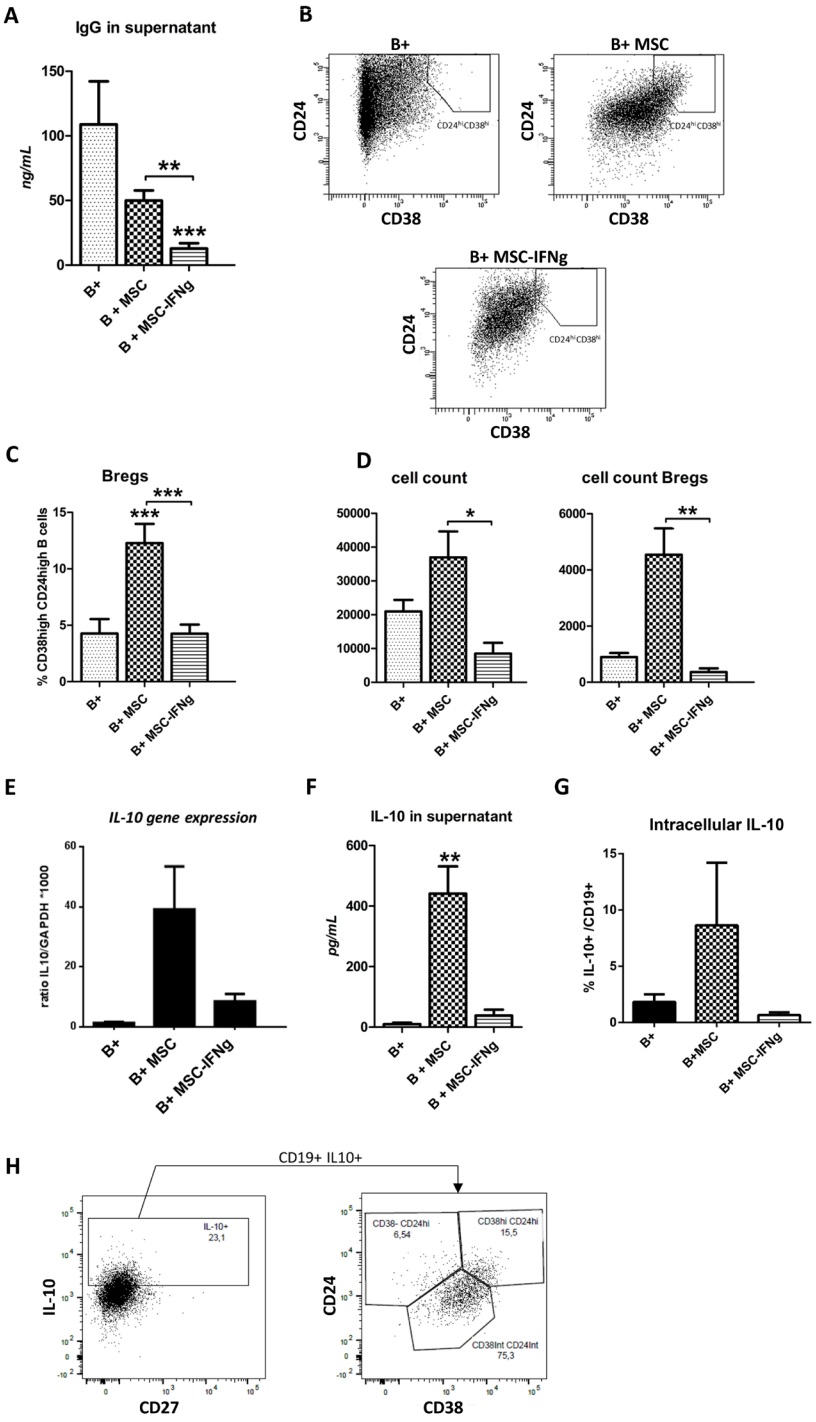
as shown in Figure 1A. Co-culture of B cells with MSC significantly increased the proliferation of the total B cell population (Figures 1B,C). MSC that were pretreated with IFN-γ did not increase the proliferation of B cells but, by contrast, inhibited B cell proliferation from 65 to 40%. Naïve and memory B cell subsets (CD27− and CD27+, respectively) showed similar increases in proliferation when co-cultured with MSC and inhibition of proliferation when co-cultured with MSC–IFN-γ. These results show that MSC need to be pre-activated with IFN-γ to bring about their anti-proliferative effect on B cells.

IFN-γ-pretreated MSC inhibit IgG production by B cells

Mesenchymal stem or stromal cells reduced IgG production by activated B cells (Figure 2A). Pretreatment of MSC with IFN-γ significantly enhanced the inhibitory effect of MSC on IgG production. In accordance with the reduced proliferation, an even stronger reduction of IgG levels was measured in the supernatant of B cells co-cultured with MSC–IFN-γ

IFN-γ conditioned MSC are poor Breg inducers

To investigate whether MSC-induced B cells with a regulatory phenotype, frequencies of CD19+ CD38high CD24high transitional B cells (Bregs), and IL-10 production were measured. After 7 days of co-culturing MSC and MSC–IFN-γ with T cell-like stimulated B cells, the percentage of Bregs was measured using flow cytometry as shown in Figure 2B. MSC significantly induced an increase of this subset. By contrast, IFN-γ-pretreated MSC were not able to induce an increase in Bregs (Figures 2B,C). In accordance with this, the absolute number of Bregs was significantly increased when MSC were co-cultured with B cells (Figure 2D). To analyze whether the induced cells had regulatory potential, the anti-inflammatory cytokine IL-10 gene expression was analyzed. MSC induced a higher trend in IL-10 gene expression, while MSC–IFN-γ did so only to a very low extent (Figure 2E). In accordance with this, IL-10 protein levels were significantly increased in the B cell and MSC co-cultures supernatants, whereas no increase in IL-10 levels was found in the supernatant of B cell and MSC–IFN-γ (Figure 2F). The proportion of IL-10-producing B cells was also analyzed in the different conditions by intracellular staining and accordingly we identified a higher percentage in the co-culture with MSC (Figure 2G). To further analyze the phenotype of the IL-10-producing B cells induced by MSC we performed intracellular IL-10 staining. The transitional CD38high CD24high subset showed the highest percentage of IL-10+ cells, although also within the naïve CD38int CD24int B cell subset significant numbers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CD38hi CD24hi
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CD38lnt CD24lnt
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CD38

of IL-10-producing cells were found (data not shown). In absolute numbers, the largest proportion of IL-10-producing B cells was observed in the CD38int CD24int subset, which corresponds to the mature naïve subset. We observed that all IL-10+ B cells produced in the presence of MSC were CD27– (Figure 2H).

The reduction of B cell proliferation by IFN-γ-pretreated MSC requires close proximity

To test whether soluble factors or cell contact-dependent mechanisms are involved in the effects of MSC and MSC–IFN-γ on B cell proliferation and Breg induction, activated B cells were cocultured with MSC and MSC–IFN-γ in a TW system to prevent cell–cell contact as shown in Figure 3A. By preventing direct cell–cell contact, the stimulatory effect of MSC on B cell proliferation was abolished (Figure 3B). Moreover, a small decrease in memory B cell proliferation was measured when B cells were cocultured with MSC in a TW setting. Interestingly, prevention of direct cell–cell contact also abolished the anti-proliferative capacity of MSC–IFN-γ both in the total B cell population and in the naïve and memory B cell populations (Figure 3B). In accordance with the lack of proliferation inhibition in co-cultures of B cells with MSC–IFN-γ in a TW system, levels of IgG were not affected by MSC and MSC–IFN-γ (Figure 3C). No Bregs were induced when B cells were co-cultured with

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Figure 2. IFN-γ-pretreated mesenchymal stem or stromal cells (MSC) prevent immunoglobulin-G (IgG) production by B cells and regulatory B cell (Breg) formation.

(A) Levels of IgG were measured in the supernatant of anti-CD40, anti-IgM and IL-2 stimulated B cells co-cultured with or without MSC or MSC– IFN-γ at a 5:1 (B cell:MSC) ratio for 7 days. (B) Representative FACS plots of the gating strategy of Bregs, identified as CD38hiCD24hi B cells, with or without MSC or MSC–IFN-γ for 7 days. (C) Percentage of Bregs of total B cells after culturing with or without MSC or MSC–IFN-γ for 7 days. (D) The absolute number of total B cells was counted after harvesting the cells from the co-cultures (left graph). The absolute number of Bregs in the culture was calculated using the percentage of Bregs measured with flow cytometry and the absolute number of total B cells (right graph). Both absolute counts refer to initial 100,000 B cells in culture. (E) Gene expression of IL-10 depicted as a ratio to GAPDH. (F) Levels of IL-10 were measured in the supernatant of anti-CD40, anti-IgM, and IL-2 stimulated B cells co-cultured with or without MSC or MSC– IFN-γ. (G) IL-10 + B cells frequencies measured by analyzing intracellular cytokine by flow cytometry. B cells co-cultured with MSc showed the higher frequencies. (H) IL-10 intracellular staining of B cells co-cultured with MSC (B + MSC group). IL-10 positive B cells are plotted to show the percentage of transitional (CD24hi CD38hi), CD24int CD38int, and CD24hi CD38– subsets. All bars indicate mean ± SEM of three experiments with three different MSC cultures and three different B cell donors.

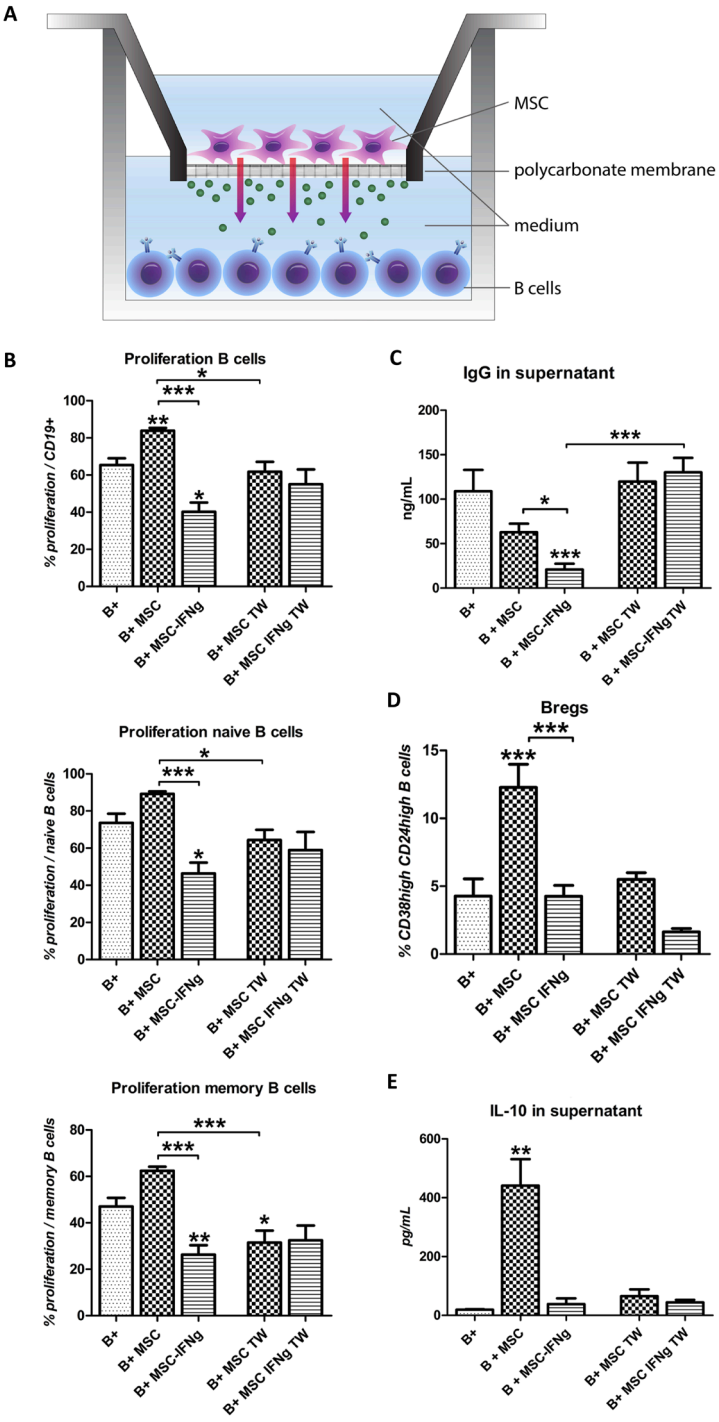

Figure 3. The reduction of B cell proliferation by IFN-γ-pretreated mesenchymal stem or stromal cells (MSC) requires close proximity.
(A) B cells were stimulated with anti-CD40, anti-IgM, and IL-2 and cultured in direct contact with MSC or MSC–IFN-γ or in transwell (TW) system to prevent direct cell contact of the B cells and MSC. (B) Proliferation of B cells was assessed through measurement of 5,6-carboxy-succinimidyl-fluoresceine-ester label dilution. (C) Levels of immunoglobulin-G measured in the supernatant with an ELISA assay. (D) Percentage of CD24hiCD38hi regulatory B cells within CD19 + cell gate measured by flow cytometry. (E) Levels of IL-10 measured in the supernatant with an ELISA assay. All bars indicate mean ± SEM of three experiments with three different MSC cultures and three different B cell donors.

MSC and MSC–IFN-γ in TW system (Figure 3D), and, in correspondence, no increase in IL-10 levels was found (Figure 3E). These results indicate that the inhibition of B cell proliferation, inhibition of IgG production, and induction of IL-10 production by MSC is dependent on cell contact or at least close proximity of MSC and B cells.

The reduction of B cell proliferation requires metabolically active MSC–IFN-γ

To examine whether the inhibition of B cell proliferation by MSC–IFN-γ requires merely interaction via membrane proteins or requires metabolically active MSC–IFN-γ, activated B cells were co-cultured with heat-inactivated MSC (HI-MSC) (Figure 4A). HI-MSC are immunophenotypically intact but release no soluble factors, as previously described [24]. Culturing B cells with HI-MSC abolishes the stimulatory effect of MSC on B cell proliferation (Figure 4B) and furthermore the proliferation of B cells was not significantly inhibited by HI-MSC–IFN-γ (Figure 4B). HI-MSC and HI-MSC–IFN-γ induced an increase in Bregs but this increase was not linked to an increase in IL-10 production (Figures 4C,D). These data indicate that the inhibition of B cell proliferation is dependent on metabolic activity of MSC–IFN-γ. Furthermore, the induction of Bregs cannot be recuperated by inactivating MSC–IFN-γ but requires metabolically active MSC. Activating MSC with IFN-γ appears to overrule the Breg inducing capacity of MSC.

Inhibition of B cell proliferation by IFN-γ stimulated MSC Is largely dependent on TRP catabolism by IDO

We hypothesized that the inhibition of B cell proliferation by MSC is mediated by IFN-γ triggered IDO induction, leading to degradation and depletion of TRP. When MSC were cultured for 4 days with IFN-γ high levels of l-kynurenine, the breakdown product of TRP, were detected (Figure 5A). When 200 μM TRP was added to B cell and MSC–IFN-γ co-cultures to counteract the effect of IDO activity, B cell proliferation increased from 17 to 48% in the total B cell population, from 19 to 52% in the case of naïve B cell proliferation, and from 16 to 36% in the case of memory B cell proliferation (Figure 5B). TRP supplementation, furthermore, reversed the effect of MSC–IFN-γ on IgG production by B cells (Figure 5C).

TRP supplementation rescues Breg induction by IFN-γ stimulated MSC

We showed that IFN-γ-pretreated MSC were not able to induce an increase in Bregs. TRP supplementation to MSC–IFN-γ and B cell co-cultures showed a

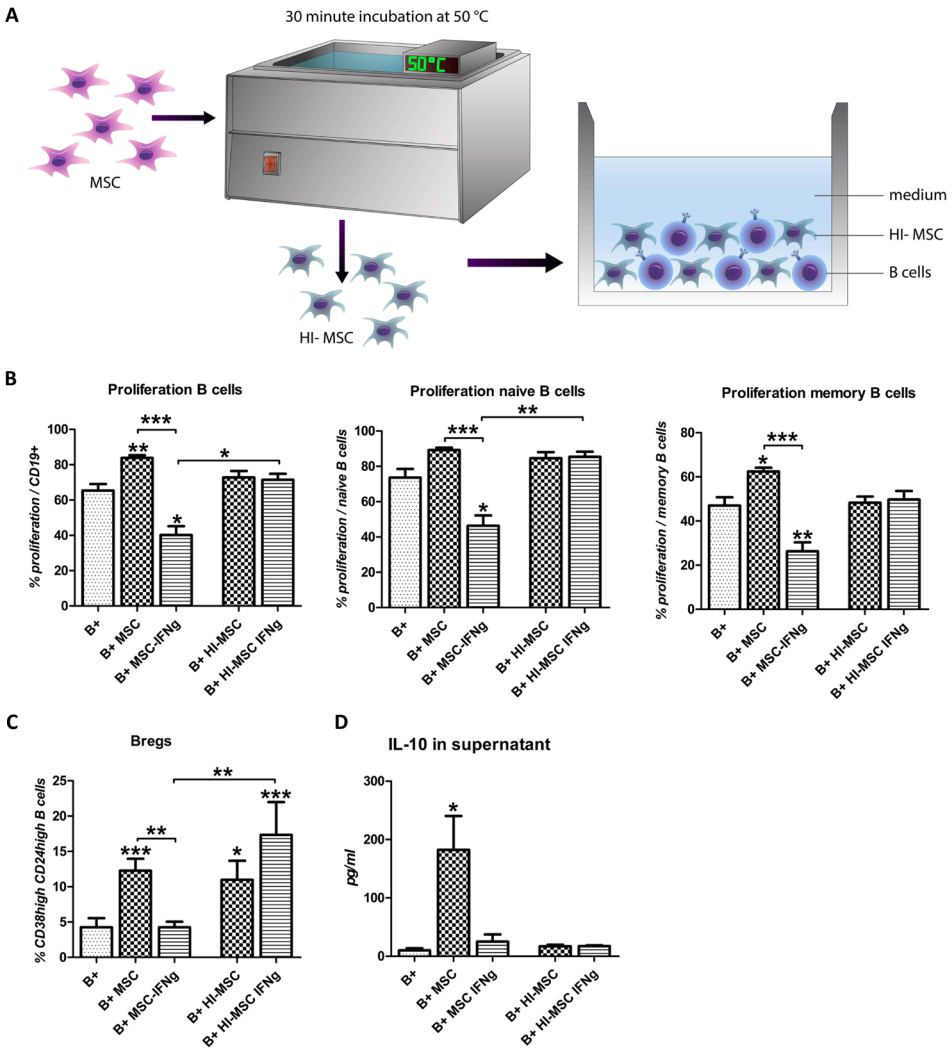

Figure 4. The reduction of B cell proliferation by IFN-γ-pretreated mesenchymal stem or stromal cells (MSC) requires viable cells.

(A) MSC were incubated for 30 min at 50°C to heat inactivate the cells (HI-MSC). B cells were stimulated with anti-CD40, anti-IgM, and IL-2 and cultured for 7 days with HI-MSC or HI-MSC–IFN-γ. (B) Proliferation of B cells was assessed through measurement of 5,6-carboxy-succinimidyl-fluoresceine-ester label dilution. (C) Percentage of induced CD24hiCD38hi regulatory B cells within CD19 + cell gate measured by flow cytometry. (D) The levels of IL-10 were measured in the supernatant of B cells cultured in the presence of viable MSC or MSC– IFN-γ or HI-MSC or HI-MSC–IFN-γ. All bars indicate mean ± SEM of three experiments with three different MSC cultures and three different B cell donors.

trend toward increased frequencies of CD38high CD24high Bregs (Figure 5D). In accordance with this, the levels of IL-10 in the supernatant of the MSC–IFN-γ cultures were significantly increased when B cell proliferation was rescued with TRP supplementation (Figure 5E). Stimulation of MSC and MSC–IFN-γ with T cell-like stimulation and TRP did not induce IL-10 secretion by MSC, eliminating the possibility that the IL-10 in the stimulated cultures is secreted by MSC–IFN-γ (data not shown). These data indicate that the incapability of MSC–IFN-γ to induce Bregs is caused by TRP depletion mediated by IFN-γ triggered IDO activity in MSC.

Discussion

The immunomodulatory properties of MSC are under strict control of pro-inflammatory factors, such as IFN-γ [13]. In this study, we show that inflammatory signals alter the effect of MSC on B cells. In the absence of immune activation, MSC promote the survival of B cells and induce the formation of Bregs, whereas they have little effect on B cell proliferation and IgG production [11]. However, after pretreatment with IFN-γ, MSC inhibit B cell proliferation, reduce IgG production, but they also lose the capacity to induce Bregs (Figure 6). During immune responses, immune cells involved in graft rejection such as T cells, monocytes, or macrophages can provide IFN-γ to MSC [26, 27]. We previously showed that in the absence of T cells, MSC fail to inhibit activated B cell proliferation [11]. Our results indicate that IFN-γ production by T cells is required to activate MSC to dampen the proliferative response of B cells. The decreased levels of IgG and Bregs found when B cells were co-cultured with IFN-γ-stimulated MSC are likely a consequence of the inhibited proliferation of B cells. These data indicate that the effects of MSC on B cells may be very different in situations where no T cells are around, such as, for instance, in patients in which T cells have been depleted with anti-thymocyte globulin after solid organ transplant rejection [28].

The interaction between MSC and B cells has been investigated in a number of studies, although study outcomes have been contrasting with respect to effects of MSC on B cell proliferation and antibody production [29]. In this study, we clarified that the effect of MSC on B cells depends on local immunological conditions. Under immunological quiescent conditions, MSC are supportive for B cells; they promote B cell survival and Breg formation. Bregs will subsequently contribute to maintenance of immunological homeostasis. Under inflammatory conditions, in our study mimicked by the addition of IFN-γ, MSC suppress the activity of B cells;

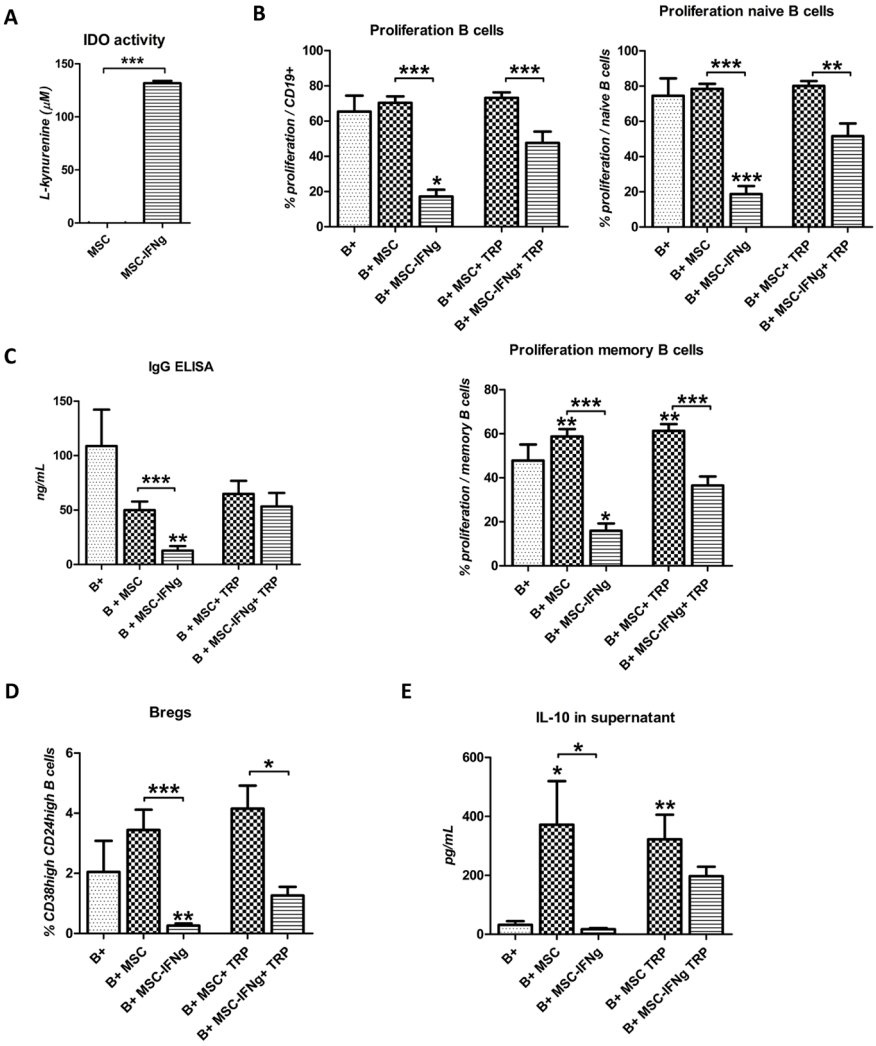

Figure 5. Inhibition of B cell proliferation by IFN-γ stimulated mesenchymal stem or stromal cells (MSC) is largely dependent on tryptophan (TRP) catabolism by indoleamine 2,3-dioxygenase (IDO).

(A) IDO activity was measured by accumulation of l-kynurenine in MSC supernatant after 4 days culture with or without 50 ng/mL IFN-γ. (B) anti-CD40, anti-IgM, and IL-2 stimulated B cells were co-cultured with MSC or MSC–IFN-γ for 7 days in the absence or presence of 200 μM TRP. Proliferation of CFSE labeled B cells is depicted as mean ± SEM of 3 experiments with different MSC cultures. IgG (C) and IL-10 (E) levels were measured in the supernatant of the cultures using ELISA. (D) Percentage of induced regulatory B cells within CD19 + cells with or without added MSC or IFN-γ in the presence or absence of 200 μM TRP for 7 days. All bars indicate mean ± SEM of three experiments with three different MSC cultures and three different B cell donors.

they inhibit B cell proliferation and reduce antibody production. At the same time, they inhibit Bregs induction. This may seem counterintuitive, but may reflect a state in which all B cell activity is shut down by MSC. Our results imply that in vivo, resident MSC are supportive for B cells and induce tolerogenic B cells under immunological quiescent conditions, whereas under inflammatory conditions MSC suppress humoral responses. For the generation of therapeutic MSC our results suggest that custom-made MSC can be generated with either B cell suppressive properties or with B cell homeostasis supportive properties. Distinct mechanisms have been described to be responsible for immunomodulation by MSC. Both soluble factors and contact-dependent ligand–receptor interaction have been proposed to participate to the MSC-mediated immunomodulation [30]. We show that MSC effects on B cells do not solely depend on soluble factors as no Bregs or IL-10 production were induced when MSC were cultured in a TW culture system. Moreover, the presence of dead but phenotypically intact MSC [24] was not enough to induce IL-10 producing B cells, implying that modulation of B cells by MSC is mediated by an active metabolic process and needs close proximity of MSC and B cells.

Indoleamine 2,3-dioxygenase-mediated TRP catabolism has been described as an important mechanism of activated MSC to modulate T cell proliferation [17]. We demonstrated that the inhibition of B cell proliferation by MSC also largely depends on the TRP depleting activity of IDO activity and can be recovered by supplementing TRP in vitro. We show that the ability of MSC to induce IL-10-producing B cells was lost when MSC were pretreated with IFN-γ but could be recovered when TRP was supplemented to the culture. Thus, in the tested experimental conditions, MSC–IFN-γ act in a similar way to non-activated MSC upon TRP supplementation, indicating that IFN-γ-induced IDO activity plays a major role in the effect of IFN-γ-activated MSC on B cells.

In this study, we named the transitional B cell subset characterized by CD19+ CD24hi CD38hi as Bregs, since this is one of the most commonly used phenotypes for this subset of B cells in humans and we have previously proven it is consistently upregulated in the presence of MSC. We further characterized this subset by quantification of IL-10 production as IL-10 is the most widely used to define Bregs function. The definition of the Breg population is an important discussion point in our manuscript and in current literature. There is no unique signature that identifies the Breg subset and probably there are many different Breg subsets with different phenotypes. In our setting, we have previously observed that MSC increase the proportion of naïve (CD19+ CD27–) and transitional (CD19+

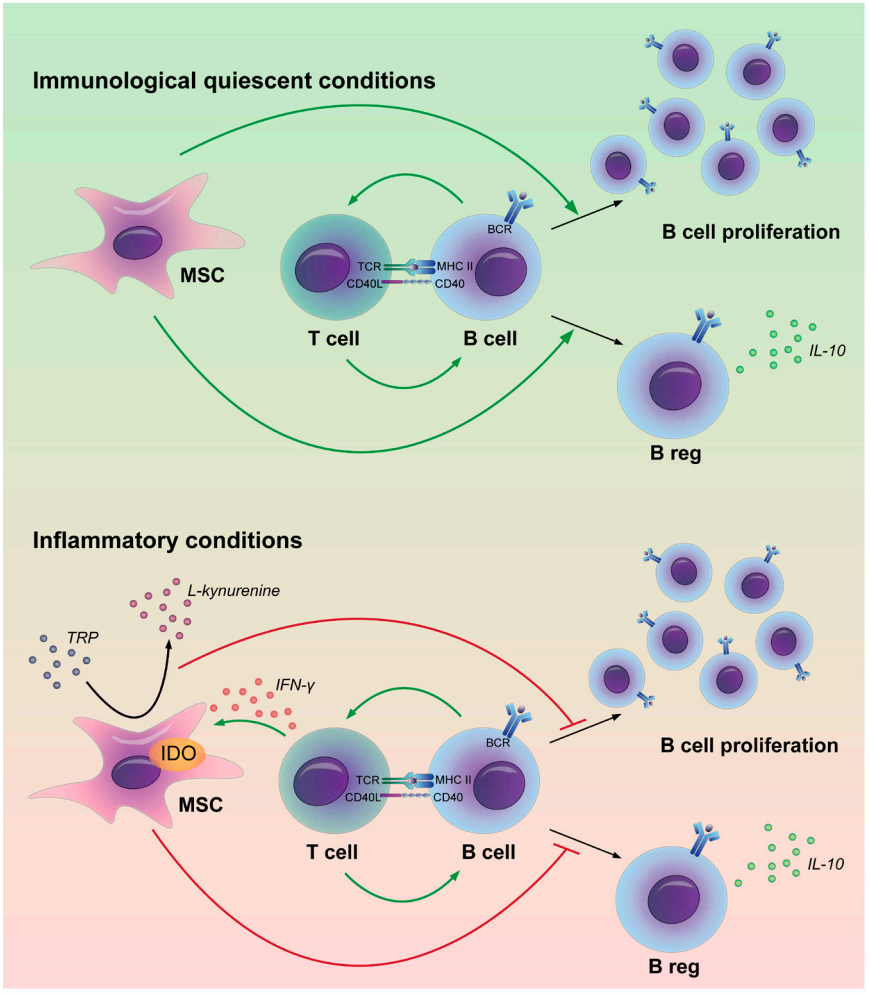

Figure 6. Model for the interactions between adipose tissue-derived mesenchymal stem or stromal cells (MSC) and B cells in immunological quiescent and in inflammatory conditions.

MSC have a stimulatory effect on B cell proliferation and regulatory B-cell formation in an immunological quiescent environment. Under inflammatory conditions, MSC break down tryptophan (TRP) through indoleamine 2,3-dioxygenase (IDO). The depletion of TRP leads to an inhibition of B cell proliferation and prevents regulatory B-cell formation.

CD24high CD38high) B cells, which was correlated to an increase of IL-10 gene expression and protein production [11]. However, the intracellular IL-10 staining in this study reveals that there is no complete match between the transitional B cells immunophenotype and IL-10-producing cells, so further marker discovery is needed to unravel a more suitable signature or a master transcription factor that would allow to properly label Bregs. While such key markers are not discovered, we used both the transitional B cell immunophenotype and the amount of IL-10 released in the culture medium to semi-quantify the Breg population in this study. Better understanding of the interaction between MSC and B cells under different immunological conditions is important for designing therapeutic approaches targeting B cells using MSC. Conventional MSC therapy can potentially be used to induce Breg formation and thereby promote tolerance such as after organ transplantation. Peng et al. show that MSC therapy in chronic graft versus host disease patients led to increased number of IL-10-producing CD5+ Bregs and increased IL-10 production by these cells [31]. On the other hand, IFN-γ-activated MSC as therapy could be beneficial in B cell-mediated diseases where suppression of B cell proliferation and IgG production is desired.

To summarize, we show that immunological conditions can dictate the effect of MSC on B cell function. MSC induce B cells with a regulatory phenotype but are not capable to dampen B cell proliferation. Under T cell-mediated inflammatory conditions, MSC strongly inhibit B cell proliferation and, as a consequence, IgG production although they do not induce formation of Bregs. This shows for the first time that MSC adapt their effect on B cells to the inflammatory climate. In vivo this means that resident MSC are supportive for B cells and induce tolerogenic B cells under immunological quiescent conditions, whereas under inflammatory conditions MSC suppress humoral responses. For therapeutic MSC, this means that we can generate MSC with either B cell suppressive properties, or MSC that support B cell homeostasis. With this knowledge specific MSC therapy can be designed for different immune disorders or transplantation.

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Chapter 4

Effects of Freeze–Thawing and Intravenous Infusion on Mesenchymal Stromal Cell Gene Expression

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Abstract

Mesenchymal stromal cells (MSC) are increasingly used as an investigative therapeutic product for immune disorders and degenerative disease. Typically, MSC are isolated from human tissue, expanded in culture, and cryopreserved until usage. The safety and efficacy of MSC therapy will depend on the phenotypical and functional characteristics of MSC. The freeze-thawing procedure may change these characteristics. Furthermore, the cells encounter a microenvironment after administration that may impact their properties. It has been demonstrated that the majority of MSC localize to the lungs after intravenous infusion, making this the site to study the effects of the in vivo milieu on administered MSC. In this study, we investigated the effect of freeze-thawing and the mouse lung microenvironment on human adipose tissue-derived MSC. There were effects of freeze-thawing on the whole genome expression profile of MSC, although the effects did not exceed inter-donor differences. There were no major changes in the expression of hemostatic regulators on transcriptional level, but significantly increased expression of procoagulant tissue factor on the surface of thawed adipose MSC, correlating with increased procoagulant activity of thawed cells. Exposure for 2 h to the lung microenvironment had a major effect on MSC gene expression and affected several immunological pathways. This indicates that MSC undergo functional changes shortly after infusion and this may influence the efficacy of MSC to modulate inflammatory responses. The results of this study demonstrate that MSC rapidly alter in response to the local milieu and disease-specific conditions may shape MSC after administration.

