Probing the Immunomodulatory Potential of Mesenchymal Stromal Cells

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Probing the Immunomodulatory Potential of Mesenchymal Stromal Cells

Optimalisatie van het immuunmodulerende vermogen van mesenchymale stromale cellen

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Voor pappa

Je bent altijd in mijn gedachten

CONTENTS

1

General introduction and Outline

Current treatments for patients with immunological disorders or whom underwent solid organ transplantation consist out of long-term use of immunosuppressive medication with adverse effects such as nephrotoxicity, diabetes, hypertension, infections and increased risk for malignancies (1, 2). In search of an effective alternative with less side effects, mesenchymal stromal cells represent a promising and novel immunotherapy due to their broad immune regulatory function. The aim of this thesis was to optimize the immune regulatory function of mesenchymal stromal cells and further elucidate their mechanism of action for therapeutic purposes.

Mesenchymal stromal cells

Stem cells have the capacity to self-renew as well as the ability to generate various types of differentiated cells. Embryonic stem cells are pluripotent cells, which means they have the ability to differentiate into all cell types (3). Adult stem cells are generally known to be restricted in their potential to differentiate and regenerate the tissues in which they reside (3). Therapeutic application of adults stem cells is currently more commonly used, because of the bigger risks (such as tumorigenic risk) as well as ethical concerns associated with the use of embryonic stem cells (4, 5).

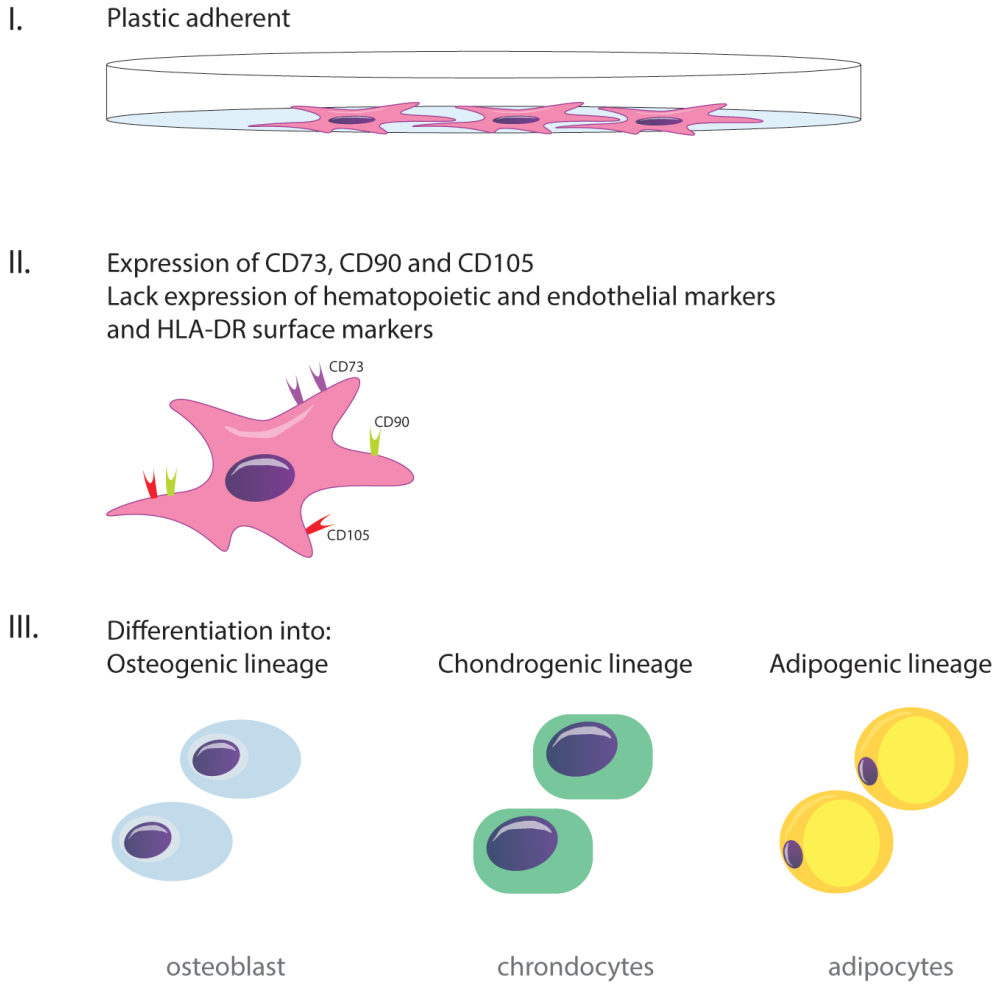
Mesenchymal stem cells are adult stem cells that are present in connective tissues throughout the body (6-13). They are also commonly referred to as mesenchymal stromal cells (MSC) and their name refers to their ability to differentiate into cells of the mesenchymal lineages. MSC are a heterogeneous population, which can be culture expanded *in vitro* after isolation. A definition of what constitutes MSC was outlined in 2005 by International society for cellular therapy (ISCT) (14). Since then, it has been widely adopted for the identification of MSC.

The ISCT guidelines state that MSC (Figure 1):

I. Must be plastic-adherent, during standard culture conditions
II. Must express CD73, CD90 and CD105, and lack expression of hematopoietic and endothelial markers such as of CD45, CD34, CD14 or CD11b. They must also lack CD79α or CD19 as well as HLA-DR surface molecules

III. Must be able to undergo osteogenic, chondrogenic and adipogenic differentiation *in vitro*

Due to their multi-lineage differentiation potential *in vitro* (15), MSC were initially widely studied for their therapeutic potential in regenerative medicine. More recently, it has come to light that MSC can play an important role in the regulation of the immune system. In addition, certain MSC subsets have been identified, based on their expression of surface markers such as Stro-1, CD73, CD90 or CD271, which possess enhanced immunosuppressive capacities (16-20). Interactions between MSC and the immune system are described in brief below. A more extended overview of the immunomodulatory properties of MSC can be found in chapter 2 of this thesis.



▲**Figure 1 ISCT guidelines. I.** MSC should be plastic adherent during standard culture conditions. II. MSC must express CD73, CD90 and CD105 and lack expression of hematopoietic and endothelial markers and HLA-DR surface markers. III. Also MSC must be able to undergo osteogenic, chondrogenic and adipogenic differentiation.

MSC and the immune system

Immunogenic characteristics of MSC

MSC are considered to be immune-privileged due to their immunophenotype: low expression of major histocompatibility complex (MHC)-I and lack of expression of MHC-II and co-stimulatory molecules (6, 21, 22). The lack of expression of co-stimulatory molecules prevents activation of T-lymphocytes. Also, complex formation of MHC-I on MSC with inhibitory Natural Killer (NK) cell receptors protects them against NK cell lysis (both in an allogeneic and autologous setting) (23). Their low immunogenicity gives rise to the opportunity to use allogeneic MSC as a therapy, without inducing allogeneic responses. However, it has come to light that MSC are not as immune-privileged as previously thought. Studies have shown that MSC can induce anti-HLA sensitization as their expression of MHC-I can lead to recognition by $CD8^+$ T-cells (24, 25).

Regulation of immune cells by MSC

The majority of what is known about interactions between the immune environment and MSC comes from *in vitro* studies with expanded MSC. MSC can reportedly regulate both cells of the innate and the adaptive immune system via secretion of soluble factors (pro- and anti- inflammatory), via their surface markers by receptor/ligand interaction, or metabolic pathways (Figure 2).

Interaction with innate immune cells

Dendritic cells (DCs) have an antigen presenting role towards naive T-cells following DC maturation. MSC have been reported to be able to inhibit the maturation of DC (26-29) as well as their antigen-presenting function, by decreasing DC expression of MHC-II, CD11c, CD83 and co-stimulatory molecules and their production of IL-12 (29-31). NK cells play a key role as effector cells in innate immunity with high cytolytic activity and production of pro-inflammatory cytokines. MSC can activate inhibitory NK cell receptors due to MHC-I expression (23, 32) and downregulate the expression of activating NK cell receptors: NKp30 and natural-killer group 2, member

D (NKG2D) (24). Furthermore, MSC have shown to have beneficial effects for neutrophils, one of the first responding phagocytes to pathogens, by promoting their survival (33, 34). Additionally, MSC also interact with other phagocytic cells: monocytes. MSC can recruit monocytes and in their presence monocytes skew towards an IL-10 producing phenotype that can induce regulatory T-cells due to IL6 and HGF secretion by MSC (35-37).

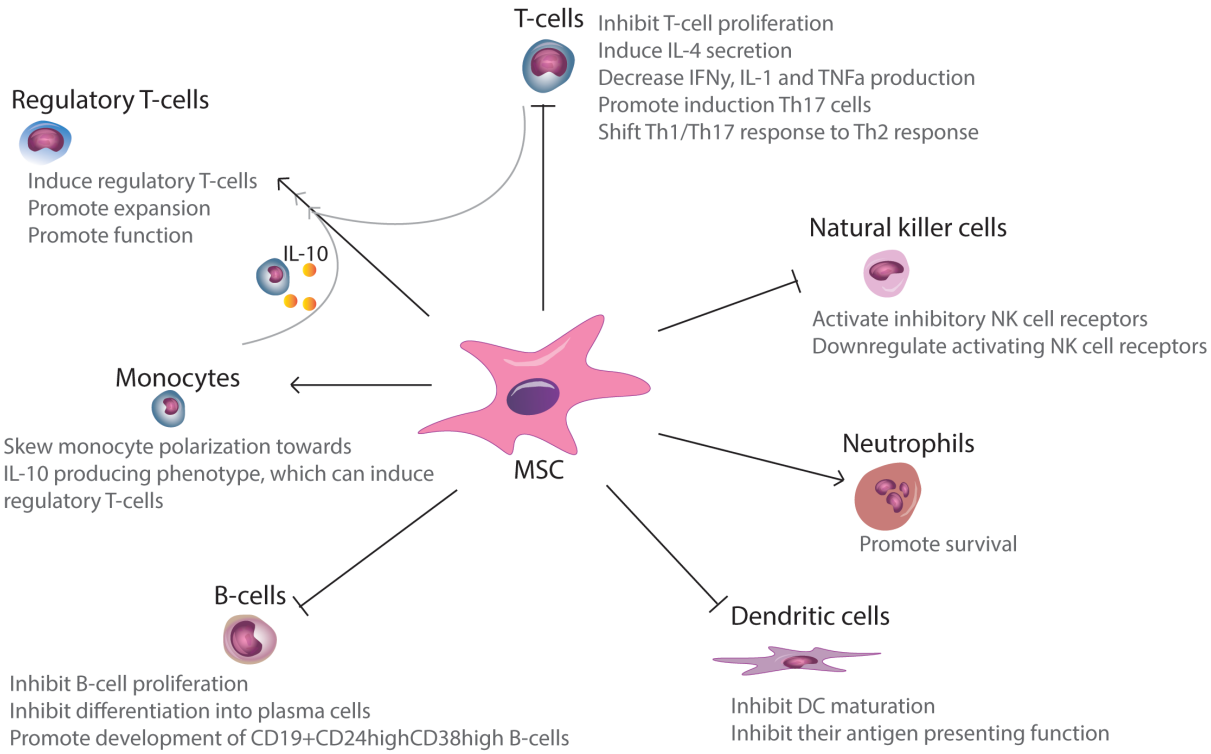
Interaction with adaptive immune cells

Next to regulating cells from the innate immune system, MSC have been reported to control cells from the adaptive immune system. MSC are able to suppress T-cell proliferation *in vitro* (38, 39), which is predominantly due to their expression of indoleamine 2,3-dioxygenase (IDO) (40). IDO is involved in tryptophan metabolism and depletes the milieu of tryptophan, which T-cells need to proliferate (39, 41). In addition, T-cells increase IL-4 secretion and decrease IFNγ, IL-2, and TNFα production in the presence of MSC (31). MSC also promote expansion and function of regulatory T cells (T-reg) (42-46). Likewise, MSC direct the induction of T-helper 17 (Th17) cells and they can shift a Th1/Th17 response to a Th2 response (47-49). Furthermore, MSC inhibit B-cell proliferation and their differentiation into plasma cells (50, 51). Also, while MSC reduce plasmablast formation, they can promote the development of $CD19^{+}CD24^{high}CD38^{high}$ B-cells) that have regulatory properties via the secretion of IL-10 (52).

Therapeutic MSC

Thus MSC have a broad immune regulatory function and can be expanded to large numbers *in vitro*, which make them suitable for immunotherapy. A variety of clinical studies have been performed with MSC isolated from bone marrow or adipose tissue and more recently from umbilical cord tissue, in graft-versus-host disease patients (53, 54), in patients after solid organ transplantation (55-58), with rheumatoid arthritis (47) or inflammatory bowel disease (59, 60). Recently, MSC have shown efficacy in a phase 3 trial in complex perianal fistulas in patients with Crohn's disease (61, 62). Furthermore, MSC are also investigated in patients with liver diseases, such as liver

cirrhosis (63-66), acute-on-chronic liver failure (67-69). Safety of systemic MSC administration has already been confirmed (70-72). And while promising results have been reported in the various studies described, most studies have not yet been able to demonstrate true efficacy of immunotherapy with MSC (73). This is partly due to a lack of understanding of the mechanisms of action of MSC therapy.



▲Figure 2 Summarizing figure of interactions between MSC and innate immune and adaptive immune cells.

Requirements for effective immunotherapy with MSC

The diversity in clinical trials makes it difficult to compare results and determine efficacy of MSC as an immunotherapy (73). This is attributed to the many considerations that need to be taken into account, which lead to a great diversity in the experimental designs. For instance, MSC can be isolated from various types of tissues (from autologous or allogeneic origin) and they may be used directly after isolation or expanded first. Expansion generates a bulk of doses per donor, but MSC alter their properties during culture expansion, which raises concerns about potential loss of their immunomodulatory properties during extensive expansion (74, 75). Furthermore, MSC may be administered directly after removal from their culture flasks or cryopreserved until administration, resembling an off the shelf product, but

again this may raise concerns on the effect of cryopreservation on MSC (76). The route of administration is a topic of discussion as it affects their bio-distribution. Commonly MSC are administered intravenously, however MSC subsequently get trapped in the lungs and hardly migrate any further (77). Alternatively, MSC may be infused intra-arterially or subcutaneously. Furthermore, frequency as well as the dose of MSC injections can be varied. Altogether, there are a lot of considerations that need to be taken into account for effective MSC immunotherapy. To complicate matters further, these considerations are likely to vary depending on the targeted disorder as disorders occur in a wide spectrum, with various pathophysiological properties that require different approaches.

The efficacy of MSC therapy may be improved by modulating MSC prior to their application. MSC have shown to be able to alter their properties in response to changes in the microenvironment. Upon *in vitro* expansion on plastic, MSC increase in size and have a high proliferation rate. Also, MSC upregulate their expression of MHC-I, MHC-II and PD-L1, increase IL6 secretion and IDO activity under inflammatory conditions (78, 79). Their potential to alter their properties can be used in our advantage for the development of a more effective therapeutic product. Activating the immunomodulatory properties of MSC prior to their clinical application may result into an immunotherapy which is highly effective.

For the development of MSC into an effective immunotherapy, elucidation of the mechanism of action of MSC is crucial. Yet to this moment, their mechanism of action is not fully understood (80). It is known that administration of MSC via the intravenous route leads to them ending up in the lung microvasculature where they are detectable up until 24 hours post infusion, after which most cells have likely died (77). However their short presence does not appear to abolish their efficacy (53, 81). Recently, an *in vivo* study by Galleu et al. demonstrated that T-cell induced cell death of MSC is crucial for the immunosuppressive effect seen by infusion of MSC (82). MSC death leads to the phagocytosis of MSC by phagocytes, which explains the rapid disappearance of MSC post infusion. Phagocytosis of MSC may affect the function of these phagocytes, which may play a part in the immunomodulatory action of MSC, even after their disappearance. Further elucidation of the fate and mechanism of action of MSC post IV infusion will be essential for predicting the clinical outcome and for the development of an effective immunotherapy with MSC.

OUTLINE OF THIS THESIS

MSC are a promising cell type for immunotherapy. They are easily obtainable, can be safely administered and they have promising immunomodulatory properties. **Chapter two** will give an overview of the current knowledge on the immunomodulatory properties of MSC and how these can be activated *in vitro*. For the development of effective immunotherapy with MSC, therapeutic properties of MSC may be potentiated *in vitro* before use. Additionally, to develop an effective MSC-based therapy and understand clinical outcome, it is crucial to understand the mechanism of immunomodulatory action of MSC after IV administration. The aim of this thesis is to optimize MSC for therapeutic purposes, by predominantly focusing on their immunomodulatory and immunogenic properties, bio distribution, and moreover to further elucidate the mechanism of action of IV infused MSC.

The yield of cells when isolating MSC from various tissues is very low. To obtain clinically relevant numbers MSC are culture expanded prior to use. Upscaling MSC production, which means increasing the duration of culture expansion, is critical to meet the demand for large numbers of therapeutic MSC. However, MSC may change their properties during culture expansion, which raises concerns about their safety and the stability of their immunomodulatory properties. Therefore, in **chapter three** the effect of long-term culture expansion on the phenotype and functionality of MSC will be determined.

After culture expansion, MSC can be cryopreserved prior to their use in the clinic or directly used from continuous culture. The use of cryopreserved MSC is a more feasible option in the clinic, because it enables the use of MSC as an off the shelf product that can be used when needed. In **chapter four** the effects of cryopreservation on MSC will be analyzed. Furthermore, as MSC are trapped in the lung microvasculature post IV infusion the influence of the lung microvasculature milieu on MSC will also be analyzed.

MSC will be primed *in vitro* in **chapter five** with various cytokines, growth factors and culture conditions with the aim of generating MSC with enhanced properties for clinical application, including reduced immunogenicity and improved immunosuppressive capacity towards T-cells. Established read-out parameters for their phenotype and functionality *in vitro*, *in vivo* and *ex vivo*, will subsequently be

used to determine the different properties of primed MSC. Furthermore, changes in bio distribution of primed MSC after IV infusion in a mouse model will be investigated.

The development of immunotherapy with MSC requires standardization and quality control as well as a thorough understanding of the effects of priming and culture expansion on MSC. All this together necessitates in-depth characterization of MSC. Hence, in **chapter six** epigenetic changes that arise upon priming as well as culture expansion of MSC will be identified.

Elucidation of the mechanism of action of MSC will help the development of MSC into effective immunotherapy and how findings of the above mentioned chapters can be implemented in this development. In **chapter seven** the mechanism of action of IV infused MSC will be investigated by examining the fate of MSC post IV infusion, interaction with cells of the recipients' immune system and how they subsequently exert their immunomodulatory effect.

The brief presence of MSC post IV infusion raises the question whether they actively exert their immunomodulatory function or whether they are purely a trigger for immune cells, which subsequently carry on the immunomodulatory effect. Therefore, **chapter eight** will investigate whether active crosstalk between MSC and immune cells is required for the immunomodulatory effect of MSC. This will be done by comparing the modulation of inflammatory responses between MSC and inactivated MSC that have preserved cell membrane protein expression, but are metabolically inactive and lost the capacity to secrete factors.

Chapter nine will combine the findings from chapter five, seven and eight and investigates the interaction between monocytes and pre-treated MSC (primed and/or inactivated).

Chapter ten and eleven summarize and discuss the results obtained within this thesis.

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Towards development of iMesenchymal Stem Cells for Immunomodulatory Therapy

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Abstract

Mesenchymal stem cells (MSC) are under development as an immunomodulatory therapy. The anticipated immunomodulatory effects of MSC are broad, from direct inhibition of lymphocyte proliferation, induction of regulatory T and B cells, to resetting the immune system via a hit-and-run principle. There are endless flavours of MSC. Differences between MSC are originating from donors variation, differences in tissue of origin, the effects of culture conditions and expansion time. Even standard culture conditions change the properties of MSC dramatically and generate MSC that only remotely resemble their *in vivo* counterparts. Adjustments in culture protocols can further emphasize properties of interest in MSC, thereby generating cells fitted for specific purposes. Culture improved immunomodulatory MSC (iMSC) can be designed to target particular immune disorders. In this review we describe the observed and the desired immunomodulatory effects of MSC and propose approaches how MSC with optimal immunomodulatory properties can be developed.

Regulation of immune cells by mesenchymal stem cells

Mesenchymal stem cells (MSC) are stromal cells present in connective tissues throughout the body. Their name refers to their ability to differentiate into cells of the mesenchymal lineages, which may be their primary function. Then why would we like to use these cells for immunomodulatory therapy?

MSC play an important role in the regulation of the immune system. They are furthermore relatively easy to isolate and can be expanded manifold in culture. Although MSC themselves are not part of the immune system according to the established definitions (1), they interact with all immune cell types. They secrete a large range of anti-inflammatory as well as pro-inflammatory factors, among them cytokines, chemokines and prostaglandins, that target immune cells and affect their function (2). Among the most highly secreted immune regulatory factors are TGF-β and IL6, via which MSC direct the induction of regulatory T cells but also Th17 cells (3), the chemo-attractants IL8, CCL2 (MCP1), CCL8 (MCP2), and prostaglandins E2 and F1 (4). In particular PGE2 appears to play a central role in the immunoregulatory activity of MSC in several disease models by reprogramming macrophages to anti-inflammatory cells and shifting Th1/Th17 responses to Th2 responses (5, 6).

In addition to regulating immune responses via their secretome, MSC express cell surface molecules that undergo interaction with immune cells. For instance, the co-stimulatory and co-inhibitory molecules CD40 and Programmed Death Ligand 1 (PD-L1), respectively, are expressed on MSC via which they modulate immune cell activity and proliferation (7, 8). MSC also express immune cell adhesion molecules ICAM-1 and VCAM-1. The expression of these adhesion molecules allows recruitment of activated immune cells to close proximity of MSC, thereby increasing their exposure to anti-inflammatory signals from MSC (9).

In addition to targeting immune cells via soluble and cell membrane signalling pathways, regulation of metabolic pathways takes a central place in the control of immune responses by MSC. MSC are involved in tryptophan metabolism via the expression of indoleamine 2,3-dioxygenase (IDO). IDO depletes tryptophan from the milieu, which results in the inhibition of lymphocyte proliferation (10). MSC furthermore catabolise immune activating ATP to immune inhibiting adenosine via ecto-5'-nucleotidase (CD73), which is abundantly expressed on MSC (11), and thereby regulate the ability of immune cells to build up immune responses. In

contrast to human MSC, mouse MSC employ iNOS instead of IDO as a key molecule for immune regulation (12). There are additional differences between species in immune regulatory mechanisms that are involved in the effects of MSC (13). This stresses the fact that there are discrepancies between human and laboratory animal MSC.

Effects of the immune environment on mesenchymal stem cells

MSC do not express unique markers and are difficult to identify in tissues. The majority of MSC research is therefore performed on cells in culture. Culture conditions themselves have a large effect on MSC phenotype and function. MSC in culture gain manifolds in size and show shifts in cell surface marker expression (14). Furthermore, culture conditions are supporting a high proliferation rate of MSC whereas MSC *in vivo* are quiescent, except in situations of repair and growth. Most of what we know about the effect of the immune environment on MSC comes from studies on *in vitro* expanded MSC. It has been demonstrated that the immunomodulatory properties of MSC are strictly regulated by local conditions. Proteins such as PD-L1, IDO and IL6 are strongly upregulated under inflammatory conditions (15). The biological function of this is likely to counterbalance ongoing immune responses and preventing them getting out of hand. In the absence of inflammatory signals MSC have a mostly supporting function for the immune system (16). MSC support the survival of B cells via yet undetermined factors (17) and prevent T cells from undergoing apoptosis via the secretion of IL7 (18).

Next to their immune regulatory properties, MSC themselves may become targets of the immune system. While MSC express low levels of HLA and co-stimulatory molecules under standard culture conditions, expression levels are increased by inflammatory stimuli. When allogeneic MSC are used for therapy, this may potentially lead to anti-MSC responses. This has indeed been demonstrated in *in vitro* assays where $CD8^+$ T cell responses can be evoked by allogeneic MSC (19). *In vivo* experiments have demonstrated that antibody responses can be detected after repeated injections of allogeneic MSC (20). This indicates that under particular conditions care has to be taken when using MSC of allogeneic origin for therapy.

MSC are not only targets of the adaptive immune system but also of the innate immune system. There is clear evidence that autologous culture expanded MSC are

lysed by activated NK cells (21, 22). The cause of this may be properties acquired by MSC during culture expansion that trigger cytotoxic responses of NK cells. NK cells may in part be responsible for the short survival time of MSC after intravenous administration (23). They are however not solely responsible for the quick disappearance of MSC after infusion as MSC administered to immune deficient mice that lack T, B and NK cells also have a short survival time (23), indicating other cell types contribute to the clearance of MSC.

Thus MSC have a broad immune regulatory function, which makes them suitable for immune therapy. The properties of MSC are modified by culture conditions and environmental factors. This can be exploited to generate MSC that have optimal immunoregulatory properties accompanied by a low immunogenicity. Below we discuss which properties of MSC would be desirable for effective and safe therapy and how such MSC can be generated.

Considerations for the use of MSC for immunotherapy

When generating MSC for clinical therapy, there are lots of flavours to choose from. Therapeutic MSC are so far mostly isolated from bone marrow or adipose tissue, but recently umbilical cord has been identified as a rich source of highly proliferative MSC. There have been different approaches to the generation of MSC batches. The approach that is used for several university initiated studies is to generate a small number of low passage MSC doses, whereas the approach that is generally employed by industry-driven studies is to generate large amounts of doses per donor (24). Furthermore, there is the option to cryopreserve MSC before usage, which is the most practical and most widely used option, and there is the option to use MSC directly from culture. Studies have indicated that MSC undergo changes in proliferation rate, adhesion and bio-distribution after infusion in response to cryopreservation (25, 26), suggesting cryopreservation may hamper the therapeutic effect of MSC (27).

The route of administration is another aspect that greatly affects the distribution and thereby the therapeutic effect of MSC. While MSC express multiple chemokine receptors (28) it is now well established that intravenously infused MSC end up in the lungs due to size restrictions and that there is limited migration of MSC from the lungs to other sites (23, 29). It is therefore questionable whether chemokine

receptors play a role in the distribution of MSC after intravenous infusion. This may be different when MSC are administered via different routes, for instance locally when they may migrate over short distances through tissues to sites of inflammation. There is evidence that endogenous MSC can migrate via the lymphatic system (30) but whether exogenously administered MSC can follow the same path is not known. For the intravenous route of administration the size of MSC may be determining for the localisation of the cells.

The immunogenicity of MSC is also likely to impact the effect of MSC. The first thought may be that non-immunogenic MSC that have a long survival time will have the best immunomodulatory effects and that a rapid disappearance of MSC after administration will impair efficacy. However, the immunogenicity of MSC may also contribute to the immunodulatory effects. We have previously demonstrated that MSC infusion leads to a mild inflammatory response, which is followed by immunosuppression (31). Furthermore, phagocytosis of MSC has been shown to lead to the development of tissue-supportive macrophages (32). There may thus be a balance between the immunogenicity of MSC and their therapeutic effects.

All clinical studies performed with MSC up to date have shown that MSC therapy is safe. There is no evidence for transformation of human MSC and only mild infusion related adverse effects have been reported (33). The real challenge for MSC immunotherapy is therefore to prove efficacy and one way to do this is to use MSC with improved immunomodulatory properties. The period of expansion of MSC offers an opportunity to steer MSC to a desired phenotype.

Improving MSC therapy

There is growing interest in the development of protocols for the generation of optimized immunomodulatory MSC (iMSC), which can be used to target specific immune disorders. These protocols include modifications of the culture medium, changes in culture conditions or selection of immunomodulatory subsets of MSC. A frequently used approach to boost the immunomodulatory properties of MSC is the addition of pro-inflammatory cytokines or Toll-like receptor (TLR) activators to the MSC culture medium. Modification of culture conditions can include the use of bioreactors and altering oxygen concentrations. Some of these MSC optimising approaches are discussed below.

Pro-inflammatory stimulation of MSC

IFNγ

IFNγ is identified as one of the most potent activators of the immunomodulatory properties MSC. IFNγ was first suggested to be a key player in activating the immunosuppressive capacity of MSC in 2006 by Krampera et al. (15). IFNγ-treated MSC demonstrated improved capability of suppressing the proliferation of $CD4^+$ and $CD8^+$ T-lymphocytes and NK cells, in a contact independent manner. Studies have shown that IFNγ induces IDO expression in MSC (10), and increases PGE2, hepatocyte growth factor (HGF) and TGFβ production (34, 35). PGE2 inhibits T-cell proliferation, induces IL-10 production and reduces TNFα, IL12, IL1β and IL8 expression in macrophages (36). The inhibitory co-stimulatory molecule programmed death ligand 1 (PD-L1) is strongly upregulated in a dose-dependent matter by IFNγ, which leads to the suppression of T-cell proliferation (37). Rafei et al. showed that IFNγ stimulation of MSC also induced CCL2 secretion and that this cytokine has a direct involvement in the inhibition of lymphocyte activation (38).

The efficacy of IFNγ-treated MSC has been investigated in several pre-clinical models. Importance of IFNγ to the immunosuppressive capacity of MSC is highlighted by the fact that MSC from IFNγ$R1^{-/-}$ mice are unable to prevent graft versus host disease (GVHD) in contrast to MSC from WT mice (39). Survival of GVHD was 100% when infusing IFNγ-treated MSC compared to the 45% with untreated MSC (40). In addition, IFNγ treatment of MSC enhances their therapeutic activity when they are used in induced colitis models (41).

The immunogenicity of MSC is differentially affected by IFNγ stimulation. Co-stimulatory molecule CD40 and the adhesion molecule CD54 (ICAM-1) are strongly upregulated as well as major histocompatibility complex-I and -II (MHC-I, -II) (34, 37, 38, 42-44). The upregulation of MHC may result in increased recognition of allogeneic MSC by $CD4^+$ and $CD8^+$ lymphocytes, which will result into increased susceptibility of MSC to be lysed by $CD8^+$ lymphocytes. As such, the consequences of IFNγ stimulation on the immunogenicity of MSC could be a reason why in some *in vivo* studies less or no immunosuppressive effects of MSC are seen after IFNγ stimulation, as MSC might already be cleared before they can exert their actions (38). On the contrary, the upregulation of MHC-I reduces lysis of MSC by NK cells

(45). Complex forming of MHC-I on MSC with receptors on NK cells leads to the activation of inhibitory signals in NK, resulting in decreased release of granules containing perforin and granzymes.

TNFα

While the effects of IFNγ on MSC have been studied most abundantly, other pro-inflammatory cytokines have potent effects on MSC as well. TNFα has similar, but less pronounced, effects on MSC as IFNγ, such as upregulation of PGE2, IDO, HGF, CD54 and MHC class-I (34, 42, 46). In addition, TNFα upregulates the production of several paracrine factors including vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2) and insulin-like growth factor 1 (IGF1) (47). Stimulation with TNFα alone, however, does not influence the immunosuppressive capacity of MSC in a mixed lymphocyte reaction (MLR) (34, 42). Ren et al. showed that IFNγ alone did not suppress T-cell proliferation, but in combination with TNFα, there was a significant suppression (39). Furthermore, IFNγ acted in concert with IL1α and IL1β to suppress T cell proliferation. Combinations of these pro-inflammatory factors lead to high expression of CD106 (VCAM-1) and iNOS in MSC and thus the production of NO, resulting in STAT5 phosphorylation in T-cells and suppression of T-cell responses (39, 44, 48). In addition, cocktails of these pro-inflammatory cytokines upregulate several chemokines (CXCL9 and CXCL10), which may bring T-cells, B-cells and DC into close proximity to the MSC so that MSC can more efficiently exert their immunosuppressive effect (39).

IL17A

High expression levels of the IL17A receptor on the MSC cell surface suggest that IL17A could be a promising stimulator of MSC immunomodulatory activity. Stimulation with IL17A increases the proliferation of MSC and has no effects on the MSC morphology or MSC surface marker expressions, including PDL1 expression. In addition, it has no effect on MHC class I and MHC class II expression, therefore it appears that MSC maintain their hypo-immunogenicity upon exposure to IL17 (43). In addition, treatment of MSC with IL17A enhanced their T-cell suppression capacity to levels observed after IFNγ-treatment (49). Furthermore, IL17-treated MSC

increased IL6 production and regulatory T cell induction from activated $CD3^+$ lymphocytes, downregulated CD25 expression by $CD4^+$ lymphocytes and inhibited Th1 cytokine secretion (IFNγ, TNFα, IL2 and IL10) (43). These results suggest that IL17 stimulation improves the therapeutic properties of MSC.

Activation of Toll-like receptors

Activation of TLR on MSC has been touched upon in a number of studies. MSC express high levels of TLR3 and TLR4 and their activation changes the phenotype and immunomodulatory properties of MSC. There is data that indicates that TLR activation stimulates the expression of immunosuppressive factors (IDO and PGE2) by MSC and enhances the inhibitory effects of MSC on immune cells (50). Other studies demonstrate that stimulation of TLR4 on MSC induces a more pro-inflammatory phenotype of MSC with high expression of pro-inflammatory factors (CXCL1, IL6, IL8 and CCL2) and adhesion molecules (VCAM-1 and ICAM-1) and a reduced ability to suppress leukocyte activation (51-53). In contrast, upon activation of TLR3 MSC exhibit a milder pro-inflammatory phenotype and show increased leukocyte affinity and maintain their ability to suppress leukocyte activation (51-53). TLR3-stimulated MSC are furthermore better protected against the cytotoxic effects of NK cells (54). It is suggested that TLR activation may represent an effective mechanism to restore immune responses in case of infection by inhibiting the immunosuppressive effect of MSC (53). Thus TLR-activated MSC may potentially find applicability as antagonists for regular of enhanced immunosuppressive MSC.

Other pro-inflammatory factors

Stimulation by pro-inflammatory factors IFNγ, TNFα, IL17A and the activation of TLR3 and TLR4 have shown to have a strong effect on the immunomodulatory properties of MSC. The effectiveness of these factors can partially be linked to the high cell surface expression levels of receptors for these factors. Next to the expression of these receptors, MSC also express various other receptors for pro-inflammatory factors on their cell surface, such as for IL1α, IL1β and IL6 as well as for prostaglandins, suggesting that these factors might as well be effective in modifying the properties of MSC. Studies on the presence of other receptors on MSC will possibly lead to the identification of additional factors that have effects on

the properties of MSC. Furthermore, the expression of some of these receptors may be regulated by stimulation with pro-inflammatory factors. This implies that MSC that are encountering a pro-inflammatory environment, become more susceptible to other factors, for example to TGFβ, IL15, TNFα and TLR3 and TLR4 activators. The use of cocktails of several pro-inflammatory factors might therefore also be of interest into further improving the immunomodulatory properties of MSC.

Optimizing culture conditions

Hypoxia

Culture conditions are far from comparable to the *in vivo* conditions. Oxygen concentration is a crucial factor influencing the properties of cells. Standard culture conditions are set at an oxygen concentration of 20%, but in MSC niches in the bone marrow and adipose tissue, depending on the blood flow, this can vary between 3 and 11% (55). Decreasing the oxygen concentration, creating a hypoxic environment, has been shown to increase MSC proliferation and enhance their secretion of VEGF and basic fibroblast growth factor (bFGF) (47, 56). Under hypoxic conditions MSC are forced to switch to anaerobic metabolism and therefore produce more lactate (46).

There is evidence that lactate polarizes macrophages to an anti-inflammatory phenotype (57). Therefore lactate production may contribute to the immune regulatory function of MSC under hypoxic conditions. Under hypoxia no signs of MSC toxicity, differences in colony forming unit (CFU) capacity, other phenotypical changes are observed (46). However, in contrast to what Liu et al. reported, Roemeling et al. saw no changes in the MSC proliferation under hypoxic conditions (46). Nevertheless, during hypoxia, MSC maintained the capacity to induce expression of IDO, PD-L1 and CXCL10 in response to IFNγ and TNF-α (46). In addition, while T cell proliferation is impaired under hypoxic conditions, T-cell inhibition by MSC under hypoxia is maintained and therefore relatively more prominent than under normoxia (46). The absence of oxygen thus appears to enhance the immunomodulatory properties of MSC in biological systems.

Bioreactors

Next to the conventional static monolayer culture of MSC, bioreactors are a novel way of expanding MSC. In bioreactors culture conditions can be accurately controlled and strains and stresses can be exerted on the MSC due to the media flow (58, 59). In these bioreactors MSC can be seeded on polymer –specific scaffolds and the dynamic media flow (due to the 2- or 3-dimensional rotation of the bioreactor) applies a mechanical stimulus to the MSC. It was seen that the proliferation and the distribution of the MSC in the scaffolds increased when the MSC were in the bioreactor. No changes in their immunogenicity were observed as MHC-I and –II expression was unaffected (58, 59). Mechanical vibrations are used to stimulate the differentiation of MSC into various lineages. In addition to affecting the differentiation potential of MSC, vibrations influenced the immunomodulatory properties of MSC by increasing expression of IL-1, TGFβ1 and TNFα (60). It has yet to be investigated what the effects of culturing in bioreactors are on the immunomodulatory properties of MSC.

Scaffolds

Scaffolds provide a surface for MSC in culture and help to retain and deliver MSC. Scaffolds made from type-I collagen, hydrogels, sponges and membranes, provide different microenvironments for MSC (61). MHC class-I and –II expression increases gradually on MSC seeded on these scaffolds, with the least increase seen in hydrogel seeded MSC. Presence of IFNγ further increases the expression of MHC class-II. In a one-way MLR, MSC seeded in a hydrogel evoked a low lymphocyte response, whereas MSC in a sponge and membrane enhanced lymphocyte proliferation, compared to MSC which were not on any scaffold (61). The use of these different scaffolds, made from the same material, has a differential effect on MSC's immunological properties.

Engineering

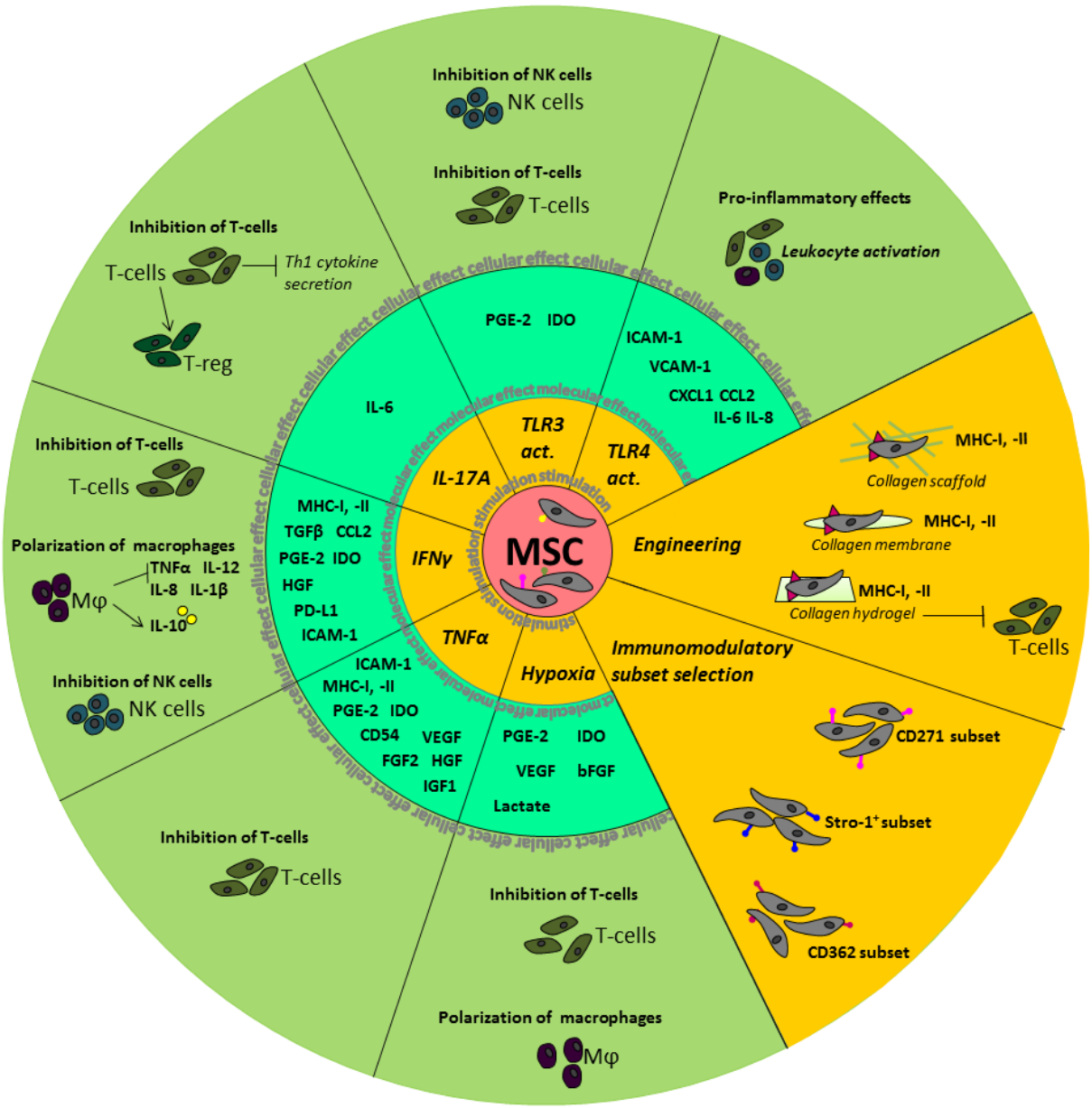
Currently, innovative techniques for engineering MSC are under development. Of interest for the development of iMSC is the use of intracellular agent-loaded microparticles (62). The technique enables the loading of MSC with particles containing agents controlling the cell's phenotype and secretome. Agents that

improve the immunogenicity and immunomodulatory properties of MSC can be incorporated in the particles, which can subsequently target the intrinsic properties of MSC. This could be useful in cases, where in the different phases of cell therapy, changes in the MSC's phenotype are required. For example, during the initial phase of injection a specific MSC phenotype with a low immunogenicity might be required, followed by localization of MSC at the site of interest, where the microparticles release their agents and inducing a change in secretome or immunogenicity, which could be necessary for the MSC to fulfill their purpose. This technique will enable the generation of iMSC, which can be customized in a time dependent manner.

Subsets of MSC with enhanced immunomodulatory properties

Freshly isolated MSC are a heterogeneous population consisting of various subsets, each with different surface marker expressions, differentiation capacity, gene expression, and secretome as well as immunomodulatory capacity (63). Among these subsets, specific subsets are identified as being immunoprivileged and/or possess superior immunomodulatory capacity. Protocols have been developed for the isolation of these specific MSC subsets. Selection based on surface marker expression results in a more homogeneous and defined MSC subset with potentially enhanced immunomodulatory efficacy. Positive selection on CD271 (low-affinity nerve growth factor receptor, LNGFR) expression generates a $CD271^+$ MSC subset with a greater immunosuppressive capacity compared to non-selected MSC (64). A subset of Stro-1^+ MSC shows enhanced support for human hematopoietic stem cell engraftment and has a greater immunosuppressive capacity, while Stro-1^- MSC have a broad distribution after infusion into tissues (65, 66). Other molecules like CD73 and CD90 are suggested to be important markers to identify MSC subsets with enhanced immunosuppressive capacity (67, 68). Recently a $CD362^+$ (Syndecan-2) subset of MSC has been identified, representing a more homogeneous population of MSC (patent number WO 2013117761 A1). Pre-selected subsets of MSC might be more susceptible to the protocols used to optimize their immunomodulatory properties. Hence, selection of a specific MSC subset may also be another way to generate iMSC.

A summary of the above described effects of manipulation of MSC is depicted in Figure 1.



▲Figure 1 Summarizing figure of the molecular and cellular effects observed after manipulation of MSC. The inner circle depicts the different stimuli used to manipulate MSC. Molecular effects, such as the upregulation of cytokines and chemokines, seen in MSC, which are the result after manipulation, are shown in the middle circle. In the outer circle, the molecular effects by stimulated MSC are depicted.

What are the desired characteristics of an iMSC?

Immunomodulatory effects of MSC are broad and depending on the disorder, iMSC will be required to possess fitted and specific immunomodulatory properties. Immunological disorders occur in a wide spectrum, with various pathophysiological properties. The design of iMSC is therefore a differential matter where several considerations have to be taken into account. Firstly, immunological diseases can be mediated by one or various immune cells. Specific targeting of T-cell proliferation and activation or manipulation of NK cells, whom play a central role in the innate immune response, will be essential in T-cell mediated diseases or innate immune diseases, respectively. In other conditions, an overall inhibition of an immune response by iMSC might be essential. Secondly, when infused intravenously MSC are prone to get trapped in the small capillaries of the lungs, due to their size (23, 29). When iMSC are required to have a local effect, manipulation of their size and the presence of tissue specific chemokine receptors will enable the cells to travel further into the body and to the designated tissue where iMSC can exert their immunomodulatory effect. On the other hand, when a systemic effect is desired, iMSC might be required to be in the circulation, or in any arbitrary location where they are trapped, and able to exert a systemic immunomodulatory effect. However, it is known that MSC are short-lived when infused intravenously (23). Within 24 hours they die and are cleared from the body. It is possible that MSC become apoptotic after administration. The clearance of apoptotic cells is known to exert immunomodulatory properties (69) and proposed to be used as immunomodulatory therapy in transplantation (70).

Nevertheless, although MSC have short term effects, long term effects have also been observed. It is suggested that their short-term effects are mediated by their secretome, while their long-term effects are due to interaction and activation of other cell types in a probable hit-and-run way of action. Several studies have reported that the modulation of T-cell responses occur indirectly via MSC-mediated induction of regulatory T-cells (Tregs) (71). These cells are known to play a role in the maintenance of self-tolerance and immune homeostasis. In addition, the induction of regulatory macrophages (Mregs) and regulatory B-cells (Bregs) is also interesting as these cells also play an important role in the regulation of autoimmune and

inflammatory diseases. Improved induction of these cells by iMSC may therefore improve the long-term effect of the iMSC therapy when used to treat autoimmune and inflammatory diseases (17, 72, 73). Depending on therapeutic purposes, iMSC might be required to have a short or long-term effect. Manipulation of the iMSC's immunogenicity and immunomodulatory properties, including their secretome, may increase their longevity as well as their immunomodulatory effect. As mentioned before, MSC therapy is proven to be safe with no transformations of the MSC with mild side effects observed (33). So therefore, focusing on the manipulation of MSC and thereby acquiring a customized phenotype will be the next step into optimized cell therapy.

Conclusions

The diversity of immunological disorders demands the generation of differential designs of iMSC. However, currently no clinical trials have been published where iMSC are used as an immunomodulatory therapy. This mainly comes down to the fact that the safety aspects of iMSC are not yet sufficiently explored and there is still a lot to be done to generate clinical grade iMSC with desired functional characteristics. Furthermore, for many immunological disorders targets for intervention have yet to be identified, and as a result the desired properties of optimized MSC are not yet known. When more information come in, the current procedure of one MSC therapy for all diseases will be refined and optimized into a customized iMSC treatment, which will be specific and cater to the needs of the targeted disorder.

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3

Ageing of bone marrow and umbilical cord derived MSC during expansion

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Abstract

Mesenchymal stromal cells (MSC) are used as experimental immunotherapy. Extensive culture expansion is necessary to obtain clinically relevant cell numbers, although the impact on MSC stability and function is unclear. Here we study the effects of long-term *in vitro* expansion on the stability and function of MSC.

Human bone marrow derived MSC (bmMSC) and umbilical cord derived MSC (ucMSC) were *in vitro* expanded. During expansion their proliferative capacity was examined. At passages 4, 8 and 12 analyses were performed to investigate the ploidy, metabolic stability, telomere length and immunophenotype. In addition, their potential to suppress lymphocyte proliferation and susceptibility to NK cell lysis was examined.

BmMSC and ucMSC showed decreasing proliferative capacity over time, whilst their telomere lengths and mitochondrial activity remained stable. Percentage of aneuploidy in cultures was unchanged after expansion. Furthermore, expression of MSC markers and markers associated with stress or ageing remained unchanged. Reduced capacity to suppress CD4 and CD8 T-cell proliferation was observed for passage 8 and 12 bmMSC and ucMSC. Finally, susceptibility of bmMSC and ucMSC to NK cell lysis remained stable.

We showed that after long-term expansion, phenotype of bmMSC and ucMSC remains stable and cells exhibit similar immunogenic properties compared to lower passage cells. However, immunosuppressive properties of MSC are reduced. These findings reveal the consequences of application of higher passage MSC in the clinic, which will help increase the yield of therapeutic MSC, but may interfere with their efficacy.

Introduction

Mesenchymal stromal cells (MSC) are increasingly used as an experimental therapy for a range of immunological and degenerative diseases [1-5] due to their immunomodulatory properties [6-10]. Clinical trials use MSC isolated from different tissues [11], most frequently bone marrow derived MSC (bmMSC) and umbilical cord derived MSC (ucMSC) [12, 13]. They are used in doses ranging from 1-2 x 10^6 MSC/kg body weight with a frequency of 1 to more than 10 infusions per clinical trial. As the frequency of MSC in tissues is very low and the amount of tissue available is limited, MSC need to be expanded *ex vivo* prior to application to obtain sufficient numbers. Depending on the origin of the MSC, the age of the donors may vary: ucMSC originate from newborns, whilst the age of bmMSC donors can vary widely. Notably, there is a positive correlation between donor age and the accumulation of mutations in human adult stem cells [14], although it is unclear whether bmMSC and ucMSC age during culture expansion and whether this affects their immunomodulatory therapeutic potential.

The use of low passage MSC for therapy is currently preferred to a higher passage MSC [15, 16] due to the impact of extended passage on immunomodulatory function. Moreover, studies which focused on the therapeutic potential of MSC for regeneration have shown that the differentiation potential of MSC is impaired after long-term *in vitro* culturing [17-20]. Notably, under conditions of stress, such as inflammation, MSC upregulate their HLA type I and II expression, rendering them more immunogenic [21] and they upregulate their programmed death-ligand 1 (PD-L1) expression [22], which is also upregulated by ageing T-cells [23].

Systematic evaluation of immunomodulatory and genetic stability over passage of MSC will definitively determine the magnitude of this effect and also define whether there is an optimal passage for immunomodulation. This will standardise MSC therapy and balance the benefits of expansion, giving more doses per MSC donor, with immunomodulatory exhaustion.

Therefore in this study, we report the effects of prolonged expansion on the stability and function of bmMSC and ucMSC. Through analysing the surface markers expression on bmMSC and ucMSC at passage 4, 8 and 12 we examined the stability of the MSC immunophenotype during prolonged culture. In addition, we evaluated

until which culture stage their proliferative capacity was maintained, which is of great importance for determining the number of potential therapeutic doses of MSC that can be obtained from a particular cell donor. To investigate whether prolonged culture would have an effect on ageing parameters, telomere length, mitochondrial activity or the number of aneuploid cells in the cultures were determined. As ageing may have effects on the functionality of MSC, the capacity of passage 4, 8 and 12 bmMSC and ucMSC to inhibit lymphocyte proliferation was tested. Finally, to examine whether prolonged culture expansion would affect the immunogenicity of MSC, the susceptibility of bmMSC and ucMSC to trigger NK cytotoxic activity was evaluated.

Material and Methods

Culture expansion of bmMSC and ucMSC

Human bone marrow samples were collected from healthy adult donors (n=3) with written informed consent according to the Ethics Ref. C.A.02/08. Isolation of $CD362^{+}$ bmMSC was according to methods described in Elliman et al. (manuscript in progress). Briefly, mononuclear cells (MNCs) were isolated by Ficoll density gradient centrifugation (GE Health Care Bio-Sciences, Buckinghamshire, UK) and ACK Lysis Buffer (Life Technologies, California, US) employed for erythrocyte lysis (n=3). MNCs were then analysed for expression of CD235-eFluor 450 (clone 6A7M, dilution 1:1000, eBioscience, Hatfield, UK), CD45-FITC (clone HI30, dilution 1:25, BD Biosciences, Oxford, UK), CD271-PE (clone ME20.4-1.H4, dilution 1:50, Miltenyi Biotec, Bergisch Gladbach, Germany) and CD362-APC (clone 305515, dilution 1:50, R&D Systems, Abingdon, UK) and the viability dye Sytox Blue was used to exclude dead cells (as per manufactures instructions, Life Technologies, California, US). Using appropriate controls including FMOs sort gates were assigned and the CD362+CD271+ population was sorted using a BD FACS Aria (BD Biosciences, Oxford UK). The mean number of CD362+CD271+ cells isolated from the donors used in this study was 5419 ± 2359 (n=3, mean ± SEM). Cells were plated, expanded in culture and cryopreserved at passage 2 for shipment to Erasmus Medical Center.

Human umbilical cord tissue was collected from virally screened healthy donors by Tissue Solutions Ltd. (Glasgow, UK) (all cord tissues provided by Tissue Solutions are obtained according to the legal and ethical requirements of the country of collection, with the approval of an ethics committee (or similar) and with anonymous consent from the donor). In the work reported here, umbilical cord tissue (n=3) was collected from Caesarean section deliveries, all tissues were transported for processing to Orbsen Therapeutics Ltd. in AQIX® solution (London, UK) at 4°C. Here, isolations were performed within 48hrs post birth. Briefly, umbilical cord tissue was washed and the whole tissue was manually dissociated before additional enzymatic digestion in a MEM Alpha (Gibco, ThermoFisher, UK) enzyme cocktail containing Collagenase 1 (2mg/ml), Hyaluronidase 1 (1mg/ml) and DNase (0.1mg/ml) (Sigma Aldrich, Ireland) for a maximum of 2 hours at 37°C. Once a single

cell suspension was obtained by filtration (100µm) cells were stained with CD362-APC (clone 305515, dilution 1:50, R&D Systems, US). After 30mins at 4°C the cells were washed and resuspended in MACs buffer 80µl/10^7cells with the addition of anti-APC beads (Miltenyi Biotec, Germany) at a concentration of 20µl/10^7cells the cells were incubated with beads for 15mins at 4°C. The $CD362^+$ cells were then isolated using MS MACs column as per manufactures instructions, (Miltenyi Biotec, Germany). Each cell fraction was counted, seeded for expansion and cryopreserved at passage 2 for shipment to Erasmus Medical Center.

BmMSC and ucMSC were thawed and cultured in minimum essential medium Eagle alpha modification (MEM-α; Sigma Aldrich, St Louis, MO, USA) containing 2 mM L-glutamine (Lonza, Verviers, Belgium), penicillin/streptomycin solution (P/S; 100IU/ml penicillin, 100IU/ml streptomycin; Lonza) and supplemented with 15% fetal bovine serum (FBS; Lonza) and 1ng/ml basic fibroblast growth factor (bFGF) (Sigma Aldrich) and kept at 37°C, 5% CO_2 and 20% O_2.

Every seven days, bmMSC and ucMSC were passaged using 0.05% trypsin-EDTA (Life technologies, Paisley, UK). The cells were seeded at in density of $1x10^5$ cells/$175cm^2$ and the amount of cells collected after trypsinisation was noted to subsequently calculate the population doublings (PD) along the passages using the formula:

$$\mathrm{PD} = \frac{\log\left(\frac{\text{Number of cells after 7 days}}{\text{Number of cells seeded}}\right)}{\log 2}. \qquad [1]$$

Flow cytometric analysis

Immunophenotyping of bmMSC and ucMSC was performed at passage 4, 8 and 12 by labelling of a set of accepted MSC surface markers: CD13 (PE-Cy7, BD Biosciences), CD73 (PE, BD Biosciences), CD90 (APC, R&D systems),CD105 (FITC, R&D systems). And hematopoietic markers CD31 (PB, BD Biosciences) and CD45 (APC-Cy7, BD Biosciences). In addition, bmMSC and ucMSC were also labelled for HLA type I (APC, BD Biosciences), HLA type II (PERCP, BD Biosciences) and PD-L1 (PE, BD Biosciences). After labelling for 30 min at 4°C in

the dark, the cells were washed and measured on the FACSCanto II flow cytometer using FACSDiva software (BD Biosciences).