Introduction

Mesenchymal stromal cells (MSC) are used as an investigative therapy for degenerative and immune disease. On the road to development of an effective therapy, MSC are being examined in numerous in vitro, preclinical, and clinical studies. The outcomes of these studies have so far shown to be variable and the efficacy of MSC in clinical studies does not always match the expectations raised by preclinical and in vitro findings [1]. There are a few factors concerning MSC functionality that could explain some of the discrepant outcomes. First, culture-expanded MSC may not be fully compatible with human blood and trigger the instant blood-mediated inflammatory reaction (IBMIR) when administered

intravenously [2]. Second, one of the recurring differences between preclinical and clinical studies is the use of MSC from continuous cultures in vitro and in most preclinical studies as opposed to the use of cryopreserved MSC in the large majority of clinical studies [3]. Third, the phenotype and functionality of MSC may change upon encounter with the in vivo microenvironment after administration. The IBMIR reaction toward MSC may on the one hand compromise therapeutic cell survival, but on the other hand also trigger their beneficial paracrine effects in vivo [2]. Triggering of IBMIR results in release of factors that can activate MSC, but may also promote priming of anti-inflammatory effector cell types (e.g., regulatory T cells, myeloid-derived suppressor cells, and alternatively activated macrophages) in response to opsonized MSC [4,5]. There are indications for significant differences between MSC that are thawed shortly before use and MSC that come straight from the culture flask. Frozen–thawed MSC have impaired immunomodulatory properties and demonstrate increased triggering of IBMIR compared to MSC from continuous culture [6]. Thawed MSC furthermore show elevated levels of heat shock proteins and impaired responsiveness to inflammatory conditions within the first 24 h after thawing [7]. It is therefore possible that cryopreserved MSC are less effective than MSC from continuous culture for certain purposes, whereas for other applications cryopreserved MSC may be particularly suitable. It is important for the advancement of MSC therapy that the effects of cryopreservation on cell functionality are mapped in detail so that optimally effective MSC can be used for therapy. Working with living cells implies that the cells can change their phenotypical and functional properties in response to environmental stimuli. The in vivo milieu that cells encounter upon administration may influence cellular function. However, insufficient knowledge of the homing habits of administered MSC, limited cell survival, and the complexity of the in vivo environment make it difficult to analyze the changes that MSC undergo after administration [8]. Some studies have demonstrated the isolation and reculture of administered MSC from mice [9,10], but none have been able to analyze the function of administered MSC in vivo. It has become clear that intravenously injected MSC initially accumulate in the lungs due to size restrictions of the lung microvasculature [11]. In the lungs, MSC encounter pulmonary microvascular endothelial cells and resident macrophages and may undergo reciprocal interactions with these cells. It has been demonstrated that MSC affect lung endothelial cells by restoring endothelial permeability by the secretion of hepatocyte growth factor [12]. We have previously demonstrated that the expression levels of multiple cytokines and chemokines in the lungs are modulated after infusion of MSC [13]. The cytokines and chemokines

are most likely derived from lung endothelial cells and lung-resident immune cells. Lung-derived factors and intercellular cell surface molecule interactions may have an effect on administered MSC. This would suggest that the functionality of MSC can change already shortly after administration when MSC are present in the lungs and this may alter the therapeutic effect of MSC. A better understanding of the interplay between MSC and the lung tissue-resident cells may lead to optimization of current MSC therapy protocols. In this study, we examined phenotypical differences between cryopreserved MSC and MSC from continuous culture and analyzed the effect of the lung microvasculature milieu on MSC properties.

Methods

Isolation and culture of human MSC

MSC were isolated from abdominal subcutaneous adipose tissue of healthy individuals that became available upon kidney donation procedure after written informed consent (protocol no. MEC-2006-190 approved by the Medical Ethics Committee of the Erasmus Medical Center). After collection, the tissue was kept in minimum essential medium-α (MEM-α) (Sigma-Aldrich, St. Louis, MO) supplemented with 1% penicillin/streptomycin solution (P/S; 100 IU/mL penicillin, 100 IU/mL streptomycin; Lonza, Verviers, Belgium) at 4C and MSC isolated within 24 h. The tissue was minced and enzymatically digested with sterile 0.5 mg/mL collagenase type IV (Life Technologies, Paisley, United Kingdom) at 37°C for 30 min under continuous shaking. The obtained cell suspension was then washed twice, resuspended in culture medium consisting of MEM-α with 1% P/S, 2 mM l-glutamine (Lonza), and 15% fetal bovine serum (FBS; Lonza) and seeded in culture flasks. After 3 days, nonadherent cells were removed. The cultures were kept at 37°C, 5% CO2, and 95% humidity and the medium refreshed once a week. When the cultures reached 90% confluence, MSC were trypsinized using 0.05% trypsin–ethylenediaminetetraaceticacid (EDTA; Life Technologies, Bleiswijk, Netherlands) and subcultured. MSC were used for experiments at passage 3.

Freeze–thawing procedure

For cryostorage, MSC were removed from their culture flasks by trypsinization and washed in MEM-α with 1% P/S and 15% FBS. They were then resuspended in MEM-α with 1% P/S and 15% FBS at 1 · 106 cells per mL and mixed 1:1 with MEM-α with 20% dimethyl sulfoxide (Merck, Darmstadt, Germany) and 20% FBS, aliquoted

in cryovials, and placed in a freezing box (Coolcell; Biocision, San Rafael, CA) at -150C. To thaw the cells, cryovials were placed in a 37C water bath until nearly all the ice was melted. The cells were then washed and kept in MEM-α with 1% P/S, 2 mM l-glutamine, and 15% FBS for 1 h at 37C before they were used in experiments.

Immunophenotyping of MSC

Flow cytometric analysis was conducted on MSC labeled with monoclonal antibodies as outlined in the supporting information (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd) and acquired on a FACS Aria (Becton Dickinson, Franklin Lakes, NJ); 2,000–5,000 gated events were quantified and analyzed with Summit v.4.1 software (Dako, Glostrup, Denmark).

In vitro blood clotting assay

The clotting time of human blood was recorded on a semiautomatic 10-channel ball coagulometer (MC10plus; Merlin Medical ABW Medizin und Technik GmbH, Lemgo, Germany), as reported earlier [14]. MSC directly from the culture flask or after thawing were washed twice and resuspended in a buffer containing 5% human serum albumin. Sodium citrate-anticoagulated human blood was obtained from healthy volunteers who had not received any medication for at least 10 days. The cuvette was filled with 100 mL of citrated blood diluted 1:1 in phosphate-buffered saline (PBS). Blood was then supplemented with 50 mL of buffer with or without 3,000 MSC or with 50 mL of positive control reagent. To initiate clotting, 50 mL of 40 mM Ca2+ solution was added to a final concentration of 10 mM. The final concentration of MSC was 15,000 cells/mL, corresponding to a dose of 1–2x106 cells/kg commonly used in clinical trials.

Administration of human MSC in mice

MSC of four healthy human donors were trypisinized, washed, put through a 40 mm cell strainer, and 1 · 106 MSC in 200 mL PBS were administered in female C57BL/6 mice (Charles River, Wilmington, MA) through tail vein injections. After 2 h, the animals were anesthetized, blood collected in EDTA tubes, and lung tissue removed and snap-frozen for RNA isolation. The animal experiments were approved by the Animal Care and Use Committee of the Erasmus Medical Center (protocol no. EMC-3004).

Human MSC gene expression analysis by RNA sequencing

MSC gene expression was analyzed immediately after removal of MSC from culture flasks, after thawing of cryopreserved MSC, and in MSC that were trapped in the lungs after intravenous administration in mice using mRNA sequencing. For the first group, RNA was isolated from MSC that were snap-frozen immediately after trypsinization (group C). For the second group, RNA was isolated from froze–thawed MSC that were snap-frozen 1 h after recovery at 37C (group FT). For the third group, RNA was isolated from lung tissue 2 h after infusion of MSC (group I). These samples contained mouse RNA mixed with human RNA from the injected MSC. For each group, MSC of the same three donors of the same passage were used. Lung tissue of a mouse that was injected with PBS was used as a negative control (sample M).

RNA was isolated using Trizol reagent (Life Technologies). Frozen lung tissue was sectioned in 20 mm slices before RNA isolation. Quantity and quality of RNA was assessed using the RNA 6000 Nano kit on a 2100 Bio-analyzer (Agilent, Palo Alto, CA). Samples with an RNA-integrity >8.5 were used. Samples were prepped with TruSeq RNA (v2, Illumina), sequenced SR43 bp on Hiseq2500 in rapid mode, and demultiplexed with CASAVA 1.8.4. Alignment was performed with TopHat 2.0.13 (large index mode, http:// tophat.cbcb.umd.edu; with Bowtie 2.2.4.0) against a reference genome containing all human (hg19) and mouse (mm10) chromosomes, with their Refseq gene annotation (Illumina iGenomes, http://support.illumina.com/sequencing/sequencing_software/igenome.html). This custom reference was intended to eliminate cross-alignment from mouse originating fragments onto their human homologous for the group I samples.

Fragment counts per gene were calculated from the TopHat BAM files, using a custom R (http://r-project.org) script based on the IRanges (Bioconductor; http://bioconductor.org) package and the RefSeq gene annotation. Differential expression analysis was performed with edgeR (Bioconductor), using only the human chromosomes and genes.

Including the mouse chromosomes in the reference genome was not enough to prevent all cross-alignment, as for regions identical between human and mouse, reads get assigned randomly to one of them. This results in human genes having a high read count for sample M, where no alignment against human genes is expected. In samples from group I, this results in highly nonuniform coverage over the transcript for human genes with regions identical between mouse and human. These genes show high peaks in the homologous regions originating from mouse RNA. These genes show up spuriously as significantly differentially expressed. We

filtered these genes out of the final list of differentially expressed genes for the comparison of group I versus group C, using a custom-built R script and the dplyr package (CRAN; http://cran.r-project.org). We used two filtering criteria; first, we removed genes that had more than five counts in the negative mouse control sample (M). Second, we removed genes where the counts were localized in less than half of their exons, and the other exons did not show any counts. Ingenuity software (Qiagen, Venlo, Netherlands) was used for pathway analysis.

Detection of C3 activation fragment a and thrombin–antithrombin complex in murine plasma

Formation of blood activation markers thrombin–antithrombin complex (TAT) and complement component C3 activation fragment a (C3a) in murine plasma was measured with enzyme-linked immunosorbent assay (Cusabio Biotech Ltd., Wuhan, China), at 2, 8, and 24 h post MSC infusion. Detection of cytokine/chemokine levels in lung tissue To detect levels of cytokines and chemokines in lung tissue, frozen lung tissue was weighted and sliced in 10-mmthick slices. The sliced tissue was centrifuged at 15,000 g and tissue fluid collected for cytokine/chemokine measurement by mouse cytokine/chemokine magnetic bead panel multiplex assay (Merck Millipore, Billerica, MA). The panel contained granulocyte colony-stimulating factor (GCSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon (IFN)g, interleukin (IL)1b, IL6, IL7, IL10, CXCL1, CXCL5, CXCL10, CCL11, monocyte chemoattractant protein 1 (MCP1), MIP1a, and tumor necrosis factor (TNF)a. The samples were measured by Luminex 100/200 cytometer (Luminex, Austin, TX) using Xponent software. Statistical analysis Data were tested for significance using analysis of variance and Student's t-test. If the data did not fit a normal distribution, the Mann-Whitney test or the Wilcoxon matched-pairs test was used (two-tailed, 95% confidence intervals). Post hoc analysis was performed using Bonferroni test for multiple comparisons. P-values <0.05 were considered statistically significant.

Results

Gene expression profiling of MSC from continuous culture versus frozen–thawed MSC

To examine the effect of freeze–thawing on MSC, human adipose tissue-derived MSC of three healthy donors from continuous culture (C1, C2, C3) and of frozen-

thawed MSC of the same donors (FT1, FT2, FT3) were used for transcriptome profiling by RNA sequencing. Gene expression clustering analysis demonstrated that inter-donor gene expression differences were larger than the effects of cryopreservation, as frozen–thawed and cryopreserved MSC clustered per donor (Figure 1A). Between the frozen–thawed MSC and continuous culture MSC groups, there were 294 genes that showed a significantly different expression. Of these genes, 167 were upregulated in the frozen–thawed MSC and 127 were downregulated (Figure 1B). The magnitude of gene expression differences was limited to a maximum increase of 10.9-fold and a maximum decrease of 5.1-fold. A summary of the most significant gene expression changes can be found in Table 1. The full data set can be accessed at GEO accession number GSE76081.

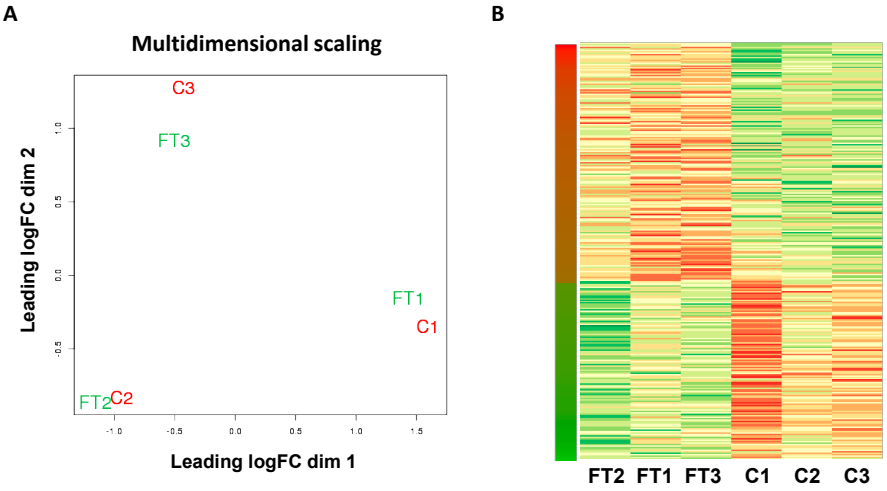

Figure 1. mRNA expression analysis of human MSC from continuous culture (C) and frozen–thawed MSC (FT).
(A) Clustering ofthe samples shows that inter-donor variation (numbers indicate donors) is larger than the variation between MSC from continuous culture and frozen–thawed MSC (left panel). (B) Heatmap depicting expression patterns of genes significantly different expressed between MSC from continuous culture and frozen-thawed MSC. Data are normalized per row.

Pathway analysis of MSC from continuous culture versus frozen–thawed MSC

A number of individual genes involved in the inhibition of cellular proliferation and induction of growth arrest were upregulated, but pathway analysis did not reveal differential expression of cell survival or cell apoptosis pathways. Freeze–thawing of MSC had an effect on innate immunity pathways (Table 1). These pathways indicated an activation of the acute phase response in frozen-thawed MSC, macrophage inhibitory factor signaling, and activation of Tolllike receptor pathways through high-mobility group protein B1 signaling. A number of genes that are involved in actin rearrangement were upregulated in frozen–thawed cells, such as a number of Rho GTPases and actin-related protein, suggesting active cytoskeletal reorganization processes in MSC after recovering from cryopreservation.

Effects of freeze–thawing on MSC immunophenotype and IBMIR induction

The cell surface expression of a panel of MSC markers was unaffected by freeze–thawing (Figure 2A). However, a small, but significant increase in the expression of the coagulation factor CD142 (tissue factor, TF) was found on thawed compared to fresh MSC (62% vs. 54% positive). Flow cytometry revealed that higher surface expression of TF on thawed cells went in hand with a small, but significant increase in the number of propidium iodide incorporating cells (84% vs. 91% viable, Figure 2B). This indicates a small increase in membrane permeability post-thawing, which may well explain the increase in TF expression, normally stored in sub-membrane intracellular granules [2]. To examine whether freeze–thawing would affect IBMIR triggering, the effect of MSC from continuous culture and frozen–thawed MSC on the clotting time of human blood was examined in vitro. The addition of MSC from continuous culture accelerated blood clotting time (Figure 2C). Frozen–thawed MSC induced a further, small but signifi- cant, acceleration of clotting time compared to continuously cultured MSC.

To corroborate our in vitro findings with the in vivo situation, we analyzed murine EDTA-plasma for formation of complement and coagulation markers at 2, 8, and 24 h post-MSC infusion (Figure 2D). Infusions of thawed cells lead to significantly higher formation of complement activation marker C3a and coagulation marker TAT at 8 h post-infusion (both $P < 0.05$).

Table 1. Summary of Gene Expression Differences Between Frozen–Thawed Mesenchymal Stromal Cells Versus Mesenchymal Stromal Cells from Continuous Culture

Gene expression changes of frozen–thawed MSC vs. MSC from continuous culture

Gene name	*Fold change up*	*Gene name*	*Fold change down*
LYPD3 Cell–matrix adhesion	10.9	*SMOC2* Extracellular matrix organization	5.1
NRARP Negative regulation of Notch signaling	10.9	*RGS5* Negative regulation of signal transduction	4.7
EGR3 Response to growth factors	7.0	*MIR17HG* Cell survival, proliferation	3.9
NPPB Diuretic hormone activity	6.2	*NEAT1* unknown	3.6
RGS2 Beta-tubulin binding	5.7	*CCDC39* Cell motility, movement	3.4
RASD1 Negative regulation of transcription	5.6	*C6orf155* Unknown	3.2
SNAI1 Development, epithelial to mesenchymal transition	5.2	*MARCH1* Antigen processing, immune response	3.1
NR4A3 Apoptosis, proliferation, survival	5.1	*CHI3L1* Inflammatory response	3.0
EGR2 Development, negative regulation of apoptosis	5.0	*CLDN1* Cell adhesion, cell–cell junction	2.8
NR4A2 Stress response	4.8	*TTC14* Unknown	2.7
DUSP5 Activation of MAPK activity	4.5	*SEL1L2* unknown	2.7
NR4A1 Positive regulation of apoptosis and proliferation	4.5	*MASP2* Complement activation	2.6
DUSP2 Inactivation of MAPK activity	4.4	*SAA2-SAA4* Acute-phase response	2.5
ID1 Angiogenesis	4.0	*EVI2B* Unknown	2.5
RRAD Negative regulation cell growth	3.9	*SAA1* Acute-phase response	2.4

Pathways differentially expressed and genes involved

MIF regulation of innate immunity↑: *z*-score 2.2, $P < 0.05$, 5 of 40 genes different; FOS↑, JUN↑, NFKBIA↑, PLA2G2A↑, PTGS2↑

ILK signaling↑: *z*-score 3.0, $P < 0.05$, 10 of 180 genes different; FOS↑, JUN↑, MYC↑, PTGS2↑, RHOB↑, RHOG↑, SNAI1↑, SNAI2↑, TMSB10/TMSB4X↑, VIM↑

Acute phase response signaling↑: *z*-score 0.82, $P < 0.05$, 9 of 166 genes different; CARBP2↑, FOS↑, JUN↑, IL6↑, NFKBIA↑, SAA1↓, SAA2↓, SOCS3↑, TNFRSF11B↓

Actin nucleation by ARP-WASP complex↑: *z*-score 1.34, $P < 0.05$, 5 of 55 genes different; ARPC1B↑, ITGA2↓, PPP1R12B↓, RHOB↑, RHOG↑

HMGB1 signalling↑: *z*-score 1.9, $P < 0.05$, 7 of 117 genes different; FOS↑, JUN↑, IL6↑, IL11↑, RHOB↑, RHOG↑, TNFRSF11B↓

Fifteen genes with the largest increases and 15 genes with the largest decreases are shown. All gene expression changes in the table are significant with *P*-values <0.05. Brief descriptions of gene functions are indicated underneath the gene names. Five pathways that showed the most significant up or downregulated activity patterns are listed with genes involved.

MSC, mesenchymal stem cells; ↑, upregulation; ↓, downregulation.

Effect of MSC from continuous culture and frozen–thawed MSC on lung immune homeostasis

To investigate whether MSC from continuous culture and frozen–thawed MSC had a differential effect on the microenvironment after infusion, 1 · 106 human MSC were injected intravenously in C57BL/6 mice. As MSC accumulate in the lungs after intravenous administration, they may interact with endothelial cells of the lung microvasculature and innate immune cells such as macrophages and granulocytes. Two hours after injection of MSC, lungs were removed and mouse cytokine and chemokine expression analyzed by multiplex assay. Injection of MSC from continuous culture as well as frozen–thawed MSC demonstrated a clear tendency for increased levels of mouse G-CSF, CXCL1, CXCL10, MCP1, and IL6 and a decrease in CXCL5 in lung tissue (Figure 3). MSC did not induce changes in the expression of IFNg, CCL11, GM-CSF, IL1b, IL7, IL10, MIP1a, and TNFa (Supplementary Figure S1). We have previously demonstrated that MSC induce a mild inflammatory response in the lungs, which may be associated with the immunomodulatory effect of MSC [13]. The present data demonstrate that there was no difference between the effects of MSC from continuous culture and frozen–thawed MSC, suggesting that the two cell preparations have a similar immunomodulatory effect in the lungs. To examine whether MSC are stabile after in vivo administration or whether they undergo changes under influence of the in vivo environment, we carried out transcriptome analysis on administered MSC. Human MSC from continuous culture (1x106) were infused in the tail vein of C57BL/6 mice. The lungs, containing entrapped MSC, were removed 2 h after infusion and total RNA isolated (I1, I2, I3). RNA of all samples was sequenced and mapped against the combined human/mouse genome, thus reflecting human MSC gene expression in the mouse lung. Lung tissue of a mouse that did not receive human MSC was used as a negative control (M). In sample M, positive expression of several human genes was detected, reflecting cross-alignment of mouse RNA on the human genome. These particular genes, which represented 30% of the originally upregulated and 60% of the downregulated genes, were disregarded in samples I1-3. Genes in which <50% of exons showed positive reads in samples I1-3, also reflecting alignment of mouse RNA on the human genome, were also disregarded (20% of the originally differentially expressed genes). The gene expression profile of the administered MSC differed considerably from both non-injected MSC groups, as can be seen in Figure 4A, where samples C1-3 and FT1-3 now appear to overlap, whereas I1-3 stand out. Negative control sample M stands out from all the other samples (Figure 4A, B). Comparison of gene expression profiles between MSC from continuous culture before infusion (C1–3) and after infusion (I1–3) revealed differential

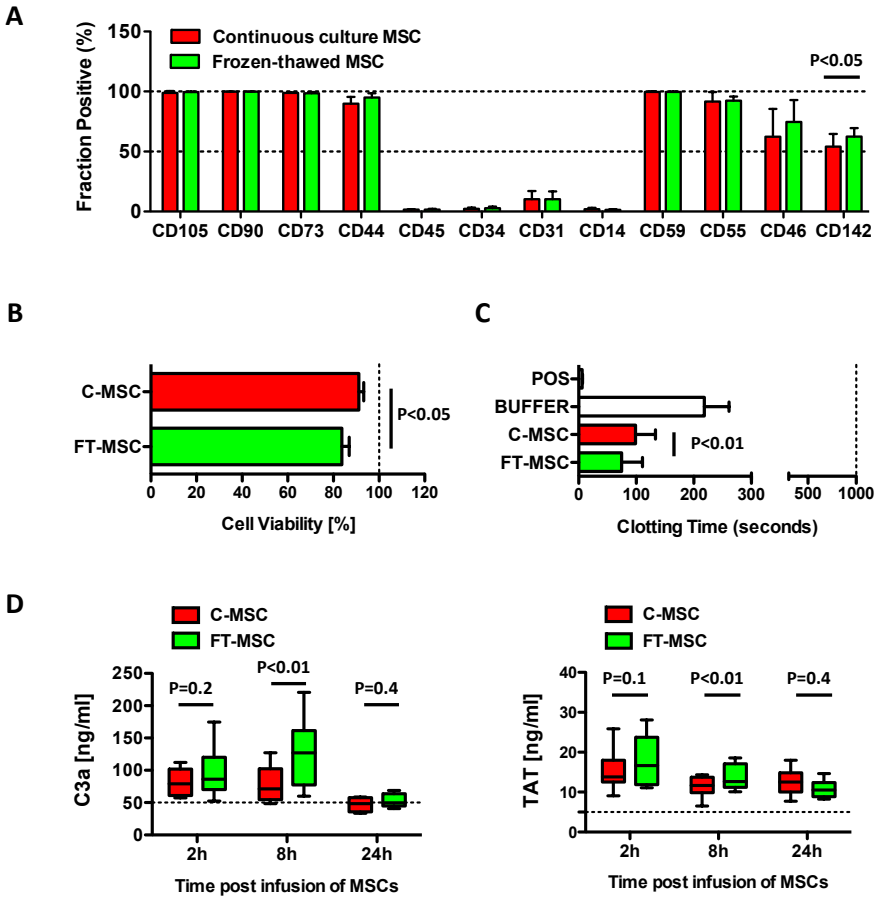

Figure 2. Effect of freeze– thawing on MSC immunophenotype and triggering of innate immune cascade activation after whole blood exposure in vitro and in vivo. (A) Cell surface marker expression. N= 7 MSC donors. (B) Cell viability measured by propidium iodide exclusion. (C) MSC from continuous culture and frozen–thawed MSC (15,000 cells per mL) were tested for their triggering of the clotting cascade by exposing them to fresh recalcified human whole blood. Blood clotting time in seconds indicated. Pos, clotting inducing control; 10 mM Ca2+ solution. Buffer: negative control; no clotting inducing factors added. (D) Quantification of complement factor C3a (ng/mL) and TAT (ng/mL) in murine plasma after systemic infusion of human MSC (n = 5 MSC donors, two tests each). Bar graphs depict mean – standard deviation. Dashed lines indicate background levels of C3a and TAT. C3a, C3 activation fragment a; TAT, thrombin–antithrombin complex

expression of 2,060 genes. Of these genes, 720 were upregulated in the infused MSC and 1,340 were downregulated (Figure 4C). The maximum increase in gene expression in the injected MSC was 1,607-fold. The decreases in gene expression were smaller, with 1,326 of the downregulated genes showing a fold change of <4. The maximum decrease was -10.3-fold. A summary of the most significant gene expression changes can be found in Table 2. The full data set can be accessed at GEO accession number GSE76081.

Pathway analysis of MSC after in vivo administration

Pathway analysis 2 h after administration demonstrated that the lung microenvironment affected pathways with an immunological function in MSC. MSC showed strongly increased expression of various human leukocyte antigen class II molecules, which are upregulated in response to inflammatory cytokines. Furthermore, there was a strong upregulation of IFNγ-induced protein 10 (or CXCL10), of the common gamma chain of the receptor for IL2, IL4, IL7, IL9, IL15, and IL21, of nuclear factor of activated T cells, which modulates gene expression during immune activation, and of IFN-regulatory factor 4. The four pathways that showed the most significantly altered patterns of activation had an immunological function. Immune signaling through the OX40 Tumor Necrosis Factor family pathway and the cytosolic pattern recognition receptor pathway showed reduced activity, whereas the TNF receptor 2 pathway and the phospho-kinase C signaling pathway showed enhanced activity (Table 2), indicating a modulation of the immunomodulatory activity of MSC upon administration. Even though it is known that MSC have a short survival after intravenous infusion, cell death pathways were not activated. The apoptosis signaling pathway showed no pattern of activation, while the HIV-induced apoptosis pathway showed reduced activity. Interestingly, multiple genes involved in tyrosine metabolism, such as tyrosinase and tyrosinase-related proteins 1 and 2, were among the most highly upregulated genes.

Production of soluble factors by injected MSC

As the therapeutic effect of MSC is thought to be partly dependent on secreted factors, the expression of genes encoding proteins with an extracellular function was analyzed. Eighteen soluble factors showed upregulated expression of at least 10-fold in injected MSC (Table 3). These factors included factors with an immune function, such as CXCL10, IL11, and IL33, and growth factors and factors that stimulate regeneration by progenitor cells, such as wingless-type MMTV integration site family member (WNTs) and bone morphogenetic protein (BMP)2.

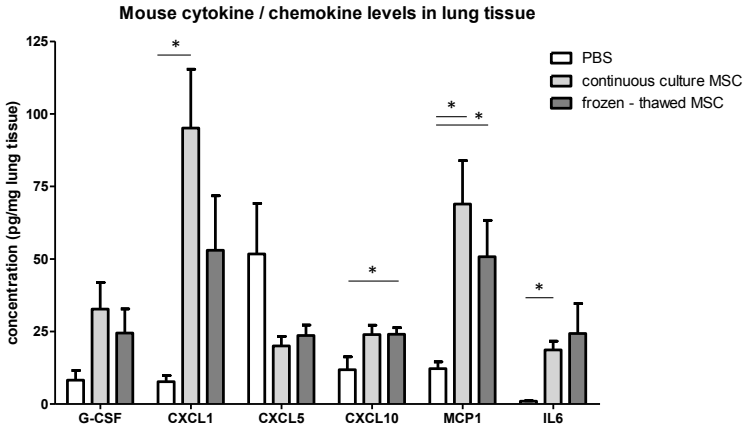

FIG. 3. Effect of infusion of human MSC from continuous culture (C) and frozen–thawed MSC (FT) on mouse cytokine and chemokine expression in the mouse lung.
1 · 106 MSC were infused through the tail vein and 2 h later, lungs removed for analysis. N = 4 MSC donors. Bar graphs depict mean – standard deviation. *P < 0.05. G-CSF, granulocyte colony-stimulating factor; MCP1, monocyte chemoattractant protein 1.

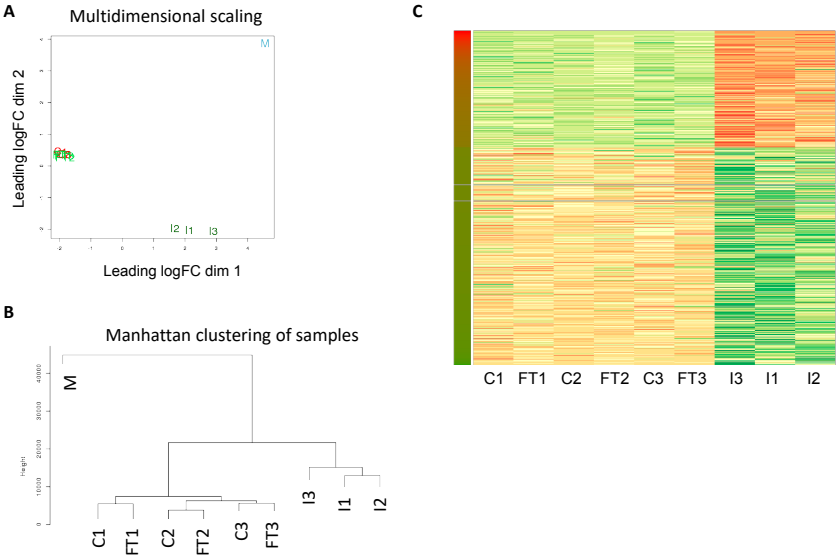

Figure 4 mRNA expression analysis of human MSC from continuous culture (C), frozen–thawed MSC (FT), and injected MSC (I).
(A) Clustering of the samples shows that the effect of the microenvironment on injected MSC is much larger than the effect of freeze–thawing on MSC. Mouse mRNA (M) clearly stands out. (B) Manhattan clustering confirms this. (C) Heatmap depicting expression patterns of genes significantly different expressed between MSC from continuous culture and injected MSC. Data are normalized per row.

Discussion

The efficiency of MSC therapy will depend for a great part on the phenotype of MSC preparations. In contrast to conventional molecular drugs that, apart from being metabolized by the recipient, do not change, cells can undergo dramatic alterations in response to changes in their microenvironment. Freeze–thawing has been indicated to affect the in vitro immunomodulatory properties of MSC [6,7]. However, MSC functionality is completely recovered after a 24-h culture period. In this study, we found limited gene expression changes in MSC 1 h after thawing. There were changes in genes involved in innate immunity pathways and cytoskeletal rearrangement. The cryopreserved cells were kept in suspension for 1 h after thawing and it is possible that these conditions induced the cells to upregulate cytoskeletal protein expression.

MSC from continuous culture and frozen–thawed MSC induced comparable immunological responses in the mouse lung, the major site of MSC embolization upon intravenous infusion. Indeed, a study by Cruz et al. demonstrated only limited differences between the effects of cryopreserved and continuously cultured MSC in ameliorating airway inflammation, supporting the concept that both MSC preparations are equally effective in vivo [15]. It is likely that the type of model used is determinative for detecting differences between frozen–thawed and continuously cultured MSC or not, as freeze–thawing may affect particular properties of MSC that are employed in particular models, but not in others. It has previously been demonstrated that islets of Langerhans [16], but also culture expanded MSC, can induce IBMIR [2]. The induction of IBMIR was weakly augmented with frozen-thawed MSC [6,17]. A strong induction of IBMIR would reduce the survival time of MSC after administration and could potentially lead to adverse effects, providing a possible explanation for the limited engraftment of therapeutic MSC [18] and adverse transfusion reactions at higher doses [14]. We found a strong induction of IBMIR for the adipose MSC used in this study, which is in agreement with other reports attributing a strong procoagulant activity to adipose tissue-derived MSC [19–21]. We measured a weak, but significantly augmented triggering of IBMIR with frozen–thawed cells. The physiological significance of this difference between continuously cultured and frozen–thawed MSC is unclear. However, most importantly, both cell types elicited strong responses. Thus, in vivo persistence will be rather limited with both continuously- and frozen–thawed adipose-derived MSC alike, not necessarily compromising their bioactivity.

Table 3. List of Soluble Factors with at Least 10-Fold Upregulated Gene Expression in Injected Mesenchymal Stromal Cells Compared to Noninjected Mesenchymal Stromal Cells

Soluble factors upregulated in injected MSC		
Gene name	*Fold change up*	*Function*
Apolipoprotein C2	1,261	Increases free fatty acid availability
CXCL10	274	Chemoattraction immune cells
Melanoma inhibitory activity	256	Migration, inhibition of adhesion
WNT7A	239	Development
Neural pentraxin 1	68.6	Innate immunity, acute phase protein
Urocortin 2	64.0	Cardiovascular stimulation
Suppressor of Ty20 homolog like 2	55.7	Nucleic acid-templated transcription
Pleiotrophin	48.5	Growth factor
Apolipoprotein E	42.2	Cholesterol metabolism
Serpin A1	34.3	Protease inhibitor
WAP four-disulfide core domain protein 1	24.3	Protease inhibitor
Parathyroid hormone-like hormone	22.6	Increases Ca^{2+} in the blood
IL11	21.1	Megakaryocyte maturation, osteotrophic
Bone morphogenetic protein 2	18.4	Osteoblast differentiation
IL33	16.0	Drives Th2 immune response
Angiopoietin 4	13.9	Angiogenesis
WNT9A	10.6	Development
Fibroblast growth factor 9	10.6	Growth factor

All gene expression changes listed are highly significant ($P < 10^{-5}$, or smaller).