Fluorescence in situ hybridization (FISH)

At passage 4 and 12, both bmMSC and ucMSC (n=3) were seeded on microscope chamber slides for 24 hours at 37°C, 5% CO_2 and 20% O_2. Subsequently dual-colour FISH analysis was performed using the bacterial artificial chromosome (BAC) clones RP11-661P17 (chromosome 15q26) and RP11-1083G9 (chromosome Xq13) or the combination of clones RP11-79F3 (chromosome 18q11) and RP11-636L7 (chromosome 21q21). Mbo1-digested BAC DNA and plasmid DNA were both labelled with Bio-16-dUTP or Dig-11-dUTP (Roche Applied Science, Indianapolis, IN, USA) using the random prime labelling system BioPrime (Invitrogen Corporation, Carlsbad, CA, USA). The microscope chamber slides containing bmMSC or ucMSC at different passages were hybridized overnight at 37°C with 10–20 ng labelled centromere probe or 40–50 ng labelled BAC probe. The next day, the chamber slides were washed at 55°C (2x saline sodium citrate [SSC] and 0.1× SSC, 0.1% Tween) and incubated with Alexa Fluor 594 Streptavidin (Life Technologies, Bleiswijk, Netherlands) and anti-digoxigenin-fluorescein isothiocyanate (Roche, Almere, Netherlands) for two hours. After washing, the slides were counter-stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Zwijndrecht, Netherlands) and mounted in anti-fade solution (DABCO-Vectashield 1:1; Vector Laboratories, Burlingame, CA, USA). Each hybridization was blindly scored by two individuals. A minimum of 200 nuclei was scored with an Axioplan 2 imaging microscope (Carl Zeiss; Sliedrecht, the Netherlands) using a chroma-sp-100 DAPI and a chroma-sp-103v1 red filter (Chroma Technology, Bellows Falls, VT, USA), and the images were captured with ISIS software (Metasystems, Altlussheim, Germany).

Telomere length assay

Flow fluorescence *in-situ* hybridization was performed on bmMSC and ucMSC at passage 4, 8 and 12 to determine the relative telomere length (RTL) with the use the telomere PNA-kit/fluorescein isothiocyanate (FITC) (Zebra Bioscience BV,

Enschede, the Netherlands). The T-cell leukemia sub-cell-line 1301 of CCRF-CEM, known for their long telomeres, was used to calculate the RTL with the formula:

$$\mathrm{RTL} = \frac{\begin{array}{c}(\text{Median FL1 sample cells with probe} - \\ \text{Median FL1 sample cells without probe}) \times \\ \text{DNA index of sample } (=2) \text{ cells} \times 100\end{array}}{\begin{array}{c}(\text{Median FL1 control cells with probe} - \\ \text{Median FL1 control cells without probe}) \times \\ \text{DNA index of sample } (=1) \text{ cells}\end{array}} \quad [2]$$

Samples were measured on a BD FACSCanto II and analysed by Kaluza Analysis 1.3 software.

MTT assay

Passage 4, 8 and 12 of bmMSC and ucMSC were seeded in 96 wells plates at 1500 cells/well/200 µl and left overnight at 37°C, 5% CO_2 and 20% O_2. The following day, 20 µl of 5 ml/ml MTT in PBS was added to each well and incubated for 5 hours at 37°C, 5% CO_2 and 20% O_2. Thereafter, the medium was removed and the formed crystals were dissolved with 100 µl of dimethyl sulfoxide (DMSO). The plate was incubated again in the incubator, for 5 minutes, followed by measuring the absorbance at 550 nm with a spectrophotometer (Victor 1420 multi-label counter; LKB-Wallac, Turku, Finland).

Peripheral blood mononuclear cells (PBMCs) isolation

Buffy coats (n=4) were obtained from healthy donors at Sanquin Blood bank (Rotterdam, The Netherlands). Subsequently peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, using Ficoll paque (GE healthcare, The Netherlands) and frozen at - 180°C until use.

CD107a NK cytotoxicity assay

To generate activated NK cells, PBMCs were seeded at 100.000 cells/well in 96-wells plates in RPMI 1640 medium (Life technologies) with penicillin/streptomycin solution (P/S; 100 IU/ml penicillin, 100 IU/ml streptomycin; Lonza), 10% heat

inactivated FBS (Lonza), interleukin 2 (IL2, 2.10^2 IU/ml; Peprotech) and interleukin 15 (IL15, 10 ng/ml; Peprotech). After 7 days, cells were collected and via a negative selection MACS procedure (NK Cell isolation kit human; MACS Miltenyi Biotec), NK cells were isolated. NK cells were then left overnight at 37°C in a polystyrene tube with IL-2 and IL-15.

The following day, NK cells were co-cultured in polypropylene tubes for one hour with either bmMSC or ucMSC of passage 4, 8 or 12 at a 1:4 ratio [MSC:NK cells]. In the presence of monensin (0.1µL/200µL; BD Golgistop; BD Biosciences) and αCD107a antibody (LAMP-1, APC, BD Pharmingen). After one hour the cells were washed and stained for CD3 (PERCP; BD Biosciences), CD16 (PE; BD Biosciences) and CD56 (PE; BD Biosciences) for 30 minutes at 4°C. The cells were then washed and analysed with the FACSCanto II flow cytometer.

αCD3CD28 lymphocyte proliferation assay

BmMSC and ucMSC of passage 4, 8 or 12 were seeded into 96-wells plates and left overnight to adhere in the incubator. The following day, PBMCs were thawed and labelled with Cell Trace CFSE (Life Technology) according to the instructions of the manufacturer and seeded at different [MSC:PBMC] ratios: [1:20], [1:10], [1:5] and [1:2.5]. The co-cultures were left for four days in the presence of αCD3/CD28 stimulation (0.5ug/ml αCD3 antibody, 0.5ug/ml αCD28 antibody and 0.5ug/ml goatαmouse antibody; Life Technology). Thereafter the PBMCs were stained for CD4 (APC; BD Biosciences) and CD8 (Pe-cy7; BD Biosciences). With the use of the FACSCanto II flow cytometer the proliferation of CD4 and CD8 T-cells was measured.

Statistical analysis

Data is analysed using IBM SPSS Statistics 21 and Prism software v5.04 (GraphPad Software Inc. La Jolla, CA). Paired Student's t-tests or one-way ANOVA for repeated measurements with Tukey multiple comparison tests are performed. Results are reported as mean ± SEM. P values <0.05 are considered significant.

Results

Characterisation of bmMSC and ucMSC

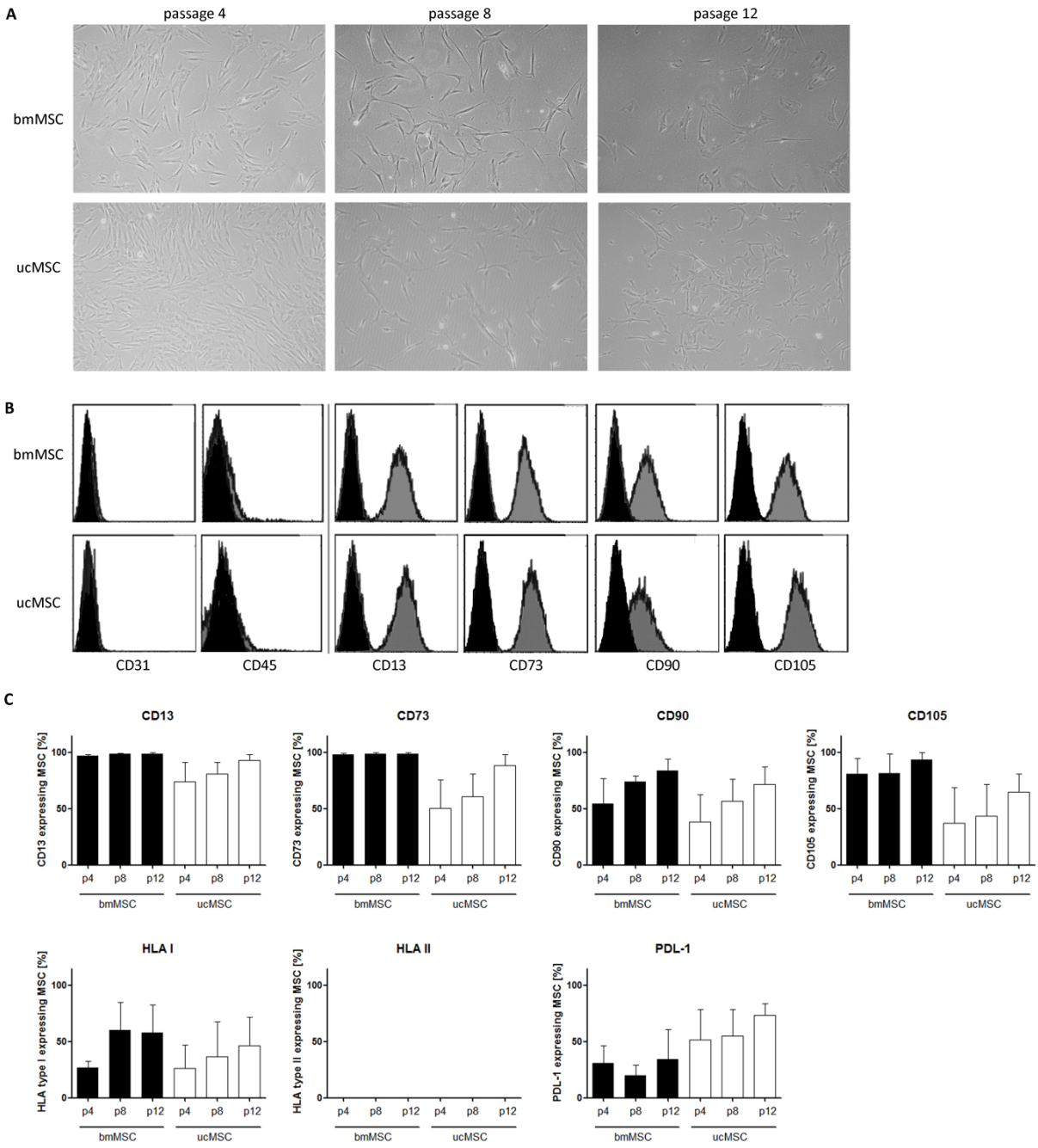
The morphology of the cells and expression of surface markers by bmMSC and ucMSC was evaluated to investigate the effect of extended culture expansion. No differences in MSC morphology were observed when comparing lower with higher passage bmMSC and ucMSC (Figure 1a). Flow cytometric analysis demonstrated expression of CD13, CD73, CD90 and CD105 and the absence of CD31 and CD45 in bmMSC and ucMSC at passage 4, confirming their mesenchymal stromal cell immunophenotype (Figure 1b). With increasing passage the percentage of bmMSC and ucMSC expressing CD13, CD73, CD105, HLA type I and II or PD-L1 remained stable (Figure 1c). These results show that the immunophenotype of bmMSC and ucMSC remains stable during extensive culture expansion.

Long term culturing reduces the proliferative capacity of bmMSC and ucMSC

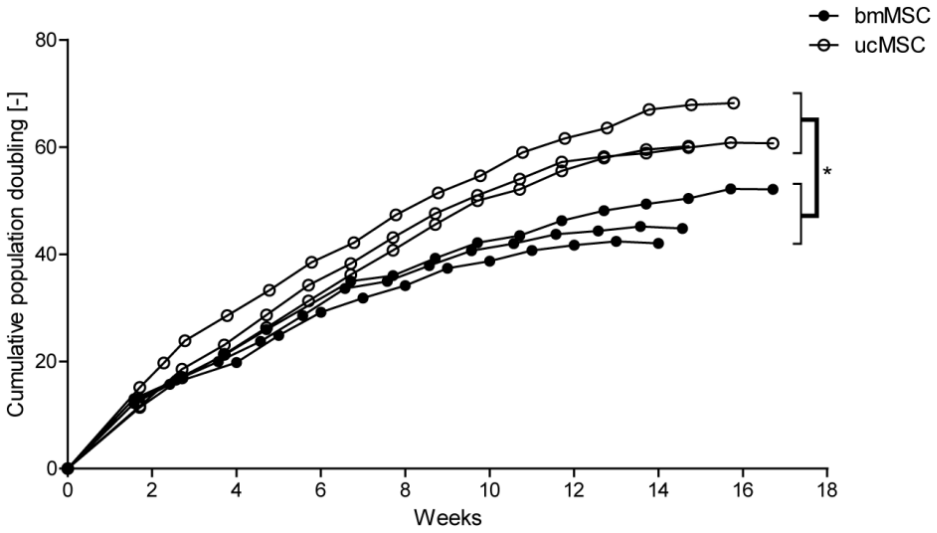
To examine the effect of extended culture expansion on the proliferative capacity of MSC, bmMSC and ucMSC were cultured for up to 17 weeks. Over time, decreasing proliferation capacity of both bmMSC and ucMSC was observed (Figure 2); on average bmMSC and ucMSC cultures underwent 4 and 6 population doublings respectively at passage 4 and 1 population doubling at passage 12. Cumulative population doublings were significantly different between bmMSC and ucMSC, whereby proliferation rate of bmMSC started to decrease from passage 7, while this decrease only occurred at passage 12 for ucMSC.

BmMSC and ucMSC maintain telomere length after extended in vitro culturing

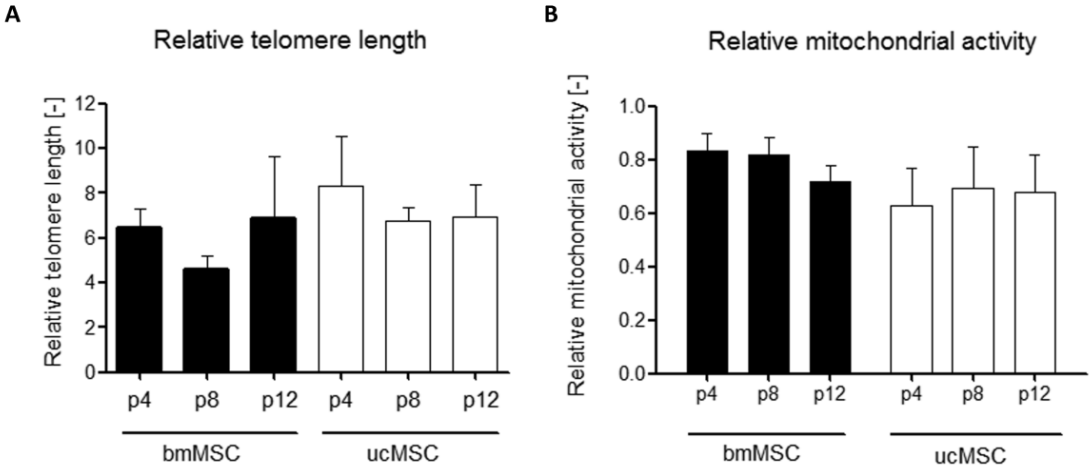
Relative telomere lengths of bmMSC and ucMSC were analysed over time to determine whether a change in length was responsible for the observed decreasing proliferation capacity. However, relative telomere lengths were not significantly different between the lower and higher passages of bmMSC and ucMSC (Figure 3a). In addition no differences in telomere lengths were observed between bmMSC and ucMSC.



▲Figure 1 Characterization of bmMSC and ucMSC at different passages: 4, 8 and 12. A Phase contrast photos of bmMSC and ucMSC at passage 4, 8 and 12. **B** Immuno phenotyping of bmMSC and ucMSC by flow cytometry. Representative histograms of expression of characteristic MSC markers CD13, CD73, CD90, CD105 and negative expressions of endothelial marker CD31 and hematopoietic marker and CD45. Isotype control (black). **C** Expression of surface markers relating to stress, ageing or the MSC phenotype, by bmMSC (black bars) and ucMSC (white bars) at passage 4, 8 and 12. Results are shown as means ± SEM (n=3).



▲**Figure 2 Population doublings.** Growth kinetics of bmMSC (closed circles) and ucMSC (open circles) during long-term culture. Each line represents one individual donor. * indicates significant difference between the average increase of population doublings over time between bmMSC and ucMSC.

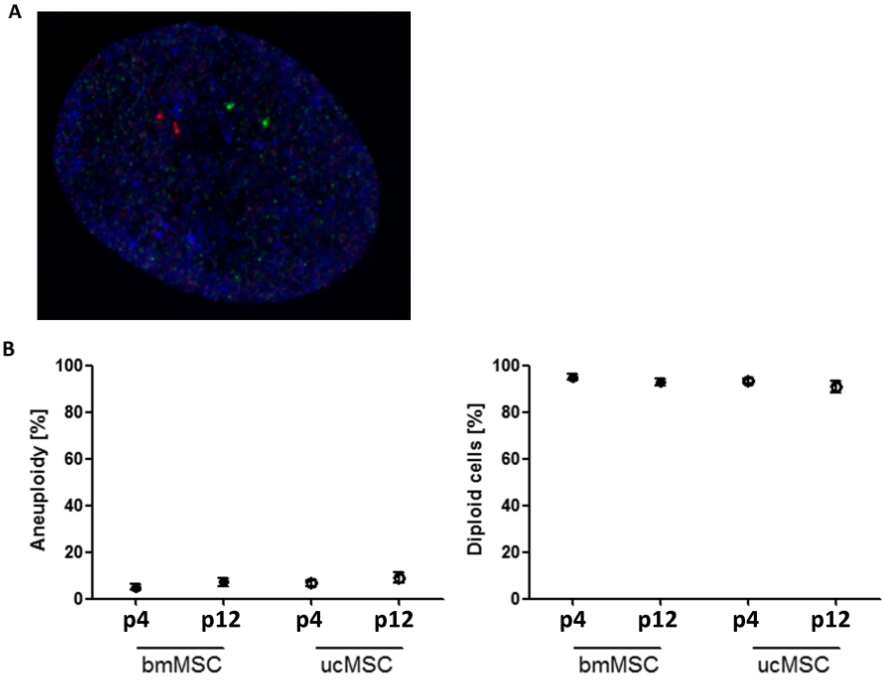


▲**Figure 3 Relative telomere length and mitochondrial activity of bmMSC and ucMSC at passage 4, 8 and 12.** Stable **A** telomere lengths and **B** mitochondrial activities of passage 4, 8 and 12 bmMSC (black bars) and ucMSC (white bars). Results are shown as means ± SEM (n=3).

Stable mitochondrial activity in bmMSC and ucMSC after extended in vitro culturing

To assess whether a change in mitochondrial activity was responsible for the decreasing proliferation capacity an MTT assay was performed to measure the mitochondrial activity. No differences were observed in the mitochondrial activity

between the lower and higher passages of both bmMSC and ucMSC (Figure 3b). In addition, no significant differences between the mitochondrial activity of bmMSC and ucMSC were observed.

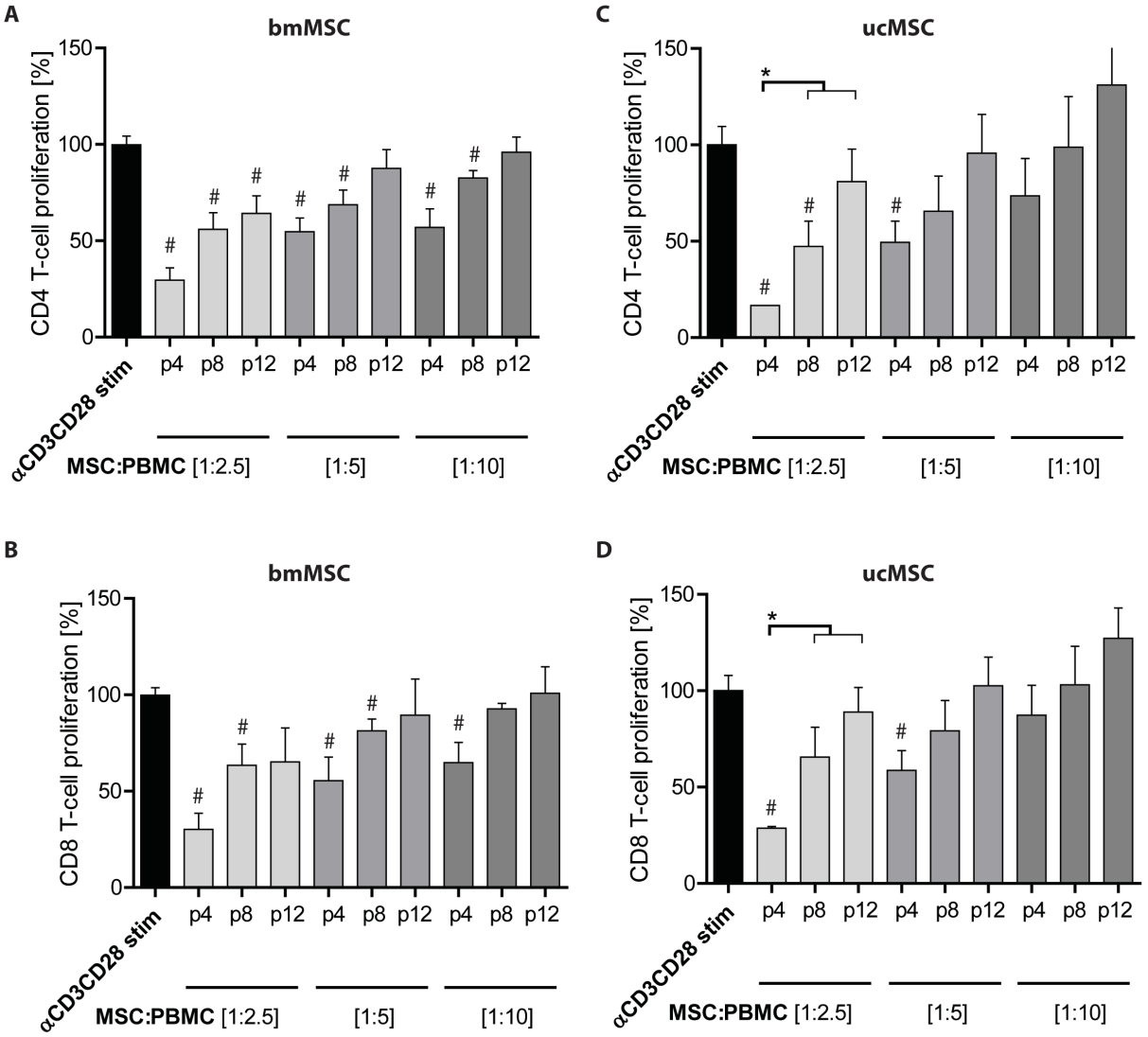


▲Figure 4 Aneuploidy in bmMSC and ucMSC at passage 4, 8 and 12. A Representative FISH staining of MSC labelled RP11-79F3 (chromosome 18q11, green) and RP11-636L7 (chromosome 21q21, red). The triple red fluorescence signal indicates aneuploidy of this particular MSC. **B** Graphs of percentage aneuploidic and diploidic bmMSC (closed circles) and ucMSC (open circles) at passage 4 and 12. A minimum of 200 cells were scored per culture. Results are shown as means ± SEM (n=3).

Aneuploidy in bmMSC and ucMSC

To examine whether the occurrence of aneuploidy in bmMSC and ucMSC increased with extensive culture expansion, FISH analysis was performed on bmMSC and ucMSC at passages 4 and 12 as depicted in Figure 4a. On average, 95±1% of bmMSC at passage 4 were diploid and 5±1% aneuploid (of which 29±10% were tetraploid) (Figure 4b). At passage 12, 93±2% of bmMSC were diploid and 7±2% aneuploidic (of which 39±12% were tetraploid). For ucMSC, 94±1% and 91±2% of the cells of passage 4 or 12, respectively, were diploid and 7±1% and 9±2% were aneuploidic (of which 54±12% and 42±7% were tetraploid respectively). No

significant differences were observed between the different MSC sources nor between lower and higher passages.

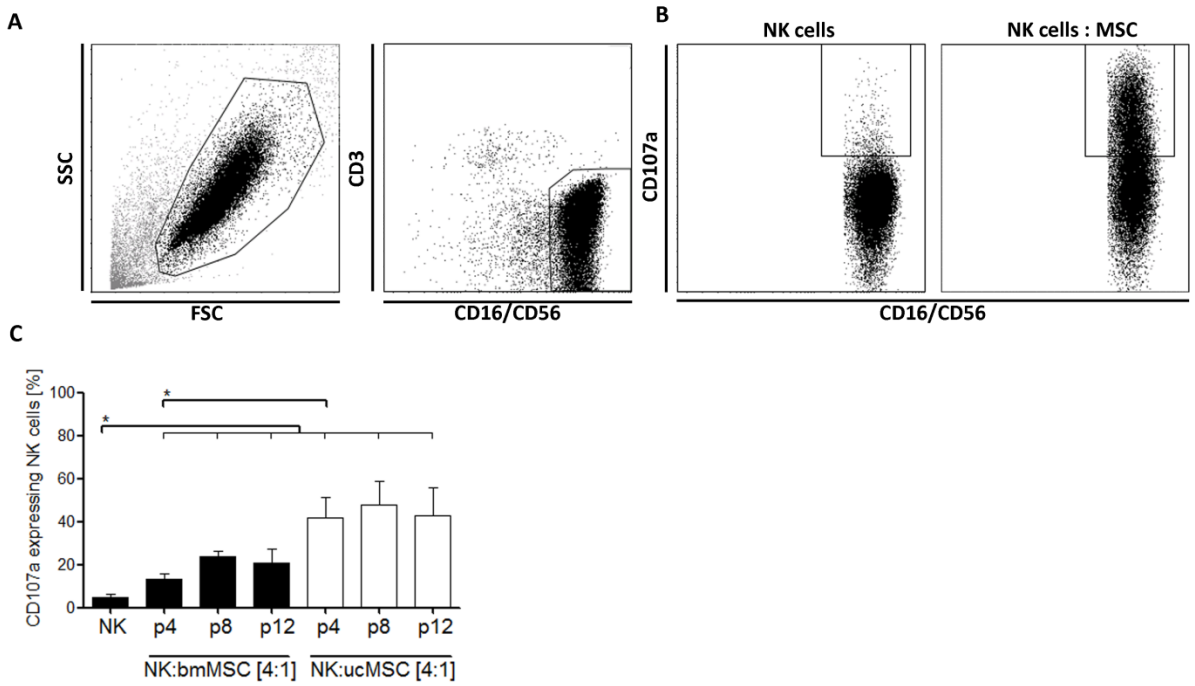


▲Figure 5 Inhibition of CD4 and CD8 T-cell proliferation. αCD3CD28 stimulated and CSFE labeled PBMCs were cocultured for 4 days with bmMSC (A and C) and ucMSC (B and D) of passage 4, 8 and 12. **A & B** CD4 and **C & D** CD8 T-cells proliferation was measured with FACS. Proliferation is expressed as the percentage proliferation CD4 and CD8 T-cell, relative to the positive control without MSC. Results are shown in means ± SEM (n=3) with * indicating $p<0.05$ compared to passage 4 MSC and # indicating significant difference compared to control without MSC

Capacity to inhibit T cell proliferation is reduced in higher passages of ucMSC

The potential of bmMSC and ucMSC, from different passages, to inhibit CD4 and CD8 T-cell proliferation was analysed by co-culturing αCD3CD28 activated PBMC at

different ratios with bmMSC and ucMSC from passage 4, 8 and 12 (Figure 5). The capacity of bmMSC and ucMSC to suppress CD4 and CD8 T-cell proliferation decreased during prolonged culture expansion. Passage 12 bmMSC lost the capacity to significantly inhibit CD4 and CD8 T cell proliferation at 1:5 and 1:10 ratios. For ucMSC this effect was even stronger; ucMSC of passage 8 and 12 showed reduced capacity to inhibit CD4 and CD8 T cell proliferation compared to passage 4 ucMSC.



▲Figure 6 CD107a expressing NK cells after exposure to bmMSC and ucMSC of passage 4, 8 and 12. A Representative FACS plot depicting selection of $CD3^-$ $CD16^+CD56^+$ (NK cells). **B** FACS plot of CD107a expression on NK cells without (left) and with(right) MSC [4:1]. **C** Graph displaying percentages of NK cells expressing CD107a after exposure to bmMSC (black bars) and ucMSC (white bars) of passage 4, 8 and 12. Results are shown in means ± SEM (n=3) with * $p<0.05$.