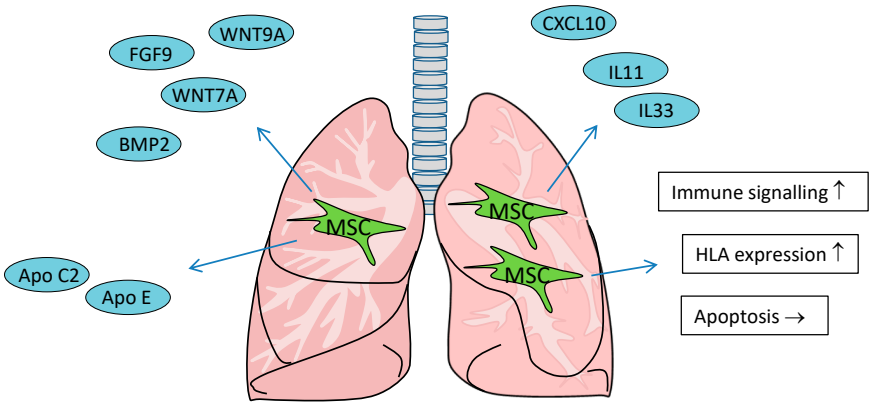

Figure 5. Schematic overview of the changes in MSC 2 h after intravenous administration. Infused MSC end up in the lungs and at 2 h, increase the expression of a range of immunomodulatory and growth factors and change the activity of immune and metabolic pathways. Apo C2, apolipoprotein C2; Apo E, apolipoprotein E; BMP2, bone morphogenic protein 2; FGF9, fibroblast growth factor 9; Wnt7A, wingless-type MMTV integration site family member 7A; Wnt9A, wingless-type MMTV integration site family, member 9A.

In contrast to the minimal effects of freeze–thawing, in vivo administration had a major effect on the transcriptional phenotype of MSC. The observed changes induced in MSC could derive from a number of factors, among them the effects of sheer-stress induced by transport through the bloodstream, factors induced by triggering of IBMIR or the effects of blood cells, and pro-inflammatory mediators on MSC. MSC accumulate in the lungs upon the first passage [11] and thus within minutes of administration, the majority of MSC are exposed to the microenvironment of the lung microvasculature, where lung endothelial cells and macrophages may undergo interactions with MSC. The gene expression changes in MSC after administration indicate a response to inflammatory signals, eliciting immunological cross talk between entrapped MSC and tissue-resident immune cells. This suggests that cells in the lung microvasculature become activated upon encounter of MSC and respond with inflammatory signals. We have demonstrated previously that lung tissue shows an inflammatory gene expression profile within hours after infusion of MSC [13] and in this study, we demonstrated that levels of the mouse inflammatory factors IL6, CXCL1, CXCL10, and MCP1 were increased in lung tissue upon administration of human MSC.

There is a possibility that the gene expression changes observed in human MSC after infusion in mice are caused by incompatibility of human cells with mouse microenvironment. However, in our earlier study, we found upregulation of immune parameters in mice infused with syngeneic mouse MSC [13]. Interestingly, in this study, we found that human MSC are well capable of responding to mouse cytokines and chemokines. Therefore, we assume that we are not merely looking at a xeno-response. The nature of the analysis used does, unfortunately, not allow administration of syngeneic cells to test this assumption.

The main question of this study is how the phenotypical changes in MSC affect their function. MSC are known to enhance their immunomodulatory function in response to inflammation [22,23], and we found evidence for increased expression of IL11 and IL33, cytokines of the IL6 and IL1 families, respectively, and for the activation of immune signaling pathways (Figure 5). There were also non-immunological pathways that were upregulated in MSC in response to the in vivo environment. There was for instance a dramatic increase in apolipoprotein expression, which is a family of soluble proteins involved in lipid transport. Apolipoproteins may, however, also have anti-inflammatory functions [24]. Furthermore, multiple genes involved in tyrosine metabolism, including tyrosinase, tyrosinase-related protein 1, and dopachrome tautomerase, were strongly upregulated. The significance of these gene expression changes is

unknown. MSC showed large increases in WNT7a and WNT9a gene expression. WNT signaling pathways have been identified to play important roles in the control of MSC proliferation and differentiation [25]. Through WNT secretion, MSC may thus target proliferation and differentiation of resident progenitor cells. These gene expression changes indicate that the microenvironment of the lung affects immunological and metabolic activity of MSC. As MSC have a short survival time after infusion, intrinsic changes in, for instance, MSC proliferation rate or differentiation potential are not relevant for the therapeutic effect of MSC. However, changes in cytokine and growth factor secretion by MSC may affect the function of resident cell types, even after MSC have disappeared. In this study, we have not examined the effect of the gene expression changes in MSC on tissues and have not determined whether concentrations of secreted factors reach effective levels. However, we can conclude from the gene expression changes that administered MSC respond rapidly to their new microenvironment and change their immunological and metabolic function. Earlier studies have shown that a large majority of intravenously infused MSC disappear within 24 h after administration [9]. We therefore expected to see an upregulation of apoptosis pathways in MSC upon intravenous administration. This was not the case, which would suggest that the disappearance of MSC is independent of the induction of apoptosis. Our data can however not completely prove this. First, apoptosis may be induced independently of gene expression changes. Second, the filter that we used to avoid cross-reactivity of RNA of the mouse lung tissue with the human genome may have filtered out apoptosis genes that are conserved between mouse and human [26]. Therefore, we cannot rule out the possibility that infused MSC undergo apoptosis in the lungs.

Over the last two decades, it has been shown that MSC therapy is safe [27], and the current challenge is to develop efficient therapy. Effort is put in identifying MSC subsets that possess superior immunomodulatory properties and in the development of culture protocols that generate MSC with optimized function. Efficacy testing should allow discrimination between more- and less potent MSC batches. The findings of this study indicate that MSC show high responsiveness and plasticity upon systemic infusion. Properties that are present in vitro are not necessarily maintained after administration, and vice versa. This is a fact to be taken into account in the development of efficient MSC therapy.

In summary, this study demonstrates that freeze–thawing procedures have little impact on MSC gene expression, but tend to sensitize the cells for stronger recognition by the IBMIR. However, MSC from continuous culture and frozen–

thawed MSC had a similar impact on immunological parameters in the lungs. Upon intravenous injection, MSC underwent major gene expression changes, reflecting a response to inflammatory activation. This study describes for the first time that MSC change phenotype and potentially function upon systemic administration, which is important for understanding MSC therapy and improving its efficiency.

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Chapter 5

Inactivated Mesenchymal Stem Cells Maintain Immunomodulatory Capacity

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Abstract

Mesenchymal stem cells (MSC) are studied as a cell therapeutic agent for treatment of various immune diseases. However, therapy with living culture-expanded cells comes with safety concerns. Furthermore, development of effective MSC immunotherapy is hampered by lack of knowledge of the mechanisms of action and the therapeutic components of MSC. Such knowledge allows better identification of diseases that are responsive to MSC treatment, optimization of the MSC product, and development of therapy based on functional components of MSC. To close in on the components that carry the therapeutic immunomodulatory activity of MSC, we generated MSC that were unable to respond to inflammatory signals or secrete immunomodulatory factors, but preserved their cellular integrity [heat-inactivated MSC (HI-MSC)]. Secretome-deficient HI-MSC and control MSC showed the same biodistribution and persistence after infusion in mice with ischemic kidney injury. Both control and HI-MSC induced mild inflammatory responses in healthy mice and dramatic increases in interleukin-10, and reductions in interferon gamma levels in sepsis mice. In vitro experiments showed that opposite to control MSC, HI-MSC lacked the capability to suppress T-cell proliferation or induce regulatory B-cell formation. However, both HI-MSC and control MSC modulated monocyte function in response to lipopolysaccharides. The results of this study demonstrate that, in particular disease models, the immunomodulatory effect of MSC does not depend on their secretome or active crosstalk with immune cells, but on recognition of MSC by monocytic cells. These findings provide a new view on MSC induced immunomodulation and help identify key components of the therapeutic effects of MSC.

Introduction

Mesenchymal stem cells (MSC) are present in most adult human tissues and can be easily obtained from adipose tissue and bone marrow. They are characterized by their ability to adhere to plastic, their rapid proliferation in culture, and their capacity to differentiate into osteoblasts, adipocytes, myocytes, and chondrocytes [1]. In addition, MSC possess immunosuppressive properties as demonstrated in experimental inflammatory disease models for autoimmune diseases, graft-versus-host disease (GvHD), and allograft rejection [2–9]. The promising results obtained from these models have triggered the investigation of MSC therapy in clinical trials for a range of immune disorders, including GvHD, Crohn's disease, diabetes mellitus,

systemic lupus erythematosus, and allograft rejection [10–15]. While some clinical trials have described positive effects of MSC treatment, others have not been able to demonstrate amelioration of disease symptoms [16,17]. The indistinct efficacy of MSC immunotherapy is debit to the lack of understanding of the mechanisms of action of MSC after administration, which hampers rational timing and dosing of MSC therapy and identification of disease conditions that can potentially benefit from MSC therapy. First, the homing characteristics of MSC after administration are not fully elucidated. Some studies have reported homing of infused MSC to sites of injury [18,19], but others showed poor homing capabilities of MSC [20]. We previously reported that intravenously (IV) infused MSC do not pass the lung barrier and have a half-life between 12 and 24 h [21]. Second, the exact nature of the interaction between MSC and immune cells after administration is not clear. In vitro studies show that under the influence of inflammatory cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), MSC inhibit the proliferation of immune cells by soluble mechanisms such as transforming growth factor beta (TGF-β), prostaglandin E2 (PGE2), and indolamine 2,3-dioxygenase [22–29]. It is therefore proposed that MSC mediate their immunomodulatory effect through their secretome [30]. There is, however, no conclusive evidence that the anti-inflammatory secretome is responsible for the immunomodulatory effects of exogenously administered MSC. The entrapment of IV-infused MSC in the lung capillaries and the short half-life of MSC after infusion [31,32] raise the questions whether administered MSC localize to the right location and live long enough to become activated by inflammatory conditions to exert their therapeutic effects through their secretome. It has become clear that MSC exert at least some of their effects after infusion through intermediate cells. For example, it has been shown that MSC have a stimulatory effect on cardiac infarct repair by activation of macrophages, since macrophage depletion partially reduced the therapeutic effect of MSC [33]. We have recently demonstrated that infusion of MSC triggers an immediate and mild systemic inflammatory response, which may be the initiator of subsequent immunosuppression [34]. It is unknown how MSC trigger such responses by host cells. In this study, we investigated whether MSC that lost the capacity to respond to inflammatory stimulation and lost the ability to secrete factors maintain their capacity to modulate immune responses. We show that such MSC maintain the ability to modulate sepsis immune responses and indicate that MSC can act as passive immunomodulatory vehicles. Our results are a step toward the development of immunomodulatory therapy based on subcellular components of MSC.

Methods

Isolation and culture of human MSC

Human MSC were isolated from subcutaneous adipose tissue that was surgically removed from the abdominal incision of healthy kidney donors. Adipose tissue was collected after written informed consent, as approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam (protocol No. MEC-2006-190). MSC were isolated from the adipose tissue as described previously [35,36]. In short, the tissue was mechanically disrupted and washed with phosphate-buffered saline (PBS). The adipose tissue was then digested enzymatically with 0.5 mg/mL collagenase type IV (Life Technologies, Paisley, UK) in RPMI 1640 Medium with glutaMAX (Gibco BRL, Life Technologies, Paisley, UK) for 30 min at 37°C under continuous shaking. The stromal vascular fraction was resuspended in minimum essential medium Eagle alpha modification (MEM-α; Sigma- Aldrich, St. Louis, MO) containing 2mM L-glutamine (Lonza, Verviers, Belgium) and 1% penicillin/streptomycin solution (P/S; 100 IU/mL penicillin, 100 IU/mL streptomycin; Lonza). MSC were cultured in a 175-cm2 cell culture flask in MEM-α supplemented with 2mM L-glutamine, P/S, and 15% fetal bovine serum (FBS; Lonza) and kept at 37°C, 5% CO2, and 20% O2. The medium was refreshed once a week and MSC were passaged at around 80% confluence using 0.05% trypsin-EDTA (Life Technologies, Bleiswijk, the Netherlands). All MSC used in experiments were between passage 2–8.

Isolation and culture of mouse MSC

Mouse MSC were isolated from the adipose tissue of male C57BL/6 mice as described previously [34] and cultured as the human MSC. The cells were frozen in 10% DMSO at -150°C at passage 1. Cells were later thawed in MEM-α supplemented with 2mML-glutamine, P/S, and 10%FBS and transferred to a 175-cm2 cell culture flask to expand. MSC used in experiments were between passage 2–9 as mouse-derived MSC maintain their capacities up to high passages (passage 10) [37].

Inactivation of MSC

MSC were inactivated in suspension in PBS in parafilm sealed tubes by 30 min incubation at 50°C in a temperature regulated water bath. The inactivated cells were then washed and used for further experiments or resuspended in MEM-α supplemented with 2mM L-glutamine, P/S, and 15% FBS and seeded in a culture plate.

Immunophenotyping of human MSC

MSC were trypsinized, washed with FACSflow (BD Biosciences, San Jose, CA), and stained with CD13-PeCy7 (clone L138), CD31-V450 (clone WM59), CD45-APC-H7 (clone 2D1), CD73-PE (clone AD2; all BD Biosciences), CD90-APC (clone Thy-1A1), and CD105-FITC (clone 166707; all R&D Systems, Minneapolis, MN). Measurements were done on a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6 software (Tree Star, Inc., Ash-land, OR).

Protein analysis by multiplex assay

Levels of vascular endothelial growth factor (VEGF), FGF2, granulocyte colony-stimulating factor (G-CSF), monocyte chemotactic protein-1 (MCP-1), and interleukin (IL)-1Ra, IFN-γ, IL-1b, IL-10, IL-6, and IL-8 were measured in a conditioned medium of human MSC after 24 h of culture in MEMa supplemented with 2mML-glutamine and P/S without FBS. In mouse serum samples, levels of IL-6, IL-10, MCP-1, CXCL1, CXCL5, G-CSF, IFN-γ, and TNF-α were measured. Cytokine and chemokine levels were quantified using a "Human cytokine/chemokine magnetic bead panel multiplex assay" (Merck Millipore, Billerica, MA) for the supernatant samples or a "Mouse cytokine/chemokine magnetic bead panel multiplex assay" (Merck Millipore) for the mouse serum samples. The samples were measured on a Luminex 100/200 cytometer (Luminex, Austin, TX) using Xponent software.

Cell viability measurements

The viability of MSC was analyzed by measuring the ability of cells to reduce MTT to formazan. Briefly, 20 mL of 5mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Munich, Germany) was added to 5,000 MSC seeded in a flat-bottom 96-well plate and incubated for 5 h at 37°C. The culture medium was then removed and formazan crystals were dissolved in 100 mL DMSO. The absorbance was measured at 550nm using a Victor Wallac 2 multilabel microplate reader (Perkin Elmer, Life Sciences, Boston, MA).

Proliferation measurement

The proliferation of MSC over time was measured using PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich Chemicals, Steinheim, Germany). Briefly, control and heat-inactivated mesenchymal stem cells (HI-MSC) were stained for 3 min with PKH26 dye. The cells were washed with FBS and 1x104 control or HI-MSC was seeded in a 12-well plate for 7 days at 37°C. Dye dilution was measured on a FACS Canto II flow cytometer (BD Biosciences).

Apoptosis staining

Early and late apoptosis of MSC was assessed by staining with Annexin V and 7-amino-actinomycin D (7-AAD) using the PE Annexin V Apoptosis Detection Kit I according to the manufacturer's guidelines (BD Biosciences). Flow cytometric analyses were performed using a FACSCanto II flowcytometer (BD Biosciences).

Mixed lymphocyte reaction

Inactivated and control MSC were plated in round-bottom 96-well plates in MEM-α supplemented with 2mM L-glutamine, P/S, and 10% heat-inactivated (30 min, 57°C) human serum in various numbers; 20, 10, 5, and 2.5x103 MSC/well. The next day, 5x104 carboxyfluorescein succinimidyl ester (CFSE)-labeled healthy donor-derived peripheral blood mononuclear cells (PBMC) and 5x104 g-irradiated (40 Gy) HLA-mismatched PBMC were added to the MSC. After 7 days, PBMC were harvested and stained for 30 min with CD3-PERCP (clone SK7; BD Biosciences). Cell proliferation was determined by CFSE dilution measured on a FACSCanto II flow cytometer (BD Biosciences).

MSC–B-cell cocultures

Splenocytes were isolated from spleens of deceased organ donors (Biobank Erasmus MC protocol No. MEC-2012-022) by Ficoll density gradient separation (GE Healthcare, Uppsala, Sweden). Quiescent B cells were obtained by negative selection using anti-CD43 magnetic beads (Miltenyi BiotecGmbH, Bergisch Gladbach,Germany). B cells were cultured for 7 days in Iscove's modified Dulbecco's medium (Lonza) supplemented with 10%HI FBS and stimulated with F(ab)2 anti-IgM (Jackson, ImmunoResearch laboratories, Inc., West Grove. PA), IL-2 (103 IU, Proleukin; Prometheus Laboratories, Inc., San Diego, CA), and 5mg/mL anti-CD40 agonistic monoclonal antibody (Bioceros, Utrecht, The Netherlands). Inactivated and control MSC were added to the culture at day 0 in a MSC:B cell ratio of 1:5. IL-10 levels in the supernatant were measured using a human IL-10 ELISA kit (U-Cytech, Utrecht, The Netherlands) according to the manufacturer's protocol.

MSC-monocyte cocultures

PBMC were isolated from the blood of healthy volunteers using Ficoll density gradient separation. Monocytes were obtained by positive selection using CD14 magnetic beads (Miltenyi Biotec GmbH). Monocytes (40,000) were co-cultured overnight with 40,000 control or inactivated MSC in round-bottom 96 wells in RPMI 1640 Medium with gluta-MAX (Gibco BRL, Life Technologies, Paisley, UK) supplemented with P/S and 10% heat-inactivated FBS.

Lipopolysaccharides (LPS; Sigma Aldrich, Gillingham, UK) were added the next day at a concentration of 100 ng/mL. TNF-α levels in the supernatant were measured 7 h after addition addition of LPS using a human TNF-α ELISA kit (U-Cytech) according to the manufacturer's protocol.

Infusion of MSC

Healthy 8-week-old female C57BL/6 mice were purchased from Charles River (Lyon, France). The mice were housed in a facility with a 12-h light–12-h dark cycle and allowed free access to food and water. All animal studies were approved by an independent institutional ethics committee on animal care and experimentation (DEC protocol EMC No. 127-12-14). In these studies, syngeneic mouse MSC were used to avoid xenogeneic and allogeneic responses.C57BL/6 adipose tissue derived MSC were trypsinized and resuspended in PBS, and one batch was inactivated by heating as described above. The MSC were then put through a 40 mm sieve and 0.3x106 cells in 200 mL PBS infused in the tail vein. Control mice received 200 mL of PBS. After 2 h, mice were sacrificed by cervical dislocation and blood was collected in serum separation tubes (Minicollect; Greiner Bio-One, Alphen a/d Rijn, The Netherlands) and spun down at 3,000 rpm for 10 min. Lungs were collected, snap frozen in liquid nitrogen, and stored at -80°C.

LPS infusion

Female C57BL/6 mice were injected with 2.5 mg/kg body weight LPS (LPS; Sigma-Aldrich, Gillingham, UK) dissolved in PBS through the tail vein. After 1 h, mice received 0.3x106 living or inactivated MSC through the tail vein. Animals were sacrificed by cervical dislocation 6 h after LPS infusion and blood was collected in serum separation tubes (Minicollect; Greiner Bio-One).

Kidney ischemia/reperfusion injury model

Unilateral ischemia/reperfusion injury (IRI) was surgically performed as described previously [38]. Briefly, female C57BL/6 mice were anaesthetized by isoflurane inhalation (5%isoflurane initially and then 2%–2.5%with 1:1 air/oxygen mixture for maintenance). Mice were kept on 37°C heating pads during the whole procedure to maintain body temperature. A midline abdominal incision was made and the left renal artery was occluded using atraumatic microvascular clamps. The incision was covered with PBS-soaked gauze and the animal was covered with aluminum foil to maintain the right body temperature. After 37 min, the clamp was released and restoration of blood flow was macroscopically confirmed by the kidney returning to normal color. The abdominal wound was closed in two layers using 5/0 sutures

and animals were given 0.5 mL PBS and 0.05 mg/kg buprenorphine as analgesic subcutaneously. Six or 24 h after clamp removal, the mice were either sacrificed by cervical dislocation and both kidneys collected, snap frozen in liquid nitrogen, and stored at -80°C or whole mice were frozen in Tissue-Tec O.C.T. Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) for MSC tracking.

MSC tracking

One batch of MSC was labeled with fluorescent Qtracker 605 beads (control MSC; Life Technologies, Grand Island, NY). Another batch of MSC was labeled with Qtracker 655 beads and heat inactivated as described previously. Inactivated and control MSC were mixed 1:1 and in total, 0.3x106 cells were injected in the tail vein of healthy mice or mice with unilateral ischemic kidney injury. After 2 and 24 h, whole mice were frozen in Tissue-Tec O.C.T. Compound and 3D anatomical and molecular fluorescence videos were generated by CryoViz_imaging. CryoViz imaging allows 3D visualization of the distribution of MSC and identification of single cells.

mRNA expression analysis

Human MSC were snap frozen directly after trypsinization, immediately after or 4 h after heat inactivation. RNA was isolated from frozen mouse lung and kidney tissues using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) and cDNA was synthesized from 1,000 ng RNA with random primers (Promega). Quantitative gene expression was determined using TaqMan gene expression master mix (Life Technologies, Carlsbad, CA) and assay-on-demand primer/probes for Hsp27 (Hs03044127_g1), Hsp70 (Hs00359163_s1), BAX (Hs00180269_m1), kidney injury molecule-1 (KIM-1; Mm00506686_m1), MCP-1 (Mm00441242_m1), macrophage inflammatory protein-1a (MIP1a; Mm00441258_m1), IL-10 (Mm00439614_m1), TGF-β (Mm01178820_m1), IL-1b (Mm01336189_m1), and housekeeping gene HPRT (Mm01545399_m1; all assay on demand primers are from: [Applied Biosystems, Foster City, CA]). Results were expressed as copy numbers (efficiency-DCT) ratio to HPRT.

Neutrophil gelatinase-associated lipocalin ELISA

Neutrophil gelatinase-associated lipocalin (NGAL) levels were measured in the serum of mice that underwent IRI to determine acute kidney injury. Serum samples were diluted 10,000x and a mouse NGAL ELISA Kit (BioPorto Diagnostics, Hellerup, Denmark) was used according to the manufacturer's protocol.

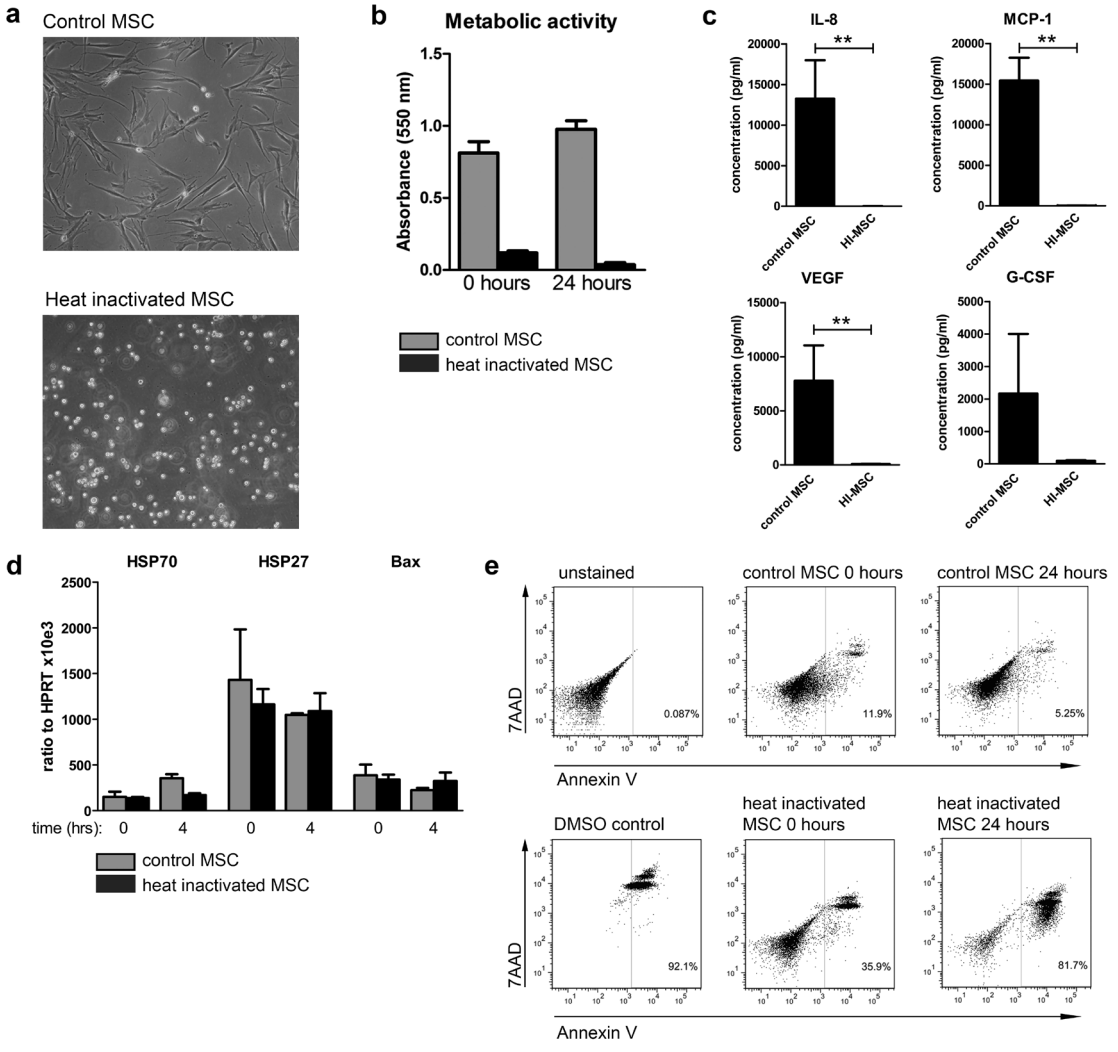
Statistical analysis

Data were analyzed using Prism software v5.04 (GraphPad Software, Inc., La Jolla, CA). Unpaired two-tailed t-tests were performed unless otherwise stated. P values were indicated as * for $P < 0.05$; **P for <0.01; and *** for $P < 0.001$. Two-tailed P values are stated.

Results

Heat inactivation of MSC

Human MSC were isolated from subcutaneous adipose tissue. To study the contribution of MSC-immune cell cross talk to the immunomodulatory effects of MSC, we generated inactivated MSC by heating human MSC for 30 min to 50°C. HI-MSC lost their capacity to adhere to plastic, whereas the majority of control MSC attached to plastic within 24 h after seeding (Figure 1A). To determine the metabolic activity of MSC after heat inactivation, the ability of cells to reduce MTT to formazan was measured. Twenty-four hours after heat inactivation, the metabolic activity of HI-MSC was not detectable (Figure 1B). The ability of MSC to secrete cytokines and growth factors was determined in conditioned medium of MSC and HI-MSC cultured for 24 h. Although control MSC secreted IL-8, MCP-1, VEGF, G-CSF, and very low levels of IL-10, as well as various other cytokines, inactivated MSC were incapable of secreting these cytokines (Figure 1C and data not shown). To examine whether heat inactivation induced cellular stress and apoptosis, we measured mRNA expression of heat shock proteins Hsp27 and Hsp70 and proapoptotic Bax immediately and 4 h after heat exposure. There were no significant differences in expression of these genes between control MSC and HI-MSC (Figure 1D), suggesting that HI-MSC are unable to respond to environmental stimuli. Moreover, staining with the apoptosis marker Annexin V and viability dye 7-AAD demonstrated that there was only minor induction of apoptosis in HI-MSC and the membrane integrity of majority of HI-MSC was intact as most of the cells were negative for Annexin V and 7-AAD, whereas DMSO-incubated MSC were 92% positive for Annexin V and 7-AAD (Figure 1E). After 24 h, the majority of HI-MSC became positive for Annexin V and 7-AAD (Figure 1E). FACS analysis of MSC surface markers CD13, CD73, CD90, and CD105 at 0 and 24 h after heat inactivation showed no difference between control and HI-MSC, indicating that the immunophenotype of MSC was preserved after heat inactivation (Figure 1F). All used MSC cultures were negative for pan leukocyte marker CD45 and


a
Control MSC
Heat inactivated MSC
b
Metabolic activity
Absorbance (550 nm)
1.5
1.0
0.5
0.0
0 hours
24 hours
control MSC
heat inactivated MSC
c
IL-8
MCP-1
VEGF
G-CSF
concentration (pg/ml)
control MSC
HI-MSC
**
d
HSP70
HSP27
Bax
ratio to HPRT x10e3
time (hrs): 0 4 0 4 0 4
control MSC
heat inactivated MSC
e
unstained
control MSC 0 hours
control MSC 24 hours
DMSO control
heat inactivated MSC 0 hours
heat inactivated MSC 24 hours
7AAD
Annexin V
0.087%
11.9%
5.25%
92.1%
35.9%
81.7%
f
0 hours after heat inactivation
24 hours after heat inactivation
CD45
0.9%
CD13
99%
96%
CD73
100%
96%
CD31
0.5%
CD90
94%
87%
CD105
99%
96%
CD13
99%
95%
CD73
99%
95%
CD90
90%
68%
CD105
99%
94%

endothelial marker CD31 (Figure 1F). These results demonstrate that heating of MSC to 50°C generates MSC that lost metabolic, proliferative, and secretory activity, but maintained cellular integrity.

MSC do not recover from heat inactivation

To determine whether the effects of heat inactivation were reversible, human MSC were heat inactivated and cultured for 7 days. HI-MSC did not recover their ability to attach to plastic within 7 days of culture (Figure 2A). Moreover, the majority (96.7%) of HI-MSC became positive for Annexin V and 7-AAD (Figure 2b) and lacked the metabolic activity 7 days after heat inactivation (Figure 2C). To determine the ability of MSC to proliferate, control MSC and HI-MSC were labeled with PKH26 and cultured for 7 days. FACS analysis showed dilution of PKH26 dye, indicating proliferation of control MSC, whereas HI-MSC lost the ability to proliferate (Figure 2D). Finally, FACS analysis demonstrated that HI-MSC maintained MSC marker expression on their cell surface after 7 days of culture (Figure 2E). Thus, MSC do not recover from heat inactivation and HI-MSC provide a useful tool for studying the mechanisms of immunomodulation by MSC.

←

Figure 1. Heat inactivation abolishes human MSC proliferation, metabolic activity, and cytokine secretion, but preserves MSC integrity and immunophenotype.
(A) Plastic adherence of control and HI-MSC 24 h after seeding. (B) Metabolic activity of control and HI-MSC was measured 0 and 24 h after heating by the ability of MSC to reduce MTT to formazan. Experiments were performed with MSC of seven donors; bars indicate mean – SEM. (C) IL-8, MCP-1, VEGF, and G-CSF secretion by control and HI-MSC after 24-h culture measured by multiplex assay. Experiments were performed with MSC of five donors; bars indicate mean – SEM. (d) Gene expression of heat shock proteins 70 and 27 and apoptotic activator Bax in control and HI-MSC 0 and 4 h after heating depicted as ratio to HPRT. Bars indicate mean – SEM. (E) Representative FACS plots depicting Annexin V and 7-AAD staining of control and HI-MSC directly and 24 h after heating. DMSO incubation (5min) was used as a positive control. (F) FACS plots of cell surface markers on control MSC (solid line and percentage top line) and HI-MSC (dotted line and percentage bottom line) compared to the unstained control (gray) directly and 24 h after heat incubation. FACS experiments were performed three times with MSC from different donors each time. 7-AAD, 7-amino-actinomycin D; G-CSF, granulocyte colony-stimulating factor; HI-MSC, heat-inactivated mesenchymal stem cells; IL, interleukin; MCP-1, monocyte chemotactic protein-1; VEGF, vascular endothelial growth factor. P values were indicated as * for $P < 0.05$; **P for < 0.01; and *** for $P < 0.001$.

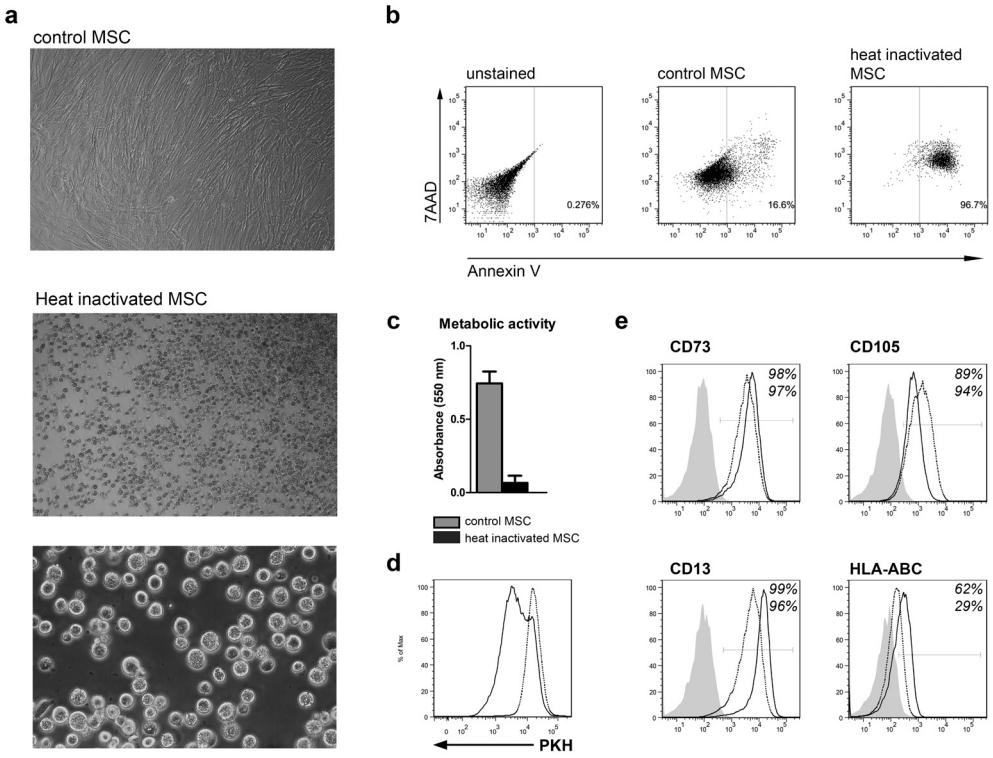


Figure 2. Heat inactivation-induced changes in MSC are irreversible. MSC were heat inactivated for 30 min at 50°C and cultured for 7 days. (A) Plastic adherence ability of control and HI-MSC after 7 days of culture 7. (B) Viability of control and HI-MSC after 7 days measured by Annexin V and 7-AAD staining. (C) Metabolic activity of control and HI-MSC at 7 day measured by the ability of MSC to reduce MTT to formazan. Bars indicate mean ± SEM. Experiments were performed with MSC from four different donors. (D) Proliferation of HI-MSC (dotted line and percentage bottom line) and control MSC (solid line and percentage top line) was assessed at day 7 by PKH26 label dilution. (E) Representative FACS plots of MSC surface markers on HI-MSC (dotted line) and control MSC (solid line) compared to the unstained control (gray) on day 7 after heat incubation. FACS experiments were performed three times with MSC from different donors each time.