No increase in susceptibility to NK cell lysis by higher passage bmMSC and ucMSC

To investigate whether the susceptibility MSC to NK cell lysis increases after extended culturing, CD107a expression on NK cells was analysed after exposure to bmMSC and ucMSC at passage 4, 8 and 12 (Figure 6). In the absence of MSC,

5±1% of the NK cells express CD107a on their surface (Figure 6C). When NK cells were exposed to bmMSC or ucMSC of passage 4, the percentage of NK cells expressing CD107a significantly increased to 13±1% and 42±5%, respectively. The percentage of NK cells expressing CD107a after exposure to passage 4 bmMSC is significantly lower compared to when NK cells are exposed to passage 4 ucMSC. However, when NK cells were exposed to bmMSC of passage 8 (24±1%) or passage 12 (21±3%) no significant differences were seen in the percentage of CD107a expressing NK cells, compared to passage 4 bmMSC. In contrast, there were no differences found when exposing NK cells to ucMSC of passage 8 (48±6%) and passage 12 (43±7%) compared to passage 4. Altogether, these results show that bmMSC and ucMSC do not become more susceptible to NK cell cytotoxicity at higher passages.

Discussion

MSC are currently, due to their immunomodulatory potential, an in demand cell source for experimental immunotherapy [6-10]. Due to the need to expand MSC before clinical use, confirmation of continued immunomodulatory function and genetic stability of higher passage MSC is required. Upscaling of their production will be critical to help meet the demand for larger numbers of MSC as required for therapeutic purposes per patient [12]. In this study, we observed some signs of ageing of bmMSC and ucMSC after extended culture expansion with a reduction in their proliferative and immunomodulatory properties. However, their phenotype remained stable after the extended culture with no increase in immunogenicity as determined by their susceptibility to NK cell lysis. An overview of the results can be found in supplementary table 1.

Although a correlation between proliferative capacity and telomere length or mitochondrial activity was anticipated [18, 24-26], the observed decrease in proliferative capacity was not correlated to changes in telomere lengths or mitochondrial activity of the cells. Stable telomere lengths could be explained by the fact that during proliferation and trypsinisation, cells with short telomere lengths disappear and culture is taken over by cells with long telomere lengths. Alternatively, telomere lengths can be maintained in cells via different telomere regulation mechanisms, such as active telomerase or telomerase-independent alternative lengthening of telomeres (ALT) mechanisms. MSC are thought to lack telomerase activity [27], however Zhao et al showed by synchronizing bmMSC in the S phase of the cell cycle that telomerase is transiently expressed [28], which might explain the observed stable telomere lengths.

In contrast to previous reports [29], stable mitochondrial activity was observed after extended culture expansion in both bmMSC and ucMSC cultures. A potential explanation for this could be that cells with a low mitochondrial activity do not propagate in the culture, whereas cells with a high mitochondrial activity will overtake the ongoing culture. On the other hand older cells, which are larger, compensate by having more mitochondria per cell albeit lower in activity [29].

Besides demonstrating that during extended culture expansion the mitochondrial activity and telomere length of bmMSC and ucMSC remain stable, we demonstrated

that in these cultures the percentage of aneuploidy remains the same. Aneuploidy of therapeutic MSC is important as association between cancer and aneuploidy was previously detected [30]. However, no tumor formation by aneuploid adipose tissue derived MSC (aMSC) was observed by Roemeling-van Rhijn et al. [31]. In both our bmMSC and ucMSC cultures no increase in aneuploidy was observed, showing that these cells have a stable ploidy after extended culture expansion.

For the application of MSC as an immunotherapy, their ability to suppress T cell proliferation and their susceptibility to NK lysis was analysed. Depending on their clinical application, for instance targeting T-cell mediated diseases or innate immune diseases, inhibition of T-cell proliferation or activation or manipulation of NK cells will be required by MSC. In this study, immunogenic properties were unaffected after extended culture expansion of bmMSC and ucMSC. On the contrary, inhibition of T cell proliferation by bmMSC and ucMSC was affected by extended culturing. The reason for this is unclear and could have multiple causes. We observed no significant differences in PD-L1 expression and IDO activity after prolonged expansion of ucMSC (data not shown), indicating that other mechanisms are responsible for the different immunomodulatory efficacy of ucMSC.

This study demonstrates that both bmMSC and ucMSC have a high proliferative capacity and can be expanded far beyond what is currently standard practise, which could lead to a higher yield of therapeutic MSC doses. In addition, they maintain their phenotype, are genetically stable and exhibit similar susceptibility to NK cell lysis after extended culture expansion. However, the immunosuppressive capacities of MSC are reduced after long term expansion. Therefore, depending on the therapeutic application, the use of lower passage MSC might still be preferred over higher passage MSC. Thus concluding, the use of higher passage MSC in the clinic will lead to an higher yield of therapeutic MSC, however consequentially this may interfere with the efficacy of MSC therapy.

Supplementary material

		Immunophenotype (% cells expressing)							Maximum population doublings	Relative telome-re length	Mitocho-ndrial activity	% Diploid MSC	Inhibition CD4 T-cell prolifera-tion [1:2.5]	Inhibition CD8 T-cell prolifera-tion [1:2.5]	Induction CD107a expressing NK cells
		CD13	CD73	CD90	CD105	HLA class I	HLA class II	PD-L1							
bmMSC	Passage 4	97(1)	98(1)	54(23)	81(14)	27(6)	0(0)	31(16)	23 (2)	6(1)	0.9(0.01)	95(1)	30% (6) #	38% (8) #	13% (3)
	Passage 8	99(1)	99(1)	74(6)	81(17)	60(25)	0(0)	20 (9)	38 (2) *	5(1)	0.8(0.04)	-	56% (9) #	63% (11) #	24% (2)
	Passage 12	99(1)	99(1)	84(11)	94(6)	58(25)	0(0)	34(27)	45 (2) *	7(3)	0.7(0.04)	93(2)	64% (9) #	65% (18)	21% (6)
ucMSC	Passage 4	74(18)	50(25)	38(25)	37(31)	26(21)	0(0)	52(27)	28 (1)	8(2)	0.3(0.02)	93(1)	17% (0) #	29% (1) #	42% (10)
	Passage 8	81(10)	61(20)	57(19)	43(28)	37(31)	0(0)	55(24)	47 (1) *	7(1)	0.5(0.07)	-	47% (13) # *	66% (15) *	48% (11)
	Passage 12	93(5)	88(10)	71(16)	65(16)	46(26)	0(0)	73(11)	59 (1) *	7(1)	0.9(0.05)	91(2)	81% (17) *	89% (13) *	43% (13)

▲**Supplementary table 1. Summary of the findings of the various experiments of this study.** Means ± SEM are shown. * indicates significant difference compared to passage 4 MSC and # indicates significant difference compared to control without MSC in the columns showing inhibition of T cell proliferation

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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 freezethawing 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 interdonor 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 1–2x10^6 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 mouseoriginating 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

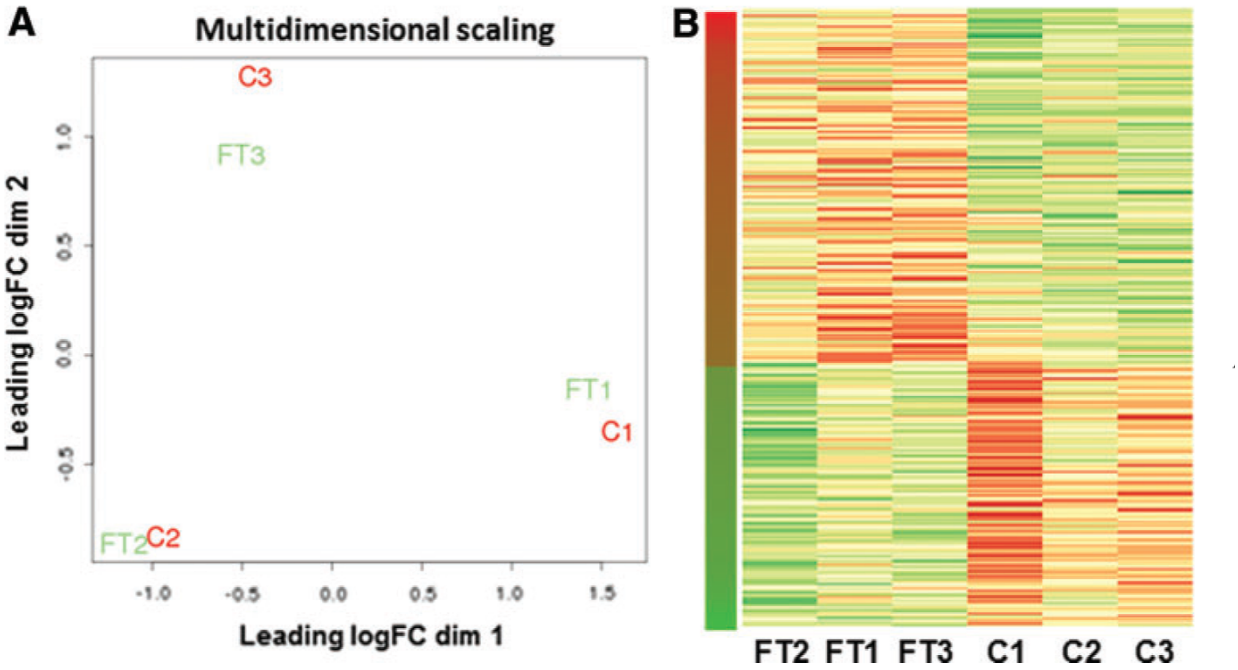
Gene expression profiling of MSC from continuous culture versus frozen–thawed MSC

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 interdonor 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 (Fig. 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.

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 signalling. 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.



▲Figure 1 mRNA expression analysis of human MSC from continuous culture (C) and frozen–thawed MSC (FT). (A) Clustering ofthe samples shows that interdonor 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.

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

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↑

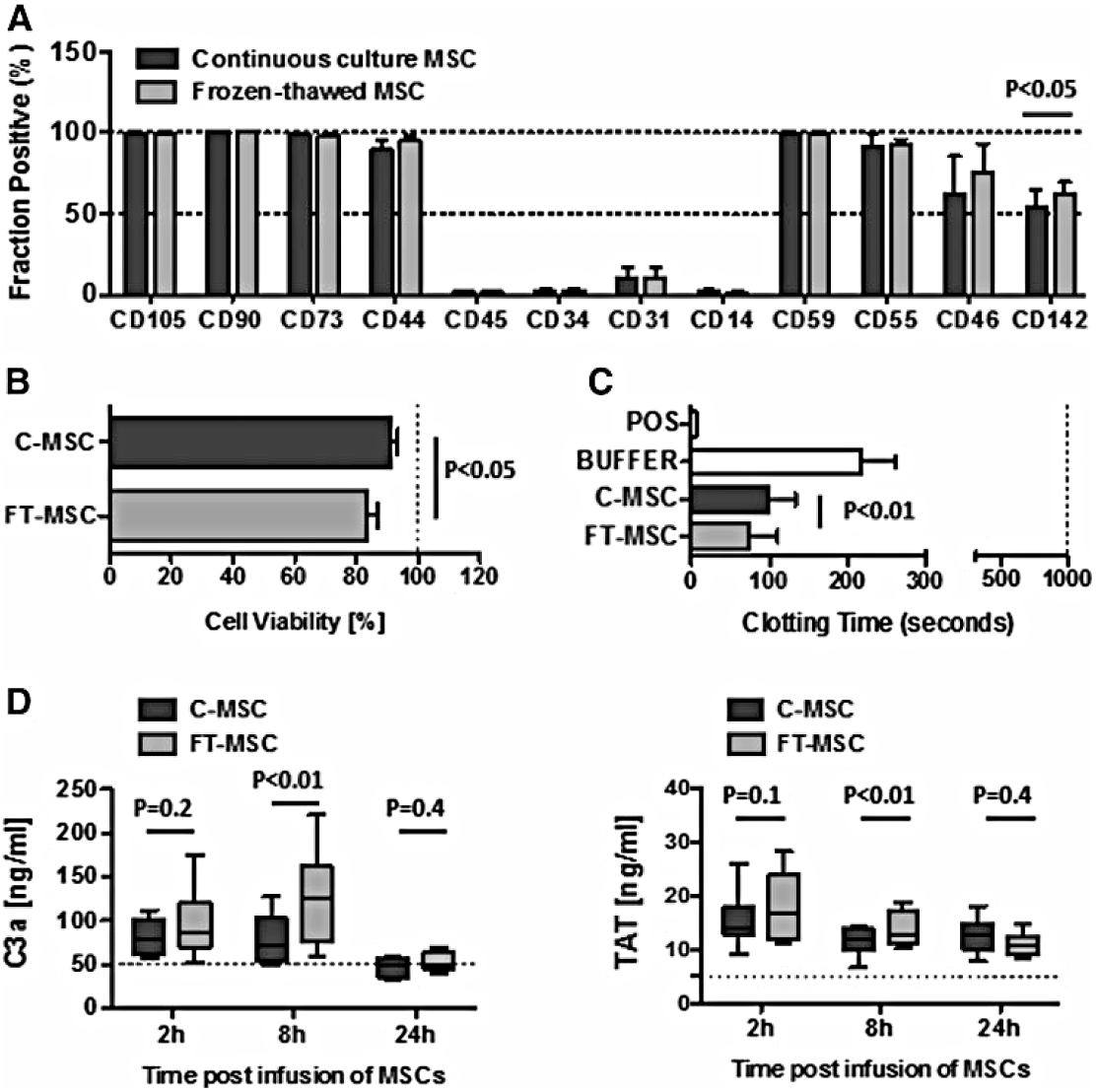
Acute phase response signaling↑: *z*-score 0.82, $P < 0.05$, 9 of 166 genes different; CARBP2↑, FOS↑, JUN↑, IL6↑, NFKBIA↑, SAA1↓, SAA2↓, SOCS3↑, TNFRSF11B↓

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.



▲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) Cellsurface 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.

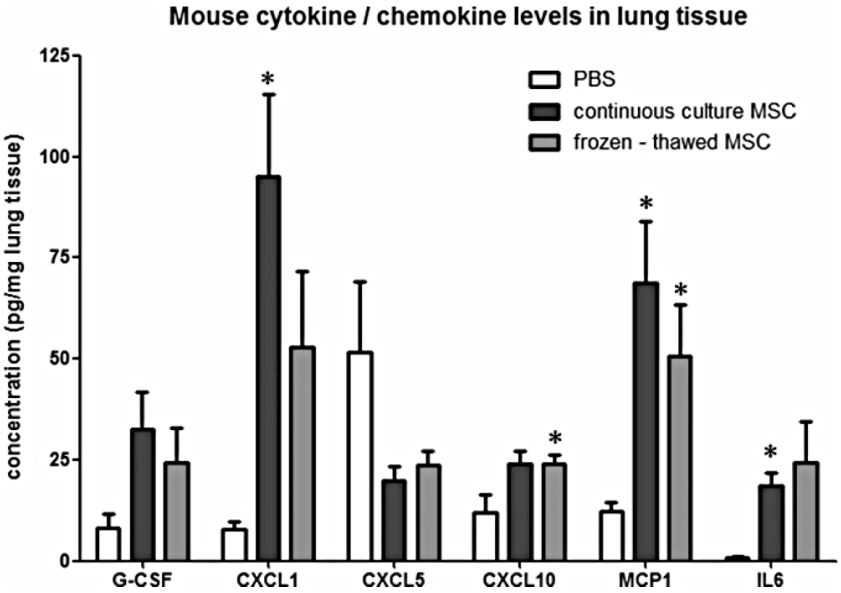
clotting time of human blood was examined in vitro. The addition of MSC from continuous culture accelerated blood clotting time *(Figure 2C)*. Frozen–thawed MSC induced a further, small but signifi- cant, acceleration of clotting time compared to continuously cultured MSC.

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 $P < 0.05$).

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 Fig. 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 ($1x10^6$) 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 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.



▲Figure 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. 1x10^6 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

Table 2. Summary of Gene Expression Differences Between Injected Mesenchymal Stromal Cells Versus Mesenchymal Stromal Cells from Continuous Culture

Gene expression changes of injected MSC vs. MSC from continuous culture

Gene name	*Fold change up*	*Gene name*	*Fold change down*
NOVA2 Regulation of RNA metabolic process	1,607	*CHAC1* Apoptosis in response to stress	10.3
LCE1A Keratinization	1,585	*EID2B* Differentiation Differentiation	7.4
TYRP1 Melanin biosynthetic process	1,252	*PPP1R35* Negative regulation of phosphatase activity	6.3
APOC2 Cholesterol homeostasis	1,218	*TIGD2* Unknown	6.1
S100B Proliferation, differentiation	1,113	*CH25H* Lipid metabolic processes	5.7
ADRB1 Adrenergic receptor	1,075	*MMAA* Lipid metabolic processes	5.1
KRT5 Cell junction assembly	1,075	*HDHD3* Metabolic processes	4.8
PRAME Negative regulation of apoptosis and differentiation	1,046	*INE1* Unknown	4.7
SOX17 Development	1,046	*KLHDC7B* Protein ubiquitination	4.5
BCL11B Differentiation	1,003	*TTC30B* Cell projection organization	4.5
ZNF366 Response to estrogen stimulus	955	*TIGD1* Unknown	4.2
HLA-DRA Antigen processing and presentation	815	*HILPDA* Positive regulation of cell proliferation, stress response	4.2
NRARP Negative regulation of Notch signaling	798	*GLI4* Regulation of transcription	4.1
CD74 (HLA-DR chain) Antigen processing and presentation	744	*CHAMP1* Protein localization to microtubule	4.0
MIR142 Cellular response to estrogen and lipopolysaccharide	657	*SERTAD4-AS1* unknown	4.0

Pathways differentially expressed and genes involved

OX40 signaling↓: z-score –0.46, $P<0.05$, 12 of 36 genes different; BCL2↑, HLA-DMA↑, HLA-DPA1↑, HLA-DQB1↑, HLA-DRA↑, HLA-DRB1↑, HLA-DRB5↑, MAPK12↓, NFKB1↑, NFKBIB↑, NFKBIE↑

TNF receptor 2 signaling↑: z-score 0.71, $P<0.05$, 8 of 26 genes different; BIRC3↑, IKBKG↓, MAP3K14↑, NFKB1↑, NFKBIB↑, NFKBIE↑, TBK1↓, TNFRSF1B↑

Activation of interferon regulatory factor by cytosolic pattern recognition receptors↓: z-score –2.50, $P<0.05$, 13 of 59 genes different; IFIH1↑, IFIT2↓, IKBKG↓, IRF3↓, IRF9↓, ISG15↑, MAPK12↓, NFKB1↑, NFKBIB↑, NFKBIE↑, SIKE1↓, STAT2↓, TBK1↓

PKCθ signaling in T lymphocytes↑: z-score 2.45, $P<0.05$, 19 of 104 genes different; HLA-DMA↑, HLA-DQA1↑, HLA-DQB1↑, HLA-DRA↑, HLA-DRB1↑, HLA-DRB5↑, IKBKG↓, MAP3K4↑, MAP3K6↓, MAP3K12↓, MAP3K14↑, NFATC1↑, NFATC2↑, NFKB1↑, NFKBIB↑, NFKBIE↑, PIK3C3↓, PIK3CB↑, PIK3R3↑

Induction of apoptosis by HIV1↓: z-score 1.16, $P<0.05$, 12 of 56 genes different; BCL2↑, BIRC3↑, DFFA↓, DFFB↓, IKBKG↓, MAP3K14↑, MAPK12↓, NFKB1↑, NFKBIB↑, NFKBIE↑, TNFRSF1B↑, TP53↓

▲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.

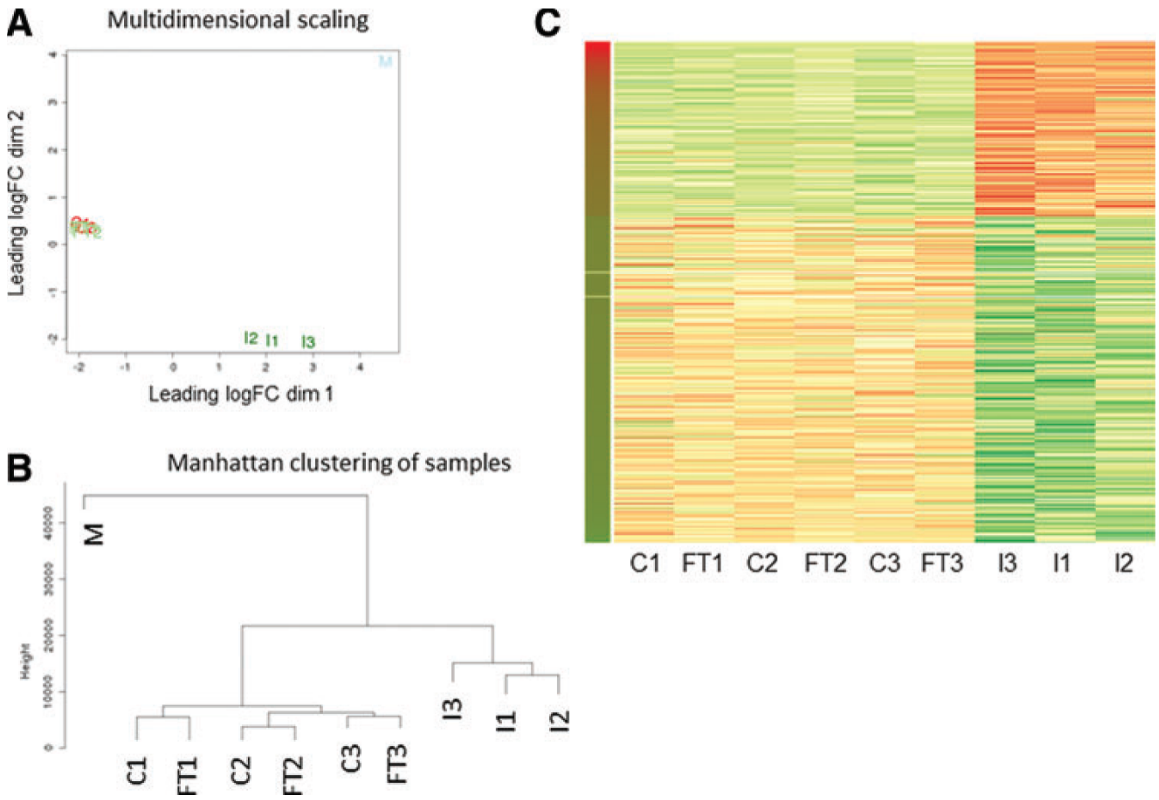
↑, upregulation; ↓, downregulation.

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.



▲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.

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).

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.

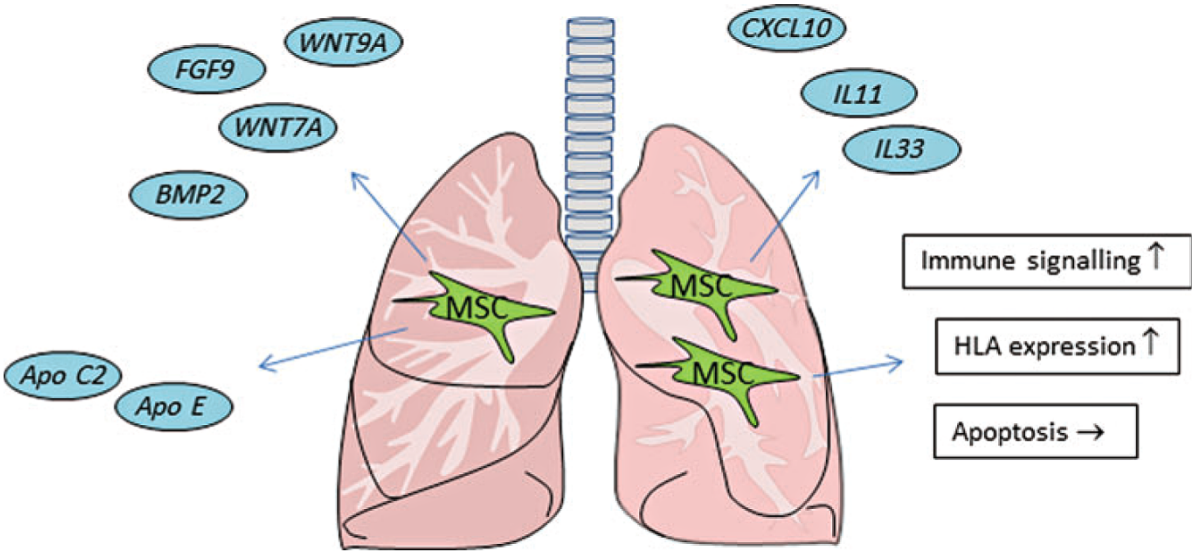
In contrast to the minimal effects of freeze–thawing, in vivo administration had a major effect on the transcriptional phenotype of MSC. The observed changes induced in MSC could derive from a number of factors, among them the effects of sheer-stress induced by transport through the bloodstream, factors 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 xenoresponse. 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



▲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.

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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5

Cytokine treatment optimises the immunotherapeutic effects of umbilical cord derived MSC for treatment of inflammatory liver disease.

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Abstract

Background

Mesenchymal stromal cells (MSC) possess immunomodulatory properties and are low immunogenic, both crucial properties for their development into an effective cellular immunotherapy. They have shown benefit in clinical trials targeting liver diseases; however the efficacy of MSC therapy will benefit from improvement of the immunomodulatory and immunogenic properties of MSC.

Methods

MSC derived from human umbilical cords (ucMSC) were treated for 3 days *in vitro* with various inflammatory factors, interleukins, vitamins and serum deprivation. Their immunogenicity and immunomodulatory capacity were examined by gene-expression analysis, surface-marker expressions, IDO activity, PGE_2 secretion and inhibition of T-cell proliferation and IFNγ production. Furthermore, their activation of NK cell cytotoxicity was investigated via CD107a expression on NK cells. The immunomodulatory capacity, bio distribution and survival of pre-treated ucMSC were investigated in a CCl_4-induced liver disease mouse model. In addition, capacity of pre-treated MSC to ameliorate liver inflammation was examined in an *ex vivo* liver inflammation co-culture model.

Results

IFNγ and a multi cytokine cocktail (MC) consisting of IFNγ, TGFβ and retinoic acid upregulated the expression of immunomodulatory factor PD-L1 and IDO activity. Subsequently, both treatments enhanced the capacity of ucMSC to inhibit CD4 and CD8 T-cell proliferation and IFNγ production. The susceptibility of ucMSC for NK cell lysis was decreased by IFNβ, TGFβ and MC treatment. *In vivo*, no immunomodulation was observed by the ucMSC. Four hours after intravenous infusion in mice with CCl_4 induced inflammatory liver injury, the majority of ucMSC were trapped in the lungs. Rapid clearance of ucMSC($VitB_6$), ucMSC(Starv+$VitB_6$) and ucMSC(MC) and altered bio-distribution of ucMSC(TGFβ) compared to

untreated ucMSC was observed. In the e*x vivo* co-culture system with inflammatory liver slices ucMSC(MC) showed significantly enhanced modulatory capacity compared to untreated ucMSC.

Conclusion

The present study demonstrates the responsiveness of ucMSC to *in vitro* optimisation treatment. The observed improvements in immunomodulatory capacity as well as immunogenicity after MC treatment may improve the efficacy of ucMSC as immunotherapy targeted towards liver inflammation.

Background

Mesenchymal stromal cells (MSC) are a novel therapeutic option for inflammatory conditions and have been studied in animal models as well as in preliminary clinical studies [1-6]. In particular, MSC have shown potential in clinical trials of patients with liver diseases, such as liver cirrhosis [7-10] and acute-on-chronic liver failure [11-13]. In addition, they have shown to be able to modulate the immune system in other various diseases, however not for all diseases there is convincing evidence [1]. MSC possess two immunological properties that make them interesting candidates for cellular immunotherapy; their immunomodulatory capacity and low recognition by the host immune system. Optimisation of these properties will enhance their efficacy as immunotherapy.

MSC express a vast range of factors, which target immune cells and influence their function, such as modulation of B cell and natural killer (NK) cell function, suppression of T-cell proliferation/activation as well as induction of regulatory T-cells (Treg) [14-17]. One of their key immunomodulatory factors is indoleamine 2,3-dioxygenase (IDO), which deprives the environment of L-Tryptophan thereby inhibiting lymphocyte proliferation [18], has been demonstrated to promote graft acceptance after solid organ transplantation [19]. In addition, prostaglandin E_2 (PGE_2), CCL2 and interleukin 1 receptor antagonist (IL1RA), which are produced by MSC have shown to have an important role in the therapeutic effects of MSC on auto-immune diseases, such as rheumatoid arthritis [3, 4, 20-23]. Besides secretion of anti-inflammatory factors, MSC express cell surface proteins that are known for their immuno-regulatory function, such as programmed death-ligand 1 (PD-L1), which inhibits lymphocyte proliferation [24].

In addition, MSC have low immunogenicity due to low levels of HLA class-I and lack of HLA class-II and co-stimulatory molecule expression on their surface, which may potentially allow the use of allogeneic MSC for treatment without inducing allogeneic responses [25]. In addition, complex formation of HLA class-I with inhibitory NK cell receptors protects MSC from NK cell lysis [26]. However, HLA class-I enables recognition of MSC by allogenic $CD8^+$ T cells, and initiation of lysis [26, 27] and Cho *et al* observed IgG antibody responses to allogeneic umbilical cord tissue-derived stromal cells [28]. Consequently, recognition by immune cells *in vivo* may reduce the

survival time of MSC and thereby restricting the duration of their effect [29]. Another *in vivo* drawback is that MSC get trapped in the lungs due to their size, restricting their migration to the site of injury [29, 30]. Improving MSC *ex vivo* in such a way that their immunogenicity is reduced and their immunosuppressive capacity is enhanced may therefore be beneficial for their survival as well as their effectiveness after administration.

MSC can be isolated from different tissues in the body [31], and often constitute a heterogeneous population of cells. The use of a more homogeneous MSC subset, will potentially increase the consistency of treatment results and improve efficacy. Studies involving specific MSC subsets, based on selection of MSC expressing Stro-1, CD73, CD90 or CD271, have shown that these subsets possess enhanced immunosuppressive capacities [32-36]. Recently, a new subset, identified by the surface marker $CD362^{+}$ (Syndecan-2) has been identified (patent number WO 2013117761 A1), which showed improved clonogenicity and immunomodulatory properties.

MSC contain an abundance of surface receptors for a large variety of factors such as inflammatory factors, interleukins, prostaglandins and several vitamins. Treatment of MSC by such factors, like interferon-γ (IFNγ), tumor necrosis factor-α (TNFα) and interleukin 17 (IL17), induces MSC to adapt towards a more immunomodulatory phenotype and alters their immunogenicity [37-39]. In addition, different culture conditions, such as serum-deprivation and hypoxia [40] may affect the function of MSC.

In this study, we exposed MSC to various growth factors, cytokines and culture conditions with the aim to generate MSC with enhanced properties; reduced immunogenicity and improved immunosuppressive capacity, for immunotherapy to target liver inflammation. Through analysing surface marker expression, gene expression and secretome we examined the immunophenotype of pre-treated MSC. In addition, the immunogenicity and immunomodulatory capacity of pre-treated MSC were evaluated by their susceptibility to trigger NK cytotoxic activity and their capacity to inhibit lymphocyte proliferation, respectively. Furthermore, their potential to ameliorate inflammation and immunomodulate the immune response was tested *in vivo* as well as *ex vivo* in a murine liver inflammation model.

Methods

Culture expansion of ucMSC

Human umbilical cord tissue was collected from Caesarean section deliveries by Tissue Solutions Ltd. (Glasgow, UK) from virally screened healthy donors. All such cord were obtained according to legal and ethical requirements of the UK, with the approval of the relevant ethics committee and with anonymous consent from the donor. Cords were harvested within 48 hours of birth and the average length of collected umbilical cord tissue (n=4-6) was 8.6 cm, with all tissues transported for processing to Orbsen Therapeutics Ltd. in AQIX® solution (London, UK) at 4°C. In short, umbilical cord tissue was washed and the whole tissue was manually dissociated before enzymatic digestion in an enzyme cocktail consisting of MEM Alpha (Gibco, ThermoFisher, UK), Collagenase 1 (2mg/ml), Hyaluronidase 1 (1mg/ml) and DNase (0.1mg/ml) (Sigma Aldrich, Ireland) for a maximum of 2 hours at 37°C. Thereafter, a single cell suspension was obtained by filtration (100µm) and the cells were stained with CD362 (Syndecan-2/ORB, APC, clone 305515, dilution 1:50, R&D Systems, US) for 30mins at 4°C. Subsequently, cells were washed and resuspended in MACs buffer 80µl/10^7cells. Next, the cells were incubated with anti-APC beads (20µl/10^7cells, Miltenyi Biotec, Germany) for 15mins at 4°C. $CD362^+$ cells were then isolated using MS MACs column according to manufactures instructions, (Miltenyi Biotec, Germany). Each cell fraction was 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 (MEM-α; Sigma, St Louis, MO, USA) containing 2 mM L-glutamine (Lonza, Verviers, Belgium), 1% penicillin/streptomycin solution (P/S; 100IU/ml penicillin, 100IU/ml streptomycin; Lonza) and supplemented with 15% fetal bovine serum (FBS; Lonza) and 1ng/ml basic fibroblast growth factor (bFGF) (Sigma) and kept at 37°C, 5% CO_2 and 20% O_2. Medium was changed once a week and ucMSC were passaged at ~80-90% confluence using 0.05% trypsin-EDTA (Life technologies, Paisley, UK). All ucMSC used in experiments were between passage 4-7.

Study design

A set of *in vitro*, *in vivo* and *ex vivo* experiments was designed to analyse the effect of pre-treatment of ucMSC with various growth factors, cytokines and culture conditions, as depicted in supplementary figure 1 and described in detail below.

Treatment of ucMSC

UcMSC were treated with (table 1 and supplementary table 1): Activin A (12ng/ml; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), budesonide (50mM; FLUKA Sigma), interferon gamma (IFNγ, 50 ng/ml; Life technologies), interferon beta (IFNβ, 5 ng/ml; Peprotech, NJ, USA), transforming growth factor beta 1 (TGFβ, 10 ng/ml; R&D systems, MN, USA), interleukin 15 (IL15, 10 ng/ml; Peprotech), interleukin 17 (IL17, 50 ng/ml; Peprotech), retinoic acid (RA, 1 μM and 10 μM; Sigma), trespostinil (20 ng/ml; Cayman chemical company, MI, USA), tumor necrosis factor α (TNFα, 10 ng/ml; Peprotech), 1α,25-Dihydroxyvitamin D_3 (vitamin D3, VitD3; 0.01 μM; Sigma) and pyridoxal hydrochloride (vitamin B_6, $VitB_6$, 1 μM; Sigma). All factors were added at day 0 and the cells were analysed at day 3, except for trespostinil, which due to its instability was added daily. In addition, ucMSC were also cultured without serum (starvation, Starv). Two further combinations were also used: starvation and vitamin B6 (Starv+$VitB_6$) and the multi cytokine combination (MC), consisting out of IFNγ, TGFβ and RA (10 μM).

Flow cytometric analysis

Flow cytometric characterisation of control and treated ucMSC was performed by labelling with standard MSC markers, according to ISSCT criteria [41]: CD13 (PE-Cy7, BD Biosciences), CD73 (PE, BD Pharmingen), CD90 (APC, R&D systems) and CD105 (FITC, R&D systems), hematopoietic markers CD31 (PB, BD Biosciences), CD45 (APC-Cy, BD Biosciences) and the surface markers: HLA class-I (Pacific blue, Biolegend), HLA class-II (PERCP, BD Biosciences), CD362 (APC, R&D systems), PD-L1 (PE, Biolegend), CD271 (PE-Cy7, BD Pharmingen), CD73 (PE, BD Pharmingen) and CD40 (PERCP, Biolegend).

L-Kynurenine Assay

IDO activity was analysed by measuring L-Kynurenine levels in the supernatants. Supernatant was harvested at day 3 and centrifuged for 10 minutes at 3000 rpm, to remove dead cells. Samples were stored at -80°C until the assay was performed. After thawing, samples were spun down, at 3000 rpm for 10 minutes. Subsequently, 100 µL 30% trichloroacetic acid (TCAA) was added to 200 µL sample, this was then vortexed and placed in a 50°C waterbath for 30 minutes. The tubes were then centrifuged at 12000 rpm for 5 minutes. 75 µL supernatant was added to 75 µL Ehrlich reagent, in duplo, and measured with a spectrophotometer (Victor 1420 multilabel plate reader; LKB-Wallac, Turku, Finland) at an absorbance of 490 nm.

PGE_2 ELISA

PGE_2 secretion by ucMSC was analysed in ucMSC culture supernatant with the PGE_2 high sensitivity ELISA kit according to the manufacturer's instructions (Enzo Life Sciences BVBA, Netherlands, ADI-930-001).

RT-PCR

UcMSC were seeded at 100.000 cells/well in 6-well plates and stimulated with the various factors. At day 3, supernatant was removed and cells were washed with PBS, RNA later (Life Technologies) added and the plates placed in the fridge. The next day mRNA was isolated with the use of the High Pure RNA Isolation Kit (Roche). mRNA was isolated from liver slices using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). cDNA was synthesized from 500ng mRNA with random primers (Promega Benelux B.V., The Netherlands). Quantitative gene expression was determined using TaqMan Gene Expression Master Mix (Life Technologies) and Assays-on-demand for IL1RA (Hs00893626_m1), MCP-1 (Mm00441242_m1), IL6 (Mm00446190_m1), TNFα (Mm00443258_m1) and IP10 (Mm00445235_m1). GAPDH (Hs 99999905_m1) was used as housekeeping gene for analysing gene expression of ucMSC. Data was expressed as the gene expression relative to GAPDH and comparing fold changes to the untreated MSC.

Gene expression levels of the liver slices are expressed as copy numbers ($\text{efficiency}^{-\Delta Ct}$).

Isolation of PBMC

Peripheral blood samples were collected from living kidney donors at Erasmus MC or isolated from buffy coats obtained from Sanquin Blood bank. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, using Ficoll-Paque (GE healthcare) and frozen at - 180°C until use.

CD107a NK cytotoxicity assay

For generation of activated NK cells, PBMCs were thawed, washed and seeded at 100.000 cells/well in 96-wells plates. They were cultured in RPMI 1640 medium (Life technologies) with 1% penicillin/streptomycin solution (P/S; 100 IU / ml penicillin, 100 IU/ml streptomycin; Lonza), 10% heat inactivated FBS (Lonza), interleukin 2 (IL2, 2.10^2 IU / ml; Peprotech) and interleukin 15 (IL15, 10 ng / ml; Peprotech). At day 7, cells were collected and washed and NK cells were isolated using a negative selection MACS procedure (NK Cell isolation kit human; MACS Miltenyi Biotec). Following MACS and a washing step, the cells were left overnight at 37°C in a polystyrene tube with IL2 and IL15.

MACS sorted NK cells were added to pre-treated ucMSC at a 4:1 ratio for 1 hour at 37°C, together with monensin (0.1 µL / 200 µL/well; BD Golgistop; BD Biosciences) and αCD107a antibody (LAMP-1, APC, BD Pharmingen) in polypropylene tubes. The cells were then washed and stained for CD3 (PERCP; BD Biosciences), CD16 (PE; BD Biosciences) and CD56 (PE; BD Biosciences) for 30 minutes at 4°C. The cells were subsequently washed and analysed with the FACSCanto II flow cytometer.

T cell proliferation assay

Pre-treated ucMSC were seeded into 96-wells plates and left overnight to adhere in the incubator. The next day PBMC were labeled with Cell Trace CFSE (Life

Technologies) according to the instructions of the manufacturer and seeded on top of the ucMSC, at different [MSC:PBMC] ratios: [1:10], [1:5] and [1:2.5]. αCD3/CD28 stimulation was added (0.5 ug / ml αCD3 antibody, 0.5 µg/ml αCD28 antibody and 0.5 µg/ml goat-α-mouse antibody; Life Technologies). The co-cultures were left for 3 days and PBMC were collected. PBMC were stained for CD4 (APC; eBioscience), CD8 (Pe-cy7; eBioscience) and intracellular IFNγ (Bv421; BD Biosciences). With the use of the FACSCanto II flow cytometer the proliferation of PBMCs was measured.

Carbon TetraChloride (CCl_4) liver injury model

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

In the first set of experiments mice received either intraperitoneal (IP) CCl_4 solution (4 µl/g bodyweight, 250 µl / ml mineral oil; 289116 Sigma) or mineral oil for controls. Three hours after CCl_4 injection pre-treated ucMSC, which were labeled with Qtracker beads, were infused intravenously (IV) in the tail vein in 200 µl suspension in PBS (250.000 cells/mice). Four hours after ucMSC infusion, mice were sacrificed using cardiac puncture under isoflurane anesthesia (Supplementary Figure 2 for experimental regimen). Whole mice were embedded in Mounting medium for Cryotomy (O.C.T. compound; VWR Chemical) and put in liquid nitrogen until frozen. The whole mice were then stored at -80°C until shipment to BioInVision for imaging.

In the second set of experiments, mice receiving IP CCl_4 solution had a similar infusion of pre-treated ucMSC three hours later. After 72 hours the mice were sacrificed by cervical dislocation and blood and livers were harvested and frozen until further use.

ALT measurement

Serum samples were thawed and centrifuged at 3000 rpm for 10 minutes. Thereafter, 50 µl of sample was diluted 1:5 with MilliQ. ALT quantification was done with an ALT measurement kit according to the instructions of the manufacturer (Roche Diagnostics; 04467388190). The samples were measured using a Roche/Hitachi cobas c analyzer.

Labelling, Quantification and Visualization of MSC

Pre-treated ucMSC were collected using 0.05% trypsin-EDTA (Life technologies) and washed. Subsequently they were labelled with Qtracker 605 cell labeling kit beads according to the manufacturer's instructions (Life technologies). UcMSC take up Qtracker 605 beads during the labelling procedure. Post labelling, ucMSC were washed to remove any beads that were not internalized by the ucMSC. 3D anatomical and molecular fluorescence videos were generated of the frozen whole mice samples by BioInVision Inc. with CryoViz^{TM} technology. The CryoViz^{TM} technology picks up the signal of clustered of Qtracker 605 beads, which are internalized in the ucMSC. Cell counts were quantified using imaging algorithms.

Ex vivo culture of liver slices

Mouse livers were obtained from healthy C57Bl/6 mice. Directly after isolation, mouse livers were sliced into 150 µm thick slices with a diameter of 1 cm with Vibr0tome (VT 1200S, LEICA). The slices were subsequently placed in 6-wells plates containing differently pre-treated MSC in high glucose DMEM with 10% serum, supplementary figure 3. In addition, lipopolysaccharide [LPS, 0.2 µg/ml; Sigma-Aldrich] was added to the medium. The tissues were kept overnight at 37°C, 5% CO_2 and 20% O_2 on a shaker (50 rpm). After 24 hours, supernatant was collected and the liver slices were snapped frozen.

Milliplex assay

Serum and tissue culture supernatant levels of IL6, IL10, granulocyte colony-stimulating factor (G-CSF), interferon gamma-induced protein 10 (IP-10), keratinocyte chemoattractant (KC, CXCL1), monocyte chemoattractant protein-1 (MCP-1, also known as CCL2), macrophage inflammatory protein-1 alpha (MIP1α) and tumor necrosis factor alpha (TNFα) were measured using a Milliplex “mouse cytokine/chemokine magnetic bead panel multiplex assay” (Merck Millipore) and Mouse Premixed Multi-Analyte kit (Magnetic Luminex assay; cat LXSAMSM; R&D systems). The samples were measured on the Luminex 100/200 cytometer (Luminex, Austin, TX, USA) using Xponent software.

Statistical Analysis

Data was analysed using IBM SPSS Statistics 21 and Prism software v5.04 (GraphPad Software Inc. La Jolla, CA), herein Mann Whitney tests were performed. P-values <0.05 were considered significant.

Results

Characterization of ucMSC

Flow cytometric analysis of ucMSC demonstrated expression of CD13, CD73, CD90 and CD105 and absence of CD31 and CD45 (Supplementary Figure 4). This is in line with the minimal ISSCT criteria and thus confirming the MSC phenotype of the used cells.

Impact of pre-treatment of ucMSC on immunogenicity and immunomodulatory phenotype

To modulate the immunomodulatory and immunogenic phenotype of ucMSC *in vitro*, ucMSC were differentially treated for 3 days and the expression of key immunomodulatory and immunogenicity molecules was examined (Table 1).

Expression of HLA class-I and -II on the ucMSC cell surface was used as a marker of ucMSC immunogenicity. The percentage of HLA class-I expressing ucMSC increased after treatment with IFNγ (8-fold; $p<0.05$), IFNβ (5-fold; $p<0.05$), Starv (2-fold; $p<0.05$), $VitB_6$ (7-fold; $p<0.05$), Starv+$VitB_6$ (9-fold; $p<0.05$), RA (4-fold; $p<0.05$) and MC (12-fold; $p<0.05$) compared to untreated ucMSC. In addition, treatment with IFNγ, $VitB_6$, Starv+$VitB_6$ and MC also increased the number of ucMSC expressing HLA class-II by 7- ($p<0.05$), 3- ($p<0.05$), 2- ($p<0.05$) and 8- fold ($p<0.05$), respectively.

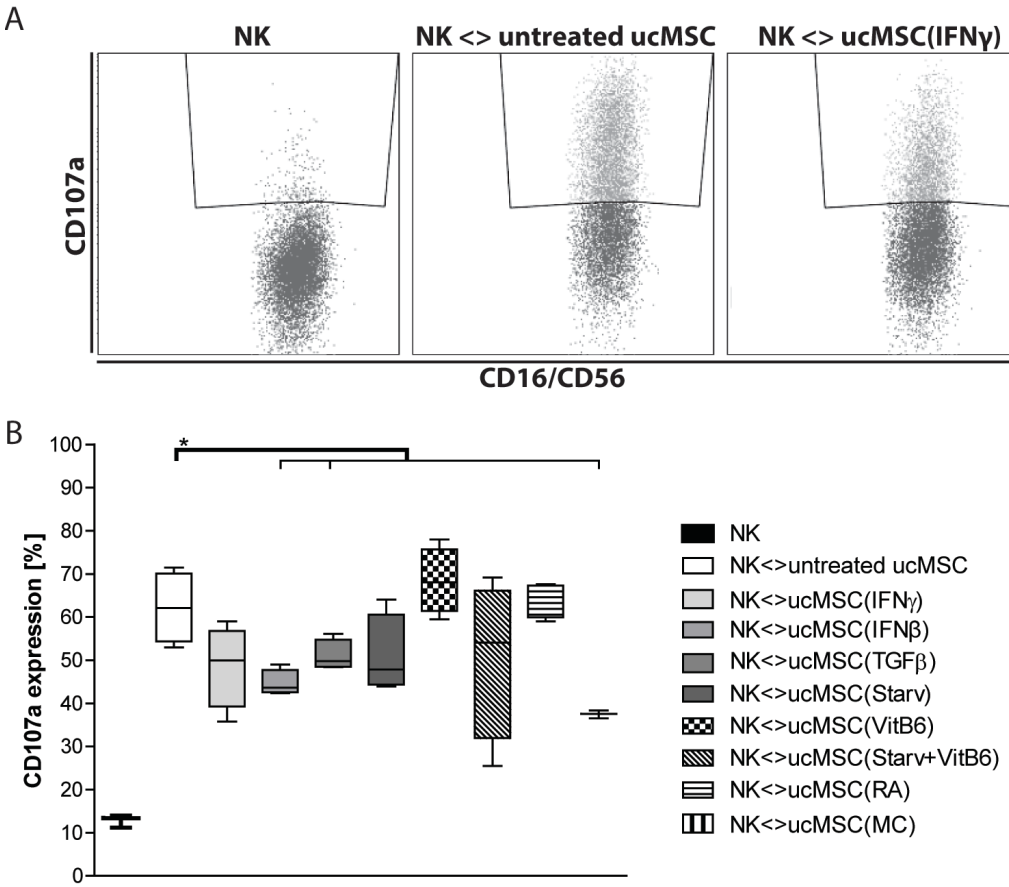
The number of ucMSC expressing the negative co-stimulatory molecule PD-L1 increased with IFNγ (96% ±3; $p<0.05$), Starv+$VitB_6$ (72% ±1; $p<0.05$) and MC treatment (97% ±1; $p<0.05$), compared to untreated ucMSC (46% ± 6). In addition, expression levels of PD-L1 per ucMSC were increased by IFNγ (median fluorescence intensity (MFI)=1947 ±263; $p<0.05$), Starv+$VitB_6$ (MFI=755 ±235; $p<0.05$) and MC treatment (MFI=1279 ±216; $p<0.05$), with respect to untreated ucMSC (MFI=219 ±22) (data not shown in Table 1). All treatments preserved the expression of CD73, which is involved in anti-inflammatory adenosine production. Gene-expression of IL1RA showed trends of upregulation after $VitB_6$ treatment and the combined treatment of Starv+$VitB_6$ (67- ($p<0.05$), and 507- fold increase

(p<0.05), respectively). IFNγ and MC treatment significantly upregulated IDO activity of ucMSC 14-fold (p<0.05) and 15-fold (p<0.05), respectively. IFNγ treatment significantly reduced the secretion of anti-inflammatory PGE_2 compared to untreated MSC (14-fold; p<0.05).

	Immunogenic read out parameters		**Immunomodulatory read out parameters**				
	HLA class I expressing cells [%]	**HLA class II expressing cells [%]**	**L-Kynurenine (correlated IDO activity) [µM]**	**PGE_2 [ng/ml]**	**PD-L1 expressing cells [%]**	**CD73 expressing cells [%]**	***Fold Increase of Gene expression compared to stimulated* IL-1RA [corrected for GAPDH]**
[-]	7±1	10±3	2±5	14±3	46±6	95±2	1±0
IFNy	60±9 *	68±9 *	27±10 *	1±0 *	95±3 *	94±3	1±0
IFNβ	36±12 *	14±5	10±10	3±2	73±12	91±5	4±1
TGFβ	9±3	10±3	3±1	3±2	58±7	89±5	4±4
Starv	15±3 *	4±2	6±3	4±3	24±8 *	100±0	1±0
VitB6	47±5 *	34±9 *	3±5	12±5	59±14	100±0	67±42
Starv + VitB6	62±7 *	22±5 *	0±3	6±3	72±7 *	100±0	507±262
RA	26±5 *	18±5	0±0	5±2	59±11	98±1	5±4
MC	85±4 *	76±7 *	29±8 *	8±3	97±1 *	98±2	3±2

▲***Table I Observed changes in immunogenic and immunomodulatory molecules after 3 days in vitro treatment of ucMSC.*** The table displays the mean ± SEM percentages of MSC expressing surface markers HLA class –I and –II, PD-L1 and CD73, measured by flow cytometry , the mean ± SEM concentration of L-Kynurenine [µM] and PGE_2 [ng/ml] measured by ELISA and fold increases in IL1RA mRNA expression compared to unstimulated MSC. n=5, * indicates p<0.05.

Treatment with IL7, IL15, IL17, budesonide, trespostinil, activin A and TNFα showed no effect on any of these parameters (supplementary table I) and thus the effects of these factors were not examined further.

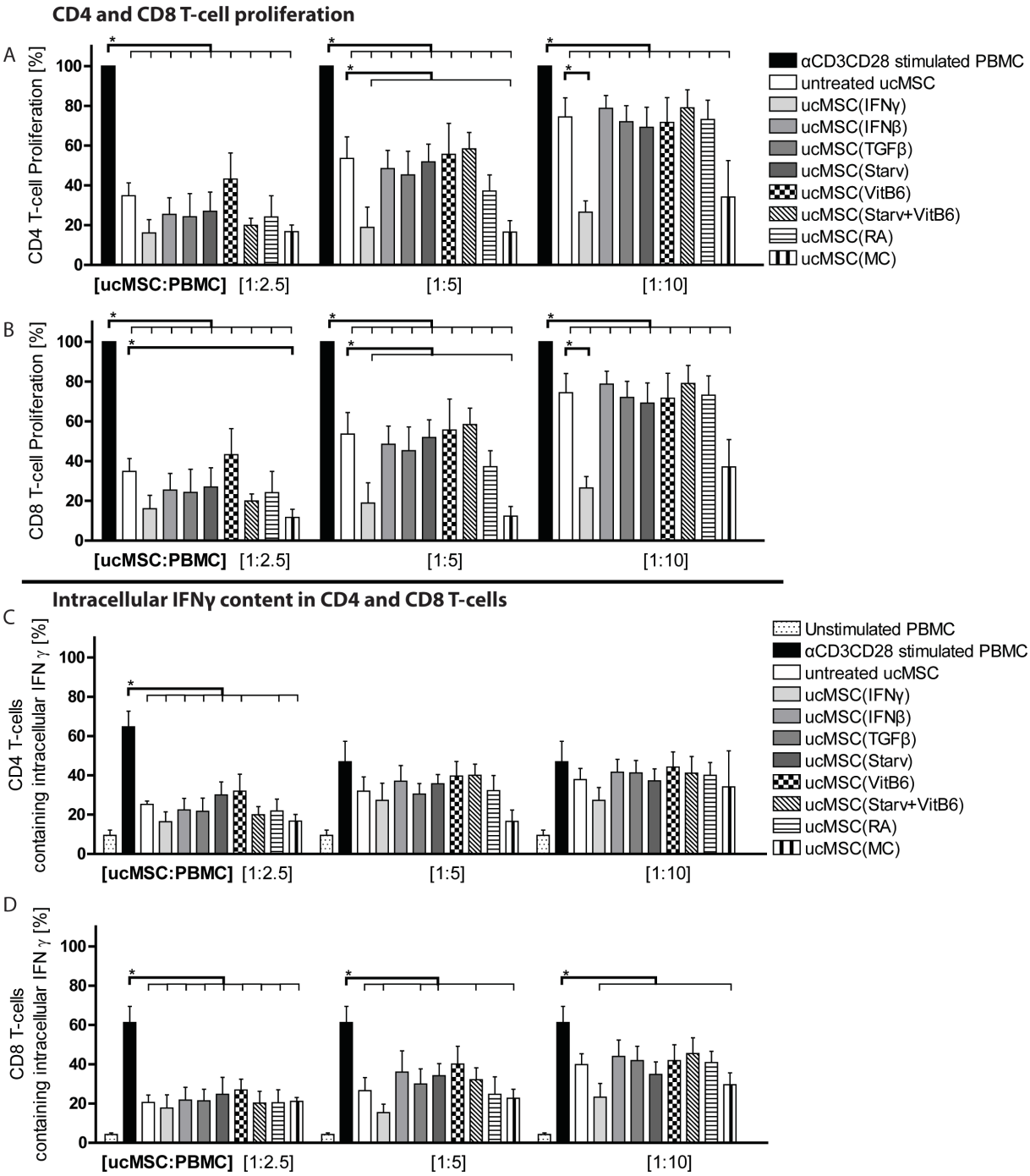


▲***Figure 1*** **CD107a expressing NK cells after exposure to pre-treated ucMSC. (a)** Representative FACS plots of CD107a expression on NK cells, without ucMSC (left), with untreated ucMSC (middle), with IFNγ treated ucMSC (right) **(b)** Graph displaying boxplots of CD107a expressing NK cells after exposure to the various pre-treated MSC (untreated, IFNγ, IFNβ, TGFβ, starvation, vitamin B6, Starv+VitB_6, retinoic acid and combination IFNγ+TGFβ+RA (MC)). Results are shown as means ± SEM (n=4). * indicates significant difference ($p<0.05$).

IFNβ, TGFβ and MC treatment of ucMSC protects against NK cell lysis.

To investigate the effect of pre-treatment of ucMSC on their susceptibility to NK cell lysis, the induction of CD107a expression on NK cells by ucMSC was analysed. The percentage of CD107a expressing NK cells increased from 13% ±1 to 62% ±4 ($p<0.05$) when NK cells were exposed to untreated ucMSC (Figure 1), however pre-treatment of ucMSC with IFNβ, TGFβ and MC reduced the increase in CD107a

expression on NK cells to 45% ±1 ($p<0.05$), 51% ±2 ($p<0.05$) and 37% ±1 ($p<0.05$) respectively.