Control and HI-MSC show the same biodistribution and persistence after intravenous infusion and do not migrate to sites of inflammation

The biodistribution and persistence of IV-infused control and HI-MSC was examined in healthy C57BL/6 mice. To avoid xenoreactivity, these studies were performed with syngeneic MSC. C57BL/6 adipose tissue MSC were labeled with fluorescent Qtracker 605 beads (control MSC) or Qtracker 655 beads before heat inactivation (HI-MSC). The beads were readily taken up by MSC and remained present in control MSC for at least 24 h (Figure 3A). HI-MSC stayed intact and maintained the beads for at least 24 h as well (Figure 3A). Control and HI-MSC were mixed at a 1:1 ratio and a total of 0.3x106 cells was IV injected in healthy C57BL/6 mice and mice imaged by CryoViz. Two hours after MSC infusion, the majority of control MSC were found in the lungs (Figure 3B and Supplementary Video S1). After 24 h, there was a >99% reduction in the number of MSC detected (Table 1). Interestingly, HI-MSC showed the same distribution pattern as controlMSC (Figure 3B and Table 1). After 24 h, >99%of HI-MSC was undetectable. To examine whether inflammatory tissue injury would provide a trigger for MSC migration, unilateral kidney IRI was induced in C57BL/6 mice. Gene expression analysis in healthy and injured kidney tissue showed that expression of KIM-1 and MCP-1 was highly upregulated in the IRI kidney, confirming the injury and inflammatory state of the kidney (Figure 3C). In accordance with this, NGAL, a marker for kidney injury, was increased in the serum of mice with kidney injury compared to healthy controls (Figure 3D). One hour after induction of IRI, mice were infused with 0.15x106 labeled control MSC mixed with 0.15x106 HI-MSC. Imaging showed that the distribution of control MSC and HI-MSC was the same as in control mice; there was no recruitment of either control or HI-MSC to the injured kidney after 2 h (Figure 3E, Table 1, and Supplementary Video S2). After 24 h, the majority of control and

TABLE 1. HEAT-INACTIVATED MESENCHYMAL STEM CELLS SHOW SIMILAR MIGRATION PROPERTIES AS CONTROL MESENCHYMAL STEM CELLS

	control MSC				*HI-MSC*			
Time point - Treatment	*Injected*	*Recovered total*	*Injured kidney*	*Healthy kidney*	*Injected*	*Recovered total*	*Injured kidney*	*Healthy kidney*
2 hours - control	150,000	47,186	-	52	150,000	82,082	-	10
24 hours - control	150,000	210	-	0	150,000	959	-	0
2 hours - kidney injury	150,000	36,801	126	129	150,000	137,723	17	13
24 hours - kidney injury	150,000	3134	11	17	150,000	11,32	0	2

Number of detected MSC recovered in whole animals and in the kidneys 2 and 24 hours after infusion of 150,000 control MSC and 150,000 HI-MSC in healthy animals and in animals with IRI in the left kidney.
HI-MSC: Heat-inactivated mesenchymal stem cells; IRI: ischemia/reperfusion injury

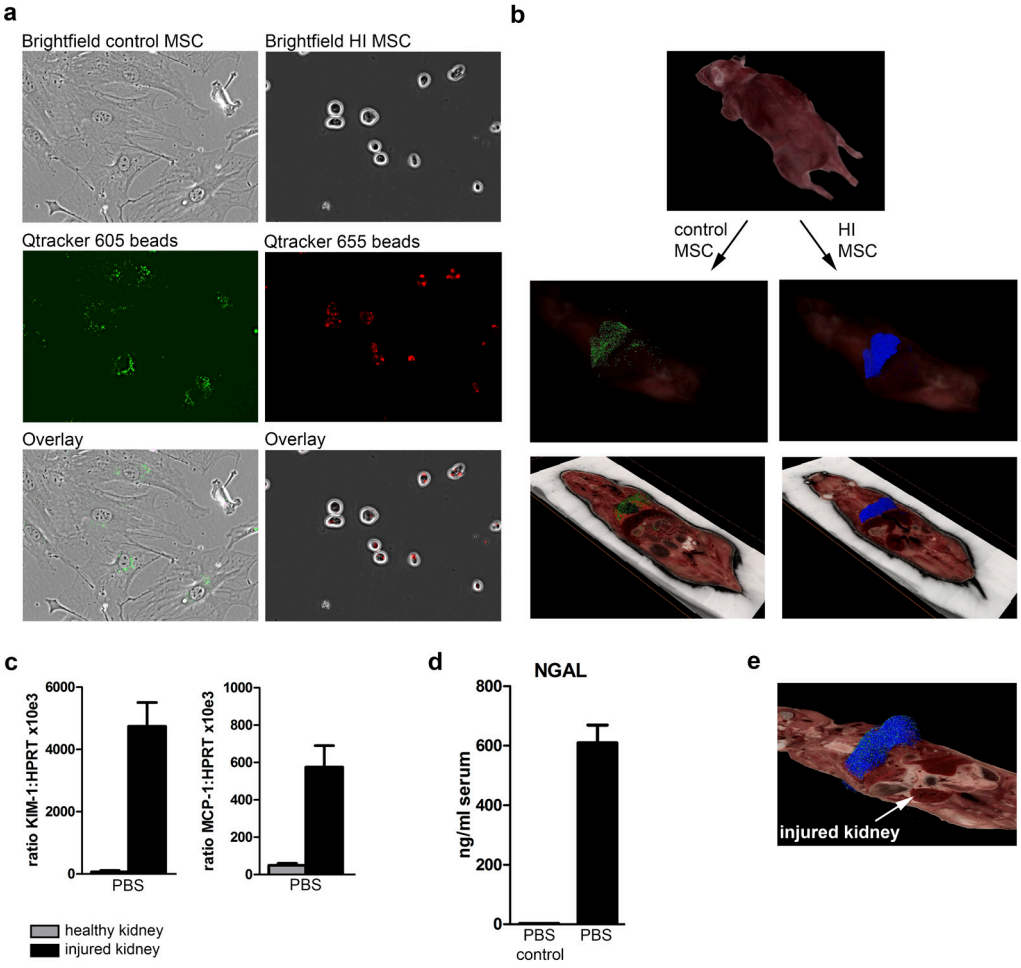


Figure 3. Control MSC and HI-MSC distribute in the same way after infusion and do not migrate to distant sites of inflammation.

MSC were labeled with fluorescent Qtracker605 beads (control MSC) or Qtracker655 beads before heat inactivation (HI-MSC) and IV infused in healthy C57BL/6 mice. (A) Beads remained visible in MSC and HI-MSC after culturing for 24 h. (B) Visualization of the distribution pattern of controlMSC(left) and HI-MSC (right) 2 h after infusion by CryoViz imaging. (C) Gene expression of KIM-1 and inflammatory MCP-1 in healthy and injured kidneys depicted as ratio to HPRT. (d) NGAL levels were measured with ELISA in the serum of LPS or PBS-treated mice. Bars indicate mean ± SEM. (e) CryoViz imaging of control MSC (green) and HI-MSC (blue) in a kidney IRI model 2 h after infusion, demonstrating the majority of MSC in the lungs. IRI, ischemia/reperfusion injury; IV, intravenously; LPS, lipopolysaccharides; KIM-1, kidney injury molecule-1; PBS, phosphate-buffered saline.

HI-MSC was no longer detectable and there was no recruitment to the injured kidney. Control MSC numbers in the healthy and injured kidney were 17 and 11, respectively (Table 1). These data indicate that administered MSC do not actively migrate to injured kidney and there is no difference in the persistence of control MSC and HI-MSC after intravenous infusion.

Control and HI-MSC induce similar immunomodulatory effects after infusion in healthy mice

As described previously, MSC induce an immunomodulatory response after IV infusion in healthy mice that can be measured both locally in the lungs and systematically in the serum [34]. To investigate whether this response is dependent on the viability of MSC, we infused 300,000 syngeneic control MSC or HI-MSC or PBS as a control into the tail vein of healthy. C57BL/6 mice Control MSC induced upregulated gene expression of pro-inflammatory MCP-1, MIP1α, and IL-1b and anti-inflammatory IL-10 and TGF-β in lung tissue (Figure 4A). Furthermore, control MSC increased serum levels of G-CSF, CXCL1, CXCL5, MCP-1, IL-6, and IL-10 (Figure 4B). Interestingly, HI-MSC induced very similar changes in circulating cytokine levels and cytokine gene expression in the lung (Figure 4A, B). IFN-γ was not detected in serum of mice treated with control MSC or HI-MSC (data not shown). These data suggest that the immune response observed after MSC infusion does not depend on the active immunomodulatory activity of MSC, but is derived from other cells that are merely triggered by the presence of exogenously administered MSC.

HI-MSC dampen inflammation in an LPS-induced sepsis model

To investigate whether HI-MSC possess some of the anti-inflammatory properties that have been reported for control MSC, C57BL/6 mice were given 2.5mg/kg LPS to induce nonlethal sepsis, followed by infusion of 300,000 control MSC or HI-MSC after 1 h. LPS induced a strong increase in serum IFN-γ levels (Figure 5). After treatment with control MSC, IFN-γ was significantly decreased. MSC also triggered a 18.4-fold increase in serum levels of IL-10 with an average of 14,000 pg/mL. TNF-α levels were threefold increased after MSC treatment. Interestingly, HI-MSC modulated the LPS-induced immune response in a similar manner as control MSC; infusion of HI-MSC significantly decreased levels of IFN-γ and increased IL-10 and TNF-α (Figure 5). Thus, without being able to respond to inflammatory stimulation and secrete anti-inflammatory factors, HI-MSC modulate LPS-induced immune responses in a similar way as control MSC.

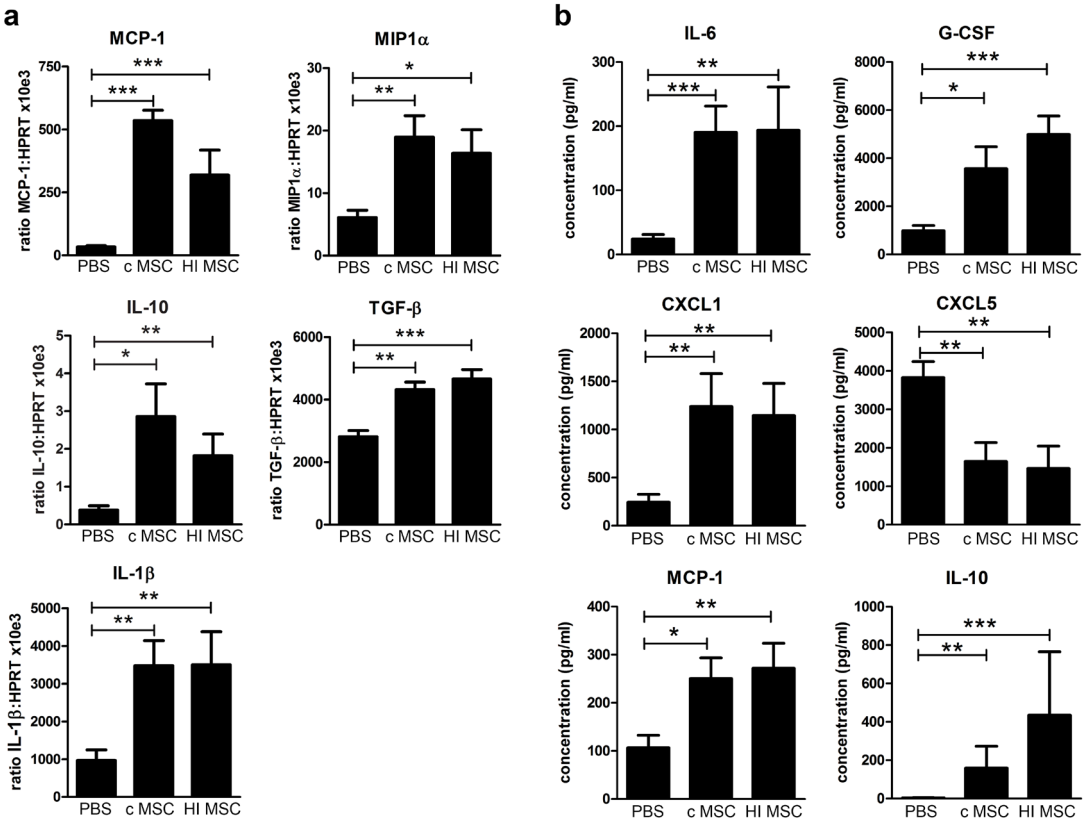


Figure 4. Control and HI-MSC induce the same immunomodulatory effect after infusion in healthy mice.

Control MSC (0.3x106 cells), HI-MSC (0.3x106 cells), or PBS was infused IV in healthy C57BL/6 mice (n = 15, n = 10, and n = 13 mice, respectively). Animals were sacrificed 2 h after infusion. (A) Gene expression of MCP-1, MIP1α, IL-10, TGF-b, and IL-1b in the lungs depicted as a ratio to HPRT. (B) Serum levels of IL-6, G-CSF, CXCL1, CXCL5, MCP-1, and IL-10 were determined with Multiplex assay. Bars indicate mean – SEM. MIP1a, macrophage inflammatory protein-1a; TGF-b, transforming growth factor beta. P values were indicated as * for $P < 0.05$; **P for < 0.01; and *** for $P < 0.001$.

HI-MSC do not inhibit T-cell proliferation

To determine how HI-MSC modulate immune responses, we examined the interaction between HI-MSC and different immune cell subsets in vitro. Traditionally, MSC have been demonstrated to have potent inhibitory effects on T-cell proliferation. Thereto, the effect of HI-MSC on T-cell proliferation was examined in mixed lymphocyte reactions. In the absence of MSC, a strong proliferative activity of allogeneic stimulated T cells was measured (Figure 6A). Coculture with third-party MSC inhibited T-cell proliferation in a dose-dependent manner. In contrast, HI-MSC did not inhibit T-cell proliferation (Figure 6A, B). At a ratio of 1:2.5, control MSC inhibited T-cell proliferation by 36.7% (±SD 14.1), whereas HI-MSC even stimulated T-cell proliferation (-5.5% inhibition, ±SD 12.3) (Supplementary Table 1). These data indicate that HI-MSC are not able to suppress T-cell proliferation.

HI-MSC do not induce regulatory B-cell formation

To examine whether HI-MSC are able to induce formation of IL-10-producing transitional B cells, as previously demonstrated for control MSC [39], control and HI-MSC were cocultured with quiescent B cells obtained from human splenocytes.

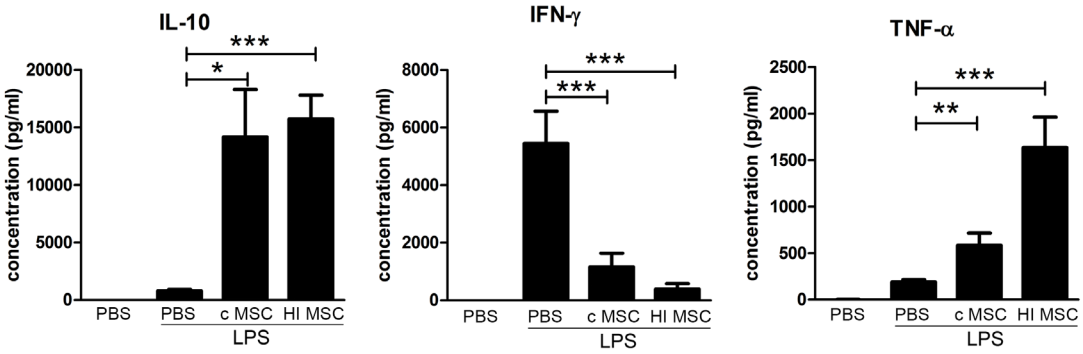


Figure 5 HI-MSC dampen inflammation in an LPS-induced sepsis model.
C57BL/6 mice received 2.5 mg/kg LPS 1 h before treatment with control MSC (0.3 · 106 cells), HI-MSC (0.3 · 106 cells), or PBS (n = 12, n = 9, and n = 11 mice, respectively). Control animals (n = 4) did not receive LPS. Animals were sacrificed 6 h after infusion of LPS. Levels of IFN-γ, IL-10, and TNF-α were determined by Multiplex assay. Bars indicate mean – SEM. IFN-g, interferon gamma; TNF-α, tumor necrosis factor alpha. P values were indicated as * for P < 0.05; **P for < 0.01; and *** for P <0.001.

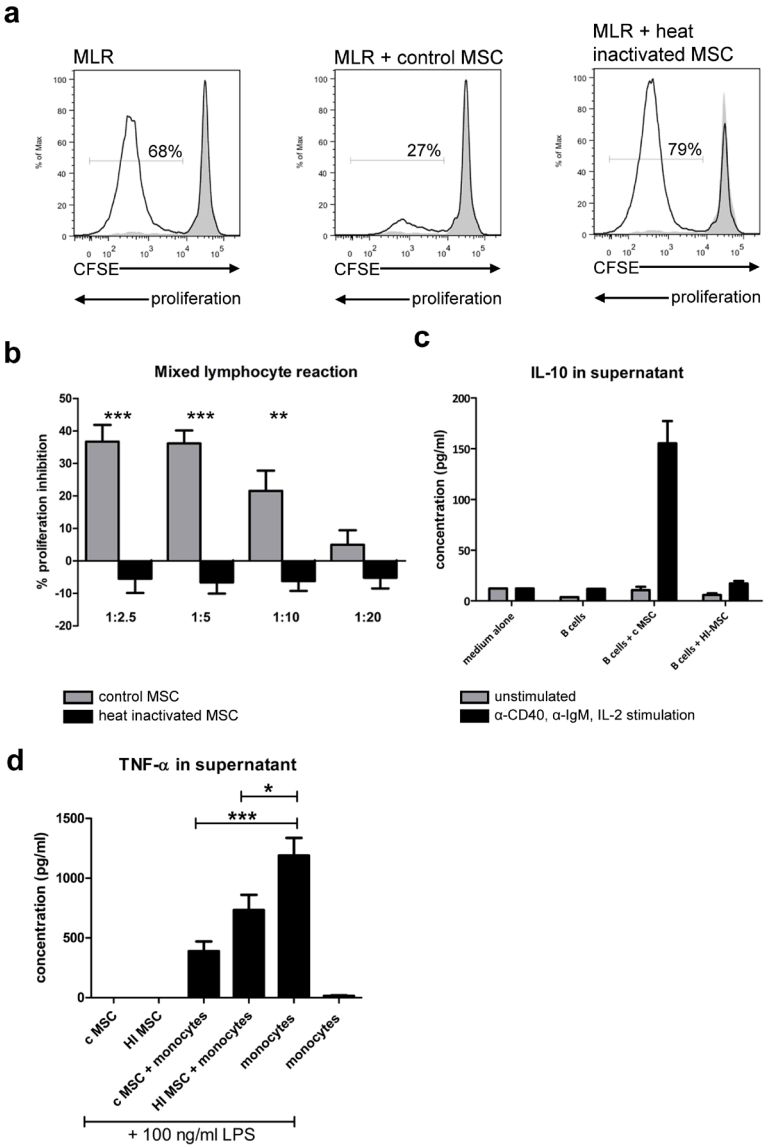


Figure 6 HI-MSC modulate monocyte function.
(A) T-cell proliferation was assessed through measurement of CFSE label dilution in an MLR with or without control MSC or HI-MSC at a 1:2.5 ratio. Representative histograms shown. Solid histograms represent unstimulated T cells. (B) Average inhibition of T-cell proliferation by control and HI-MSC in an MLR of 4 different experiments. Bars indicate mean – SEM. (C) Effect of control MSC and HI-MSC on the induction of IL-10-producing B cells. B cells were stimulated with anti-IgM, anti-CD40, and IL-2 and MSC added at a 1:5 ratio. IL-10 levels in supernatants were measured by ELISA. Bars indicate mean – SEM. (d) Effect of control MSC and HI-MSC on CD14+ monocytes. MSC were cocultured with CD14+ monocytes at a 1:1 ratio and after 24 h, 100 ng/mL LPS was added. TNF-a levels were measured by ELISA. Bars indicate mean – SEM. CFSE, carboxyfluorescein succinimidyl ester; MLR, mixed lymphocyte reaction. P values were indicated as * for $P < 0.05$; **P for < 0.01; and *** for $P < 0.001$.

B cells were activated by anti-IgM, anti-CD40 agonistic antibody, and IL-2. In contrast to control MSC, HI-MSC did not induce IL-10-producing regulatory B cells (Figure 6C).

HI-MSC modulate monocyte function

To determine whether the observed immunomodulatory effects of HI-MSC were mediated by monocytes, CD14+ monocytes were isolated from PBMC. Monocytes were cocultured with control and HI-MSC for 18 h. After 18 h, LPS was added to stimulate TNF-α secretion by monocytes. Control MSC significantly decreased LPS-induced TNF-α production by monocytes (Figure 6D). Interestingly, monocytes cocultured with HI-MSC also produced significantly less TNF-α in response to LPS (Figure 6D). These results demonstrate that HI-MSC can modulate monocyte function and indicate that in vivo immunomodulating effects of HI-MSC may be mediated by monocytes.

Discussion

MSC are widely studied as a potential treatment option for a range of immune disorders. However, surprisingly, little is known about the mechanisms of immunomodulation by MSC after infusion. It is generally considered that the in vitro immunomodulatory effects of MSC translate to their effects after in vivo administration and MSC thus play an active role in immunomodulatory processes by responding to inflammatory challenge with the production of anti-inflammatory factors. In this study, we demonstrate that MSC that are unable to respond to inflammatory stimulation or secrete anti-inflammatory factors are effective in vivo immune modulators.

One of the controversies in the field of MSC is the effects mediated by secreted molecules versus those mediated by cell membrane contact. Secreted molecules can easily be studied using a transwell system and then contact-dependent effects are inferred. However, directly demonstrating the effects of membrane contact, separate from secreted molecules, has not been possible. We have developed a system to specifically assess the role of the MSC surface membrane. By heat inactivating MSC, the cells have ceased normal function, but the plasma membrane remains intact. Hence, the cell has become a "bag" of cytoplasm. This model affords the opportunity to specifically investigate the role of MSC membrane contact without the possibility of confounding effects due to secreted

molecules. While HI-MSC are on a course to overt cell death, they remain intact during the time frame of our assays, validating our experimental model to assess the role of the membrane.

Up to now, the disease-modulating activity of MSC was credited primarily to the secretion of anti-inflammatory factors. In vitro lymphocyte proliferation assays in transwell culture systems or with MSC-conditioned medium demonstrate that the suppression of T-cell proliferation is to a large extent dependent on soluble factors [23,26,40]. Moreover, the MSC conditioned medium has been shown to enhance ischemic cardiomyocyte recovery in vitro and limit infarct size in rat hearts [41], and offers protection against acute kidney injury [42]. Our data confirm that the ability of MSC to respond to inflammatory stimulation and secrete anti-inflammatory factors is instrumental for the suppression of T-cell proliferation and induction of regulatory B cells in vitro. Our data, however, also demonstrate that the in vivo immunomodulatory effects of MSC depend on very different mechanisms. HI-MSC were equally efficient as control MSC in modulating the LPS induced inflammatory response. This demonstrates that the observed immunomodulatory effects of MSC were independent of soluble factors. Furthermore, it demonstrates that MSC do not have to be able to respond to environmental challenges to mediate their effects. In contrast, it suggests that other cells can obtain immunomodulatory properties merely by encounter with MSC.

This study contributes to understanding the in vivo immunomodulatory effect of MSC by suggesting that MSC act as a fast trigger for immunomodulation, which is subsequently carried on by other cells. Other groups have already indicated that macrophages may play a role in the immunomodulatory effect of MSC. Phagocytosis of dead MSC by macrophages has been demonstrated to induce an immunosuppressive phenotype [43]. Nemeth et al. have shown that the therapeutic effects of MSC in a sepsis model depend on reprogramming of macrophages to release lower amounts of TNF-α and increased amounts of IL-10 by MSC-produced PGE2 [44]. Our data demonstrated that control as well as inactivated MSC dramatically increased systemic IL-10 levels in LPS-induced sepsis mice. In coculture experiments, control MSC did not induce IL-10 production by LPS activated monocytes, whereas inactivated MSC marginally increased IL-10 production (data not shown). In this setup, however, TNF-levels were significantly decreased, suggesting that monocytes are able to adapt their function in response to inert MSC and may carry on some of the immunosuppressive effects of MSC after infusion.

A recurring matter of concern in the field of MSC therapy is the short half-life of MSC after infusion [31,45]. Furthermore, there is debate about the ability and necessity of MSC to migrate to sites of inflammation. In this study, we investigated the persistence and distribution of MSC after infusion by CryoViz imaging of whole mice and compared it with HI-MSC. We found no difference in the distribution of HI-MSC and control MSC in mice with unilateral kidney IRI, indicating that MSC are distributed by passive mechanisms. Less than 10% of the administered control or HI-MSC were detected 24 h after administration. As the labeling beads can only be detected by the CryoViz imaging system when they are concentrated in the MSC, the loss of signal indicates that MSC either fell apart or were phagocytosed by host cells. In conclusion, we show that HI-MSC induce immunomodulatory responses in vivo. These responses are similar to those induced by control MSC. This indicates that at least part of the immune modulatory response induced by MSC is independent on activation of MSC by inflammatory challenge and subsequent production of anti-inflammatory factors. Instead, passive interactions with host cells, potentially monocytes, are likely to mediate these effects. This has implications for the development of MSC immune therapy. First, it suggests that MSC surface phenotype is determinative of the clinical effect of MSC. Second, the possibility to use inactivated cells could reduce recurring concerns about the stability of therapeutic MSC. Finally, understanding the immunomodulatory mechanisms of MSC provides tools for the development of effective MSC immune therapy by allowing the induction of key properties of MSC to generate optimal effective cells.

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Chapter 6

Immunomodulation Induced by Mesenchymal Stem Cells (MSC) is Triggered through Phagocytosis of MSC by Innate Immune Cells

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Abstract

Mesenchymal stem or stromal cells (MSC) are under investigation as a potential immunotherapy. MSC are usually administered via intravenous infusion, after which they are trapped in the lungs and die and disappear within a day. The fate of MSC after their disappearance from the lungs is unknown and it is unclear how MSC realize their immunomodulatory effects in their short lifespan. We examined immunological mechanisms determining the fate of infused MSC and the immunomodulatory response associated with it. Tracking viable and dead human umbilical cord MSC (ucMSC) in mice using Qtracker beads (contained in viable cells) and Hoechst33342 (staining all cells) revealed that viable ucMSC were present in the lungs immediately after infusion. Twenty-four hours later, the majority of ucMSC were dead and found in the lungs and liver where they were contained in monocytic cells of predominantly non-classical Ly6Clow phenotype. Monocytes containing ucMSC were also detected systemically. In vitro experiments confirmed that human CD14++/CD16- classical monocytes polarized toward a non-classical CD14++CD16+CD206+ phenotype after phagocytosis of ucMSC and expressed programmed death ligand-1 and IL-10, while TNF-α was reduced. ucMSC-primed monocytes induced Foxp3+ regulatory T cell formation in mixed lymphocyte reactions. These results demonstrate that infused MSC are rapidly phagocytosed by monocytes, which subsequently migrate from the lungs to other body sites. Phagocytosis of ucMSC induces phenotypical and functional changes in monocytes, which subsequently modulate cells of the adaptive immune system. It can be concluded that monocytes play a crucial role in mediating, distributing, and transferring the immunomodulatory effect of MSC.

Introduction

Mesenchymal stem or stromal cells (MSC) are currently being investigated in various animal models [1-7] and clinical trials [8-13] for their immunotherapeutic potential. Around 700 clinical trials with MSC were registered with clinicaltrials.gov in early 2017. The in vitro immunomodulatory properties of MSC are well documented, but their mechanism of action after administration is largely unknown [14]. Administration of MSC is most commonly performed via intravenous infusion, after which they are known to end up in the micro-vasculature of the lungs from where the majority are lost within 24 hours [15]. The assumed short survival of

MSC does not appear to interfere with their effectiveness, as beneficial effects of MSC are seen in a variety of settings long after the cells have been cleared [12, 16-21]. Yet, how MSC modulate the host immune system during their short lifespan is still unclear.

Recently, we observed that inactivation of MSC in which their immunophenotype remained intact while their secretome and active crosstalk with immune cells was disabled, retained the cells' immunomodulatory capacity in a lipopolysaccharide sepsis model [22]. In this model, the therapeutic effect of MSC appears to be independent of their cellular activity and depends on a mechanism potentially involving recognition and phagocytosis of MSC by monocytic cells [22, 23].
Monocytes can induce long-term adaptive immune responses upon differentiation into macrophages; moreover, in vitro studies have shown that MSC stimulate monocytes to adapt an anti-inflammatory IL-10 producing phenotype [24, 25]. In addition, we have recently shown that membrane particles that were generated from MSC are able to modulate the immune response by targeting pro-inflammatory monocytes and inducing apoptosis [26]. Furthermore, intravenous administration of MSC has been shown to lead to the induction of regulatory monocytes that are capable of suppressing allo- and autoimmune responses independently of regulatory T cells (Tregs) [27].
In the present study, we elucidated the fate of infused MSC and their immunomodulatory effects after administration and demonstrated that infused MSC are rapidly cleared through phagocytosis by monocytes. This results in the polarization of monocytes toward an immunosuppressive phenotype, which then impacts on adaptive immune cells. Moreover, MSC-activated monocytes relocate via the systemic route to other body sites, in particular to the liver, thereby distributing their adapted immune status. This suggests that at least part of the immunomodulatory response seen after infusion of MSC is independent of the cellular activity of MSC.

Methods

Culture expansion of ucMSC

Human umbilical cord tissue was collected from Caesarean section deliveries by Tissue Solutions Ltd. (Glasgow, U.K.) from healthy donors without known active viral infections. All cord tissue was obtained according to the legal and

ethical requirements of the country of collection, with the approval of an ethics committee (or similar body) and with anonymous consent from the donor. Isolation of CD362+ ucMSC was performed as previously described by de Witte et al. [28, 29]. After isolation, cells were counted, seeded for expansion and cryopreserved at passage 2 for shipment to Erasmus Medical Center. Here, ucMSC were cultured in minimum essential medium Eagle alpha modification (Sigma-Aldrich, St Louis, MO) containing 2 mM l-glutamine (Lonza, Verviers, Belgium), 1% penicillin/streptomycin solution (P/S; 100 IU/ml penicillin, 100 IU/ml streptomycin; Lonza) and supplemented with 15% fetal bovine serum (FBS; Lonza) and 1 ng/ml basic fibroblast growth factor (Sigma-Aldrich) and kept at 37°C, 5% CO2 and air O2. The medium was refreshed once a week and ucMSC were passaged using 0.05% trypsin-EDTA (Life technologies, Paisley, U.K.) at ~80%–90% confluence. UcMSC were used in experiments between passage 3 and 6.

Generation of Conditioned Medium

For the generation of conditioned medium from ucMSC, 100,000 ucMSC were seeded per 6 wells plate well in 2 ml of standard culture medium. Medium was refreshed the following day. UcMSC were cultured for 3 days in the same medium, whereafter medium was collected and centrifuged for 10 minutes at 3,000 RPM to remove cell debris and stored at −80°C until further use.

Labeling ucMSC with Qtracker 605 beads, Hoechst33342 and PKH26

For in vivo tracking experiments of viable and dead cells using CryoViz imaging, ucMSC were dual labeled with Qtracker 605 beads (Life technologies) and Hoechst33342 (ThermoFisher, Bleiswijk, The Netherlands) as these labels were properly detected by the available detectors. UcMSC were labeled with Qtracker 605 beads according the manufacturer's instructions. Qtracker beads are actively taken up and contained within viable cells, while they disperse when cells die (Supporting Information Figure S1). After labeling, ucMSC were thoroughly washed to remove any beads that were not internalized. Subsequently, ucMSC were incubated with Hoechst33342 (1 μg/ml), which binds to DNA and remains bound even after cells die. For monocyte phagocytosis experiments, ucMSC were labeled with the membrane dye PKH26 (PKH26 Red Fluorescent Cell Linker Kit, Sigma-Aldrich, Zwijndrecht, The Netherlands) according to the manufacturer's instructions.

Mice

Healthy male C57BL/6 mice (8 weeks old) were purchased from Charles River, (Freiburg, Germany). The mice had free access to food and water and were kept at a 12-hour light-dark cycle. Animal housing conditions and all procedures were carried out in strict accordance with current EU legislation on animal experimentation. All procedures were approved by the Institutional Committee for Animal Research (protocol EMC No. 127-12-14).

Cell tracking by CryoViz imaging

Healthy male C57BL/6 mice were infused with ucMSC [150,000 ucMSC/200 µl phosphate buffered saline (PBS)] that were dual labeled with Qtracker 605 beads and Hoechst33342 via tail vein injections. Five minutes, 24 and 72 hours after ucMSC infusion, the mice were euthanized with carbon dioxide. Subsequently, whole mice were embedded in mounting medium for Cryotomy (O.C.T. compound, VWR Chemical, Amsterdam, The Netherlands), frozen in liquid nitrogen and stored at −80°C until shipment to BioInVision, OH, for imaging. At BioInVision 3D anatomical and molecular fluorescence videos were generated with CryoViz technology. The signals of Qtracker 605 beads and Hoechst33342are spectrally separated from each other. Hence, a combination of hardware (optical filters) and software (machine learning based cell detector) was used to differentiate between them. UcMSC positive for Qtracker605 beads were detected by the fluorescent signal that arises from clustered beads present in viable cells. Non-viable ucMSC are not capable of containing the beads intracellular and as a consequence the beads will disperse and the signal may no longer be picked up. Hoechst33342, in contrast, is present in viable and dead cells, but its signal is not detected in live ucMSC as the Qtracker605 signal outshines the Hoechst33342 signal. As a result, the Hoechst33342 signal is detected only in dead ucMSC. Cell counts for Qtracker 605 positive cells (live ucMSC) and Hoechst33342 positive cells (dead ucMSC) were quantified using imaging algorithms by BioInVision Inc.