▲***Figure 2*** **Inhibition of CD4 and CD8 T-cell proliferation and intracellular IFNγ production.** MSC and αCD3CD28 stimulated and CSFE labeled PBMCs were co-cultured at different ratios for 7 days. Thereafter proliferation of **(a)** CD4 and **(b)** CD8 T-cells was measured utilizing CFSE dilution with FACS. Proliferation is expressed as the percentage of proliferating cells relative to the positive control in the absence of ucMSC. **(c)** IFNγ production by CD4 and **(d)** CD8 T-cells was measured using FACS and an intracellular labeling of IFNγ. The IFNγ containing CD4 and CD8 T-cells are represented as a percentage from the CD4 or CD8 T-cell population. Results are shown as means ± SEM (n=5). * indicates significant difference ($p<0.05$).

Pre-treated MSC inhibit T-cell proliferation and IFNγ production.

To examine the potential of MSC to inhibit CD4 and CD8 T-cell proliferation and IFNγ production, αCD3CD28 activated PBMCs were co-cultured at different ratios with pre-treated ucMSC. CD4 and CD8 T-cell proliferation was inhibited in a dose-dependent manner by all ucMSC (Figure 2a/b). UcMSC treated with IFNγ and MC suppressed CD4 and CD8 T-cell proliferation more potently than untreated MSC (Figure 2a/b). However, none of the pre-treated ucMSC were able to significantly reduce the intracellular IFNγ content of CD4 and CD8 T-cells, compared to the untreated MSC (Figure 2c/d).

Effect of ucMSC in CCl_4 induced liver inflammation model

To examine the immunomodulatory capacity of pre-treated ucMSC in CCl_4 treated mice, serum levels of inflammatory associated cytokines MCP-1 and IP-10 were measured 3 days after cell infusion (Figure 3a/b). Whilst, significantly elevated levels were observed when comparing healthy to liver injured mice, no significant differences were observed with ucMSC infusion. Similarly, infusion of pre-treated ucMSC did not reduce ALT levels as a marker of liver damage with respect to the CCL4 control or compared to infusion with untreated ucMSC (Figure 3c). As no immunomodulation was observed; bio distribution of ucMSC was subsequently examined.

Pre-treatment of ucMSC alters their retention and bio distribution *in vivo.*

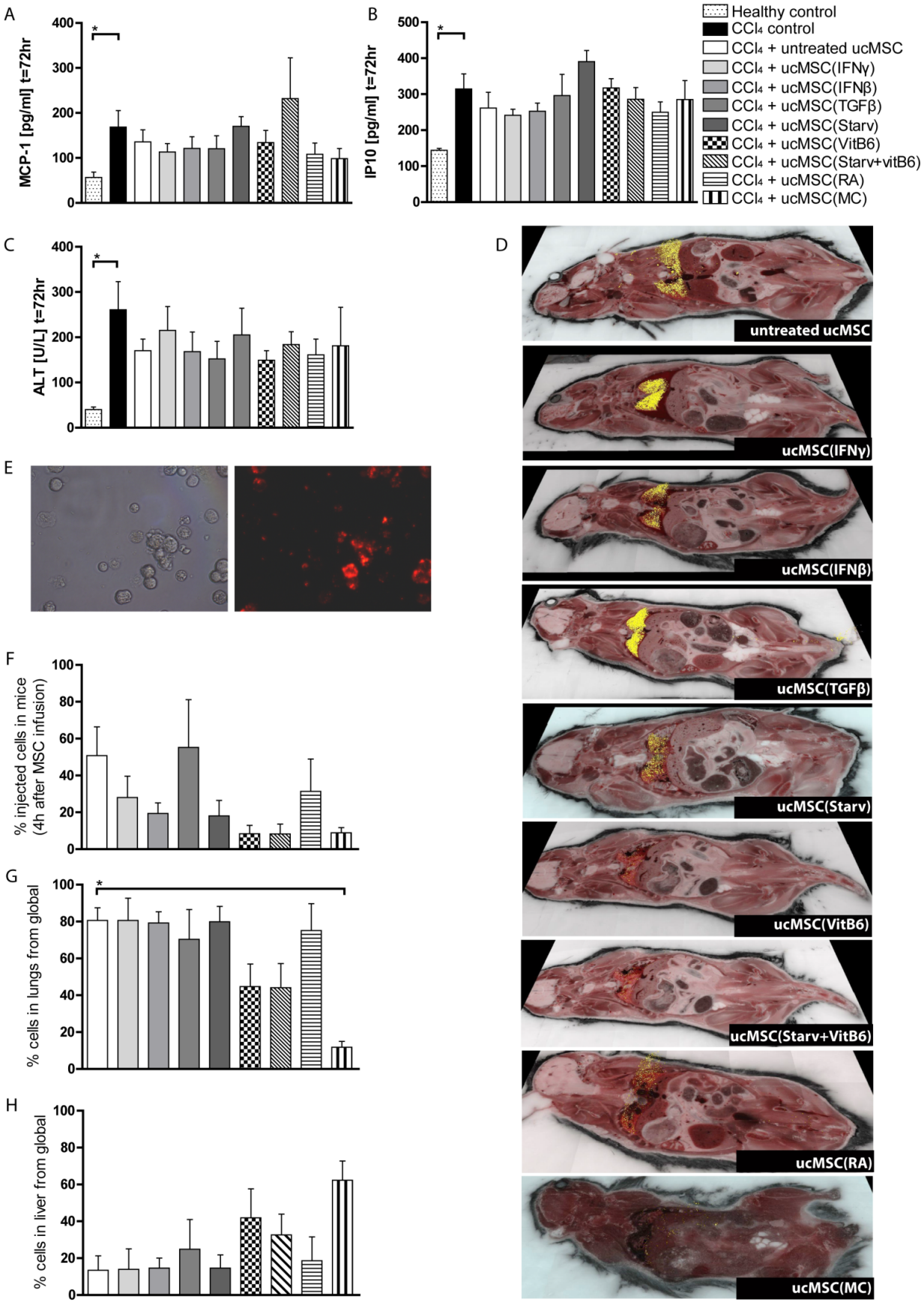
UcMSC were labelled with Qtracker605 beads to enable analysis of their bio distribution at a single cell level by CryoViz imaging. Representative bright field and fluorescent images of ucMSC, four hours post labelling with Qtracker605 beads are shown in Figure 3e. Four hours after infusion of 250.000 labelled ucMSC in CCl_4 treated mice, 51% of untreated ucMSC were detectable within the mice (Figure 3d/f). The majority of these ucMSC were located within the lungs (81%) with a small percentage located within the liver (13%) (Figure 3g/h). Pre-treatment with MC (12%) led to a significant lower distribution of ucMSC to the lungs compared to untreated ucMSC (81%) (Figure 3g). Furthermore, a larger percentage of TGFβ-treated MSC

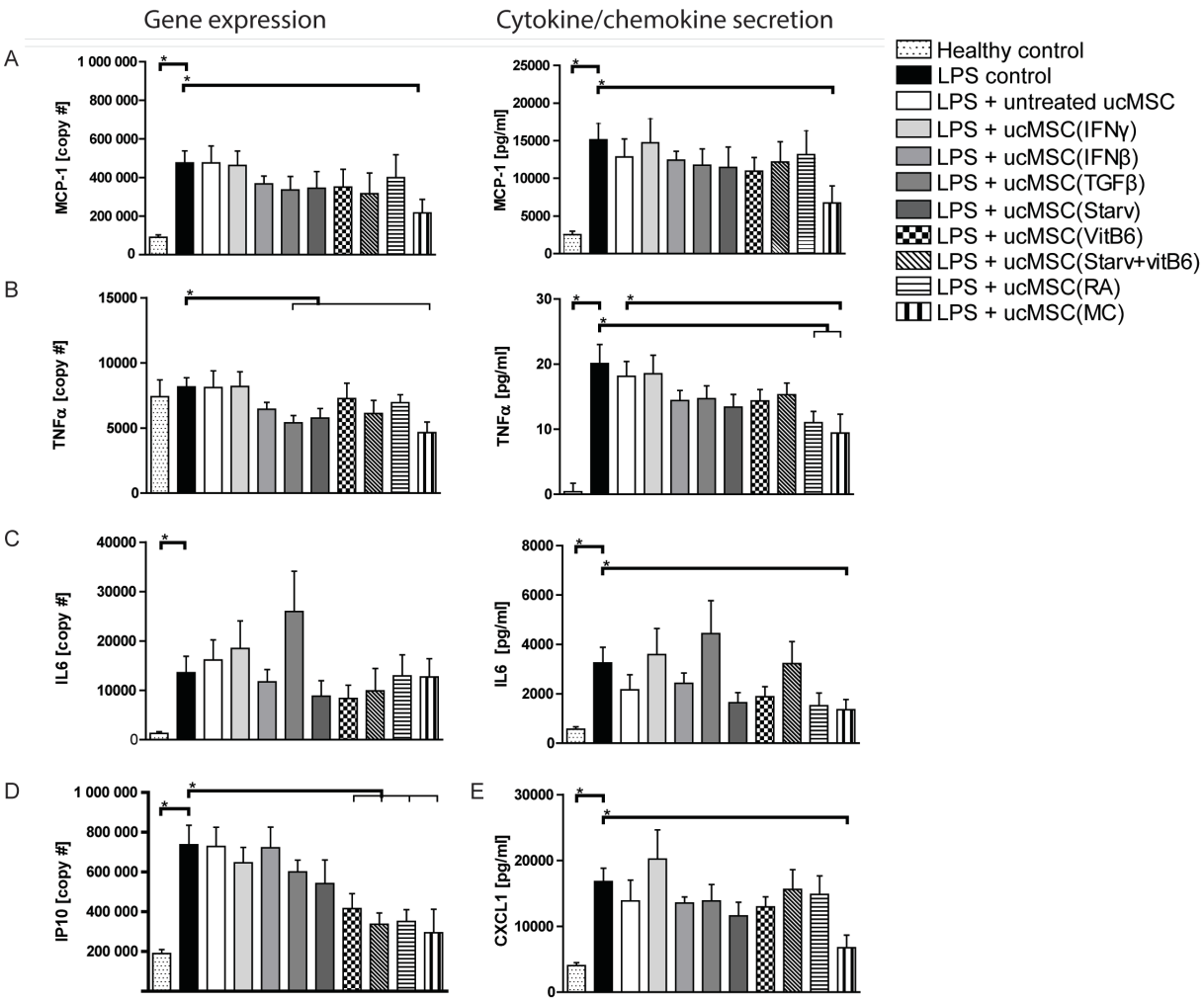
were found within mice (62%) compared to untreated ucMSC, of which a larger percentage were found in the liver (25%) compared to untreated ucMSC (13%) (Figure 3h). These observations were not a consequence of cell size differences as MC- and TGFβ-treated ucMSC were the same size as untreated ucMSC (Supplementary Figure 5). Pre-treatment with IFNγ, IFNβ, Starvation, $VitB_6$ and MC resulted in the detection of lower numbers of ucMSC 4 h after administration compared to untreated ucMSC (Figure 3f).

UcMSC(MC) resolve liver inflammation in an *ex vivo* liver tissue model

As intravenously administered ucMSC poorly distribute to the liver, we assessed the efficacy of ucMSC in ameliorating liver inflammation in an *ex vivo* liver tissue slice, where cells were placed in direct contact with injured liver tissue. Significantly elevated gene expression levels and secretion levels of inflammatory factors (MCP-1, TNFα, IL6, IP-10 and CXCL1) were observed in the diseased control liver tissue compared to the healthy control (Figure 4). Pre-treatment of MSC with MC resulted in a marked reduction in expression levels of several inflammatory genes (MCP-1, TNFα and IP10), with more limited effects seen on TNFα (ucMSC(IFNβ)) and IP10 (ucMSC($VitB_6$), ucMSC(starvation) and ucMSC(RA)). Moreover, MC pre-treatment decreased secretion of MCP-1, TNFα, IL6 and CXCL1 (all $p<0.05$), whereas RA pre-treatment only reduced TNFα secretion.

► ***Figure 3*** **Effects on inflammation and liver damage in CCL4-induced liver injury and bio distribution of pre-treated MSC.** Pretreated MSC were labeled with fluorescent Qtracker 605 beads and IV infused in C57BL/6 mice. **(a)** Levels of MCP-1, **(b)** IP10 and **(c)** ALT were measured in the serum 72 hours after ucMSC infusion. **(d)** Representative images of the bio-distribution of pretreated MSC *in vivo* 4 hours after ucMSC infusion. Yellow dots correspond with a cluster of observed MSC. **(e)** Representative image of ucMSC labeled with Qtracker 605 beads, still visible in MSC 4 hours after labeling. **(f)** Diagram showing the percentage of infused ucMSC 4 hours after administration. Figures **(g)** and **(h)** show the relative number of ucMSC in lungs and liver, respectively, calculated from the total number of detected ucMSC. Results are shown as means ± SEM (n=4). * indicates significant difference ($p<0.05$) with respect to the CCL_4 control.


A
MCP-1 [pg/ml] t=72hr
400
300
200
100
0
*
B
IP10 [pg/ml] t=72hr
500
400
300
200
100
0
*
Healthy control
CCl4 control
CCl4 + untreated ucMSC
CCl4 + ucMSC(IFNγ)
CCl4 + ucMSC(IFNβ)
CCl4 + ucMSC(TGFβ)
CCl4 + ucMSC(Starv)
CCl4 + ucMSC(VitB6)
CCl4 + ucMSC(Starv+vitB6)
CCl4 + ucMSC(RA)
CCl4 + ucMSC(MC)
C
ALT [U/L] t=72hr
400
300
200
100
0
*
D
untreated ucMSC
ucMSC(IFNγ)
ucMSC(IFNβ)
ucMSC(TGFβ)
ucMSC(Starv)
ucMSC(VitB6)
ucMSC(Starv+VitB6)
ucMSC(RA)
ucMSC(MC)
E
F
% injected cells in mice (4h after MSC infusion)
100
80
60
40
20
0
G
% cells in lungs from global
100
80
60
40
20
0
*
H
% cells in liver from global
100
80
60
40
20
0



▲***Figure 4 Effect of ucMSC on ex vivo liver slices.*** Gene expression levels and cytokine/chemokine levels were measured in liver slices treated with LPS and supernatant, respectively. **(a)(b)(c)** Both gene expression levels and secretion levels were measured for MCP-1, TNFα and IL6. In addition, **(d)** gene expression levels of IP10 and also **(e)** CXCL1 were measured in the medium. Results are shown as means ± SEM (n=6). * indicates significant difference ($p<0.05$).

Discussion

MSC have shown to be a promising cell type for treating various immune disorders in numerous disease models [3, 4, 19, 21, 22], including clinical trials of patients with liver disease [7-13]. However, the immunosuppressive effects of MSC need to be induced by inflammatory stimulation, and MSC get trapped in the lungs after intravenous infusion and have a short *in vivo* survival time [29, 30]. Therefore, to enhance their therapeutic efficacy, improvement of their immunomodulatory properties and immunogenicity is necessary. The present study demonstrates that ucMSC are responsive to a variety of treatments, including inflammatory factors, vitamins and serum-deprivation. Their responsiveness may be employed to improve the properties of ucMSC to generate cells with enhanced therapeutic efficacy.

In vitro experiments showed that treatment with IFNγ or MC, increases the immunosuppressive capacity of ucMSC. In addition, treatment with IFNβ, TGFβ and MC strongly decreases their susceptibility to lysis by NK cells. *In vivo* tracking results showed differential effects of the pre-treatments on the MSC's bio-distribution and survival, where treatment with vitB_6, Starv+VitB_6 and MC ensured rapid clearance while treatment with TGFβ resulted in prolonged presence of ucMSC. No immunomodulatory effects were observed by pre-treated ucMSC in a CCl_4 liver inflammation model, possibly due to the fact that the majority of the cells was trapped in the lungs. In the *ex vivo* culture system in which liver slices were cultured in the presence of ucMSC, significant amelioration of the inflammation by ucMSC(MC) was demonstrated by reduced inflammatory cytokine production. In summary, most effectively, pre-treatment of ucMSC with the multi cytokine combination MC, resulted in enhanced immunomodulation and reduced immunogenicity.

Application of optimised ucMSC as an immunotherapy depends on the therapeutic requirements necessary to target the disorder. For example, strong immunomodulatory properties in MSC such as high IDO activity are favourable when helping graft acceptance after transplantation [19]. Secretion of PGE2 and CCL2 has shown to be important to attenuate sepsis, local inflammation and mediates auto-immune disorders [3, 4, 21, 22]. In the present study we observed that ucMSC are very susceptible to treatment by IFNγ and MC, whereby they are induced into a

strong immunomodulatory phenotype with high IDO activity, PD-L1 expression and potential to inhibit lymphocyte proliferation. Interestingly, in contrast to IFNγ treatment, MC treatment also decreases the susceptibility of ucMSC to NK cell lysis and ameliorates inflammation in liver tissue slices.

Our results demonstrate that the phenotype and function of ucMSC is not fixed. Recently, we observed that the lung microvasculature wherein MSC get trapped initiate upregulation of various pathways in MSC and thus alter their phenotype after infusion [42]. It is conceivable that the induced changes by the various pre-treatments will be influenced after infusion. The interactions between pre-treated ucMSC and the micro-environment are therefore a determinant on the effectiveness of these ucMSC.

Efficacy of MSC therapy depends on various aspects, one of which is the bio-distribution of MSC after infusion. Although no direct correlation has been established, it is suggested that short-term effects of MSC are mediated by their secretome and their long-term effects are a result of activation and interaction with other cells types, via a hit-and-run mechanism [3, 15, 18, 21, 43]. Thus, the bio distribution of MSC determines which cell types they may encounter and depending on the aim of the therapy a specific distribution will be favoured. Tracking of labelled ucMSC by CryoViz enables in depth visualization of MSC after administration and subsequently gives an interpretation of not only their bio-distribution, but also their survival. Previously, localization in the lungs and short survival time of intravenous injected bone marrow derived MSC was observed [29]. The present study confirms that the majority of infused ucMSC accumulate in the lungs and that there is a rapid clearance of cells. The retainment of ucMSC was improved by pre-treatment with TGFβ*,* which corresponds with the observed reduced recognition of ucMSC(TGFβ) by NK cells *in vitro*. Interestingly, ucMSC(TGFβ) showed no differences in their expression of HLA type-I and –II whereas they were less recognized less by NK cells. This suggests that TGFβ potentially modulates the expression of ligands on MSC that were not analysed in this study (e.g. NKG2D, DNAM1 and natural cytotoxicity receptors [44-46]). Depending on the therapeutic requirements, longevity or rapid clearance of administered MSC may be desirable. Our results demonstrate that longevity and bio-distribution of MSC can be modulated by pre-treatment of the cells.

Lack of immunomodulation by any of the pre-treated ucMSC *in vivo* could be credited to the fact that the ucMSC were unable to travel towards the liver. As for when the cells were in contact with targeted liver tissue, in the inflammatory *ex vivo* model, strong amelioration of the inflammation by ucMSC(MC) was observed. Possibly, this *in vivo* inflammatory liver model requires ucMSC to be in direct contact with the tissue for them to be able to exert their immunomodulatory capacities. Therefore for future studies, administration of ucMSC via the portal vein or hepatic artery may be a more efficient approach for treatment of liver inflammation.

Conclusions

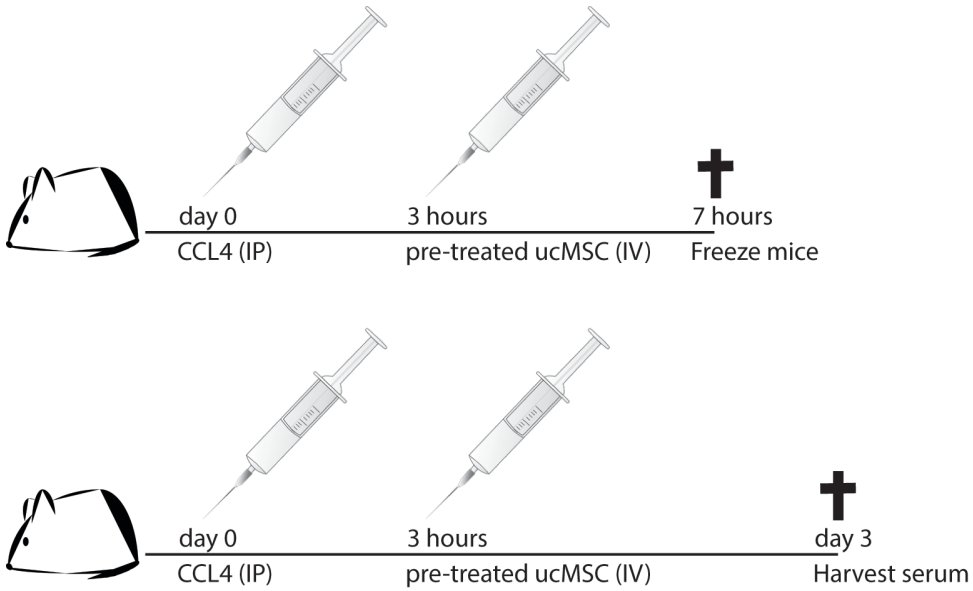
To conclude, these findings demonstrate that ucMSC are responsive to *in vitro* treatment, whereby their immunomodulatory properties and their immunogenicity can be differentially modulated. TGFβ treatment of ucMSC reduced their immunogenicity and altered their bio distribution and IFNγ treatment enhanced their immunomodulatory capacities. Most interestingly, treatment with the multi cytokine combination MC resulted in reduced immunogenicity, increased immunomodulatory capacity as well as enhanced effectiveness to ameliorate liver inflammation. Therefore, *in vitro* pre-treatment of ucMSC MC will make them more suitable as an effective immunotherapy targeted for liver inflammation.

Supplementary materials

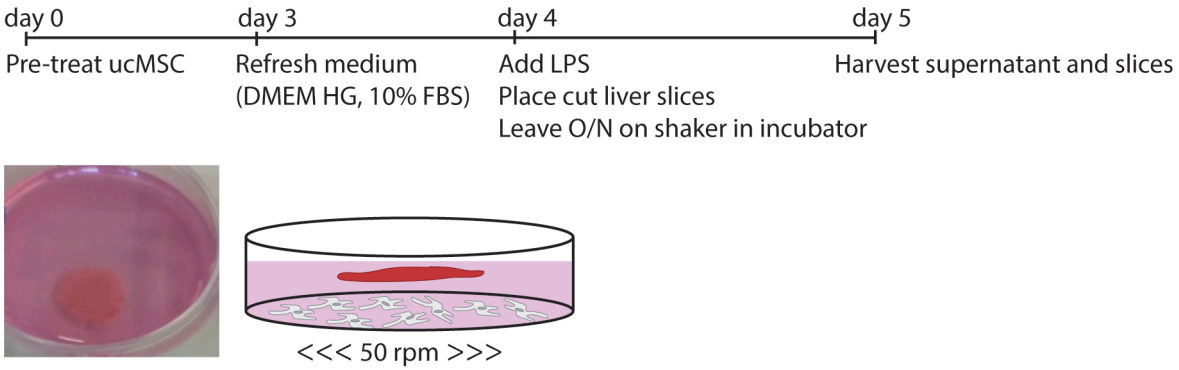
Study design

	in vitro	*in vivo*	*ex vivo*
Set up	*Treatment of ucMSC with factors*	*CCl4-induced liver inflammation mouse model*	*Inflammatory liver tissue slices*
Read out parameters	Immunomodulatory and immunogenic markers (FACS, PCR and secreted factors) Immunosuppresive capacity assay (inhibition of lymphocyte proliferation) Immunogenicity assay (CD107a expression on NK cells)	Immunomodulatory factors in serum Bio distribution of pre-treated ucMSC	Inflammatory markers in liver slices (PCR and secreted factors)

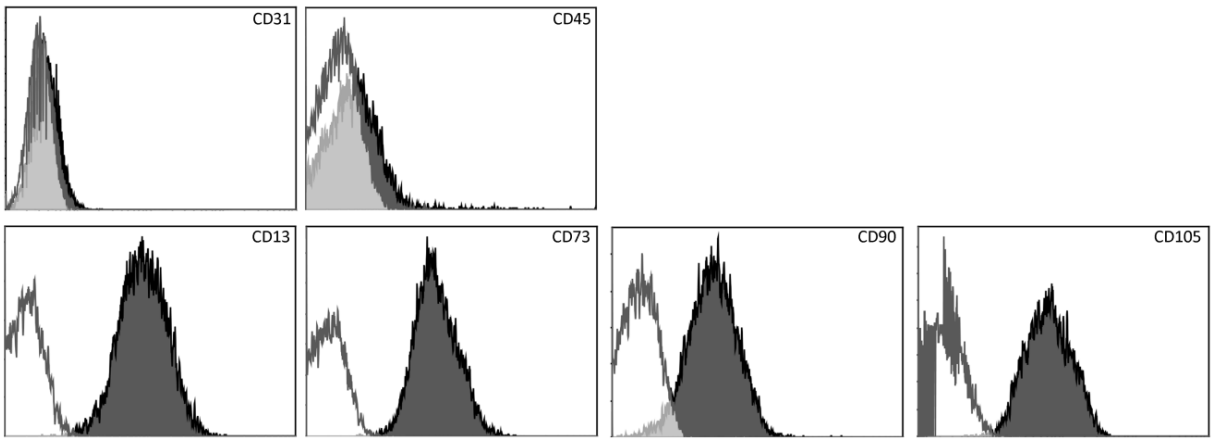
▲***Supplementary figure 1*** **Study design.** This study is organized in three sections: *in vitro*, *in vivo* and *ex vivo.*



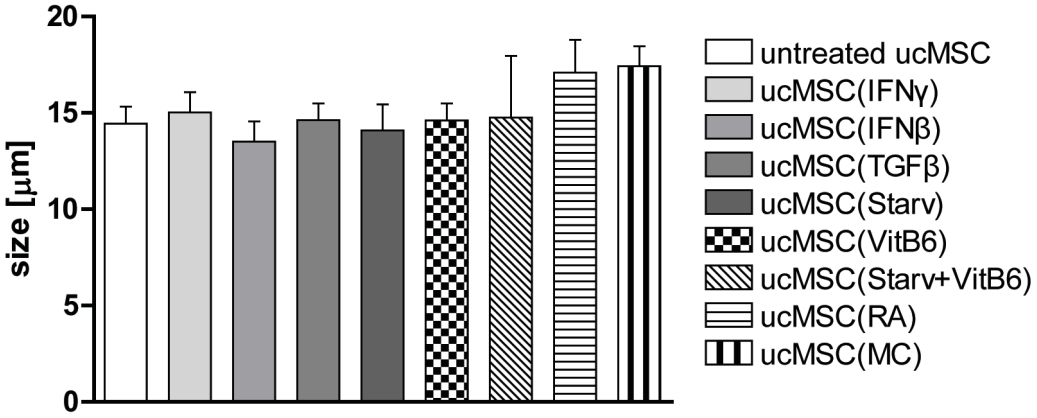
▲***Supplementary figure 2*** **In vivo experimental schemes.** (Top) In the first set of experiments mice were treated with CCl_4 and 3 hours later pre-treated ucMSC (untreated, IFNγ, IFNβ, TGFβ, starvation, vitamin B6, Starv+VitB_6, RA and MC), which were labeled with Qtracker605 beads, were infused IV. Four hours after ucMSC infusion the mice were sacrificed and prepared for imaging. (Bottom) In the second set of experiments mice were sacrificed 72 hours after CCL4 injection and serum was collected.



▲***Supplementary figure 3*** **Ex vivo experimental scheme.** UcMSC were pre-treated for 3 days. Medium was then refreshed and the cells were left overnight in the incubator. The following day, livers were collected from healthy C57BL/6 mice and directly after isolation cut into slices (Diameter = 1 cm and thickness = 150 µm) and placed in the wells on top of the pre-treated ucMSC in the presence of LPS. The liver slices were left overnight in the incubator on a shaker (50 rpm). After 24 hours, supernatant and liver slices were harvested.



▲***Supplementary figure 4*** **Characterization ucMSC by flow cytometry.** Representative histograms of expression of MSC markers CD13, CD73, CD90, CD105 and negative expression of the endothelial marker CD31 and hematopoietic marker CD45. Stained ucMSC (grey) and isotype control (white).