Detection of ucMSC phagocytosis by monocytes in vivo

The mice were infused via the tail vein with PKH26-labeled ucMSC (150,000 ucMSC/200 ul PBS). Twentyfour hours after the ucMSC infusion, the mice were sacrificed by cervical dislocation and the lungs, blood, and liver were harvested. The lungs and livers were digested by collagenase type IV (0.5 mg/ml, Life Technologies, Paisley, U.K.) for 30 minutes at 37°C to obtain a single cell suspension.

Red blood cells were lysed with red blood cell lysis buffer (ThermoFisher) and the cells suspensions were then washed with FACS buffer (PBS ± 0.1% BSA +0.1% sodium azide). Single cell suspensions of lung tissue and heparinized whole blood (100 µl) were stained for CD11b-APC, Ly6C-Bv450BD (both BD Biosciences, San Jose, CA), CD45-Pe-Cy7, CX3CR1-PERCPCy5.5 (all Biolegend) and lung cells were stained in addition for CD68-PE (Biolegend) for 30 minutes at 4°C. The blood samples were subsequently lysed for 10 minutes with Lyse/Fix buffer (BD Biosciences) and washed twice with FACS buffer. Liver samples were stained for CD11b-APC, Ly6C-Bv450, CD45-Pe-Cy7, and CLEC4F-PE (kindly provided by Xifeng Yang, Biolegend) for 30 minutes at 4°C. Samples were then washed with FACS buffer and measured on a FACSCanto II flow cytometer.

Detection of phagocytosis of ucMSC by human immune cells

Human peripheral blood samples were collected from healthy volunteers. 50,000 PKH26-labeled ucMSC were added to 200 µl whole blood for 1, 4, and 24 hours in polypropylene tubes at 37°C, 5% CO2 and air O2. In addition, peripheral blood mononuclear cells (PBMC) were isolated from blood by density gradient centrifugation using Ficoll-Paque (GE healthcare). Monocytes were isolated from PBMC via the positive selection of CD14+ cells by MACS using CD14 microbeads (Miltenyi, Bergisch Gladbach, Germany), according to the manufacturer's recommendations. Subsequently, 200,000 monocytes were co-cultured with 50,000 PKH26-labeled ucMSC for 1, 4, and 24 hours in polypropylene tubes in RPMI medium supplemented with 2 mM l-glutamine, 1% P/S and 10% heat-inactivated FBS at 37°C, 5% CO2 and air O2.

Whole-blood or isolated monocytes incubated with ucMSC were stained for CD14-Pacific Blue (BD Biosciences), CD15-FITC (BD Biosciences) and CD45-APC (BD Biosciences) or CD14-Pacific Blue (BD Biosciences), CD16-FITC (Bio-Rad, The Netherlands), CD90-APC (BD Biosciences), HLA-DR-Amcyan (BD Biosciences), PD-L1-PeCy7 (BD Biosciences), CD206-Pacific Blue (BD Biosciences), CD163-FITC (Bio-rad antibodies), and Via-Probe (BD Biosciences) respectively, for 30 minutes at 4°C. Whole-blood samples were then fixed and red blood cells lysed for 10 minutes at 4°C with BD FACS Lysing solution (BD Biosciences). Samples were washed and measured on a FACSCanto II flow cytometer with FACSDiva software (BD Biosciences).

Detection of Monocyte Phenotype Shift due to Phagocytosis ucMSC or Cytokines Secreted By ucMSC

CD14+ selected monocytes were cultured in 50% ucMSC conditioned medium or co-cultured with ucMSC at a 4:1 ratio in standard culture medium for 24 hours. Subsequently, samples were stained for CD45-APC, CD14-Pacific Blue and CD16-FITC or CD90-APC (BD Biosciences), PD-L1-PeCy7, CD206-Pacific Blue and CD163-FITC, for 30 minutes at 4°C. Samples were washed and measured on a FACSCanto II flow cytometer with FACSDiva software (BD Biosciences).

Confocal microscopy imaging of ucMSC phagocytosis by monocytes

Monocytes were isolated from PBMC via positive selection of CD14+ cells as described above and labeled with PKH67 (PKH67 Green Fluorescent Cell Linker Kit, Sigma-Aldrich) for 10 minutes at 37°C. The monocytes were cultured at 37°C on gelatin-coated glass slides for 1 and 16 hours in the presence of PKH26 labeled ucMSC at a 1:4 ratio (ucMSC:monocytes) in RPMI medium supplemented with 2 mM l-glutamine, 1% P/S and 10% heat-inactivated FBS. As a negative control, monocytes were co-cultured with ucMSC for 16 hours at 4°C.

Confocal microscopy analysis of phagocytosis of PKH26-labeled ucMSC by monocytes was carried out on a Leica TCS SP5 confocal microscope (Leica Microsystems B.V., Eindhoven, The Netherlands) equipped with Leica Application Suite – Advanced Fluorescence software, DPSS 561 nm lasers, using a ×60 (1.4 NA oil) objective. The microscope was equipped with a temperature-controlled incubator (incubator settings: 37°C and 5% CO2). Images were processed using ImageJ 1.48 (National Institutes of Health, Washington).

Addition of ucMSC Primed Monocytes to Mixed Lymphocyte Reaction (MLR)

CD14+ monocytes were isolated from PBMC via MACS separation as described above. To prime CD14+ monocytes, the cells were co-cultured for 24 hours with ucMSC at a 1:4 ratio (ucMSC:monocytes). Thereafter, ucMSC were manually separated from monocytes using biotin anti-human CD73 (clone AD2, Biolegend Inc., San Diego, CA) and MagniSort Streptavidin Positive Selection Beads (MSPB-6003, eBioscience, Affymetrix Inc, San Diego, CA) and the EasySep Magnet (StemCell technologies, Germany). The obtained untouched primed monocytes showed a purity of >98% (Supporting Information Figure S2).

Primed and non-primed monocytes (10,000) were added to MLR of 50,000 carboxyfluorescein succinimidylester (CFSE)-labeled PBMC (autologous to monocytes) and 50,000 γ-irradiated (40 Gy) HLA-mismatched PBMC in RPMI supplemented with 2 mM l-glutamine, 1% P/S and 10% heat-inactivated FBS. After 7 days, PBMC were harvested and stained for 30 minutes at room temperature with CD3-PERCP (BD Biosciences), CD4-Pacific Blue (Biolegend Inc.), CD8-APC-Cy7 (BD Pharmingen), CD25-PE-Cy7 (BD Pharmingen), and CD127-PE (BD Pharmingen). In addition, intracellular staining for Foxp3 (eBiosciences) was performed with anti-human FoxP3-APC staining kit (BD Biosciences). Cell proliferation was determined by CFSE dilution, measured on a FACSCanto II flow cytometer (BD Biosciences).

Real time qPCR

mRNA was isolated from primed and non-primed monocytes using the High Pure RNA Isolation Kit (Roche). Complement DNA was synthesized from 500 ng mRNA with random primers (Promega Benelux B.V., The Netherlands). Quantitative gene expression was determined using TaqMan Gene Expression Assays-on-demand for IL1β (Hs00174097.m1), IL6 (Hs00174131.m1), IL8 (Hs00174114.m1), IL10 (Hs00174086.m1), TGFβ (Hs00171257.m1), and TNFα (Hs99999043.m1; all Applied Biosystems, Foster City, CA). Results were expressed as copy number.

Statistical Analysis

Statistical analysis was performed by unpaired t tests using Prism software v5.04 (GraphPad Software Inc., La Jolla, CA). p values of $<.05$ were considered significant.

Results

UcMSC accumulate in the lungs after intravenous infusion

To investigate the bio-distribution of intravenously infused ucMSC, cells were dual labeled with Qtracker605 beads and Hoechst33342 prior to infusion to enable detection of live and dead ucMSC in vivo, respectively. Live ucMSC were identified by Qtracker605 signal (Qtracker605 signal outshines the Hoechst33342 signal), whereas dead ucMSC were detected by Hoechst33342 signal. Detection of Qtracker605 signal 5 minutes post infusion revealed that the majority of ucMSC were alive and present in the lungs (Figure 1A, 1E). In addition, few dead ucMSC were observed in the lungs and liver as detected by Hoechst33342 signal (Figure 1B, 1E).

Dead ucMSC re-localize to the liver prior to their disappearance

At 24 hours post-infusion, a large decrease in the number of live ucMSC was observed in the lungs (Figure 1C, 1E). The number of dead cells in the lungs was however increased and interestingly, there was an accumulation of dead ucMSC in the liver (Figure 1D, 1E). No living ucMSC were detected in the liver and by 72 hours post-infusion, minimal numbers of cells were detected, which were all dead (Figure 1E).

UcMSC Are Phagocytosed and Redistributed By Host Innate Immune Cells

To examine how ucMSC disappear from the lungs and reappear in the liver 24 hours after infusion, whole blood, lungs and liver were harvested from mice that were infused with 150,000 PKH26-labeled ucMSC, single cell suspensions were prepared and stained for leukocyte markers and analyzed by flow cytometry. PKH26+ cells were found in the lungs (3.4% ± 0.13% of total cells), blood (0.7% ± 0.05%), and liver (2.9% ± 0.11%) (Figure 2A, 2B). In the cell suspensions from lungs and blood, PKH26+ cells were mostly CD11b++, whereas in the liver, PKH26 signal was mostly found in CD11b+ cells (Figure 2A, 2C), indicating that ucMSC were phagocytosed by host-innate immune cells. A minority of PKH26+ cells in the lungs were CD68+CD11b+ lung-resident macrophages (12.6% ± 1.0%), whereas 32.1% ± 0.9% were CX3CR1+CD11b++ blood-derived monocytes and 47.5% ± 1.1% were SSC++CD11b++ neutrophils (Figure 2A, 2D). In the blood, 89.3% ± 1.3% of PKH26+ cells were CX3CR1+CD11b++ monocytes and 5.7% ± 0.7% were neutrophils (Figure 2A, 2E). In the liver, PKH26+ cells were mainly CLEC4F+CD11b+ Kupffer cells (83.8% ± 0.4%), whereas 3.8% ± 0.15% were CLEC4F-CD11b++ and 10.1% ± 0.5% were neutrophils (Figure 2A, 2F).

Monocytes express a regulatory phenotype after phagocytosis of ucMSC

Thus, monocytes and neutrophils contribute to the clearing of infused ucMSC. In addition to their phagocytic activity, monocytes may play immune-activating as well as immune-regulatory roles. To examine the function of monocytes that phagocytosed ucMSC, PKH26+ monocytes in lung, blood, and liver cell suspensions were subdivided into classical (pro-inflammatory) and non-classical (anti-inflammatory) monocytes, based on their expression of Ly6C (Figure 3A). In addition, CD68, CDX3CR1, or CLEC4F were used to indicate lung resident macrophages, blood circulating monocytes and Kupffer cells, respectively. In the

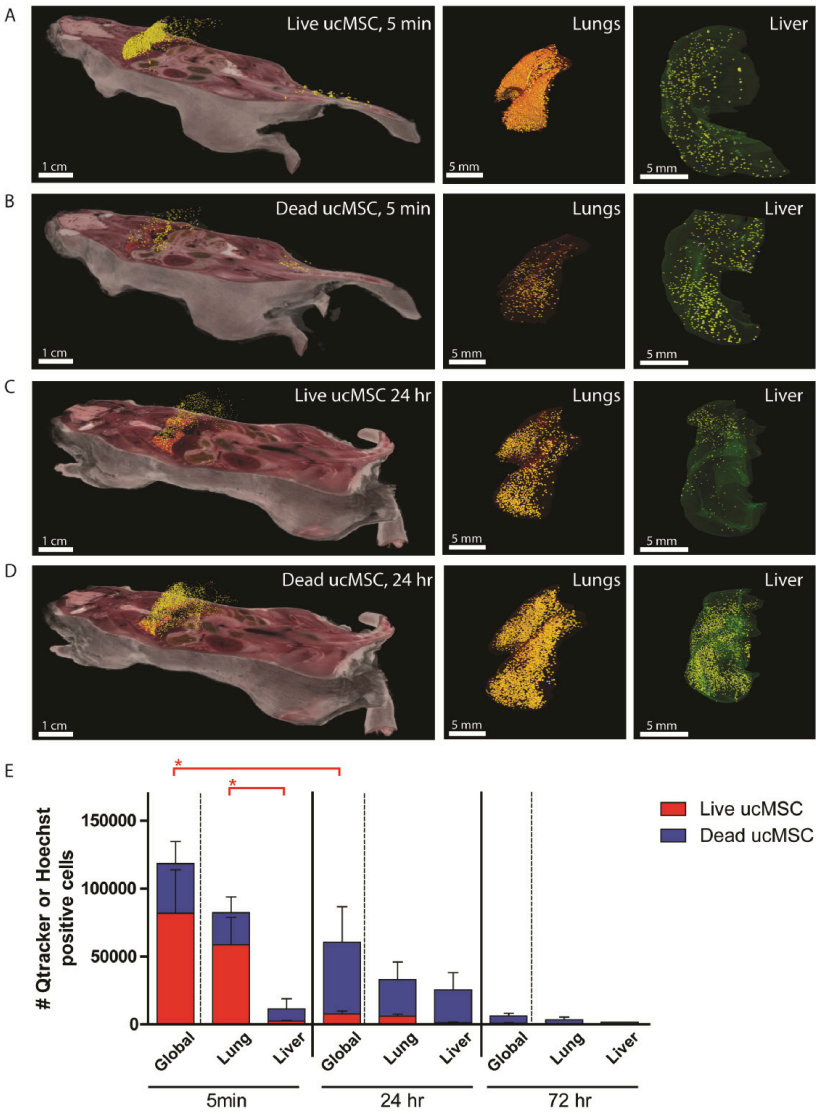


Figure 1. UcMSC strand in the lungs after infusion and re-localize to the liver prior to their disappearance.

CryoViz images (left: whole body, middle: lungs, right: liver) of mice after tail vein infusion of 150,000 live ucMSC. (A) Qtracker 605 bead signal, corresponding to live ucMSC 5 min post ucMSC infusion and (B) Hoechst33342 signal, corresponding to dead ucMSC 5 min post ucMSC infusion. (C) Qtracker 605 bead signal 24h post ucMSC infusion and (D) Hoechst33342 signal 24h post ucMSC infusion. (E) Number of Qtracker 605 bead (red) positive live ucMSC and Hoechst33342 (blue) positive dead ucMSC at 5 min, 24h and 72h post ucMSC infusion, globally, in the lungs and in the liver. Results are shown as means ± SEM (n=6). * indicates significant difference ($p<0.05$).

lungs, non-classical blood circulating monocytes (Ly6C-CD68-) are the biggest population within the PKH+ cells (Figure 3B). Next, lung resident macrophages make up a big portion. In the blood, the majority of PKH+ monocytes demonstrate a non-classical Ly6C- CX3CR1+CD11b+ phenotype (Figure 3B). Furthermore, PKH+ cells in the liver consist mainly out of Kupffer cells (CLEC4F+) followed by monocytes with a non-classical (Ly6C-CLEC4F-) phenotype (Figure 3B).

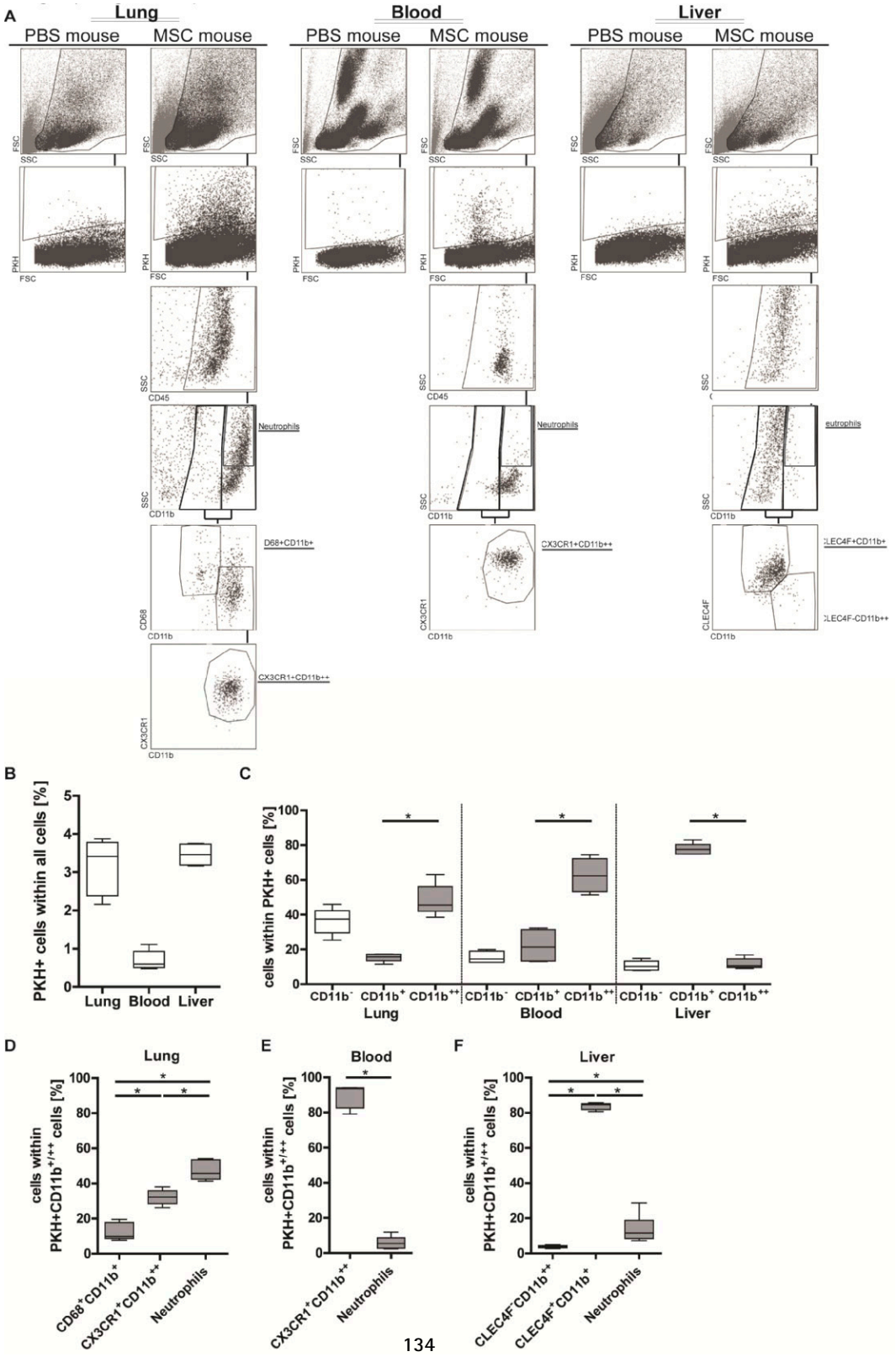
ucMSC are actively phagocytosed by monocytes in vitro

To further study the interaction of ucMSC with human innate immune cells, PKH26-labeled ucMSC were added to human whole blood in vitro. After 24 hours of incubation, 21% ± 8% of CD45+CD15+ neutrophils and 91% ± 3% of CD45+CD14+ monocytes had become positive for PKH26 (Figure 4A), thereby confirming the results from the in vivo experiments. In contrast, no significant uptake of ucMSC was measured in CD45+ SSClow lymphocytes at all time points (Supporting Information Figure S3).

PKH26-labeled ucMSC were subsequently incubated with human blood-derived CD14+ monocytes. Nearly all monocytes became positive for PKH26 within 24 hours as measured by flow cytometry (19% ± 2% at 1 hour, 34% ± 3% at 4 hours and 92% ± 1% 24 hours) (Figure 4B). To visualize the phagocytosis of ucMSC by human monocytes, serial confocal images of co-cultures of PKH67-labeled monocytes and PKH26-labeled ucMSC were produced. It was observed that monocytes actively migrated toward ucMSC within 1 hour (Figure 4C). Monocytes with internalized fragments of PKH26-labeled ucMSC were observed 3 hours after the start of the co-cultures (Figure 4D). At 16 hours, the majority of monocytes contained PKH26-labeled ucMSC fragments (Figure 4E). In the control co-culture, which was left at 4°C for 16 hours, no phagocytosis of ucMSC by monocytes was observed (Figure 4F), demonstrating that phagocytosis of ucMSC by monocytes is an active process.

Phagocytosis of ucMSC activates monocytes and induces polarization

UcMSC are rapidly recognized and phagocytosed by human monocytes. To investigate whether phagocytosis of ucMSC affects monocyte properties, expression of PD-L1, CD90, IL-6, IL-1β, IL-8, TGF-β, TNF-α, and IL-10 was analyzed. Monocytes significantly upregulated cell surface expression of PD-L1 (from 40% ± 9% to 73% ± 3%, $p<.05$) and CD90 (from 21% ± 4% to 47% ± 3%, $p<.05$) after 24 hours of co-culturing with ucMSC (Figure 5A, 5B). Moreover, mRNA expression


A
Lung
Blood
Liver
PBS mouse
MSC mouse
FSC
SSC
PKH
CD45
CD11b
Neutrophils
CD68
D68+CD11b+
CX3CR1
CX3CR1+CD11b++
CLEC4F
CLEC4F+CD11b+
CLEC4F-CD11b++
B
PKH+ cells within all cells [%]
Lung
Blood
Liver
C
cells within PKH+ cells [%]
CD11b⁻
CD11b⁺
CD11b⁺⁺
D
Lung
cells within PKH+CD11b⁺/⁺⁺ cells [%]
CD68⁺CD11b⁺
CX3CR1⁺CD11b⁺⁺
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CLEC4F⁺CD11b⁺
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levels of IL-1β, IL-6, IL-8, IL-10, and TGFβ significantly increased in the presence of ucMSC, whereas expression of pro-inflammatory TNF-α decreased (Figure 5C).
Activation of human monocytes is associated with a phenotype shift from CD14++CD16- to CD14+CD16+ [30, 31]. After co-culture of human monocytes with ucMSC for 24 hours, the predominant monocyte population shifted from CD14++CD16- (84% ± 4%) to CD14++CD16+ (55% ± 2%), known as immune regulatory intermediate monocytes (Figure 5D). Furthermore, after co-culture with ucMSC, monocytes significantly increased expression of CD163 and CD206, markers associated with an immune regulatory function of monocytes (Figure 5E). These results support the observation of the in vivo experiments that monocytes that had phagocytosed ucMSC were predominantly of an anti-inflammatory phenotype.

Skewing of Monocytes By Phagocytosis of ucMSC Differs from Skewing By ucMSC Conditioned Medium

Monocytes that have phagocytosed ucMSC exhibit a different phenotype than prior to phagocytosis. To investigate whether this is caused by factors secreted by ucMSC or by phagocytosis of ucMSC, monocytes were cultured in ucMSC conditioned medium or co-cultured with ucMSC. After 3 days the phenotype of monocytes (CD14, CD16, CD163, CD206, CD90, and PD-L1 expression) was analyzed. CD14, CD16, CD90, and PD-L1 expression by monocytes that phagocytosed ucMSC or monocytes that were cultured in ucMSC conditioned

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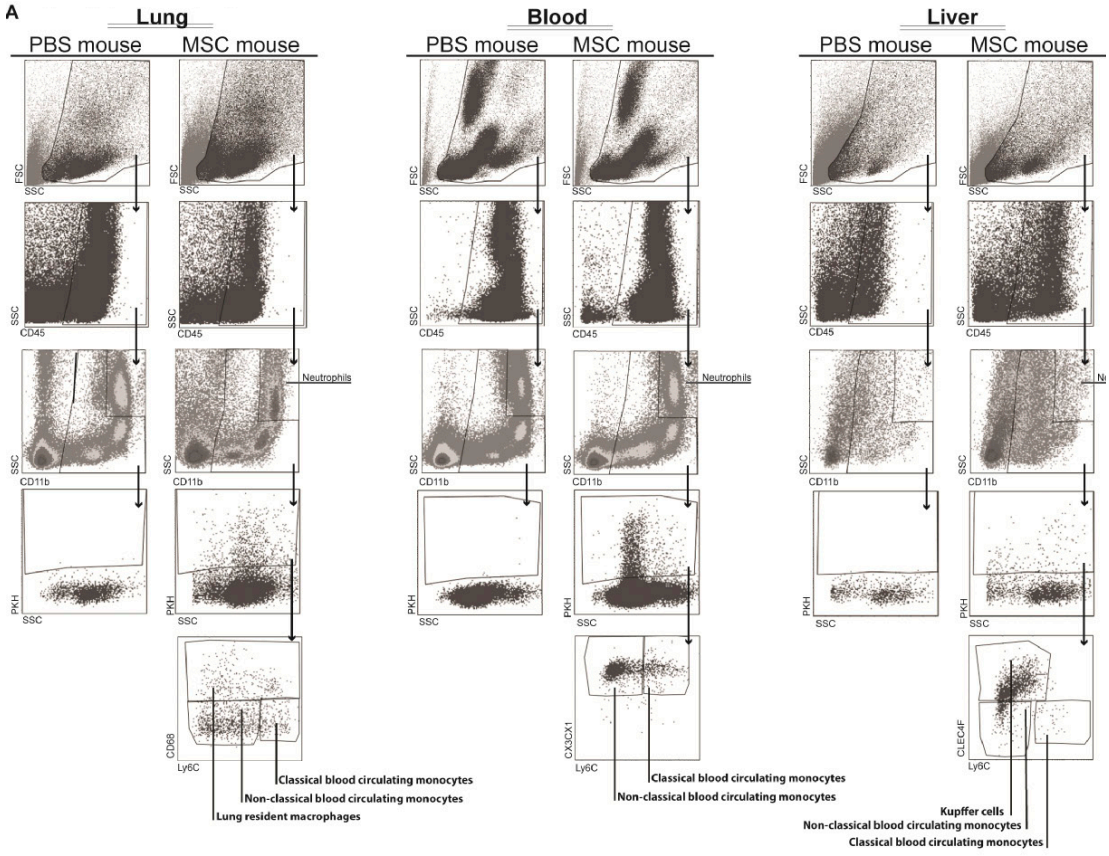
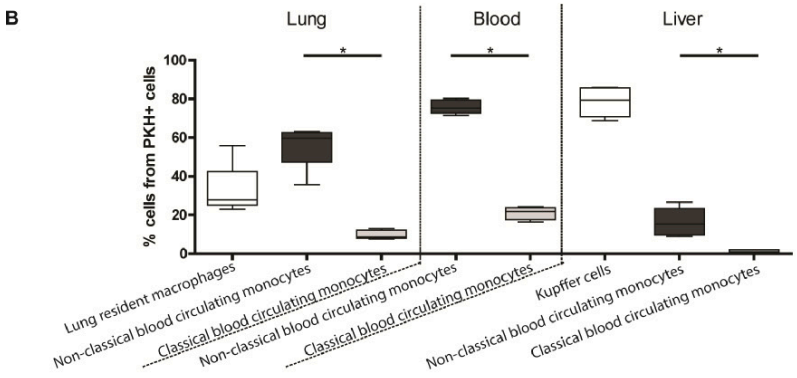
Figure 2. UcMSC are phagocytosed after infusion by host immune cells and distributed to blood and liver.
PKH26-labeled ucMSC were administered to mice via the tail vein and after 24h cells of the lungs, blood and liver were analyzed by flow cytometry. (A) Gating strategy for lungs, blood and liver cell suspensions to investigate the origin of PKH26 signal based on CD11b, CX3CR1, CD68 and CLEC4F expression of PHK26+ cells. (B) Proportion of PKH26+ cells in the lungs, blood and liver. (C) Proportion of CD11b+, CD11b++ and CD11b- PKH26+ cells in the lungs, blood and liver. (D) Proportion of lung resident macrophages (CD68+CD11b+), circulating monocytes (CX3CR1+CD11b++) and neutrophils (SSChighCD11b+) of PKH26+CD11b+/++ cells in the lungs. (E) Proportion of CX3CR1+CD11b++ and neutrophils in PKH26+CD11b+/++ cells in the blood. (F) Proportion of CLEC4F-CD11b++, CLEC4F+CD11b+ (Kupffer cells) and neutrophils in PKH26+CD11b+/++ cells in the liver. Results are shown as means ± SEM (n=5). * indicates significant difference ($p<0.05$).


A
Lung
PBS mouse
MSC mouse
Blood
PBS mouse
MSC mouse
Liver
PBS mouse
MSC mouse
FSC
SSC
CD45
Neutrophils
CD11b
PKH
CD68
Ly6C
CX3CX1
CLEC4F
Classical blood circulating monocytes
Non-classical blood circulating monocytes
Lung resident macrophages
Kupffer cells


B
Lung
Blood
Liver
% cells from PKH+ cells
100
80
60
40
20
0
*
Lung resident macrophages
Non-classical blood circulating monocytes
Classical blood circulating monocytes
Non-classical blood circulating monocytes
Classical blood circulating monocytes
Kupffer cells
Non-classical blood circulating monocytes
Classical blood circulating monocytes

medium was similar (Figure 5F). However, in contrast to monocytes that phagocytosed ucMSC, monocytes cultured in ucMSC conditioned medium did not upregulate CD163 expression, nor CD206 expression (Figure 5F). The percentage of monocytes expressing CD163-CD206+ was significantly higher when monocytes phagocytosed ucMSC (31.6% ± 3%) compared to when they are cultured in ucMSC conditioned medium (5.3% ± 2%). Likewise, significantly more monocytes expressed CD163+CD206+ after phagocytosis of ucMSC (9.4% ± 2%) compared to after culturing in ucMSC conditioned medium (1.9% ± 1%).

Monocytes primed by ucMSC induce regulatory T cells

Upon phagocytosis of ucMSC, monocytes are activated and polarized towards an immune regulatory phenotype. We investigated whether these primed monocytes would subsequently alter the adaptive immune response in vitro. UcMSC primed and unprimed monocytes were added to mixed lymphocyte reactions, in which the responder cells were autologous to the added monocytes. Addition of ucMSC primed monocytes led to a significant increase in Foxp3+ regulatory T cells from 8.9 ± 2% to 13 ± 2% of CD4+CD25hiCD127- cells (Figure 6A,B).

In contrast, addition of ucMSC primed monocytes to the mixed lymphocyte reaction led to a significant reduction in activated CD4+ T cells (Foxp3-CD4+CD25hiCD127-). Finally, the ratio of Foxp3+/Foxp3- CD4+CD25hiCD127- T cells increased from 0.1 to 0.2 upon addition of ucMSC primed monocytes (Figure 6B).

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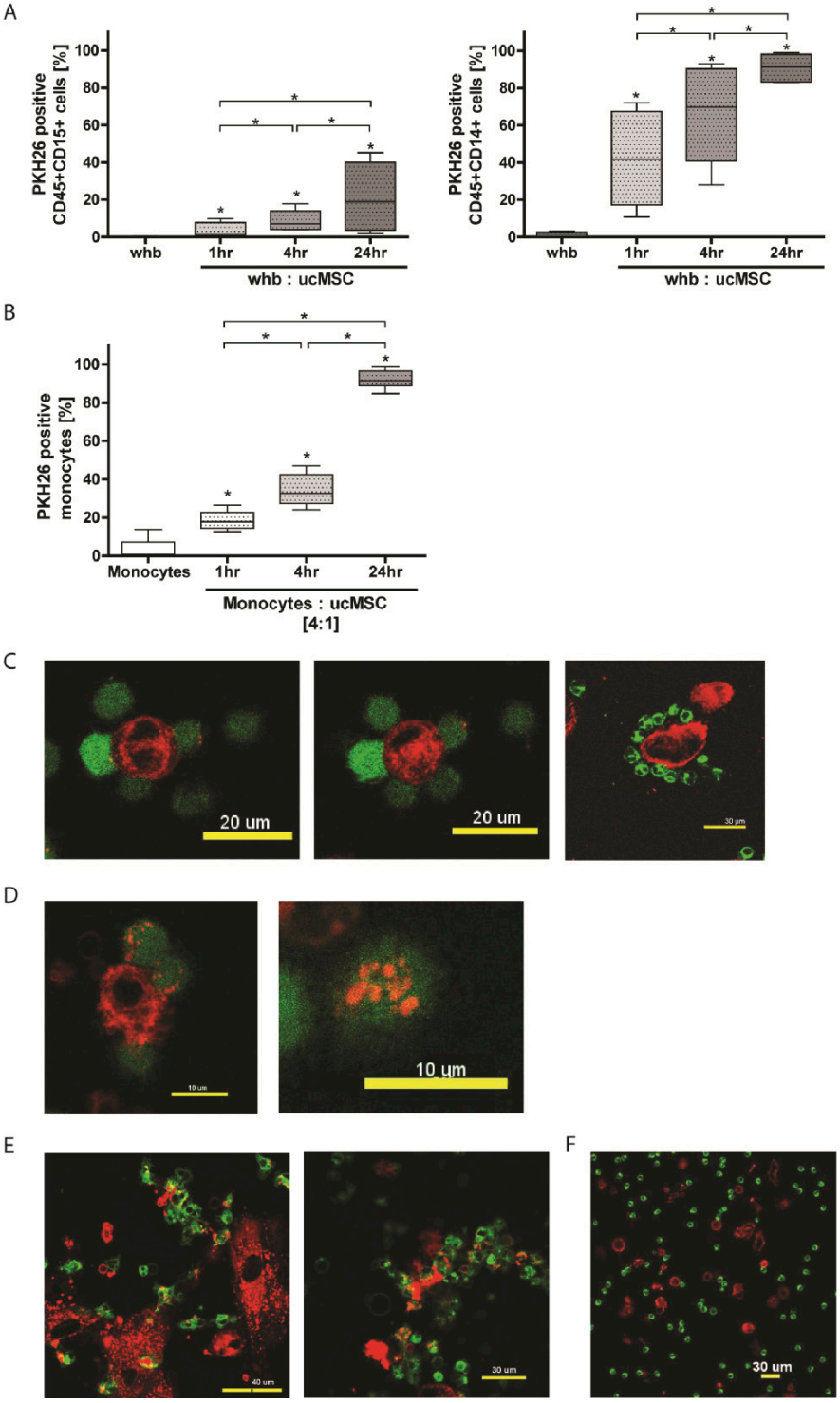
Figure 3. Monocytes that have phagocytosed ucMSC predominantly express a Ly6C- regulatory phenotype.

Monocytes that have phagocytosed umbilical cord mesenchymal stem or stromal cells (ucMSC) predominantly express a Ly6C- regulatory phenotype. (A): Representative flow cytometry plots of PKH positive classical (pro-inflammatory) and non-classical (anti-inflammatory) monocytes based on SSC and CD11b and Ly6C expression in the lungs, blood, and liver. Non-classical monocytes are predominantly positive for PKH26 signal (indicating phagocytosis of MSC). (B): Distribution of PKH positive cells in the lungs, blood, and liver 24 hours after PBS or ucMSC infusion. Results are shown as means±SEM (n=3 PBS mice and n=5 ucMSC mice). * Indicates significant difference ($p<.05$). Abbreviations: MSC, mesenchymal stem or stromal cells; PBS, phosphate buffered saline.


A
PKH26 positive CD45+CD15+ cells [%]
whb
1hr
4hr
24hr
whb : ucMSC
PKH26 positive CD45+CD14+ cells [%]
whb
1hr
4hr
24hr
whb : ucMSC
B
PKH26 positive monocytes [%]
Monocytes
1hr
4hr
24hr
Monocytes : ucMSC [4:1]
C
20 um
20 um
30 um
D
10 um
10 um
E
40 um
30 um
F
30 um

Discussion

Previous work has demonstrated that intravenously administered MSC accumulate in the lungs and have a short survival time [15, 29, 32, 33]. The present study shows that monocytes and neutrophils contribute to the clearance of MSC from the lungs by phagocytosing MSC. Subsequently, these cells migrate via the blood stream to other body sites, in particular to the liver. Our in vitro data show that phagocytosis of MSC induces phenotypic and functional changes in monocytes, which then modulate the adaptive immune cell compartment.

The brief presence and restricted distribution of intravenously administered MSC appears to be in contrast with the short-term and long-term effects of MSC administration observed in numerous pre-clinical studies and in a number of clinical trials [8, 12, 16-21]. The short lifespan of MSC after intravenous infusion challenges the hypothesis that the effects of MSC are mediated via their secretome. MSC may lack time to secrete sufficient levels of immunomodulatory factors before they are cleared, although it is possible that disintegration of MSC leads to the release of intracellularly contained cytokines and growth factors. This phenomena might not be specific for MSC and may also be induced by other cell types as well. However, MSC have shown to be effective in several clinical trials as such we explored the fate of MSC after infusion into further depth. Previously, we showed that expression of the macrophage markers CD68 and F4/80 is significantly increased in the lungs of mice 2 hours after MSC infusion, suggesting recruitment of macrophages to the lungs after MSC infusion [34]. These cells are likely to play a key role in the effects of MSC infusion.

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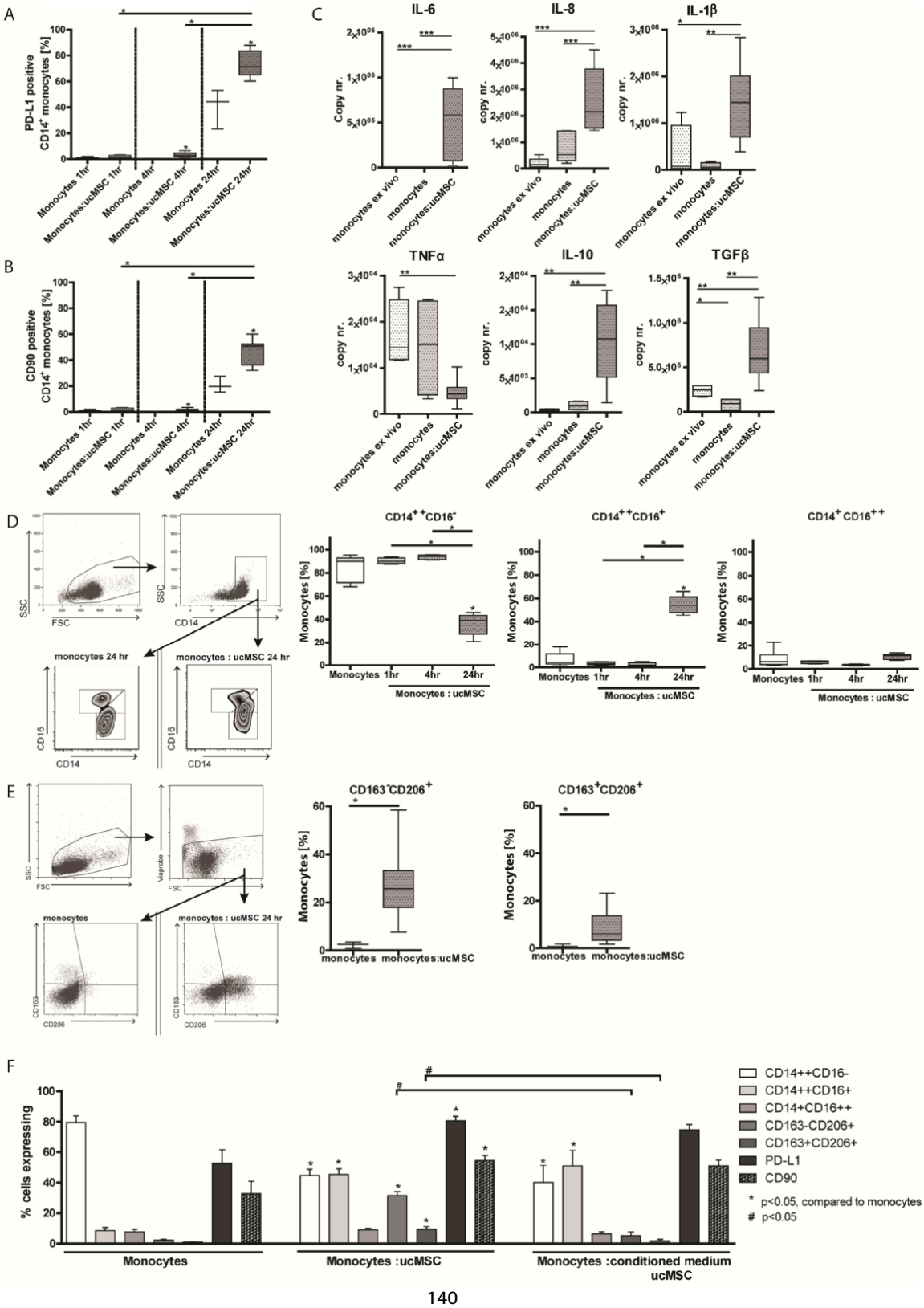
Figure 4. UcMSC are phagocytosed by human monocytes in vitro.
(A) Frequency of PKH26+ neutrophils (left) and monocytes (right) after addition of PKH26+ ucMSC to human whole blood. An increase in PKH26+ neutrophils and monocytes can be observed over time. (B) Percentage of PKH26+ monocytes after co-culture of isolated CD14+ monocytes with PKH26+ ucMSC. (C) Confocal images 1h after adding PKH26+ ucMSC (red) to PKH67+ monocytes (green). (D) UcMSC are phagocytosed by monocytes and fragments of ucMSC are visible intracellularly. (E) Overview image of co-culture at 16h. (F) Image of co-culture kept at 4 ºC for 16h, showing a lack of phagocytosis of ucMSC by monocytes, indicating phagocytosis is an active process. Results are shown as means ± SEM (n=3). * indicates significant difference ($p<0.05$).


A
PD-L1 positive CD14+ monocytes [%]
Monocytes 1hr
Monocytes:ucMSC 1hr
Monocytes 4hr
Monocytes:ucMSC 4hr
Monocytes 24hr
Monocytes:ucMSC 24hr
B
CD90 positive CD14+ monocytes [%]
C
IL-6
IL-8
IL-1β
TNFα
IL-10
TGFβ
copy nr.
monocytes ex vivo
monocytes
monocytes:ucMSC
D
SSC
FSC
CD14
CD15
monocytes 24 hr
monocytes : ucMSC 24 hr
CD14++CD16-
CD14++CD16+
CD14+CD16++
Monocytes [%]
Monocytes 1hr
4hr
24hr
Monocytes : ucMSC
E
Viaprobe
monocytes
monocytes : ucMSC 24 hr
CD163
CD206
CD163-CD206+
CD163+CD206+
monocytes
monocytes:ucMSC
F
% cells expressing
Monocytes
Monocytes :ucMSC
Monocytes :conditioned medium ucMSC
CD14++CD16-
CD14++CD16+
CD14+CD16++
CD163-CD206+
CD163+CD206+
PD-L1
CD90
* p<0.05, compared to monocytes
# p<0.05

The data of the present study confirm that monocytic cells play a role in the clearance of infused MSC [35, 36]. Braza et al. showed a similar phenomenon of phagocytosis of IV infused MSC in the lungs by cells of the monocyte/macrophage lineage (F4/80+CD11c+). In their study different markers and terminology were used to define the phagocytosing cells of the monocyte/macrophage lineage, yet their results were in line with our data. Recently, Dazzi et al. showed that for MSC-induced immunosuppression to occur, T cell induced cell death of MSC is essential, which triggers phagocytes to engulf MSC [37].

After phagocytosis of MSC, monocytes migrate to other body sites via the blood stream (summarized in Figure 7). In addition, some MSC may disintegrate and the remnants may be transported out of the lungs via the blood stream. We found accumulation of MSC remnants in the Kupffer cells of the liver. Kupffer cells line the liver sinusoids and are likely to encounter passaging MSC remnants. Kupffer cells are professional clean-up cells through phagocytosis of cellular debris and may thus contribute to the clearance of MSC.

The clean-up of infused MSC leaves a clear footprint in the monocyte compartment. We observed that monocytes that phagocytosed MSC were of a Ly6C- regulatory phenotype. Ly6C- monocytes containing remnants of MSC were

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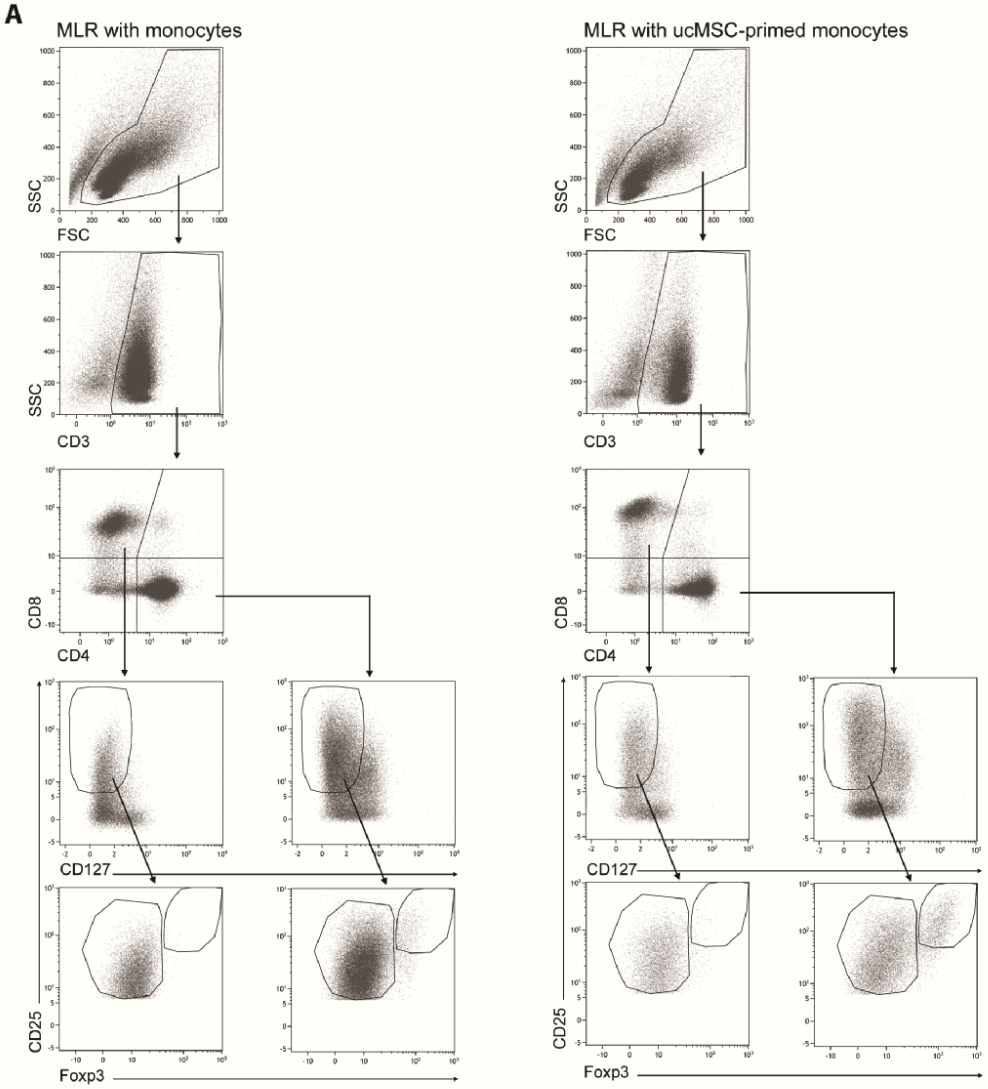
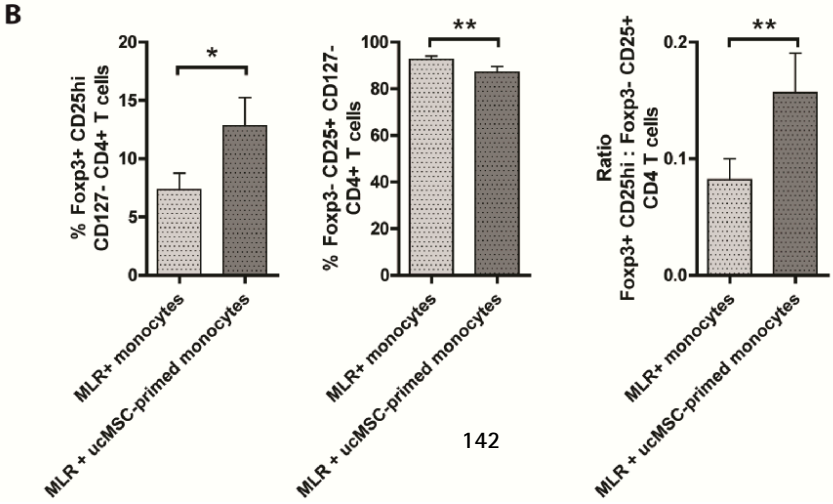
Figure 5. Human monocytes adapt phenotype upon phagocytosis of ucMSC in vitro. Human monocytes adapt phenotype upon phagocytosis of ucMSC in vitro. Protein expression of the surface proteins (A) PD-L1 and (B) CD90 on CD14+ monocytes is increased upon co-culture with ucMSC. * Indicates significant difference ($p<.05$). (C): mRNA expression levels of IL-6, IL-8, IL1β, TNF-α, IL10, and TGF-β in CD14+ monocytes increase upon co-culture with ucMSC. * Indicates significant difference ($p<.05$). (D): Representative flow cytometry plot demonstrating changes in monocyte subset composition based on CD14 and CD16 expression 24 hours after co-culture with ucMSC. During co-culture, the frequency of CD14++CD16- monocytes decreased whereas CD14++CD16+ monocytes increased. * Indicates significant difference ($p<.05$). (E): Representative flow cytometry plot demonstrating increases in the frequency of CD163-CD206+ and CD163+CD206+ monocyte subsets after 24 hours of co-culture of monocytes with ucMSC. * Indicates significant difference ($p<.05$). (F): Percentage of monocytes expressing CD14++CD16+, CD14++CD16-, CD14+CD16++, CD163-CD206+, CD16+CD206+, PD-L1, and CD90 when monocytes are cultured alone, when monocytes phagocytosed ucMSC and when monocytes are cultured in ucMSC conditioned medium. * Indicates significant difference compared to monocytes cultured alone ($p<.05$) and # indicates significant difference ($p<.05$). Results are shown as means ±SEM ($n=3$). Abbreviation: UcMSC, umbilical cord mesenchymal stem or stromal cells.


A
MLR with monocytes
MLR with ucMSC-primed monocytes
SSC
FSC
CD3
CD8
CD4
CD127
CD25
Foxp3


B
% Foxp3+ CD25hi CD127- CD4+ T cells
% Foxp3- CD25+ CD127- CD4+ T cells
Ratio Foxp3+ CD25hi : Foxp3- CD25+ CD4 T cells
MLR+ monocytes
MLR + ucMSC-primed monocytes

observed in the lungs but also in the blood and in the liver of MSC treated animals. This demonstrates that MSC infusion induces the distribution of monocytes with immunoregulatory properties throughout the body. This is in line with previous findings by Miteva et al. where MSC were also seen to induce the distribution of anti-inflammatory monocytes in mice with Coxsackievirus B3-induced myocarditis [38]. It is however unclear why Ly6C- monocytes specifically localize to the liver, but this may be part of an established clean-up route. It appears clear, however, that by recruitment of anti-inflammatory monocytes that phagocytosed MSC and by phagocytosis of MSC remnants by Kupffer cells, the liver is a target for MSC immune therapy.

The question remains whether Ly6C- monocytes selectively phagocytose MSC, or whether Ly6C+ monocytes undergo phenotypic changes after phagocytosis of MSC. Our in vitro data suggest the latter. We showed that upon phagocytosis of ucMSC, human monocytes increased surface expression of the co-inhibitory molecule PD-L1 and polarized from CD14++CD16- classical monocytes toward CD14++CD16+ intermediate monocytes. We have previously also observed this phenomena in our lab when using adipose derived MSC instead of umbilical cord derived MSC (data not shown). This phenomena when co-culturing monocytes with ucMSC was accompanied by an increased expression of CD206 on a subpopulation of monocytes. This is in accordance to what Cutler et al. observed when co-culturing ucMSC together with human adult PBMC [39]. In our hands, upregulation of CD206 solely occurred when monocytes were able to phagocytose ucMSC and not by exposure to soluble factors that were secreted by ucMSC. CD206 is a known marker for alternatively activated monocytes [40, 41]. Along with an increased CD206+ monocyte population, a population of CD206+CD163+ co-expressing monocytes was significantly increased upon phagocytosis of ucMSC. This again exclusively occurred when monocytes were

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Figure 6. UcMSC primed monocytes induce regulatory T cells.
CD14+ monocytes were co-cultured for 24h with or without ucMSC and subsequently separated from the ucMSC using MACS separation. Primed and unprimed monocytes were added to a mixed lymphocyte reaction. (A) Gating strategy of mixed lymphocyte reaction with primed monocytes after 7 days. (B) Frequencies of Foxp3-CD25+CD127-CD4+ activated T cells and Foxp3+CD25hiCD127-CD4+ regulatory T cells of CD4 T cells (left two graphs) and fluorescence intensity of Foxp3 within Foxp3+CD25hiCD127-CD4+ T cells (right graph). Plots indicate means ± SEM (n≥4).

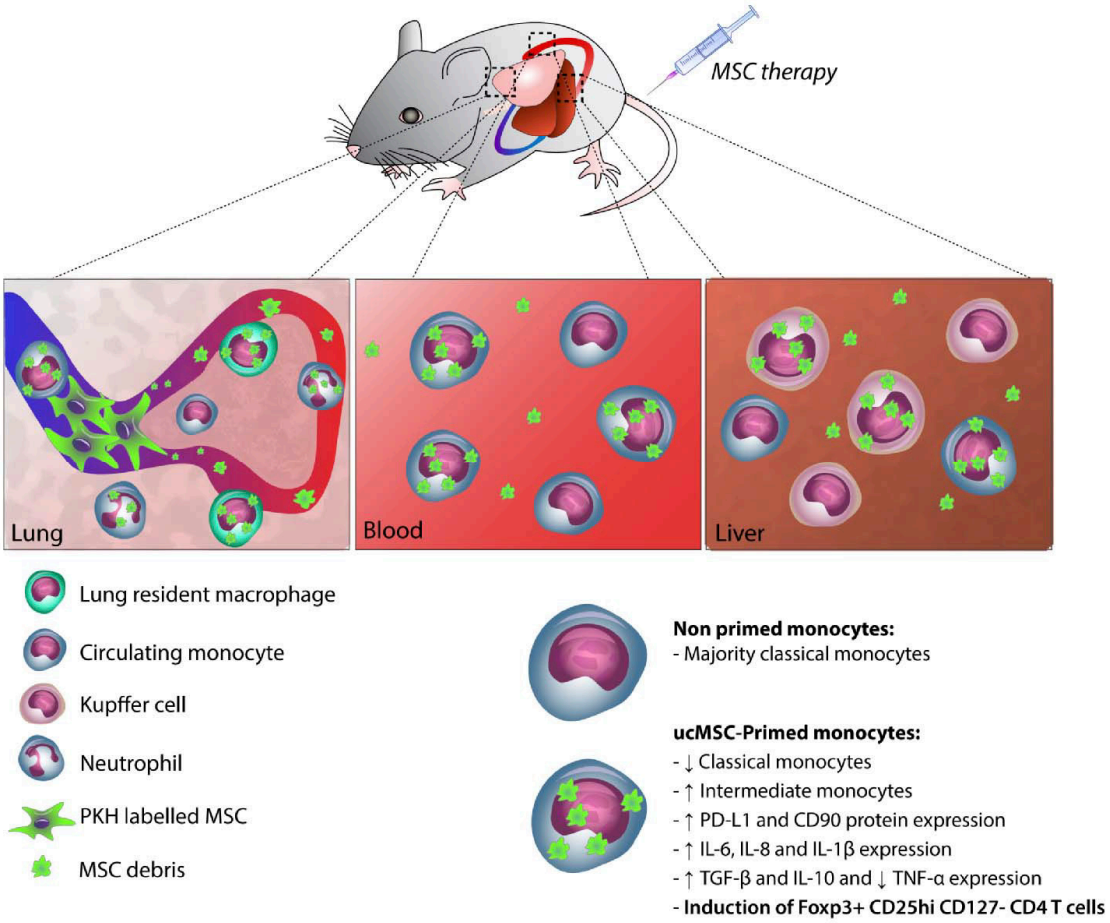

Figure 7. Overview of the interaction of monocytic cells with infused MSC.
UcMSC get entrapped in the lungs after intravenous infusion and are rapidly cleared from the system through phagocytosis by neutrophils, lung resident macrophages and circulating monocytes. Monocytes containing ucMSC migrate via the blood stream to other sites, in particular to the liver. In addition, debris of ucMSC ends up in the liver where it is phagocytosed by liver-resident Kupffer cells. Phagocytosis of ucMSC induces an immunomodulatory phenotype in monocytes and ucMSC-primed monocytes induces Foxp3+CD25hiCD127-CD4+ regulatory T cells.

able to phagocytose ucMSC. These CD206+CD163+ co-expressing monocytes have been described in the literature as important cells for the generation of CD4+CD25hiFoxP3+ T cells and as high IL-10 producing cells with the capacity to take up apoptotic cells [42-44]. In our study, we observed significant increases in IL-10 production by monocytes upon co-culture with ucMSC, alongside a decrease in TNFα and increase in IL-6 and TGFβ. This is well in conformity with earlier studies that demonstrated that phagocytosis of MSC induces an immunosuppressive phenotype in macrophages [23, 35]. These cells produce increased amounts of IL-10 and IL-6 while their production of IL-12 and TNF-α decreases [45, 46]. Other studies have shown different ways in which monocytes are immunomodulated by MSC in vitro, by the secretion of soluble factors [25, 44, 47]. These studies were performed in different experimental settings such as whereby MSC were plastic adhered, which is in contrast to our setting as we used polypropylene tubes to avoid baseline activation of monocytes adhering to the plastic. Moreover, in vivo when MSC are infused they remain in suspension the first time frame, hence usage of polypropylene tubes more closely resembles more the in vivo setting.
Clearance of infused MSC leaves a phenotypical and functional footprint in the monocyte compartment. To examine whether these changes affected monocyte function, ucMSC-primed monocytes were added to mixed lymphocyte reactions. We were able to show that ucMSC-primed monocytes increased Foxp3+CD25hiCD127-CD4+ Tregs cells. Multiple studies have reported increased frequencies of Tregs cells in experimental animal studies [48, 49] and in patients treated with MSC [50-53]. It has furthermore been shown that immunosuppressive macrophages (M2) can induce Tregs cells in vitro [54]. Our results give insight in how MSC driven polarization of monocytic cells may mediate increasing Tregs cell numbers after MSC infusion.

In conclusion, we have demonstrated that the rapid clearance of infused MSC is largely mediated by phagocytosis by monocytes, which subsequently relocate from the lungs to the bloodstream and the liver. UcMSC-primed monocytes change their phenotype and function and change the course of immune responses. The described mechanisms are likely to play a role in the immunomodulatory response after MSC infusion in disease models and clinical trials. Future studies will determine whether monocyte polarization can be attributed to specific components of MSC. This could eventually lead to more defined therapies based on the most active components that can be produced in an efficient and controlled manner.

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Chapter 7

Membrane Particles Generated from Mesenchymal Stromal Cells Modulate Immune Responses by Selective Targeting of Pro-inflammatory Monocytes

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Abstract

Mesenchymal stromal cells (MSC) are a promising therapy for immunological disorders. However, culture expanded MSC are large and get trapped in the capillary networks of the lungs after intravenous infusion, where they have a short survival time. Hypothetically, living cells are a risk for tumor formation. To reduce risks associated with MSC infusion and improve the distribution in the body, we generated membrane particles (MP) of MSC and MSC stimulated with IFN-γ (MPγ). Tracking analysis and electron microscopy indicated that the average size of MP was 120 nm, and they showed a round shape. MP exhibited ATPase, nucleotidase and esterase activity, indicating they are enzymatically active. MP and MPγ did not physically interact with T cells and had no effect on CD4+ and CD8+ T cells proliferation. However, MP and MPγ selectively bound to monocytes and decreased the frequency of pro-inflammatory CD14+CD16+ monocytes by induction of selective apoptosis. MP and MPγ increased the percentage of CD90 positive monocytes, and MPγ but not MP increased the percentage of anti-inflammatory PD-L1 monocytes. MPγ increased mRNA expression of PD-L1 in monocytes. These data demonstrate that MP have immunomodulatory properties and have potential as a novel cell-free therapy for treatment of immunological disorders.

Introduction

Mesenchymal stromal cells (MSC) are studied as an experimental therapy for immunological disorders due to their diverse immunomodulatory properties [1–3]. Multiple clinical trials with MSC in inflammatory disease and transplantation have been conducted, such as in graft versus host disease [4], kidney transplantation [5], and Crohn's disease [6]. The outcomes of several of these trials hint towards a beneficial immunomodulatory effect of MSC, but they are not conclusive [7]. This is partly due to the small patient numbers, to the lack of understanding of the effects of MSC after administration, and perhaps because MSC derived from different tissue sources are used which display distinct paracrine potential and immune regulatory properties. Several authors have compared the capacity of MSC from various tissue sources to suppress peripheral blood B, T and NK cells, and it has been reported that adipose tissue-derived MSC (AT-MSC) have a stronger immunomodulatory effect than MSC from other tissue sources [8,9].

The function of MSC as immunomodulatory agent has been attributed to a variety of mechanisms, notably cytokine and chemokine secretion [10,11]. Multiple pathways have been identified to play a role in in vitro assays, but it is unknown whether they play a role in the immunomodulatory effects of MSC administered to animals or patients. Intravenous infusion has been used as the route of MSC delivery for most preclinical studies [12,13] and clinical trials [7]. It was the assumption that intravenous infusion of MSC would lead to a broad biodistribution of MSC. However, tracking studies have shown that the majority of MSC localize to the lungs after intravenous infusion. The detainment of MSC in the lungs is due to their size (>20 μm in diameter) [14,15], which exceeds the width of the micro-capillaries of the lungs. It has furthermore become clear that MSC have a short-term survival after infusion [16,17]. Over 90% of infused MSC are lost within 24h after infusion. Even though infused MSC end up in the lungs and disappear rapidly, they exert immunomodulatory effects. The short lifespan of MSC after intravenous infusion questions the contribution of secreted anti-inflammatory factors by MSC to the modulation of immune responses.

Recent work demonstrated that heat inactivated MSC that lost their capacity to secrete factors maintain their immunomodulatory capacity after intravenous infusion in an LPS-induced sepsis model, suggesting that cell membrane dependent interactions with immune cells are responsible for the immune regulatory effects [18]. MSC express immunomodulatory molecules on their membrane such as Toll-like receptors (TLRs) [19], ATPases [20] and CD73 (ecto-5'-nucleotidase, Ecto5'NTase) which dephosphorylate ATP into AMP and AMP into adenosine, respectively [21]. This is an important immunomodulatory function as adenosine has immunosuppressive properties [22]. MSC also express receptors involved in differentiation pathways such as CD90 (Thy-1 membrane glycoprotein) that is known for its participation on the differentiation of MSC by acting as an obstacle in the pathway of differentiation commitment [23]. The ability of MSC to modulate the immune system can be enhanced by treatment of MSC with pro-inflammatory cytokines, in particular interferon-γ (IFN-γ) and tumor necrosis factor (TNF)-α [24–26]. Under inflammatory conditions MSC upregulate the expression of cell surface proteins with immune regulatory function, such as programmed death ligand 1 (PD-L1), and Fas ligand via which they directly target immune cells and inhibit their activation and function [27].

Despite of the great potential, several factors including the practical difficulties that come with the use of living cells, their short survival after intravenous infusion and their poor biodistribution, have been major technical challenges to

be overcome before MSC based therapy can be used for clinical application in a consistently therapeutic manner [28]. A modification in the treatment that avoids these complications but preserves the diverse immunoregulatory properties of MSC would therefore improve the applicability of this therapy. We propose a new cell-free therapy based on the generation of small plasma membrane particles (MP) from AT-MSC cultured under different conditions. Therefore, the aim of this study was to generate and characterize MP derived from MSC cultured with and without IFN-γ, analyze their immunomodulatory properties, and their interaction with the immune system.

Methods

Ethics statement and human tissue samples

The MSC were provided by Internal Medicine Department, Transplantation laboratory of the Erasmus MC (The Netherlands). The cells were isolated from subcutaneous adipose tissue from healthy donors that became available during the kidney donation procedure. The tissues were not procured from prisoners, and were collected after obtaining written informed consent for all patients, as approved by the Medical Ethical Committee of the Erasmus University Medical Centre Rotterdam (protocol no. MEC-2006-190). All experiments were performed in accordance with the approved guidelines.

Isolation and culture of MSC from adipose tissue

Subcutaneous adipose tissue from five healthy human kidney donors became available during the donation procedure. The adipose tissue was collected in minimum essential medium-α (MEM-α) (Sigma-Aldrich, St. Louis, MO) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin (P/S) (Lonza, Verviers, Belgium), and 2 mM L-glutamine (Lonza).

The tissue was mechanically disrupted and enzymatically digested with 0.5 mg/ml collagenase type IV in RPMI for 30 min at 37 °C under continuous shaking. Thereafter, the cells were resuspended in MEM-α with 15% fetal bovine serum (FBS; Lonza), 2 mM L-glutamine and 1% P/S, filtered through a 100 µm cell strainer, and transferred to a 175 cm2 culture flasks (Greiner Bio-one, Essen, Germany). Cultures were kept at 37 °C, 5% CO2, and 95% humidity, at 90% confluence; adherent cells were removed from culture flasks by incubation in 0.05% trypsin-EDTA (Life Technologies, Bleiswijk, The Netherlands) at 37 °C.

Two MSC culture conditions were used for the experiments: unstimulated MSC, and pretreated with IFN-γ (50 ng/ml, Sigma-Aldrich). After incubation for 3 days, MSC were collected to generate cell membrane particles. MSC were used for experiments between passages 2 and 6.

Immunophenotypic characterization of AT-MSC

Unstimulated and IFNγ-stimulated AT-MSC were trypsinized and washed with FACS Flow (BD Biosciences, San Jose, CA). Cell suspensions were incubated with mouse-antihuman monoclonal antibodies against CD13-PE-Cy7; HLA-DR-PERCP; HLA-ABC-APC; CD31-FITC; CD73-PE; PD-L1-PE (all BD Biosciences); CD90-APC and CD105-FITC (R&D Systems, Abingdon, UK) at room temperature in the absence of light for 30 min. After two washes with FACS Flow, flow cytometric analysis was performed using FACSCANTO-II with FACSDIVA Software (BD Biosciences).

Generation of cell Membrane Particles

Unstimulated and IFN-γ stimulated AT-MSC were trypsinized and washed twice with PBS. Then, the MSC were incubated in milliQ water at 4 °C until the cells exploded and liberated the nuclei (about 20 min). This step of the protocol was checked by microscopy. Then, the plasma membrane of cells was fractionated by passing them through a 29G needle several times.

Cell extracts were cleared of unbroken cells and nuclei by centrifugation at 2,000 × g for 20 min. The obtained supernatant was transferred to an Amicon Ultra-15 100 kDa device and concentrated by centrifugation at 4,000 x g at 4 °C. The concentrated pellet consisted of crude plasma membrane and was diluted in 1 ml of 0.2 µm filtered PBS, cell culture medium or water. All procedures were performed on ice.

Nanoparticle tracking analysis (NTA)

Analysis of absolute size distribution and concentration of MP was performed using NanoSight NS300 (NanoSight Ltd.). With NTA, particles are automatically tracked and sized based on Brownian motion and the diffusion coefficient. The analysis settings were optimized using as control filtered PBS and bovine serum albumin (BSA, Sigma-Aldrich) solution and kept constant between samples. The NTA measurement conditions were: detect threshold 3 (determined with the BSA solution), three measurements per sample (30 s/measurement), temperature 23.61 ± 0.8 °C; viscosity 0.92 ± 0.02 cP, frames per second 25. Each video was analyzed to give the mean, mode, median and estimated concentration for each particle

size. The samples were diluted to obtain the right number of particles (1 × 108 particles/ml) in accordance with the manufacturer's recommendations.

Transmission electron microscopy examination of MP

After fixation with paraformaldehyde (2%), all the samples were adsorbed for 20 min to glow-discharged carbon coated grids by floating the grids on 10 µL drops on parafilm. Grids with adhered MP were washed with water, stained with 2% uranyl acetate in water and examined in the electron microscope Tecnai T12 Spirit equipped with an Eagle CCD camera 4kx4k (FEI Company, Eindhoven, The Netherlands).

ATPase assay

ATPase activity from MP and MPγ was measured using an ATPase assay kit according to the manufacturer's instructions (Sigma-Aldrich). A phosphate standard was used for creating a standard curve. MP (1 × 1012, 1 × 1011, 1 × 1010 and 1 × 109 particles/ml) were incubated with 4 mM ATP for 30 min at room temperature in assay buffer with malachite green reagent. The formation of the colorimetric product that formed in the presence of free phosphates was measured with a spectrophotometer at 620 nm.

As a control for possible phosphate contamination, the four MP concentrations were incubated in assay buffer without ATP. The signal from these samples was subtracted from the samples incubated with ATP.