▲***Supplementary figure 5*** **MSC diameter.** Measured diameter of pre-treated ucMSC in µm. Results are shown as means ± SEM (n=5). * indicates $p<0.05$.

	Immunogenic read out parameters		**Immunomodulatory read out parameters**				
	HLA class I expressing cells [%]	**HLA class II expressing cells [%]**	**L-Kynurenine (correlated IDO activity) [µM]**	**PGE_2 [ng/ml]**	**PD-L1 expressing cells [%]**	**CD73 expressing cells [%]**	*Fold Increase of Gene expression compared to stimulated* **IL-1RA [corrected for GAPDH]**
[-]	7±1	10±3	2±5	14±3	46±6	95±2	1±0
Vitamin D3	13±5	15±6	2±1	-	19±6	100±0	NAV
IL7	6±2	4±0	2±4	-	38±8	80±10	NAV
IL15	3±1	4±0	-1±0	-	43±11	76±14	1.1±0
IL17	3±1	4±1	2±1	-	41±8	74±13	1.0±1
Budesonide	10±2	20±5	2±2	-	18±3	99±0	NAV
Trespostinil	15±6	17±6	3±0	-	21±6	100±0	1.1±0
Activin A	14±5	14±5	2±1	-	19±5	99±0	1.7±1
TNFα	11±4	13±5	3±4	-	21±1	99±0	1.1±0

▲ ***Supplementary Table I Key immunogenic and immunomodulatory molecules after 3 day in vitro treatment of ucMSC.*** The table displays the mean ± SEM percentages of MSC expressing surface markers HLA class –I and –II, PD-L1 and CD73, measured via flow cytometric analysis. Mean ± SEM concentration of L-Kynurenine [µM] and PGE2 [ng/ml]. Fold increase of IL1RA gene expression compared to unstimulated ucMSC. n=5, no significant differences were observed.

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6

Epigenetic changes in umbilical cord mesenchymal stromal cells upon stimulation and culture expansion

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Abstract

Mesenchymal stromal cells (MSC) are studied for their immunotherapeutic potential. Prior to therapeutic use MSC are culture expanded to obtain the required cell numbers and to improve their efficacy MSC may be primed *in vitro*. Culture expansion and priming induce phenotypical and functional changes in MSC and thus standardisation and quality control measurements come in need. We investigated the impact of priming and culturing on MSC DNA methylation and examined the use of epigenetic profiling as a quality control tool.

Human umbilical cord-derived MSC (ucMSC) were cultured for three days with IFNγ, TGFβ or a multi-factor combination (MC; IFNγ, TGFβ and retinoic acid). In addition, ucMSC were culture expanded for 14 days. Phenotypical changes and T-cell proliferation inhibition capacity were examined. Genome-wide DNA methylation was measured with Infinium MethylationEPIC Beadchip.

Upon priming, ucMSC exhibited a different immunophenotype and ucMSC(IFNγ) and ucMSC(MC) had an increased capacity to inhibit T-cell proliferation. DNA methylation patterns were minimally affected by priming, with only one significantly differentially methylated site (DMS) in IFNγ and MC-primed ucMSC associated with autophagy activity. In contrast, 14 days after culture expansion ucMSC displayed minor phenotypical and functional changes but showed more than 4000 significantly DMS, mostly concerning genes involved in membrane composition, cell adhesion and transmembrane signalling.

These data show that DNA methylation of MSC is only marginally affected by priming, whereas culture expansion and subsequent increased cellular interactions have a large impact on methylation. On account of this study we suggest that DNA methylation analysis is a useful quality control tool for culture expanded therapeutic MSC.

Introduction

Mesenchymal stromal cells (MSC) have been extensively examined in clinical trials regarding their immunotherapeutic potential [1-4]. Prior to their application in the clinic, MSC are commonly expanded to obtain clinically relevant numbers. However, during long-term *in vitro* culture expansion the phenotype and function of MSC is affected [5-7]. Previous studies have shown that during long-term expansion their proliferative capacity decreases [7,8]. In addition, long-term culture expansion affects the immunomodulatory properties of MSC, for instance their capacity to inhibit of T-cell proliferation [8]. Recently, there has been a growing interest in the optimization of the immunomodulatory properties of MSC *in vitro*. MSC can be primed with stimuli to enhance their immunomodulatory properties with the aim to improve their therapeutic efficacy [9-17]. Prior to their clinical application, MSC are routinely tested for multiple parameters to assess their safety and functionality, such as karyotype, morphology (spindle-shape) and viability as well as their cell surface protein expression and differentiation capacity according to the recommendations of the International Society for Cellular Therapy [1-4,18-21]. These tests give a global indication of the state of MSC. However, MSC have a great ability to adapt and culture expansion and priming may therefore modify MSC on a different level. Therefore, we endeavored to perform a more in depth analysis of the effects of culture expansion and stimulation on MSC.

Epigenetic modifications of the genome can be both hereditary as well as environmentally influenced. These epigenetic modifications affect gene expression without altering the genomic sequence and are important regulators of cellular function [22-25]. Methylation of cytosines at cytosine-phosphate-guanine (CpG) sites in the DNA, is one of the main mechanisms of epigenetic modifications. Methylation at a CpG site may block the start of transcription and in particular methylation of CpG islands at transcriptional start sites (TSSs) is associated with long-term gene silencing [22]. *In vitro* procedures may affect DNA methylation; potentially resulting in changes in their gene expression and subsequently their phenotype and function.

Previously, it was demonstrated that there is an association between osteogenic differentiation of MSC and their DNA methylation pattern [26-29]. In addition, other studies demonstrated that during long-term culture expansion, where MSC were cultured over 10 passages, MSC became senescent and their DNA methylation

patterns changed [30,31]. However, no study to date has addressed the effect of priming MSC *in vitro* with various stimuli to optimize their immunomodulatory properties on the DNA methylation. Furthermore, it remains unclear whether during culture expansion DNA methylation patterns of MSC are affected. Elucidation of the effects of MSC expansion and priming on DNA methylation may result in additional quality control tools that help in development and application of MSC therapy in clinical setting. This will ensure the use of better standardized MSC therapeutic products. Therefore in this study we investigated the changes in methylation in the epigenome of MSC during priming by various stimuli and also after two weeks culture expansion.

Material & Methods

Mesenchymal stromal cells

Human umbilical cord tissue was collected by Tissue Solutions Ltd. (Glasgow, UK) from Caesarean section deliveries from virally screened healthy donors. Whole cord tissue of the neonatal side was used for MSC isolation. All cord tissues provided by Tissue Solutions were obtained according to the legal and ethical requirements of the country of collection, with the approval of an ethics committee (or similar) and with anonymous consent from the donor. Isolation of the $CD362^+$ subset of ucMSC was performed according to previous manuscripts by de Witte et al. [8,10]. After isolation, each cell fraction was 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 (MEM-α; Sigma-Aldrich, St Louis, MO, USA) containing 2 mM L-glutamine (Lonza, Verviers, Belgium), 1% penicillin/streptomycin solution (P/S; 100IU/ml penicillin, 100IU/ml streptomycin; Lonza) and supplemented with 15% batch tested fetal bovine serum (FBS; Lonza) and 1 ng/ml basic fibroblast growth factor (bFGF) (Sigma) and kept at 37°C, 5% CO_2 and 20% O_2. Once a week medium was refreshed and ucMSC were passaged using 0.05% trypsin-EDTA (Life technologies, Paisley, UK) at ~80-90% confluence. All ucMSC used in experiments were between passage 3-6.

Characterization of ucMSC was performed by flow cytometric analysis of the cell surface markers: CD31 (PB, BD Biosciences), CD45 (APC-Cy, BD Biosciences), CD13 (PE-Cy7, BD Biosciences), CD73 (PE, BD Pharmingen), CD90 (APC, R&D systems) and CD105 (FITC, R&D systems). After labeling the cells were washed and measured on the FACSCanto II flow cytometer (BD Biosciences) (Supplementary figure 1).

Experimental design

The experimental design consists of two parts: ‘Priming of MSC’ and ‘Culture expansion of MSC’, see also figure 1.

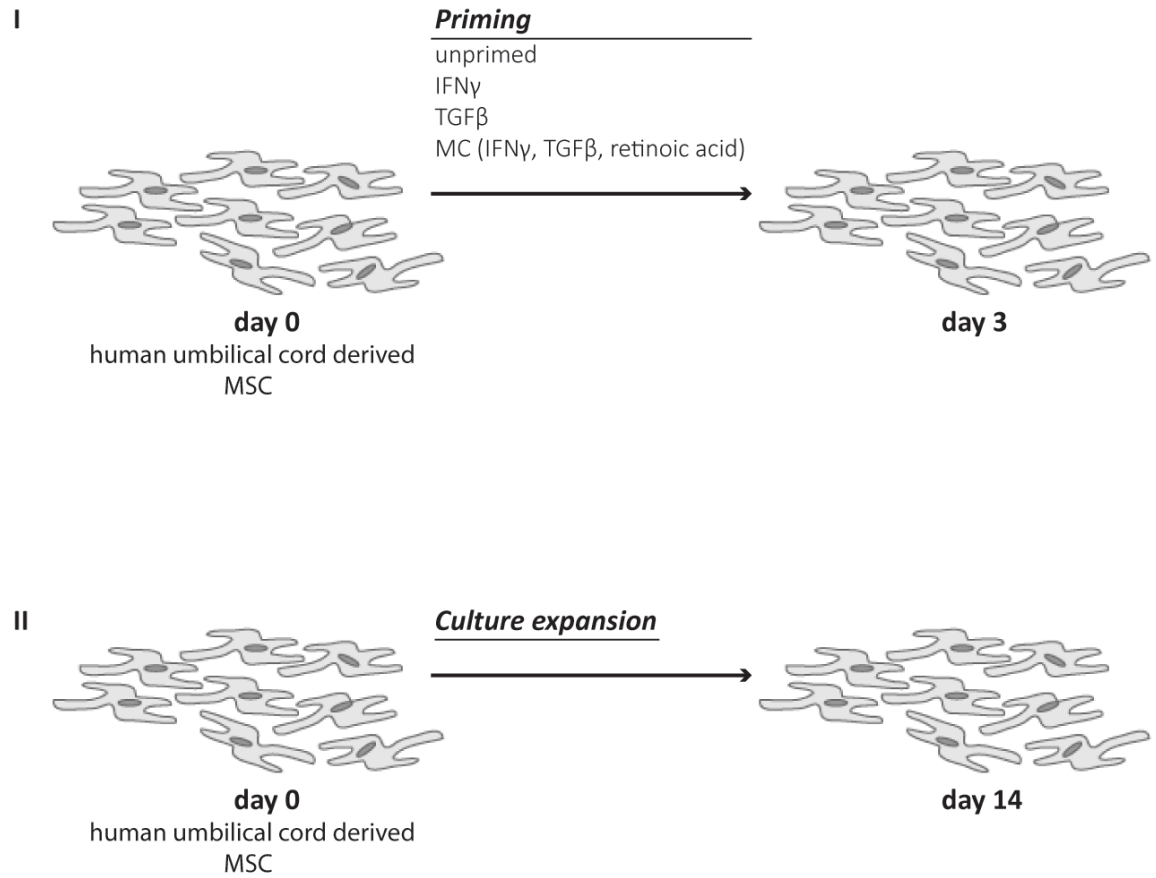
Priming of ucMSC

ucMSC of 4 different umbilical cord donors were stimulated with factors were known to modify MSC function or phenotype, as demonstrated in previous studies [10,32].

At day 0, MSC (confluent culture) were stimulated for three days with interferon gamma (IFNγ, 50 ng/ml; Life technologies, transforming growth factor beta 1 (TGFβ, 10ng/ml; R&D systems, MN, USA) or a multi-factor combination (MC) of IFNγ, TGFβ and retinoic acid (RA, 100 µM; Sigma). At day 3, cells were trypsinised and either used for experiment or snap frozen in pellets containing 300,000 cells and stored at -80°C.

Culture expansion of MSC

At day 0, MSC of 4 different umbilical cord donors were seeded (250,000 cells/T175 flask). Medium was partly refreshed (50%) every 5/6 days. At day 14, cells were used for experiment or snap frozen as pellets for future use.



▲ ***Figure I Study design.*** *(Top) In the first part of the experimental design umbilical cord derived MSC (ucMSC) were left unprimed or primed with IFNγ, TGFβ or a multifactor combination of IFNγ + TGFβ + Retinoic Acid (MC) for 3 days. Thereafter, whole-genome DNA methylation analysis was performed on unprimed and primed ucMSC. (Bottom) In the second part, ucMSC were culture expanded for fourteen days. Whole-genome DNA methylation analysis was performed on ucMSC before and after culture expansion.*

DNA extraction procedure

DNA was isolated from ucMSC with the QIAamp DNA Micro kit (Qiagen; Germany) according to the manufacturer's instructions. DNA concentration was measured with spectrophotometry (Nanodrop spectrometer, Thermo Scientific, USA). DNA quality of the samples was estimated by measuring the ratio of absorbance at 260/280nm (between 1.7-2) and with agarose gel electrophoresis.

Bisulfite treatment and DNA methylation measurement

To determine DNA methylation profiles, samples underwent bisulfite conversion. During this conversion unmethylated cytosines were converted into uracil (supplementary figure 2). The bisulfite conversion was performed using 500 ng genomic DNA per sample and using the EZ-96 DNA Methylation Kit (Shallow; Zymo Research, CA, USA). Bisulfite converted samples were then hybridized to the Illumina 850k DNA methylation array (Infinium MethylationEPIC Beadchip; Illumina; USA) according to the manufacturer's instructions.

In short, the Infinium MethylationEPIC BeadChip applies both Infinium I and II assay chemistry technologies. The infinium I assay uses two bead types: methylated (M) and unmethylated (U). Whereas Infinium II assay uses a single bead type, with the methylated state determined at the single base extension step after hybridization. This array provides methylation data of over 850,000 CpG sites in the genome. These CpG sites are located in CpG islands, shores and shelves, the 5′UTR, 3′UTR and bodies of RefSeq genes, FANTOM5 enhancers, ENCODE open chromatin and ENCODE transcription factor binding sites.

The raw data of the DNA methylation arrays is deposited at the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI) under accession number GSE113527 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE113527).

Analysis

Analysis of the methylation data was performed using Rstudio (RStudio Desktop 1.1.3.83) and comb-p software. R Bioconductor packages DMRcate [33,34], limma [35,36], minfi [37] and missMethyl [38] were used. Firstly, the raw methylation data was imported in R. Subsequently the data were normalized (Subset quantile

normalization: SQN (within and between array)). Probes on the sex chromosomes were removed from the dataset, resulting in a remaining 810,005 sites. Subsequently M-value (equation 1) and β-value (equation 2) were calculated.

$$M = log2(\frac{methylated}{unmethylated}) \quad [1]$$

$$\beta = \frac{methylated}{methylated + unmethylated} \quad [2]$$

The M-value is the log2 ratio of the intensities of methylated probe versus unmethylated probe. When M=0 there are equal amounts of methylated and unmethylated sites, when M>1 there are more methylated than unmethylated molecules and when M<1 there are more unmethylated than methylated molecules. β-value is the ratio of the methylated probe intensity and the overall intensity *(with β=0: completely unmethylated and β =1: fully methylated)*. According to Du et al. the use of M-values is more appropriate when doing differential methylation analysis [39]. Therefore, M-values of 810,005 CpG sites were used for further analysis.

To determine differences in DNA methylation after culture expansion or priming, paired analyses were performed. Firstly, differences in methylation between the different conditions for each ucMSC donor were identified, followed by joining the differences across ucMSC donors to determine (significant) differences in the mean methylation level of each CpG site (paired testing). Sites with a p_{adj}<0.05 were considered significantly differentially methylated.

The lists of significantly differentially methylated CpG sites were subsequently used to perform gene ontology testing, using the gometh function in R. Furthermore, the list was used to find differentially methylated regions (DMRs) in a command line tool and python library: Comb-P [40]. By calculating auto-correlation, combining adjacent p-values Stouffer-Liptak-Kechris correction, performing false discovery adjustment, finding regions of enrichment (i.e. series of adjacent low P-values) and assigning significance to regions with irregularly spaced p-values, Comb-P enables identification of significant DMRs. The size of the regions analyzed was set to 500 basepairs (bp) with the seed at p<0.01. Multiple testing was taken into account by correcting using a Šidák correction.

T cell proliferation assay

Primed and culture expanded ucMSC were seeded into 96-wells plates and left overnight to adhere in the incubator. The next day PBMC were labeled with Cell Trace CFSE (Life Technologies) according to the instructions of the manufacturer and seeded on top of the ucMSC, at different [MSC:PBMC] ratios: [1:10], [1:5] and [1:2.5] in RPMI supplemented with 2 mM L-glutamine, 100IU/ml penicillin, 100IU/ml streptomycin and 15% FBS. αCD3/CD28 stimulation was added (0.5 µg / ml αCD3 antibody, 0.5 µg/ml αCD28 antibody and 1 µg/ml goat-α-mouse antibody; Life Technologies). The co-cultures were left for 3 days and PBMC were collected. PBMC were stained for CD4 (APC; eBioscience) and CD8 (Pe-cy7; eBioscience). With the use of the FACSCanto II flow cytometer the proliferation of PBMCs was measured.

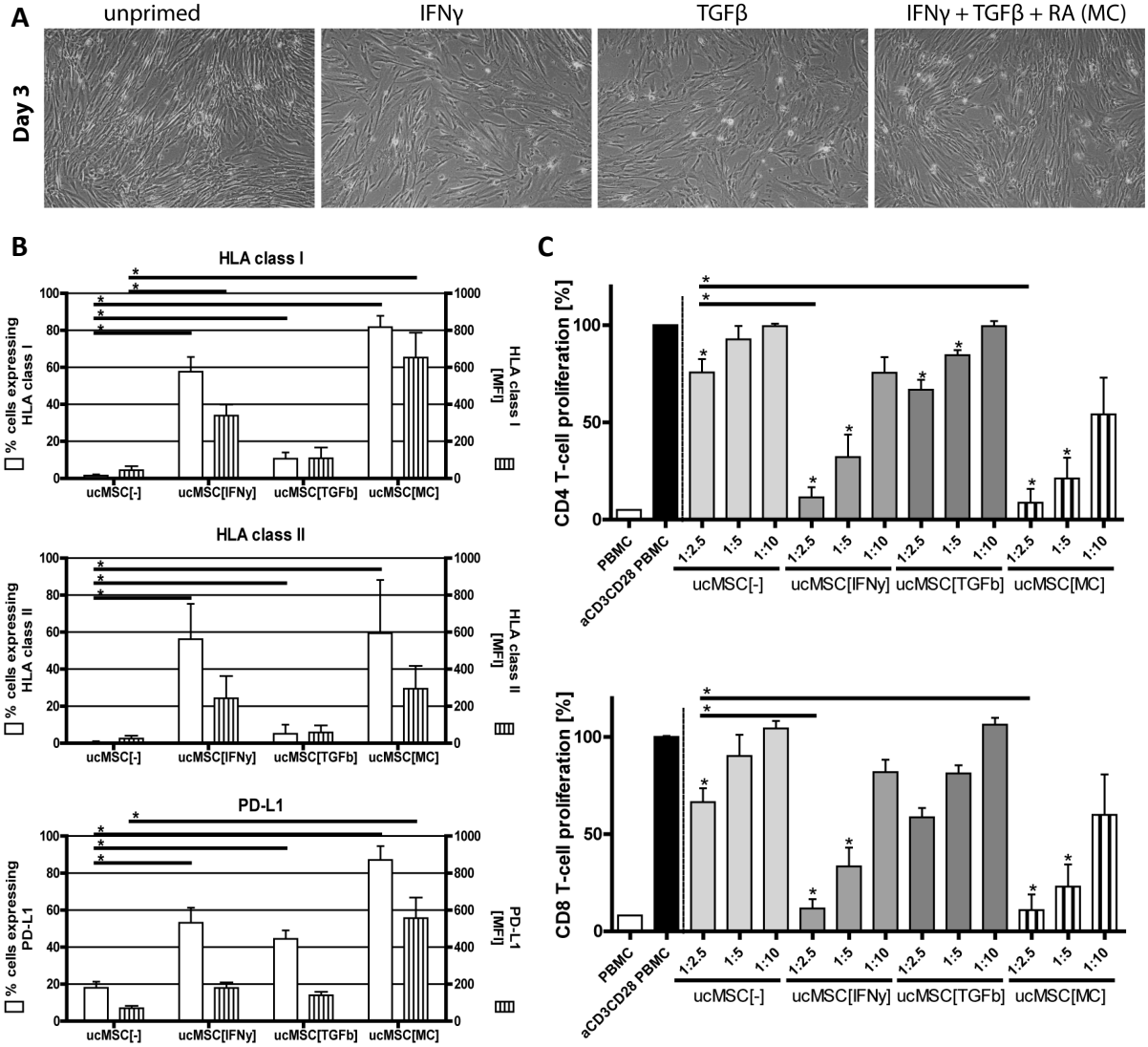
RT-PCR

mRNA was isolated from ucMSC, from the same samples as the DNA extraction, using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA). cDNA was synthesized from 500ng mRNA with random primers (Promega Benelux B.V., The Netherlands). Quantitative gene expression was determined using TaqMan Gene Expression Master Mix (Life Technologies) and Assays-on-demand for HS1BP3 (Hs00916454_m1).

Results

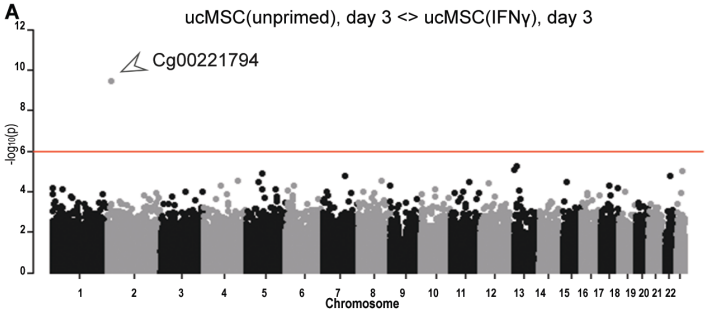
Priming alters ucMSC immunophenotype and functionality

UcMSC were cultured for three days in the presence of IFNγ, TGFβ or a combination of IFNγ, TGFβ and RA (MC). We observed that whilst ucMSC maintained their spindle shaped morphology (figure 2a) and expression of MSC markers CD13, CD73, CD90 and CD105 (supplementary figure 1) upon priming, expression of HLA type I, II and PD-L1 was increased (percentage expressing cells as well as the MFI) (figure 2b). The immunomodulatory capacity of ucMSC, determined by their potential to inhibit CD4 and CD8 T-cell proliferation, significantly increased after priming ucMSC with IFNγ and MC (figure 2c and supplementary figure 3).

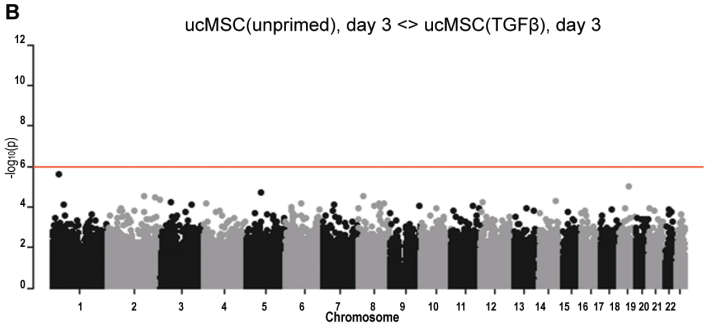


▲ ***Figure 2.*** *Morphology, surface marker expression and immunosuppressive capacity of primed ucMSC. (A) Representative bright field photos of unprimed ucMSC and after priming with IFNγ, TGFβ or the multi factor combination. (B) Expression of HLA class I (top), HLA class II (middle) and PD-L1 (bottom) in percentage of positive cells (clear bars, on left axis of graph) or in MFI (striped bars, on right axis of graph). (C) Inhibition of CD4 T-cell proliferation (top) and CD8 T-cells (bottom) by unprimed and primed ucMSC, in different ratios. * p<0.**05.***

A



B



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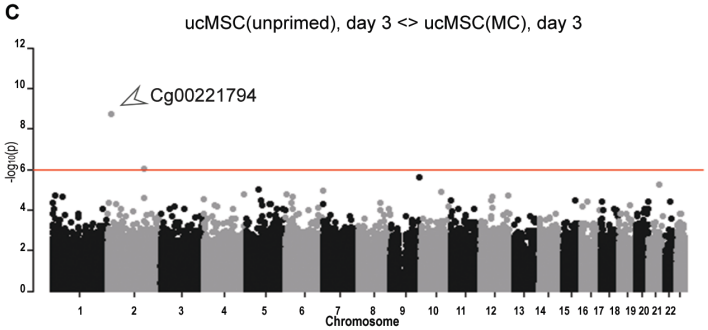
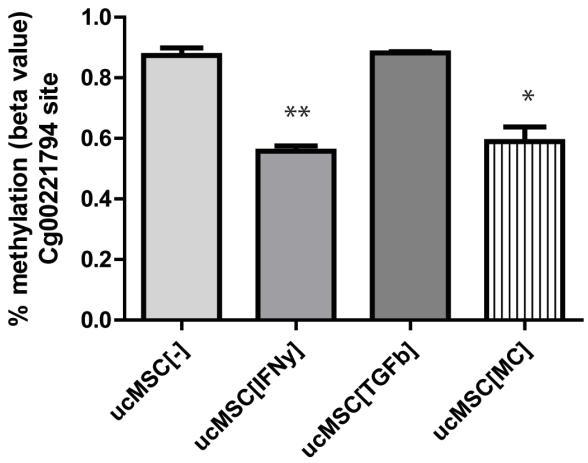


D

Comparison between		# Differentially methylated site (DMS)	# Differentially methylated region (DMR)
ucMSC[-] ***day 3***	ucMSC[**IFNγ**] ***day 3***	1	0
ucMSC[-] ***day 3***	ucMSC[**TGFβ**] ***day 3***	0	0
ucMSC[-] ***day 3***	ucMSC[**MC**] ***day 3***	1	0

E



◄ ***Figure 3.*** *Analysis of differentially methylated sites and regions of primed ucMSC. Manhattan plots of differentially methylated sites of ucMSC 3 days after priming with (A) IFNγ, (B) TGFβ or (C) a multi factor combination (MC) compared to unprimed ucMSC. Genome-wide significance levels of 1.0×10−6 from the Bonferroni correction were used. Horizontal lines mark significance cut offs. Arrow indicates significantly methylated site. (D) Summarizing table of the number of differentially methylated sites (DMS) and regions (DMR) after priming of ucMSC. (E) Percentage methylation (beta values) of Cg00221794 site at day 3 after priming. N = 4 per condition. * p<0.05, ** p<0.005 compared to ucMSC[-].*

Impact of priming of ucMSC on DNA methylation

To examine whether priming of ucMSC leaves an epigenetic imprint that can be used to identify MSC potency or as a inclusion or exclusion criterion of MSC for clinical use, genome-wide DNA methylation profiles were generated of ucMSC after 3 days priming with IFNγ, TGFβ or MC. We compared DNA methylation profiles of unprimed ucMSC to those of primed ucMSC and demonstrated that priming of ucMSC with IFNγ and MC but not TGFβ led to differential methylation at a single site located on chromosome 2 (figure 3a-d). This site, Cg00221794, was hypomethylated in ucMSC primed with IFNγ and MC compared to unprimed ucMSC (figure 3e). No differentially methylated regions were detected in any of the primed ucMSC (figure 3d).

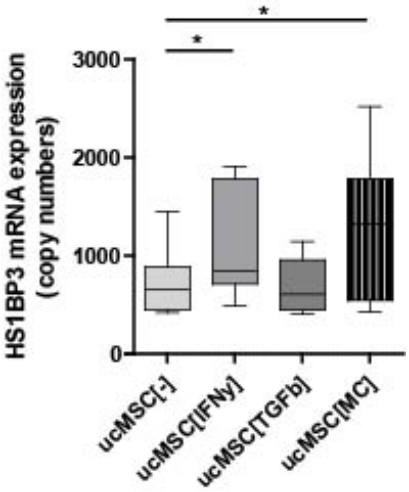
Modified expression near the IFNγ and MC induced hypomethylated site Cg00221794

The CpG site Cg00221794 is located near an area annotated as the Hematopoietic Cell-Specific Lyn Substrate 1 binding protein 3 (HS1BP3) gene. To investigate whether hypomethylation of this site affects HS1BP3 gene expression levels, mRNA levels of HS1BP3 were analyzed in unprimed and IFNγ, TGFβ or MC primed ucMSC. Gene expression levels of HS1BP3 were significantly upregulated upon priming of ucMSC with IFNγ or MC compared to unprimed ucMSC (figure 4).