CD73 activity assay

A modified protocol of CD73 inhibitor screening assay kit (BPS Bioscience) was used to determine whether MP were able to degrade AMP into adenosine plus phosphate. MP and MPγ (1 × 1012, 1 × 1011 and 1 × 1010 particles/ml) were incubated with AMP (500 µM) during 25 min at 37 °C. Then, colorimetric detection reagent was added to measure the free phosphate from the CD73 reaction. Samples without AMP were measured as a control for free phosphate contamination. CD73 enzyme (2 and 1 ng) was used to calculate the concentration of CD73 in the MP, and MPγ.

Esterase activity by CFSE

CFDA-SE, which is non-fluorescent, enters the cytoplasm of cells where intracellular

esterases remove the acetate groups and convert the molecule to a fluorescent ester (CFSE). This conversion was used to detect whether MP have esterase activity. After MP generation, 1 × 1010, 1 × 109, 1 × 108 and 1 × 107 particles/ml were labeled with 50 μM of CFDA-SE and incubated at 37 °C for 30 min. Dilution of the MP was performed to obtain a proper stoichiometry of the CFSE staining. PBS + CFDA-SE and non-stained MP were used as controls.
CFSE fluorescence was measured by flow cytometry (FACS Canto II, BD Biosciences). Due to the small size of the MP, reliable FSC and SSC measurements could not be obtained. Instead, MP were identified by setting a fluorescence threshold triggering on the CFSE fluorescence so that events above the threshold could be identified as CFSE-loaded MP.

CD3/CD28 T cell proliferation assay

To evaluate the immunomodulatory capacity of MP, PBMC were labeled with 1 μM of CFSE and plated in round bottom 96-well culture plates at a density of 5 × 104 cells/well. T cell proliferation was stimulated by adding human anti-CD3/anti-CD28 antibodies (1 μl/ml each) with a linker antibody Ig (2 μl/ml) (BD Biosciences). PBMC were incubated with different ratios of MP, or MPγ (1:5,000, 1:10,000, 1:40,000, 1:80,000) for 4 days. On the fourth day, non-adherent PBMC were removed from the plate, washed with FACS Flow and incubated with monoclonal antibodies against CD4-PerCP and CD8-PE-Cy7 (antibodies were purchased from BD Biosciences) at room temperature for 30 min. When a CFSE-labeled cell divides, its progeny are endowed with half the number of CFSE-tagged molecules and thus each cell division can be assessed by measuring the corresponding decrease in cell fluorescence by flow cytometry.

Interaction of MP with monocytes

CD14+ cells were purified from PBMC using auto-MACS Pro by positive-selection. Monocyte purity was measured by flow cytometry after staining with mouse-antihuman monoclonal antibodies against CD14-PerCP (BD Biosciences) and CD3-PacBlue (BD Biosciences). Isolated CD14+ monocytes (2 × 105 cells/200 μl) were cultured in RPMI 1640 medium (Life Technologies), supplemented with 10% FBS and 1% P/S. Monocytes were cultured with MP, or MPγ at different ratios (1:10,000, 1:40,000, 1:80,000) in polypropylene tubes. After 24 h of incubation, monocytes were collected for PCR analysis or flow cytometry after staining with CD14-PacBlue, CD3-PerCP, CD16-FITC, PD-L1-PE and CD90-APC (all BD Biosciences).

Quantitative RT-PCR analysis

Monocytes were harvested, washed with PBS-diethylpyrocarbonate (DEPC; Sigma-Aldrich) and stored at −80 °C. Total RNA was isolated and 500 ng used for complementary DNA (cDNA) synthesis. Gene expression was determined by Quantitative Real-Time PCR (qPCR) using the TaqMan Universal PCR Master Mix (Life Technologies), and the assay-on-demand primer/probes for CD90 (Hs00264235_s1), PDL-1 (Hs00204257.m1), interleukin-6 (IL-6; Hs00174131.m1), IL-10 (Hs00174086.m1), tumor necrosis factor-α (TNF-α; Hs99999043.m1) (Thermo Fisher), and indoleamine 2,3-dioxygenase (IDO; Hs00158627.m1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA served as housekeeping gene for normalization (Hs99999905.m1; Thermo Fisher).

Apoptosis of monocyte subsets

Monocytes were cultured with MP, or MPγ at different ratios (1:10,000, 1:40,000 and 1:80,000) in polypropylene tubes overnight. Then, cells were incubated with monoclonal antibodies against CD14-Pacific Blue and CD16-FITC (antibodies were purchased from BD Biosciences) at room temperature for 30 min. After washing step, cells were stained with fluorochrome-conjugated Annexin-V for 15 min at RT to assess the apoptotic cells. All data were measured on a FACSCanto II flow cytometer (BD) and analyzed using FACSDiva software.

MP uptake assays

To obtain fluorescent MP, MSC were labeled with the red fluorescent PKH-26 dye (PKH-MP), which intercalates into lipid bilayers, according to the manufacturer's instructions (Sigma-Aldrich).

Human PBMC from healthy donors were isolated by density gradient centrifugation (Ficoll Isopaque, Sigma Aldrich) and cultured with PKH-MP (ratio 1:40,000). The incubation conditions were 37 °C, 5% CO2, and 95% humidity. As a control for the uptake process, PBMC were incubated with PKH-MP at 4 °C. PKH-MP uptake by lymphocytes and monocytes was analyzed by flow cytometry (FACS Canto II, Becton Dickinson) at 1 h, and 24 h.

Confocal microscopy analysis of PKH-MP uptake by monocytes was carried out by isolating CD14+ cells from PBMC using auto-MACS Pro by positive-selection (Miltenyi Biotec, Leiden, The Netherlands). Then, monocytes were labelled with PKH-67 (Life Technologies) and cultured with PKH-MP (ratio 1:4 × 104) for 24 h. The nuclei of the monocytes were stained with DAPI. Images of monocytes were performed on a Leica TCS SP5 confocal microscope (Leica Microsystems B.V.,

Science Park Eindhoven, Netherlands) equipped with Leica Application Suite – Advanced Fluorescence (LAS AF) software, DPSS 561 nm lasers, using a 60 X (1.4 NA oil) objective. Images were processed using ImageJ 1.48 (National Institutes of Health, Washington, USA).

Statistical Analysis

Data were analyzed for statistical significance either by Student's t-test or one-way ANOVA analysis using GraphPad Prism 5 software. $P < 0.05$ was considered significant.

Results

Characterization of adipose tissue derived mesenchymal stromal cells

Commonly used AT-MSC surface markers were analyzed in unstimulated and IFN-γ stimulated AT-MSC by flow cytometry (Figure 1A). Both types of cells were negative for the markers CD45 and CD31, and positive for CD13, CD73, CD90 and CD105. There was no statistical significant difference in the percentage of unstimulated and IFN-γ stimulated AT-MSC expressing these markers. However, stimulation with IFN-γ significantly increased the percentage of cells positive for immune-markers such as HLA-I, HLA-II, and PD-L1 (Figure 1B). The mean fluorescence intensity of the various markers was determined and a significant increase in the expression of CD105, HLA-II, and PD-L1 was observed after IFN-γ treatment (Figure 1C).

Generation and characterization of Membrane Particles (MP)

MP were generated from unstimulated and IFN-γ stimulated AT-MSC. The number of cells used for each analysis was between 1 × 106–1.5 × 106 cells (80% confluency). The size distribution of the obtained MP was studied using Nanoparticle tracking analysis (NTA). The size of the particles ranged from 63 to 700 nm (Figure 2A), and the mode size of the samples was 121.7 ± 35.5 nm for MP and 138.3 ± 62.1 nm for MPγ (Figure 2B). The percentage of particles with a size larger than 200 nm was lower than 5% in every sample.

Based on the particle concentration per ml, the average number of particles generated from each MSC was 1.2 × 105 ± 2.7 × 104 for MP and 1.1 × 105 ± 2.8 ×

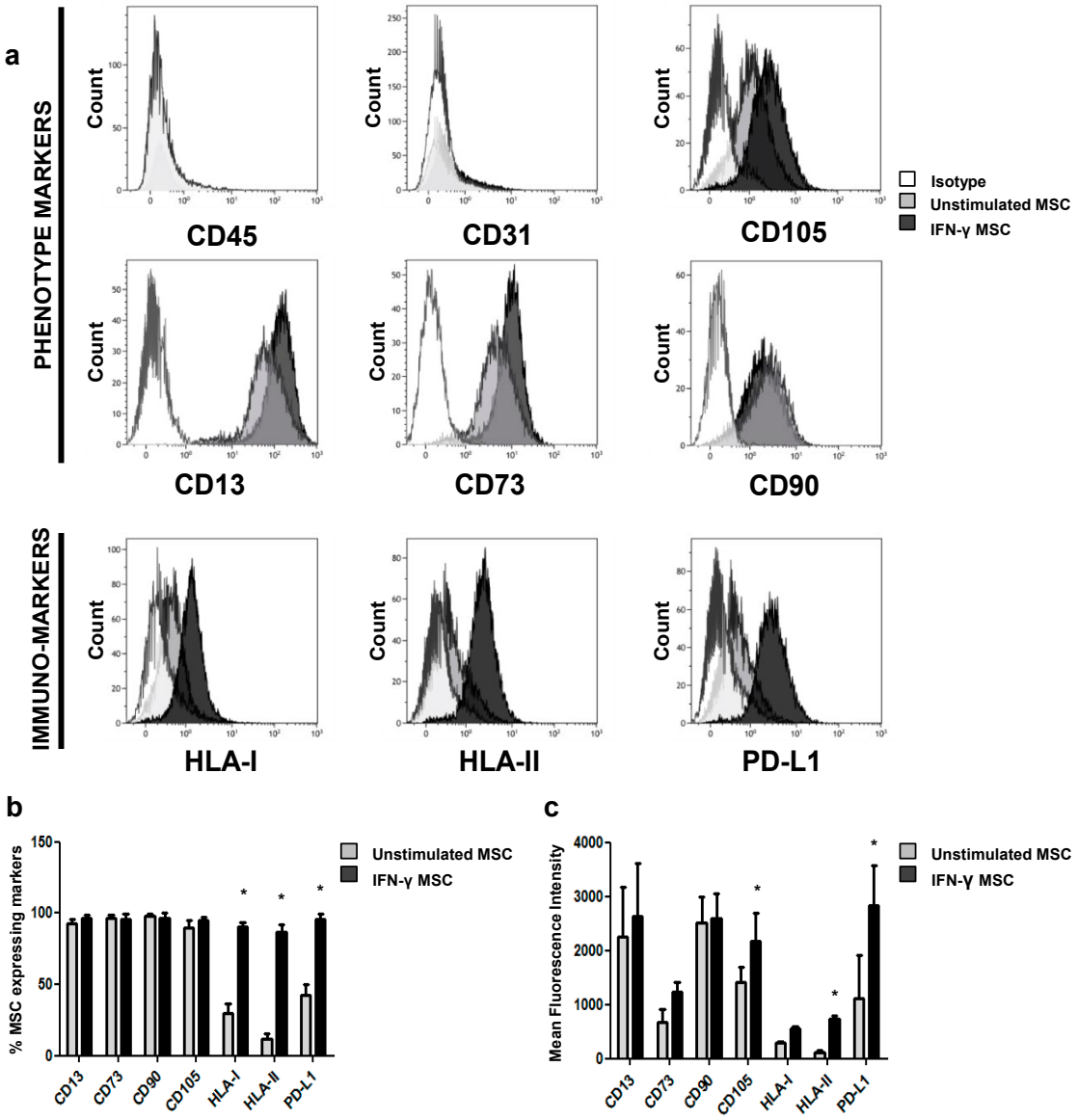

Figure 1 Immunophenotype of unstimulated and IFN-γ stimulated AT-MSC.
(a) Representative flow cytometry analysis of the commonly used markers for MSC (CD45 and CD31, both negative, and CD105, CD13, CD73, CD90), and the immune-markers HLA-I, HLA-II, and PD-L1. Isotype (white histograms), unstimulated AT-MSC (grey histograms) and IFN-γ AT-MSC (black histograms). (b) Percentage positive cells and (c) Mean fluorescence intensities (MFI) of the markers on unstimulated and IFN-γ stimulated AT-MSC. Data are presented as mean ± SD from 5 independent experiments. P values refer to the condition without IFN-γ. Unpaired t-test was used for statistical analysis.

104 for MPγ (Figure 2C). There was no significant difference in size distribution or concentration (MP/MSC) between MP and MPγ.
The transmission electron microscopy images illustrate that MP consist of a population of particles heterogeneous in size with most of the particles showing a size of less than 200 nm (Figure 2D) but some showing larger sizes. This result confirms the NTA analysis. It can be clearly observed that both the larger and smaller MP have a round shape.

Membrane Particles from AT-MSC possess enzyme activity

To analyze whether MP have enzyme activity, we examined the ability of MP and MPγ to convert ATP to ADP by ATPase activity, and AMP to adenosine by the nucleotidase activity of CD73. The last product of these two reactions is free phosphate, so the samples for these assays were prepared in milliQ water to avoid contamination with free phosphates from saline buffers. Before measurement of enzyme activities, MP (diluted in milliQ water) were analyzed by NTA for determination of their concentration.
Figure 3A shows the ATPase activity (units/l) calculated from the standard curve generated with known free phosphate concentrations. MP and MPγ were able to convert ATP to free phosphate and the level of free phosphate was dependent on the concentration of MP. There was no statistical difference between MP and MPγ.
To examine whether MP and MPγ possess CD73 activity, the production of free phosphates by 2, and 1 ng of purified CD73 was compared with different concentrations of MP, and MPγ. Both types of MP were able to produce free phosphates after adding the substrate (AMP). The detection of free phosphate was dependent on concentration of MP and the amount of CD73 present in MP was calculated through the CD73 controls (Figure 3B).

Esterase activity was measured by the conversion of non-fluorescent CFDA-SE to fluorescent CFSE by MP using flow cytometry based on a FITC fluorescence triggering strategy (Figure 3C). This fluorescent-based flow cytometry protocol allows detection of particles based on positive fluorescence signals, not on size, as the average MP size of 120 nm is too small to be detected by most flow cytometers. Controls used for this flow cytometry protocol were PBS + CFDA-SE, and non-labeled MP (top 2 graphs). As expected, these controls were negative as no CFSE fluorescence can be expected. When MP were incubated with CFDA-SE, they converted CFDA to fluorescent CFSE, as demonstrated by the detection of fluorescent events (lower 4 graphs) showing that MP have esterase activity. As

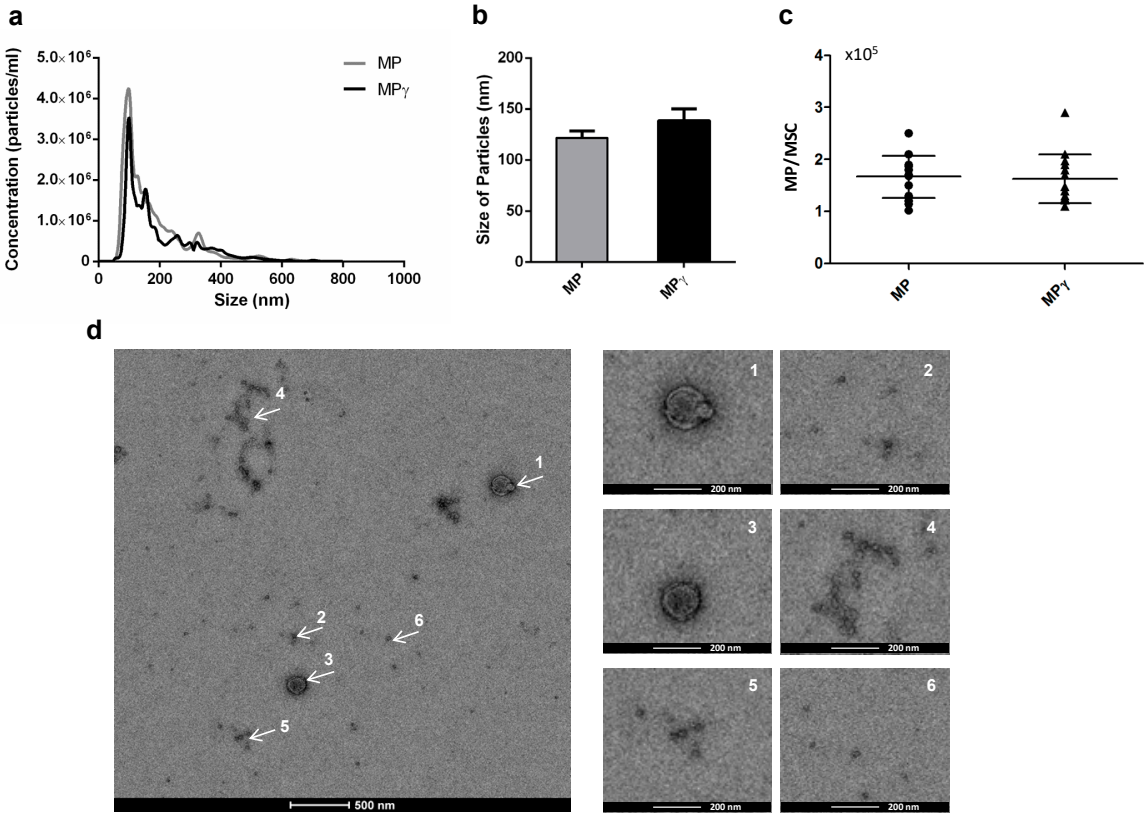

Figure 2. Characterization of Membrane Particles generated from unstimulated and IFN-γ stimulated AT-MSC (MP and MPγ, respectively).
(A) Nanoparticle tracking analysis (NTA) profiles of MP and MPγ. The NTA software generates a distribution graph on a particle-by-particle basis, a count (in terms of absolute number and concentration), and (B) size distribution of MP and MPγ. (C) The average number of particles generated per MSC. Data are presented as mean ± SD from 10 independent preparations of MP. There was no statistical difference with respect to concentration and size between MP and MPγ. The statistic test used was unpaired t-test. (D) Transmission electron microscopy analysis of MP. White arrows point to areas zoomed in on at the images on the right side. Most of the MP showed a round shape and a size below 200 nm.

an additional control, MP were diluted before CFSE staining. The results shown indicate the recording of all samples during 1 min. The number of detected particles decreased for more diluted samples, but the MFI of the CFSE staining of the particles remained the same. This means that single MP can be detected with the used flow cytometry strategy. Fluorescent-based flow cytometry protocols were recently described in literature [29,30].

Effects of Membrane Particles on T cell proliferation

CFSE loaded human peripheral blood mononuclear cells (PBMC) stimulated with anti-CD3/antiCD28 antibodies were cultured with different ratios of MP for 4 days (1:5,000, 1:10,000, 1:40,000, 1:80,000). To analyze lymphocyte proliferation, CFSE dilution was measured in CD4+ and CD8+ T cells. Addition of increasing concentrations of MP or MPγ did not affect the proliferation of CD4+ and CD8+ T cells (Figure 4a and b).

Membrane Particles decrease the proportion of CD16+ monocytes and increase CD90+ and PD-L1+ monocyte subsets

Monocytes were cultured with different ratios of MP for 24 h (1:10,000, 1:40,000, 1:80,000) to determine whether MP could affect monocyte cell surface markers expression and immune function. Monocytes were cultured in polypropylene tubes to avoid the adherence of the cells and differentiation into macrophages. Culture of monocytes in the presence of MP or MPγ treatment decreased the frequency of pro-inflammatory CD14+CD16+ cells at ratios of 1:40,000 (by 45% and 49%, respectively) and 1:80,000 (by 48% and 35%, respectively) (Figure 5A). Monocytes treated with MP at ratios of 1:40,000 and 1:80,000 furthermore increased the expression of CD90 by 17% and 25%, respectively. Meanwhile, the MPγ group showed an increase in CD90 expression at ratios of 1:10,000 by 8%, 1:40,000 by 16% and 1:80,000 by 20% (Figure 5B). Moreover, MPγ treatment induced anti-inflammatory PD-L1 expression in monocytic cells by 16% at a 1:10,000 ratio, 43% at a 1:40,000 ratio and 62% at a 1:80,000 ratio. MP had a smaller effect on PD-L1 expression with a 15% increase at a ratio of 1:40,000 (Figure 5C).

Membrane Particles affect the expression of pro- and anti-inflammatory genes in monocytes

In order to examine the effect of MP on monocyte immune function, and to examine whether the immunophenotypic changes observed were a result of protein transfer or of gene expression regulation, mRNA expression of a number of

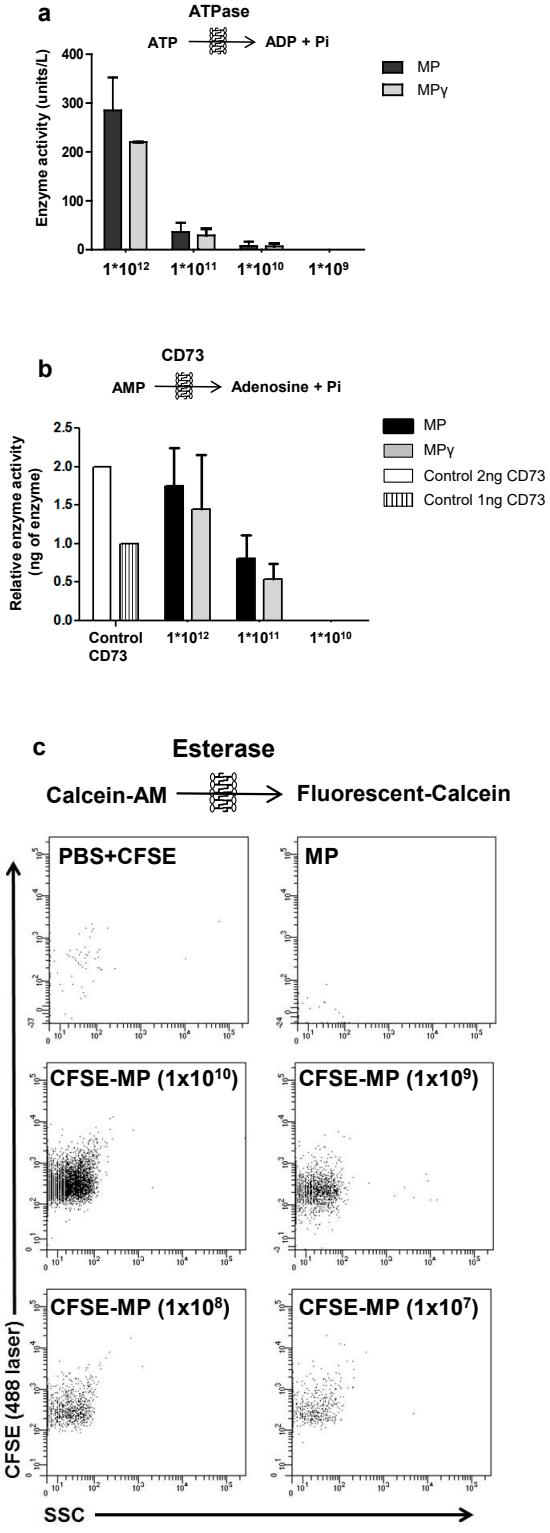

Figure 3. Enzymatic activity of Membrane Particles

(A) ATPase activity was measured at four different concentrations of MP (1 x 1012, 1 x 1011, 1 x 1010 and 1 x 109 particles/ml). MP and MPγ were able to catalyze the breakdown of ATP and the detection of free phosphate was dependent on the concentration of MP. (B) The nucleotidase activity of the MSC marker CD73 was measured for three concentrations of MP (1 x 1012, 1 x 1011 and 1 x 1010 particles/ml). MP and MPγ were able to produce free phosphates after adding AMP substrate in a dose-dependent fashion. CD73 enzyme (2 and 1 ng) was used to calculate the concentration of CD73 in the MP. There was no statistical difference in enzyme activity between MP and MPγ. (C) Esterase activity of three concentrations of MP (1 x 109, 1 x 108 and 1 x 107 particles/ml) was measured by the conversion of CFDA-SE to CFSE by flow cytometry. Fluorescent events were observed in MP labeled with CFSE (CFSE-MP), and the number of CFSE-MP detected was dependent on the concentration of MP. There was no statistical difference between MP and MPγ in esterase activity. Controls (PBS+CFSE and non-labeled MP) were negative. Data are presented as mean ± SD. Enzyme activities were detected in MP generated from 5 different MSC donors.

genes with pro- and anti-inflammatory function was analyzed in monocytes by qPCR after 24 h of stimulation with MP. Upregulation of CD90 gene expression as a result of particles stimulation was observed in MP and MPγ treated monocytes ($p < 0.05$) (Figure 5D). Moreover, expression of the anti-inflammatory factors IDO and PD-L1 was increased in monocytes treated with MPγ, but not MP ($p < 0.05$) (Figure 5D). There was a trend for increased expression of IL-6 after MP and MPγ treatment, but this was not significant. Significant changes in gene expression were also not observed for the pro-inflammatory cytokines TNF-α and anti-inflammatory cytokine IL-10.

Membrane particles induce selective apoptosis of pro-inflammatory CD14+CD16+ monocytes

Monocyte incubated for 24 h with MP and MPγ (1:10,000, 1:40,000, and 1:80,000 ratios) were analyzed by flow cytometry for apoptosis by Annexin V staining. MP and MPγ did not significantly induce apoptosis in classical monocytes (CD14+CD16–) (Figure 6A). However, pro-inflammatory monocytes (CD14+CD16+) showed an increase ($p < 0.05$) in apoptosis after incubation with MPγ at a ratio of

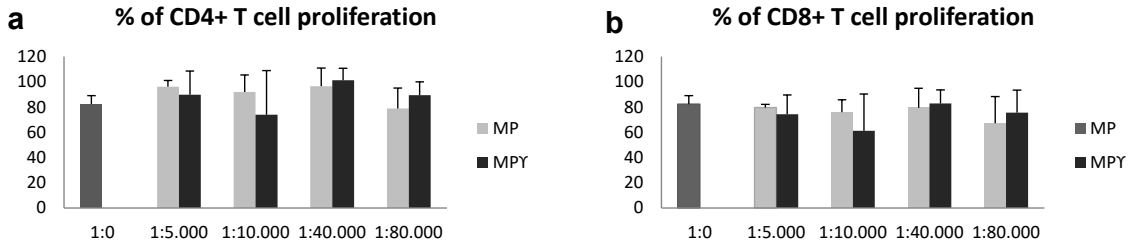

Figure 4. Effect of Membrane Particles on lymphocyte proliferation.
CFSE loaded PBMC stimulated with anti-CD3/antiCD28 antibody were cultured with different ratios of MP for 4 days (1:5,000, 1:10,000, 1:40,000 and 1:80,000). CFSE dilution in CD4+ and CD8+ T cells was measured. (A and B) Addition of MP or MPγ did not affect the proliferation of CD4+ and CD8+ T cells. (n = 8; mean ± SD). Two-way ANOVA was used for statistical analysis.

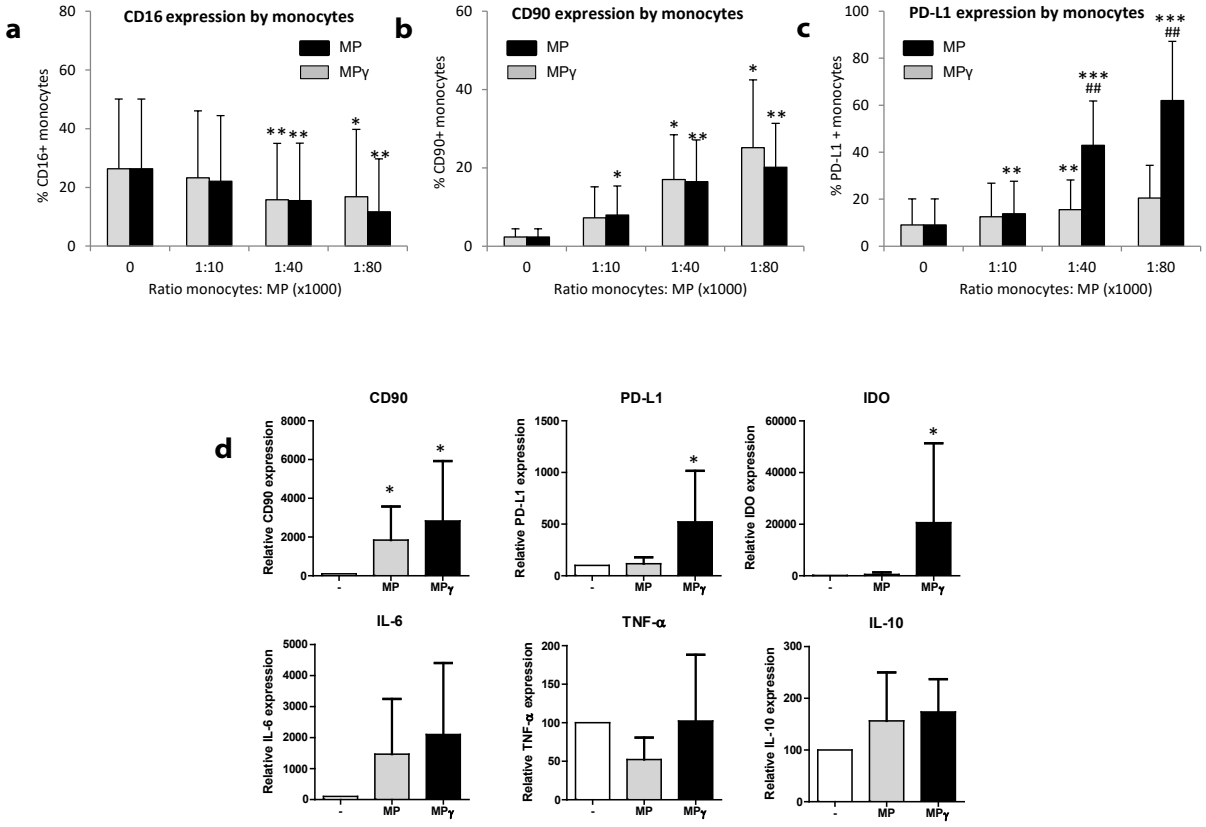

Figure 5. Effect of MP on CD14+ cells.

Monocytes were cultured with different ratios of MP for 24 h (1:10,000, 1:40,000 and 1:80,000) to determine the effect of MP on monocyte immunophenotype. (A) Expression of CD16 on monocytes cultured in the presence of MP or MPγ (n = 6; mean ± SD). (B and C) Monocyte cell surface levels of CD90 and PD-L1 in the presence of MP or MPγ (n = 7; mean ± SD). (D) mRNA expression of monocytes after culture with MP. After 24 h of culture with MP or MPγ, monocytes were separated from MP and assessed by real-time RT-PCR for CD90, IDO, PD-L1, IL-6, TNF-α and IL-10 expression (n = 6; mean ± SD). Multiple comparison test (two-way ANOVA) was used for statistical analysis, *p < 0.05, **p < 0.01 and ***p < 0.001 vs control; #p < 0.05 and ##p < 0.01 vs MP group.

1:40,000, and after incubation of MP and MPγ at ratios of 1:80,000 (Figure 6B). This indicated that MP specifically induce apoptosis of pro-inflammatory monocytes.

Monocytes but not lymphocytes are able to take up Membrane Particles

Since the previous results showed that MP had immunomodulatory properties on monocytes but not on lymphocytes, we analyzed the interaction of MP with both types of immune cells. For that purpose, MP labeled with PKH membrane dye were added to PBMC (ratio 1:40,000) and incubated during 1 h and 24 h at 37 °C. As a control the cells were incubated at 4 °C, at which temperature no active uptake of MP is expected. A representative flow cytometry analysis is showed in Figure 7A and B.

1 h after the addition of MP, a small percentage of CD3-lymphocytes (1.3 ± 0.2%) were positive for PKH-MP (Figure 7C) while 20 ± 5.3% of CD14-monocytes was able to uptake MP ($p < 0.05$) (Figure 7D). The difference between the MP uptake by monocytes and lymphocytes was higher after 24 h (lymphocytes: 5.2 ± 1.4%,

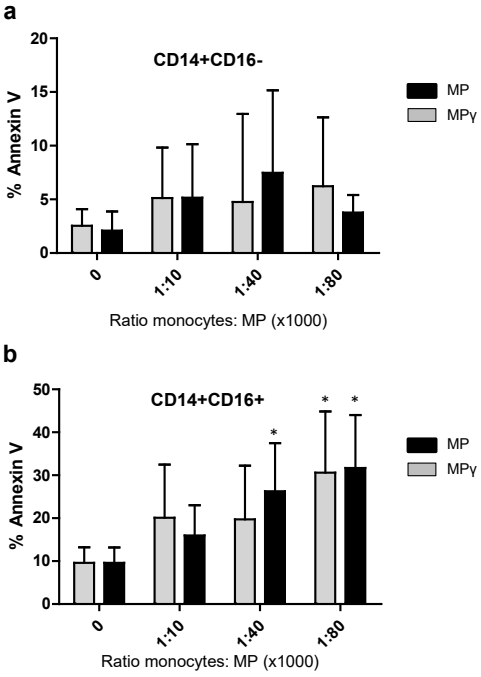

Figure 6. Effect of MP on apoptosis of monocyte subsets measured by Annexin V staining.
Monocytes were cultured overnight with 3 ratios of MP or MPγ (1:10,000, 1:40,000 and 1:80,000). (A) Percentage of Annexin V positive CD14+CD16- classical monocytes, and (B) percentage of Annexin V positive CD14+CD16+ pro-inflammatory monocytes. Data represent mean ± SD of 5 experiments using MP from 3 different donors. Two-way ANOVA was used for statistical analysis. P values (*$p < 0.05$) refer to the control without MP.

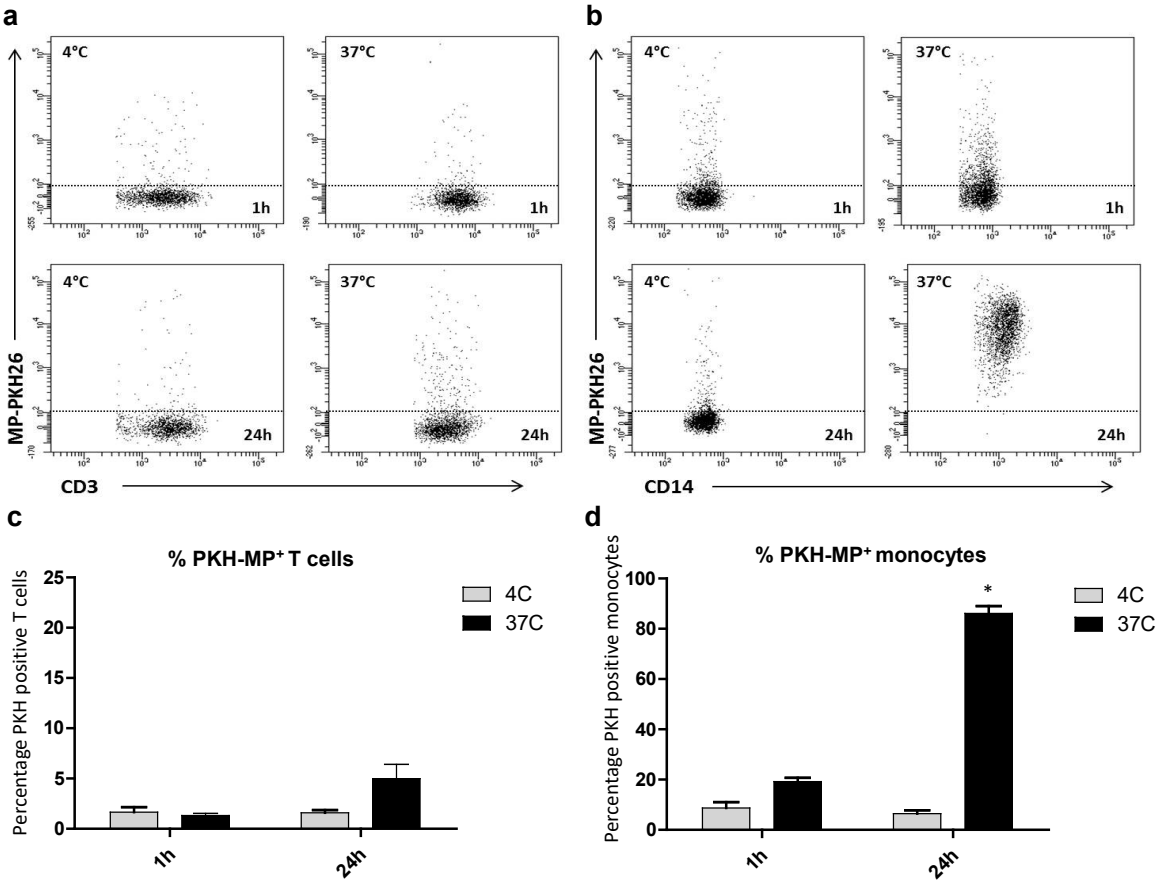

Figure 7. Uptake of MP by monocytes. MSC were labeled with PKH-26 before generation of MP (PKH-MP).

PKH-MP were added to PBMC (ratio 1:40,000) and incubated for 1 h and 24 h at 37°C. As a control the experiment was incubated at 4°C. (A and B) Representative flow cytometry analysis of PKH-MP uptake by lymphocytes (CD3) and monocytes (CD14) at time points 1 h and 24 h at 4°C and at 37°C. (C) Percentage of CD3+ T cells positive for PKH-MP, and (D) Percentage of CD14+ monocytes positive for PKH-MP. Data are presented as mean ± SD from 6 experiments. Two-way ANOVA was used for statistical analysis. P values (*$p < 0.05$) refer to the 4°C control at the 1 h time point.

monocytes: 93 ± 4.3%; $p < 0.05$). The 4 °C control for uptake was always below 3% for monocytes and lymphocytes in all the time points. This result indicated that MP uptake was mediated in an energy-dependent process.
To examine whether MP could be internalized by monocytes, confocal immunofluorescence microscopy was performed with isolated CD14+ cells from PBMC. The membrane of the monocytes was labeled with PKH-67 and cultured with PKH-MP (1:40,000). Time-lapse recordings showed that MP bound to the plasma membrane of the monocytes but they were not internalized. To look in detail at the localization of MP on the monocytes, z-stack images were analyzed by confocal microscopy (Figure 8). These images confirmed that MP remained localized to the cell surface of the monocytes.

Discussion

The immunomodulatory capacity of MSC is often attributed to the secretion of soluble factors [11]. We recently demonstrated that inactivated MSC without the capacity to secrete factors can modulate immune responses in vitro and in vivo [9]. Inactivated MSC showed similar bio-distribution as living MSC as both are trapped in the lungs following intravenous administration. Here, we went one step further and generated nanoparticles from the membranes of adipose tissue MSC with diverse immunomodulatory properties by induction of regulatory proteins on the plasma membrane after treating the MSC with IFN-γ.

To generate MP, supernatants of MSC cultures were discarded and the cells were washed several times with PBS. Hereby the inclusion of soluble proteins in the MP preparations is avoided, which is a major challenge in the field of natural extracellular vesicles (EV) and causing misinterpretation of results [31]. The isolation methods for obtaining EV allow the co-precipitation of proteins, and RNA associated to lipoproteins secreted by the cells [32]. These contaminations mask the functional properties of EV and hamper their therapeutic application. With our novel protocol, we avoid the inclusion of artefacts from soluble molecules, and make MP a good alternative to EV.

Nanosight technology and electron microscopy were used for the characterization of MP. Most of the MP showed a size below 200 nm, and a round shape. Both

5 µm

characteristics make the MP an attractive therapeutic tool. Firstly, because their small size MP can easily maneuver through the capillary network of the lungs and reach sites of action beyond the lungs. Secondly, their morphology (closed circular structures) would allow loading of MP with compounds of interest and use MP as a delivery vehicle for future applications. Sun et al. provided evidence that anti-inflammatory drugs can be loaded into EV from myeloid cells and thereby enhance the delivery of the drug to activated monocytes in a LPS-induced septic shock model [33]. The use of MP from MSC as a natural delivery vehicle would have the advantage that the vehicle per se show immunomodulatory properties, which gives the carrier additional value. It is also important to consider that the production and manipulation of MP is easier and cheaper than the methodology used for the collection of EV, as it is possible to generate about 1.5 × 105 particles/cell.

In addition to their morphological characteristics, MP were shown to possess enzyme activity. It has been reported that extracellular vesicles from MSC have a cargo rich in enzymatically active glycolytic enzymes, ATPases, and ATP-generating enzymes, such as adenylate kinase and nucleoside-diphosphate kinase [34]. Enzymatic activity has been demonstrated to be important for modulating the conditions in the vesicle nano-environment by consuming or generating metabolic energy. Katsuda et al. demonstrated the unique potential of extracellular vesicles from adipose tissue derived MSC for treatment of Alzheimer's disease. These authors found that these extracellular vesicles carry Neprilysin, a metalloprotease, which ameliorates the disease's symptoms [35]. We showed that MP possess nucleotidase and esterase activity, which are major enzymes regulating immunity and inflammation [21,22].

Lymphocyte proliferation is the most commonly used assay to demonstrate the immunomodulatory capacity of MSC and it has been used as a standard assay

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Figure 8. Confocal microscopy analysis of MP uptake by monocytes at 24 h.
Z-stack images were collected at 1.2 µm intervals ranging from 0 to 17.6 µm. Staining for monocyte membrane (green), MP (red), and nucleus (blue) shows that MP are localized on the membrane of the monocytes (white arrows) and are not internalized. Scale bars: 5 µm.

to compare the immunosuppressive effect of MSC from different tissue sources. Comparative studies have sometimes however produced conflicting results. Puissant et al. have reported similar inhibition of T cell proliferation by bone marrow and adipose tissue MSC [36], whereas Ribeiro et al. found that adipose tissue MSC to have stronger suppressive effects than bone marrow and umbilical cord MSC8. In pilot experiments, we generated MP from bone marrow derived MSC. These MP demonstrated similar properties as MP from adipose tissue MSC.

The mechanisms through which MSC suppress lymphocyte proliferation are largely dependent on soluble mediators. In our study, we found no effect of MP on lymphocyte proliferation. This can be explained by the fact that MP cannot secrete soluble factors, but also because lymphocytes were shown to be unable to bind or uptake MP. However, MP induced modulation of monocyte cell surface markers expression and changed their immune function. Furthermore, MP and MPγ induced the selective apoptosis of proinflammatory CD14+CD16+ monocytes.

CD16+ monocytes are major producers of inflammatory cytokines such as TNF-α and IL-12 [37,38] and high numbers of CD16+ monocytes are associated with acute and chronic inflammatory conditions [39]. Our results therefore suggest that MP act as immunomodulators that eliminate pro-inflammatory monocytes. Importantly, we also found that the immunomodulation induced by MP and MPγ is different. MPγ but not MP increased PD-L1 in the membrane of the monocytes and the mRNA expression of the anti-inflammatory factor IDO. Thus, the modification of the membrane protein composition of MSC by treatment of the cells with various stimuli provides us the opportunity to generate MP adapted for treatment of a specific immunological disorder. For example, MPγ with their enhanced capacity to induce PD-L1 and IDO by monocytes may be suitable for treatment of more severe immune responses involving inflammatory monocytes, while MP derived from MSC pre-treated with factors that induce proteins with regenerative function may be useful for inducing regenerative processes after resolving inflammation. As there is a lot of knowledge about modulation of MSC properties by cytokine treatment, there are tools in hand to control the make-up of MP. Thus, the potential therapeutic applications of MP are far reaching.

We demonstrated that the interaction of MP with monocytes is by binding and fusion with the plasma membrane of the monocytes. This is an active and specific mechanism for monocytes because at low temperatures MP were unable to fuse

with the monocyte membranes. It is furthermore specific because MP do not bind to lymphocytes. The confocal microscopy images showed that there is no internalization of MP into monocytes, indicating phagocytosis plays no role in the uptake of MP. The mechanism of binding and fusion of MP with monocyte membranes supports the idea that MP can be a natural delivery vehicle for monocyte-targeting drugs.

In conclusion, MP represent a therapeutic strategy that combines the potential of MSC therapy with reduced risks associated with the use of living cells and improved ability to reach sites beyond the lungs. Our data demonstrates that MP target monocytes, via which they may have a broad immunomodulatory effect (Figure 9). These data suggest that MP can serve as a novel cell-free therapeutic for treating immunological disorders. Additional studies, both in vitro and in vivo, are needed to improve our understanding the mechanisms of action of this potential immunosuppressive tool.

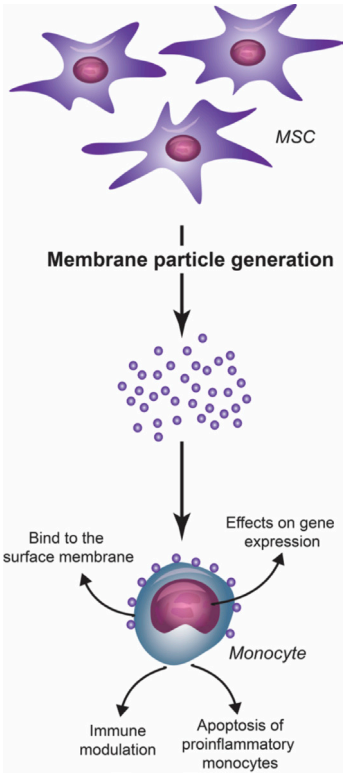

Figure 9. Schematic overview of the interaction of MP with monocytes
MP generated from MSC bind to monocyte plasma membranes. As an effect of the MP-monocyte interaction, MP modulate monocyte function by affecting gene expression and inducing apoptosis of pro-inflammatory monocytes.

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Chapter 8

Summary and Discussion

Summary

Mesenchymal stem or stromal cells (MSC) are adult stem cells that possess immunosuppressive capacities. Over the last decade, MSC have been under the investigation as immunotherapy in a wide range of immunological diseases. The most common route of administration of MSC is via IV injection. Upon IV injection MSC are trapped in the micro capillary network of the lungs and within 24 hours the majority of the MSC have disappeared. Despite this short survival time and limited bio-distribution, long term immunomodulatory effects have been observed in experimental animal models **(chapter 1)** as well as in clinical trials. However, clinical MSC trials are often limited to small number of patients and tested in wide variety of diseases that are caused by different immune cells and have different disease readouts. In **chapter 2** a systematic review was conducted of clinical studies using MSC with the purpose of immunomodulation to investigate the efficacy of MSC therapy. Although some of the included studies showed amelioration of disease symptoms after treatment with MSC therapy, other studies failed to show a positive effect of MSC therapy. Moreover, measurements of immunological parameters in blood of patients after MSC treatment were largely inconsistent between studies, mainly because of the large differences in study design, patient population and timepoints of the measurements. The results of this review underline that currently there is still a lack in understanding of the mechanisms of action of MSC immunotherapy.

In **chapter 3** the effect of an inflammatory environment on MSC was examined as they are likely exposed to inflammatory factors upon infusion in patients that suffer from acute or chronic inflammatory diseases. In this chapter, it was shown that inflammatory signals alter the effect of MSC on B cells. MSC have a stimulatory effect on B cell proliferation and regulatory B-cell formation in an immunological quiescent environment. Under inflammatory conditions, MSC inhibit B cell proliferation and plasmablast formation, while the induction of regulatory B cells is reduced. One of the molecular pathways involved is the tryptophan catabolic pathway via break down of tryptophan (TRP) through indoleamine 2,3-dioxygenase (IDO). The depletion of TRP leads to an inhibition of B cell proliferation but also prevents regulatory B-cell formation. These data show that immunological conditions can dictate the effect of MSC on B cell function.

Apart from the inflammatory signals that MSC might encounter, cells are also greatly altered by culture conditions. In **chapter 4** we show that cryopreservation of MSC induced minor gene expression changes involved in innate immunity

pathways and cytoskeletal rearrangement and increased triggering of the instant blood-mediated inflammatory reaction. In this chapter, we furthermore showed that upon infusion, the lung microenvironment has a major effect on MSC genes reflecting a response to inflammatory signals. Upon IV administration, MSC change their phenotype and may potentially change their function.

These data show that upon infusion, there is immunological cross talk between entrapped MSC and tissue-resident immune cells and cells in the lung microvasculature seem to become activated upon encounter of MSC. In **chapter 5** we further investigated the response of host cells triggered by infused MSC. By infusion of MSC that were unable to respond to inflammatory signals or secrete immunomodulatory factors but preserved their cellular integrity [heat-inactivated MSC (HI-MSC)], we show that the immunomodulatory effect of MSC does not depend on their secretome or active crosstalk with immune cells, but on recognition of MSC by monocytic cells.

In **chapter 6** we further elucidated the interaction of MSC with monocytic cells upon infusion. Here we show that MSC are rapidly cleared upon infusion through phagocytosis by hosts neutrophils, lung resident macrophages and circulating monocytes. Subsequently, MSC-primed monocytes change their phenotype towards an immunosuppressive phenotype and migrate from the lungs to the bloodstream and the liver. These data show the fate of MSC upon IV infusion.

Immunotherapy with living MSC comes with challenges as in vitro expansion of MSC is labor intensive and time consuming. In **chapter 7** we generated membrane particles from MSC, which are smaller than MSC and will thus pass the lung microvasculature and are easier to store. In in vitro cultures, these particles bind to monocyte plasma membranes and modulate monocyte function by affecting gene expression and inducing specific apoptosis of pro-inflammatory monocytes.

Discussion

In this thesis the mechanisms of actions of MSC immunotherapy were investigated. Promising results of in vitro experiments and experimental animal models have led to clinical studies investigating primarily safety and secondly immunomodulatory efficacy of MSC therapy in immunological diseases. Although the safety of clinical MSC therapy is well established by now [1] the efficacy of MSC immune therapy for the treatment of immunological diseases is not yet clear. Difficulty in drawing decisive conclusions is mainly caused by lack of sufficient

sample size and/or well controlled control groups in the majority of the clinical trials. Moreover, underreporting of clinical trials with neutral or negative outcomes leads to publication bias [2], variation in study setup, administrated cell dose, immunosuppressive co-medication and follow up time makes it difficult to draw conclusions about the immunological impact of MSC treatment. These studies underline that to obtain reproducible and consistent data on MSC therapeutic efficacy, larger studies including appropriate endpoints and standardized immune monitoring assays should be initiated [3]. Knowledge on the efficacy of MSC therapy is also hampered by the lack of understanding the mechanisms of action of MSC treatment upon infusion in patients. Better understanding of MSC based therapy helps to design optimal MSC therapeutic product and better tailored MSC therapy for different immunological diseases.

The present thesis and previous work show that the in vitro immunosuppressive and anti-proliferative effects of MSC are dependent on their pre-activation with inflammatory factors [4-7]. Priming of MSC with inflammatory factors is likely to occur after MSC administration as patients treated with MSC often suffer from acute or chronic inflammatory diseases. Tailor made MSC can be generated by mimicking inflammatory conditions in vitro by the addition of IFN-γ. Under these conditions, MSC suppress the activity of B cells, they reduce antibody production and inhibit B cell proliferation, but they also lose the capacity to induce Bregs. On the other hand, MSC promote B cell survival and Breg formation under immunological quiescent conditions. This way custom-made MSC can be generated with either B cell suppressive properties, or MSC that support B cell homeostasis that can be used for different immune diseases.

Culture expansion, necessary to obtain sufficient cells for MSC therapy, greatly affects MSC phenotype. Culture medium and plastic adherence have a major impact on MSC size and expression of (adhesion) molecules [8]. Due to these phenotypical changes, MSC get trapped in the lung capillaries upon IV infusion [9-11]. In the lung microenvironment, infused cells can change their phenotypical and functional properties in response to environmental stimuli [12]. As the majority of MSC disappears within 24 hours after infusion, intrinsic phenotypical changes may be of limited relevance for the therapeutic effect of MSC. Therefore, outcomes of in vitro studies of the effect of MSC on other cell types, for example on B cells, might differ from in vivo outcomes. Nonetheless, upon in vivo IV infusion, changes in secretion of soluble factors by MSC might affect tissue-resident host cells and these changes might even persist after disappearance of MSC. In this thesis it is shown that that upon infusion, the host lung microenvironment reacts

to MSC by secretion of inflammatory factors. Moreover, gene expression of both pro- and anti-inflammatory factors as well as neutrophil and macrophage markers was increased in the lung tissue just hours after MSC infusion [11, 13]. In this thesis it is shown that infusion of inactivated MSC that lost their capacity to actively secrete soluble factors evoked the same response on the lung microenvironment as living cells did. Moreover, even in a lipopolysaccharide induced sepsis model inactivated MSC retained their immunomodulatory capacity. Although it would be possible that disintegration of inactivated MSC leads to release of intracellularly contained cytokines and other factors, the disappearance of the cells within 24 hours indicates that these cells are actively cleared. These data imply that MSC affect host cells upon infusion and this affect is independent of the secretion of soluble factors. Furthermore, it demonstrates that MSC do not have to be able to respond to environmental challenges to mediate their effects. In chapter 6, data show that upon coculture, monocytes rapidly migrate towards and phagocytose MSC. This same phenomenon is seen in vivo where remnants of MSC can be found in lung resident macrophages and circulating monocytes as well as liver-resident Kupffer cells. This suggest that although the bio-distribution of infused MSC is limited to the lungs, MSC-derived signals travel throughout the body. Cleaning up the remnants of MSC polarizes monocytes towards an immunosuppressive "MSC primed" phenotype as also described by other papers [14, 15]. Monocytes can directly trigger immunosuppression through secretion of cytokines and chemokines affecting the local microenvironment but also indirectly trigger immunosuppression through induction of adaptive regulatory cells. It has previously been shown that MSC can induce Treg formation in vitro through secretion of a whole range of soluble factors such as HLA-G5CCL1, leukemia inhibitory factor (LIF), TGF-β, IL-1β and IL-2 [16, 17] well as through direct cell-cell contact (Notch-1 pathway). However, the induction of Tregs by MSC might differ in vivo as their short survival time prevents MSC to actively induce Tregs. Despite their limited presence, increased numbers of Tregs can be found in patients even weeks to months post MSC infusion [18-22]. In this thesis we show that "MSC primed" monocytes can increase Foxp3+CD25hiCD127-CD4+ Tregs. The capability of "MSC primed" monocytes to increase Foxp3+CD25hiCD127-CD4+ Tregs in vitro indicates that MSC merely function as catalysts for inducing long lasting immunoregulation in treated patients.

If the induction of immunosuppression by MSC upon IV infusion is caused by their rapid clearance, cell membrane dependent interactions of MSC with host immune cells seem to be largely responsible for the immunomodulatory effects.

The generation of small membrane particles from MSC can provide for a therapy that is safer, easier and less time consuming to generate. These particles may be able to pass the lung barrier and migrate throughout the body. Using particles eliminates the risks that come with the use of living cells. They can be tailored to bind to specific cell types, such as for instance endothelial cells or specific myeloid cell subtypes. This could lead to novel therapy with the same immunomodulatory effect as conventional MSC therapy.

Conclusion

Understanding the mechanisms of action of MSC immunomotherapy contributes to generating more effective and safer therapy and can help to better treat patients with different immunological diseases. This thesis shows that upon the most common way of infusion, MSC function mainly as catalysts inducing immunosuppression through the host's own immune cells. Monocytic cells seem to play an important role in the first step of inducing immunosuppression by altering their phenotype upon phagocytosis of MSC. Long term immunosuppressive effects seen in patients treated with MSC suggests that MSC primed innate immune cells induce a long term immune response by interaction with adaptive immune cells. Better understanding of this process to induce long term immunosuppression helps to provide novel and cell-free therapeutics to treat immunological disorders.

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Chapter 9

Nederlandse samenvatting

Mesenchymale stamcellen (MSC), ook wel Mesenchymale stromale cellen genoemd, zijn voorloper cellen die kunnen uitgroeien tot volwassen steunweefsels zoals bot-, kraakbeen- en vetcellen wanneer zij de juiste signalen hiervoor ontvangen. Op het lab kunnen we deze cellen isoleren uit vrijwel alle weefsels van zowel embryo's als volwassenen. Doordat MSC nog kunnen uitgroeien tot de verschillende celtypen van het Mesenchym is er veel onderzoek gedaan naar het gebruik van deze cellen voor regeneratieve therapie met het idee dat de MSC disfunctionele cellen in zieke organen van patiënten zouden kunnen vervangen. De laatste decennia is duidelijk geworden dat MSC ook in staat zijn om afweerreacties van het immuunsysteem te remmen. Deze eigenschappen maken MSC interessant als celtherapie voor bijvoorbeeld patiënten met immunologische ziekten of orgaantransplantatie patiënten Vooral in het laboratorium is veel onderzoek gedaan naar de werkingsmechanismen achter hoe MSC cellen van het immuunsysteem kunnen onderdrukken. Daarnaast laten klinische trials met MSC-therapie zien dat MSC-celtherapie veilig is voor patiënten. Echter, er is minder bekend over de werkzaamheid van MSC als behandeling van patiënten. Zo wijzen studies met diermodellen erop dat na het intraveneus (IV, in de ader) inspuiten van MSC, de grote meerderheid van de cellen na 24 uur niet meer aanwezig is in het lichaam. Desondanks de korte aanwezigheid van de cellen in het lichaam laten sommige klinische trials langdurige positieve effecten zien bij patiënten nadat zij met MSC behandeld zijn (**hoofdstuk 1**). Om een beter beeld te krijgen van de effectiviteit van MSC-celtherapie werden in **hoofdstuk 2** 62 klinische trials waarbij MSC als celtherapie gegeven werd aan patiënten met verschillende ziekten vergeleken. Er werd gekeken naar data die vermindering van symptomen bij patiënten beschreef en parameters die veranderingen van het afweersysteem van patiënten aanduiden. Sommige van de beschreven studies lieten verlichting van symptomen zien na behandeling met MSC maar bij andere studies werd geen zichtbaar effect gemeten. Daarnaast is het lastig om een conclusie te baseren op de studies omdat de studies erg verschillen in studie-opbouw, patiëntpopulatie en het moment van het meten van symptoomveranderingen. Ook zijn de patiënt groepen vaak klein en ontbreekt een goede controlegroep. De resultaten van deze review benadrukken dat er momenteel nog steeds een gebrek aan inzicht is in wat er gebeurd met het afweersysteem van de patiënten na het toedienen van MSC-celtherapie en hoe patiënten baat hebben van MSC-therapie.

In **hoofdstuk 3** werd het effect van een inflammatoire omgeving op MSC beschreven. Wanneer MSC worden ingespoten in patiënten is de kans groot dat zij blootgesteld worden aan een inflammatoire omgeving omdat de behandelde

patiënten vaak lijden aan acute of chronische inflammatoire ziekten. In dit hoofdstuk werd beschreven dat inflammatoire signalen het effect van MSC op B-cellen veranderen. B-cellen zijn immuuncellen die betrokken kunnen zijn bij orgaanafstoting na transplantatie door bijvoorbeeld de productie van donorspecifieke antistoffen en cytokines (celsignalererende moleculen). Na activatie kunnen B-cellen uitrijpen tot plasmablasten en plasmacellen die grote hoeveelheden antistoffen uitscheiden. Op basis van o.a. cytokine productie kunnen B-cellen worden onderverdeeld in verschillende populaties, variërend van effector B-cellen en regulatoire B-cellen die de immuunreactie kunnen onderdrukken. Onder normale omstandigheden hebben MSC een stimulerend effect op B-cel expansie en regulatoire B-celvorming. Echter, onder inflammatoire omstandigheden remmen MSC B-celproliferatie en plasmablastvorming, terwijl de inductie van regulatoire B-cellen wordt verminderd. In een inflammatoire omgeving zetten MSC tryptofaan (TRP) om in L-kynurenine via indoleamine 2,3-dioxygenase (IDO). De depletie van TRP in de omgeving leidt tot een remming van B-cel expansie maar voorkomt ook de aanmaak van regulatoire B-cellen. Deze gegevens tonen aan dat immunologische factoren het effect van MSC op B-celfunctie beïnvloeden.

Afgezien van de inflammatoire signalen die MSC kunnen tegen komen hebben ook de kweekomstandigheden in het laboratorium grote invloed op de cellen.

In **hoofdstuk 4** werd het effect van invriezen (cryopreservatie) op MSC onderzocht. Uit vergelijking van MSC uit kweek en vers ontdooide MSC bleek dat cryopreservatie weinig invloed heeft op de MSC genexpressie. Echter, cryopreservatie leidt tot een iets grotere kans op het in gang zetten van een directe bloed-gemedieerde ontstekingsreactie. Deze ontstekingsreactie zou de overlevingstijd van MSC na toediening verkorten en zou mogelijk kunnen leiden tot negatieve effecten op de gezondheid van patiënten. Na het inspuiten van MSC werden belangrijke veranderingen in de genexpressie van MSC gemeten die een reactie op inflammatoire activering weerspiegelen. Deze data laten zien dat er na het inspuiten van MSC sprake is van interactie tussen MSC en cellen in het long micromileu en dat immuuncellen in de long lijken te worden geactiveerd na het in aanraking komen met MSC.

In **hoofdstuk 5** is meer onderzoek gedaan naar de respons van long residente-cellen na interactie met MSC. Door infusie van hitte geïnactiveerde MSC (HI-MSC) werd laten zien dat het immuunmodulerende effect van MSC niet afhankelijk is van productie van cytokines of actieve interactie met immuuncellen, maar op herkenning van MSC door monocytische cellen. Deze cellen zijn gespecialiseerd

in het opruimen van resten van dode of beschadigde cellen, dit proces wordt fagocytose genoemd.
Deze interactie tussen MSC en monocytische cellen is vervolgens verder onderzocht en beschreven in **hoofdstuk 6**. Hier werd beschreven dat MSC binnen korte tijd na infusie worden opgeruimd door verscheidene fagocyten. Na het opruimen van MSC zien we dat monocyten (een type monocytische cel) van fenotype veranderen naar cellen met een regulatoir fenotype. Dit hoofdstuk toont het lot van MSC na infusie en het mogelijke werkingsmechanisme van MSC-celtherapie.
Aan immunotherapie met levende MSC zijn risico's verbonden zoals infectie van de kweek of mutatie van de cellen, daarnaast is het in vitro kweken van MSC een arbeidsintensief en tijdrovend proces. In **hoofdstuk 7** werden membraandeeltjes van de celwand van MSC gegenereerd. Deze deeltjes zijn kleiner dan MSC en zijn in tegenstelling tot MSC in staat om de haarvaten van de long te passeren. Tevens kan het genereren van membraandeeltjes van MSC zorgen voor een therapie die veiliger, gemakkelijker en minder tijdrovend is om te ontwikkelen. In kweekschaaltjes bonden deze deeltjes aan monocyten waarna zij de functie van deze monocyten konden moduleren door genexpressie te beïnvloeden en specifieke celdood van pro-inflammatoire monocyten te induceren. Door het op maat maken van membraandeeltjes die kunnen binden aan specifieke celtypen kan een nieuwe therapie met hetzelfde immuunmodulerende effect als conventionele MSC-therapie maar die minder risico's met zich mee brengt worden ontwikkeld.

Het begrijpen van de werkingsmechanismen van MSC-therapie draagt bij aan het genereren van een effectievere en veiligere therapie en kan helpen om patiënten met verschillende immunologische aandoeningen of na orgaantransplantatie beter te behandelen.

Samenvattend laat dit proefschrift zien dat MSC na IV inspuiten voornamelijk functioneren als katalysatoren die immunosuppressie induceren door de eigen immuuncellen van de gastheer. Monocytische cellen lijken een belangrijke rol te spelen in de eerste stap van het induceren van immunosuppressie door hun fenotype te veranderen na fagocytose van MSC. Langdurige immunosuppressieve effecten waargenomen bij patiënten behandeld met MSC suggereren dat fagocterende immuuncellen van de host na contact met MSC in staat zijn

een langdurige immuunrespons te induceren door interactie met andere immuuncellen.
Meer onderzoek over hoe deze langdurige immunosuppressie wordt geïnduceerd is noodzakelijk voor de ontwikkeling van betere en mogelijk cel-vrije therapieën voor de behandeling van immunologische ziekten en voor patiënten na orgaantransplantatie.

Appendices

Curriculum vitae auctoris
List of publications
PhD portfolio
Acknowledgements (Dankwoord)

Curriculum Vitae auctoris

Franka Luk was born on July 2nd 1988, in Gouda, the Netherlands. Together with her two younger sisters she was raised by her parents Joost Luk and Elise de Paus. In 2007 she graduated HAVO from the Goudse Scholengemeenschap Leo Vroman and started studying biology and medical laboratory research at the Hogeschool Leiden. In 2011, after obtaining her Bachelor of applied science, she started the research master Infection and Immunity at the Erasmus University in Rotterdam. In September 2013 she obtained her Master's degree after which she started her PhD project at the Transplantation Laboratory of the Internal Medicine Department, Division of Nephrology and Transplantation, at the Erasmus Medical Center, under supervision of dr. Martin J. Hoogduijn and prof. dr. Carla C. Baan. This research is presented in this thesis. In October 2018 she started her new job as project leader on education innovation at the Internal Medicine department of the Leiden University Medical Center under the supervision of prof. dr. Marlies E.J. Reinders.

List of publications

De Leur, K, **Luk F**, van den Bosch TPP, Dieterich M, van der Laan LJW, Hendriks RW, Clahsen - van Groningen MC, Issa F, Baan CC, Hoogduijn MJ. *The effects of an IL-21 receptor antagonist in a humanized skin transplant mouse model.* Manuscript in preparation

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* Both authors contributed equally to this study

Fabiany da C. Gonçalves, **Luk F**, Korevaar SS, Bouzid R, Paz AH, López-Iglesias C, Baan CC, Merino A, Hoogduijn MJ. *Membrane particles generated from mesenchymal stromal cells modulate immune responses by selective targeting of pro-inflammatory monocytes*. Sci Rep. 2017, 1:12100

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Hoogduijn MJ, de Witte SF, **Luk F**, van den Hout-van Vroonhoven MC, Ignatowicz L, Catar R, Strini T, Korevaar SS, van IJcken WF, Betjes MG, Franquesa M, Moll G, Baan CC. *Effects of Freeze-Thawing and Intravenous Infusion on Mesenchymal Stromal Cell Gene Expression*. Stem Cells Dev 8: 586-597, 2016.

Luk F, de Witte SF, Bramer WM, Baan CC, Hoogduijn MJ. *Efficacy of immunotherapy with mesenchymal stem cells in man: a systematic review*. Expert Rev Clin Immunol 11: 617-636, 2015.

Franquesa M, Hoogduijn MJ, Ripoll E, **Luk F**, Salih M, Betjes MG, Torras J, Baan CC, Grinyó JM, Merino AM. *Update on controls for isolation and quantification methodology of extracellular vesicles derived from adipose tissue mesenchymal stem cells*. Front Immunol 5: 525, 2014.

Eggenhofer E, **Luk F**, Dahlke MH, Hoogduijn MJ. *The life and fate of mesenchymal stem cells*. Front Immunol 5: 148, 2014.

PhD Portfolio

Summary of PhD training and teaching

Name PhD student:	Franka Luk
Erasmus MC Department:	Internal medicine
Research School:	Postgraduate school Molecular Medicine
PhD period:	2013 - 2017
Promotor:	Prof.dr. Carla C Baan
Co-promotor:	Dr. Martin J Hoogduijn

1. PhD training

General courses

2015	Research Integrity
2016	Biostatistical Methods I: Basic Principles (CC02)

Specific courses (e.g. Research school, Medical Training)

2014	Course on R
2014	Analysis of microarray and RNA seq expression data using R
2017	Adobe Indesign course

Seminars and workshops

2016	Transplantation seminar (Erasmus)	oral

Presentations at (inter)national conferences

2014	Mesenchymal stem cells in Solid Organ Transplan–tation (MiSOT), Bergamo, Italy	oral
2015	Science days, Dept. of internal medicine, Antwerp, Belgium	oral
2015	Molmed day, Rotterdam	poster
2015	Joined British transplantation society & Nederlandse Transplantatie Vereniging, Bournemouth, UK	oral
2015	European Society for Organ Transplantation (ESOT) congress, Brussels	oral
2016	International Society for Cellular Therapy (ISCT) congress, Seville, Spain	poster
2016	Bootcongres, nederlandse transplantatie vereniging, Groningen	oral
2016	Nantes Actualités Transplantation (NAT) annual event, Nantes, France	oral, chair and poster
2016	International congress of The Transplantation Society (TTS), Hong Kong	oral
2017	MiSOT, Regensburg, Germany	oral
2017	Science days, Dept. of internal medicine, Antwerp, Belgium	poster
2017	Molmed day, Rotterdam	poster
2017	bootcongres, Nederlandse Transplantatie Vereniging (NTV), Utrecht	oral
2018	ESOT, Barcelona, Spain	oral
2018	TTS, Madrid, Spain	oral

Travel grants and awards

2018	Mentor – mentee Award TTS, Madrid, Spain

Attendance at (Inter)national conferences

2014	Science days, Dept. of internal medicine, Antwerp, Belgium
2014	Molmed day, Rotterdam
2014	Annual meeting NTV (bootcongres), Leiden

2. Teaching

Supervising practicals and excursions, Tutoring

2014-2017	Lab rotations masters' I&I students
2014	Junior med school students
2015	Junior med school students

Other

2017	Published article for 'Frontiers for young minds' (children aged 13-15)
2016	Cover design for "Stem Cells and Development"

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