Culture expansion alters the immunophenotype of ucMSC

To assess the effects of culture expansion the morphology, immunophenotype and capacity to suppress T cell proliferation of ucMSC were assessed before and after 14 days of culture expansion. After 14 days of culture expansion, the cultures were

>90% confluent (figure 5a). There was no change in expression of the MSC markers CD13, CD73, CD90 and CD105 (supplementary figure 1) while there was significant increased protein expression of HLA type II and PD-L1 on ucMSC at day 14 compared to prior to culture expansion (day 0) (figure 5b). Furthermore, there was no significant difference in the ability of day 0 versus day 14 ucMSC to suppress T cell proliferation (figure 5c).



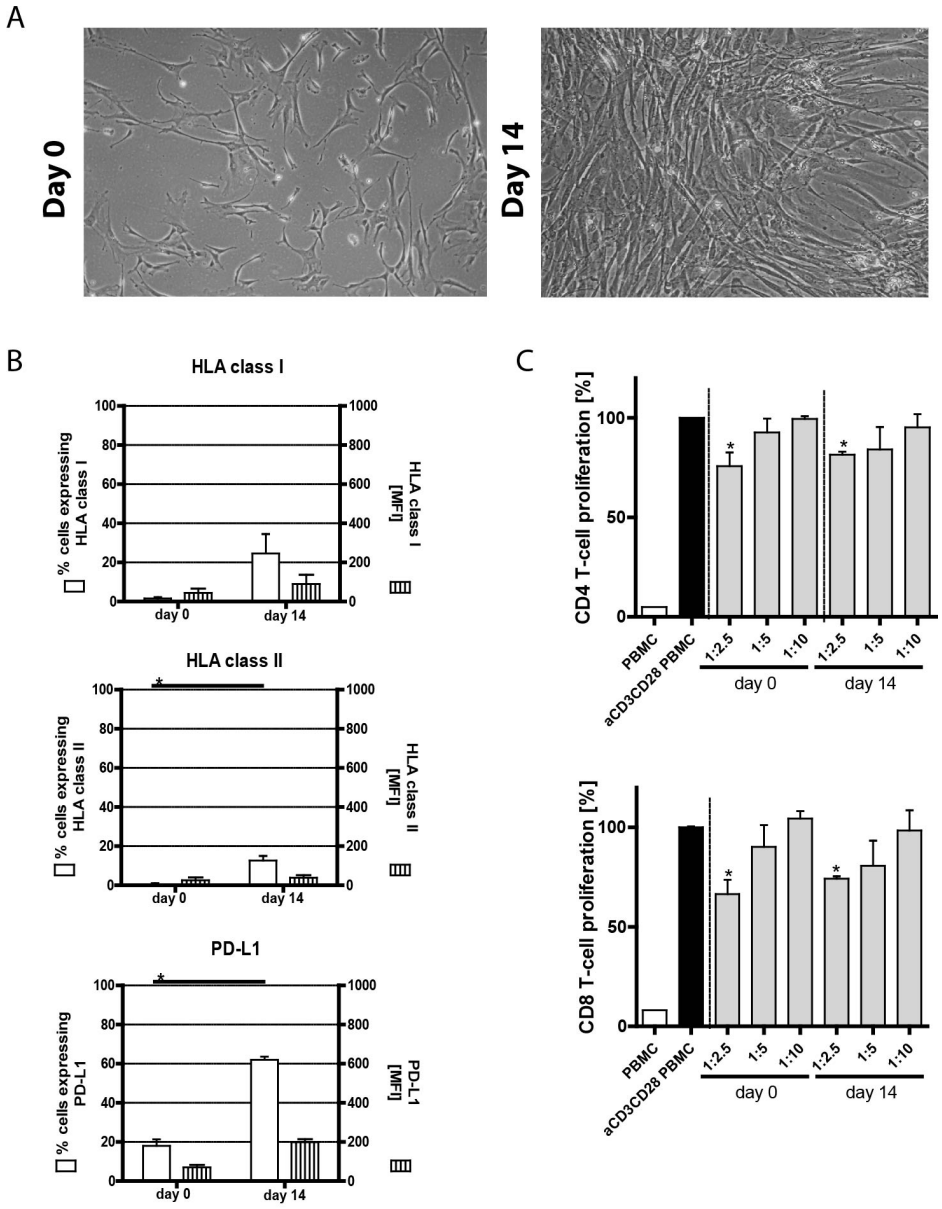
▲ ***Figure 4.*** *mRNA expression levels of HS1BP3. mRNA expression level of HS1BP3 in ucMSC after 3 days priming. * p<0.05.*

UcMSC undergo major epigenetic changes during culture expansion

DNA methylation patterns were determined in MSC 14 days after culture expansion. In contrast to the minor effects of priming of ucMSC with IFNγ, TGFβ or MC on DNA methylation, 14 days of culture expansion led to 4831 significantly differentially methylated sites (DMS) (figure 6a). Gene ontology analyses revealed these differences were located in genes involved in plasma membrane composition, cell adhesion and transmembrane signaling. We furthermore observed 545 differentially methylated regions (DMR) (figure 6b), of which 47 were hypermethylated and 498 hypomethylated (figure 6c). This suggests in general elevated expression of cell membrane associated proteins upon increase confluency following 14 days of culture of ucMSC.

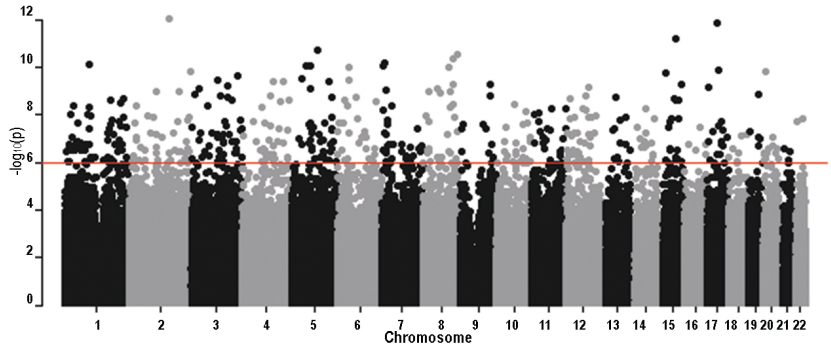
The top 10 most significantly hyper or hypo-methylated regions are shown in supplementary tables 1 and 2. These results demonstrate that in contrast to priming of ucMSC with IFNγ, TGFβ or MC, culturing ucMSC for 14 days has a major impact on DNA methylation profiles of ucMSC. The significant changes in DNA methylation

between newly seeded MSC and MSC cultured for 14 days suggest that methylation profiling can be used to differentiate between MSC cultures of different culture phase and potentially as an inclusion / exclusion assay for MSC for clinical therapy.



▲ ***Figure 5.*** *Morphology, surface marker expression and immunosuppressive capacity of culture expanded ucMSC. (A) Representative bright field photos of ucMSC after fourteen days of culture expansion. Magnification 50x. (B) Expression of HLA class I (top), HLA class II (middle) and PD-L1 (bottom) in percentage of positive cells (clear bars, on left axis of graph) or in MFI (striped bars, on right axis of graph). (C) Inhibition of CD4 T-cell proliferation (top) and CD8 T-cells (bottom) by ucMSC prior culture expansion (day 0) or post culture expansion (day 14), in different ratios. * p<0.05 compared to aCD3CD28 PBMC.*

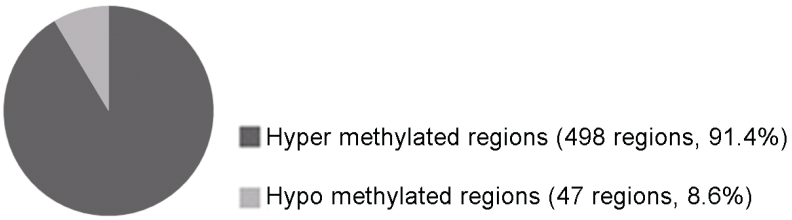
A



B

Comparison between		# Differentially methylated site (DMS)	# Differentially methylated region (DMR)
ucMSC ***day 0***	ucMSC ***day 14***	4831	545

C *Differentially methylated regions after 14 days of culture expansion*



▲ ***Figure 6.*** *Analysis of differentially methylated sites and regions of ucMSC at day 0 and after 14 days of culture. Manhattan plot of differentially methylated sites of (A) ucMSC after 14 days of culture expansion compared to ucMSC before 14 days of culture expansion. Genome-wide significance levels of 1.0×10−6 from the Bonferroni correction were used. (B) Summarizing table of the number of differentially methylated sites (DMS) and regions (DMR) after culture expansion of ucMSC. (C) Pie chart revealing the distribution of observed hypermethylated and hypomethylated regions. N = 4 per condition.*

Discussion

Our data demonstrate that priming of ucMSC, despite inducing immunophenotypical and functional changes, does not induce major epigenetic changes. In contrast, culture expansion over 14 days (one passage) has lesser effects on ucMSC phenotype and function, but has a major impact on the epigenetic profile of the cells. This suggests that ucMSC that are immunophenotypically identical may represent cells of a different standard. Epigenetic analysis of MSC may therefore represent a useful tool to validate MSC according to set epigenetic profile standards.

Culture expansion is a necessity when working with MSC to generate sufficient numbers of cells, although preferably MSC are used at a low passage for research and for clinical trials to minimize risks associated with their stability, safety and functionality. Long-term *in vitro* culture expansion of MSC increases the probability of genetic instabilities, and studies have reported increasing aneuploidy of MSC cultures during long-term expansion [41-43]. Recently, we showed that during long-term culture expansion, ucMSC remain genetically and phenotypical stable, but their immunosuppressive capacity decreases [5]. This is not observed for short-term culture expansion. In the present study, despite minor effects on immunophenotypical parameters, we identified major differences in the DNA methylation pattern of ucMSC after 14 days of culture expansion.

We postulate that clonal expansion, aging of the cells or confluency of the culture may contribute to the major changes observed in DNA methylation. Firstly, ucMSC are a heterogeneous population, such that across MSC cultures there are differences in their secretome, surface marker expression, gene expression and also in their epigenome. Throughout the experiment, ucMSC were seeded and cultured in the same flask for 14 days, during which period extensive proliferation took place. It is plausible that certain ucMSC have higher proliferation rates, which would lead to an enrichment of this population, representing clonal expansion [44]. Secondly, the amount of proliferation and duration of the expansion may have led to ageing of the cells. DNA methylation levels have been demonstrated to change during cellular senescence of MSC [45,46], with Dahl et al reporting a shift towards more DNA methylation over time in culture of MSC [47]. In contrast with these findings, we detected more hypomethylation after 14 days of culture expansion. During 14 days of culture expansion a high cell density (up to >90% confluency) was reached. Under

these conditions the cells are forced to increase intercellular interactions, which is likely to affect matrix and membrane composition and intercellular signaling. This is supported by our findings that genes related to membrane composition, cellular adhesion and transmembrane signaling were hypomethylated, suggesting increased expression. Confluency upon harvest will therefore affect MSC and DNA methylation analysis is a tool to monitor this in a quantitative manner.

DNA methylation affects gene expression, but this is not necessarily a direct consequence of methylation changes of the gene of interest itself [22,23]. When priming ucMSC with IFNγ or MC a single hypomethylated site, namely Cg00221794 was identified, which is located near the HS1BP3 gene. Hypomethylation in a promotor region is suggested to lead to increased gene expression [22]. Although site Cg00221794 is not located in the promotor region of the HS1BP3 gene but in close proximity, HS1BP3 gene expression was increased in ucMSC after priming with IFNγ or MC, which suggests a role for site Cg00221794 in the regulation of HS1BP3. HS1BP3 has recently been identified as a regulator of autophagy [48]. Its depletion inhibits autophagosome formation by interacting with phosphatidic acid on endosomes thereby preventing endosomal development into autophagosomes [49]. MSC display a high level of autophagy under homeostatic conditions, which is up or downregulated under stress or during differentiation [50]. The role of HS1BP3 in MSC is unknown, but it may well be implicated in the regulation of MSC autophagy.

Whereas priming of ucMSC with IFNγ or MC had only minor effects on DNA methylation, it had a significant effect on the T cell inhibition capacity of MSC and their expression of PD-L1 and HLA class I and II. On the contrary, 14 days of culture induced significant changes in DNA methylation, but had no effect on T cell inhibition capacity of MSC and small effects on PD-L1 and HLA expression. It is however possible that other functions of MSC that were not investigated, such as their capacity to modulate monocyte function or secrete trophic factors, are affected by the DNA methylation changes induced after 14 days of culture. Depending on the functional requirements of MSC for different types of applications, it becomes important to test the behavior of primed or prolonged culture expanded MSC in relevant assays.

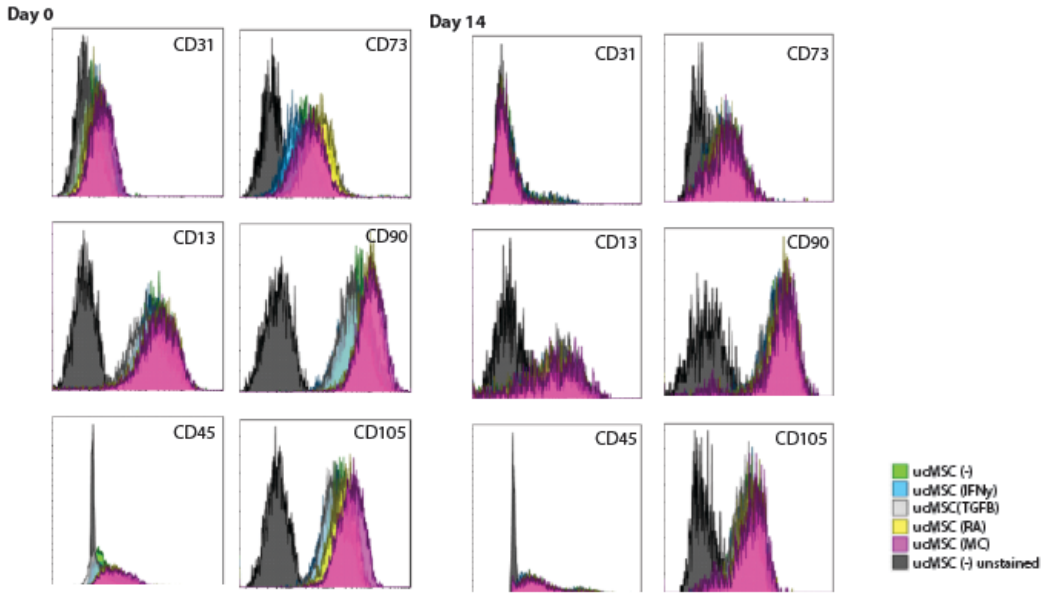
The NIH Roadmap Epigenomics Consortium generated reference epigenetic profiles of various human cell types, which supplies data concerning annotation and functional information of genomic sites and regions [51], which we used to annotate

functions to differentially methylated sites in ucMSC (supplementary tables 1,2). However, the Roadmap relates to adipose tissue derived MSC and bone marrow derived MSC and there is no data available for ucMSC. Although MSC from various tissue sites resemble each other, it is also clear that there are tissue specific differences. Therefore it is possible that particular methylation sites have a different function in ucMSC than described in the Roadmap.

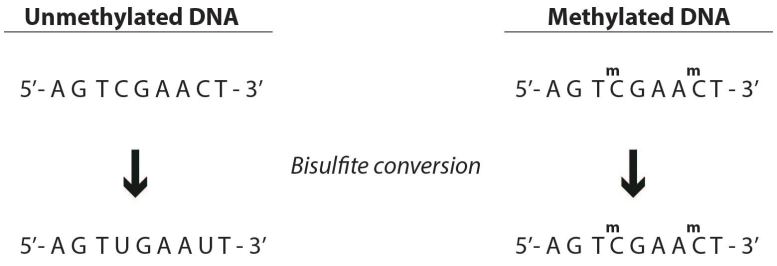
The use of a standardized therapeutic MSC product, is crucial to guarantee safety and predictable functionality. Although, it is unclear what the full impact of changes in DNA methylation of ucMSC on safety and functionality is, methylation status offers a global view on the state of a cell culture. In our hands, T cell inhibitory capacity of ucMSC did not change in response to changes in methylation induced by prolonged culture, but it is likely that other properties of the cells were affected. The mechanisms of MSC therapy have not been fully elucidated and therefore at this moment it is not possible to test relevant functional properties of the cells in relation to methylation status. When more is known about the mechanisms of action of MSC therapy, the effect of epigenetic changes on these particular mechanisms can be determined. Therefore, DNA methylation profiling could be used as a part of the characterization of therapeutic MSC to ensure the use of MSC of a fixed state, for standardization properties.

To conclude, MSC can be subjected to *in vitro* manipulations that lead to various phenotypical and functional changes. Our data showed that priming MSC with various stimuli has a minor impact on their DNA methylation, whereas during *in vitro* culture expansion MSC exhibit more extensive changes in DNA methylation profiles. These major changes in DNA methylation may influence the safety and efficacy of MSC therapy, which needs to be further investigated. Our results reveal that epigenetic profiles may be used as a quality control measure for MSC for experimental and in particular clinical use. Additional assessment of their DNA methylation pattern prior to their (pre)-clinical use, next to testing e.g. their karyotype, viability and phenotype will give a more in-depth analysis of their state. Moreover, assessment of their DNA methylation pattern as a quality control will contribute to the standardization of therapeutic MSC.

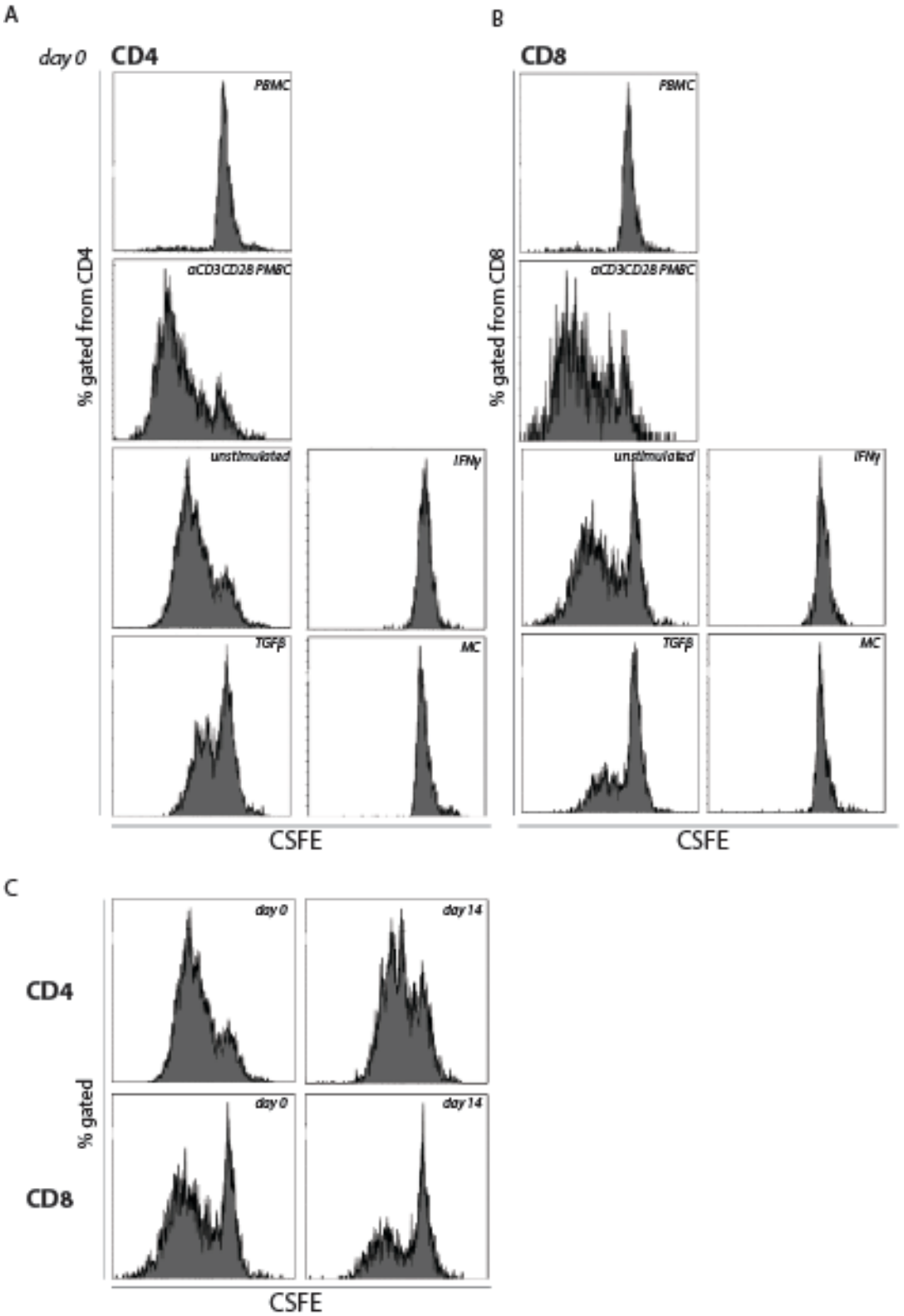
Supplementary Figures



▲ ***Supplementary figure 1 Characterization of ucMSC.*** *Immunophenotyping of ucMSC by flow cytometry. Representative histograms (green: ucMSC(-), blue: ucMSC(IFNy), light grey: ucMSC(TGFb), yellow: ucMSC(RA), pink: ucMSC(MC) and dark grey: unstained ucMSC(-)) of expression of characteristic MSC markers CD13, CD73, CD90, CD105 and negative expression of endothelial marker CD31 and hematopoietic marker and CD45 by ucMSC post 3 day priming (Left) and after 14 days of culture expansion (Right).*



▲ ***Supplementary figure 2 Example of bisulfite conversion.*** *All samples underwent bisulfite conversion: unmethylated cytosines are converted into uracil.*



▲ ***Supplementary figure 3 T cell suppression by ucMSC.*** *UcMSC and αCD3CD28 stimulated CSFE labeled PBMCs were co-cultured at a 1:2.5 ratio for 3 days. Representative histograms of CSFE in gated CD4 (A) and CD8 T-cells (B) co-cultured with primed ucMSC and with 14 days culture expanded ucMSC (C).*

Supplementary Tables

▼ ***Supplementary table 1 Top 10 hypomethylated regions in ucMSC after 14 days of culture expansion.*** *Annotated to ROADMAP reference data for adipose derived MSC and bone marrow derived MSC. The p-values of the regions and the number of CpG sites within the significantly hypomethylated regions are indicated.*

day 0 *ucMSC* <> day 14 *ucMSC*

#	Chr	Start	*In region*	p_{region}	#CpG
1	7	23387365	This DMR is located in an area annotated to the Insulin like growth factor 2 mRNA binding protein 3 (**IGF2BP3**) gene and is present in a region known to be a strong enhancer region and an active TSS.	6.01 x10^{-18}	*5*
2	5	159894868	This DMR is located in an area annotated to the MIR3142 Host Gene (**MIR3142HG**) gene and is present in a region known to be (near) an active TSS.	2.39 x10^{-17}	*5*
3	5	73928997	This DMR is located in an area annotated to the Ectodermal-Neural Cortex 1 (**CCL28, ENC1**)gene and is present in a region known to be a strong enhancer and transcription region.	9.40 x10^{-18}	*3*
4	9	118135710	This DMR is located in an area annotated to the Deleted In Esophageal Cancer 1 (**DEC1**) gene and is present in a region known to be a strong enhancer region and an active TSS.	3.34 x10^{-17}	*2*
5	4	160319500	This DMR is located in an area annotated to the **RP11-138A23.2** gene and is present in a region known to be an enhancer and transcription region	4.34 x10^{-15}	*3*
6	4	123693559	This region was not annotated to a gene by	8.26	*2*

			ROADMAP reference data	$x10^{-15}$	
7	7	18548468	This DMR is located in an area annotated to the Histone deacetylase 9 (**HDAC9**) gene and is present in a region known to be an active TSS.	5.80 $x10^{-14}$	*4*
8	6	29454623	This DMR is located in an area annotated to the MAS1 Proto-Oncogene Like (**MAS1L**) gene and is present in a region known to be quiescent.	1.68 $x10^{-13}$	*12*
9	4	74606107	This DMR is located in an area annotated to the Interleukin-8 **(IL-8, CXCL8)** gene and is present in a region known to be a enhancer region and an active TSS.	7.83 $x10^{-13}$	*4*
10	6	29429909	This DMR is located in an area annotated to the Olfactory receptor 2H1 **(OR2H1**) gene and is present in a region known to be quiescent.	8.34 $x10^{-13}$	*6*

▼ ***Supplementary table 2 Top 10 hypermethylated regions in ucMSC after 14 days of culture expansion.*** *Annotated to ROADMAP reference data for adipose derived MSC and bone marrow derived MSC. The p-values of the regions and the number of CpG sites within the significantly hypermethylated regions are indicated.*

day 0 *ucMSC* <> day 14 *ucMSC*

#	Chr	Start	*In region*	p_{region}	#CpG
1	12	14996143	This DMR is located in an area annotated to the ADP-Ribosyltransferase 4 (**ART4**) gene and is present in a region known to be an enhancer and transcription region, quiescent and near a TSS.	5.74 x10^{-12}	*11*
2	17	77018501	This DMR is located in an area annotated to the C1QTNF1 Antisense RNA 1 (**C1QTNF1-AS1**) gene and is present in a region known to be a strong enhancer region, quiescent and a repressed polycomb.	1.51 x10^{-11}	*8*
3	8	72757787	This DMR is located in an area annotated to the MSC Antisense RNA 1 (**MSC-AS1**) gene and is present in a region known to be an enhancer region, near a TSS, bivalent promotor and a repressed polycomb.	4.67 x10^{-9}	*5*
4	15	74466337	This DMR is located in an area annotated to the Immunoglobulin superfamily containing leucine rich repeat (**ISLR**) gene and is present in a region known to be an active TSS.	4.67 x10^{-9}	*6*
5	12	16760040	This DMR is located in an area annotated to the LIM Domain Only 3; Microsomal Glutathione S-Transferase 1 (**LMO3**) gene and is present in a region known to bea repressed polycomb, bivalent promotor and near a TSS.	1.50 x10^{-8}	12

6	2	239799314	This DMR is located in an area annotated to the Twist Family BHLH Transcription Factor 2 (**TWIST2**) gene and is present in a region known to be a transcription region and quiescent.	1.53 $x10^{-8}$	*4*
7	4	111561070	This DMR is located in an area annotated to the Paired Like Homeodomain 2 (**PITX2**) gene and is present in a region known to be repressed polycomb and quiescent.	1.55 $x10^{-8}$	*3*
8	12	3259078	This DMR is located in an area annotated to the Tetraspanin 9 (**TSPAN9-IT1/TSPAN9**) gene and is present in a region known to be a enhancer region.	5.27 $x10^{-8}$	*2*
9	8	27468684	This DMR is located in an area annotated to the Clusterin (**CLU**) gene and is present in a region known to be a strong enhancer region and near a TSS.	1.83 $x10^{-7}$	*9*
10	4	99417260	This DMR is located in an area annotated to the Tetraspanin 5 **(TSPAN5)** gene and is present in a region known to be a enhancer and transcription region.	1.96 $x10^{-7}$	*4*

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7

Immunomodulation by therapeutic mesenchymal stromal cells (MSC) is triggered through phagocytosis of MSC by monocytic 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 $Ly6C^{low}$ phenotype. Monocytes containing ucMSC were also detected systemically. *In vitro* experiments confirmed that human $CD14^{++}/CD16^{-}$ classical monocytes polarized towards 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

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 (LPS) 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 towards 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.

Materials and methods

Culture expansion of ucMSC

Human umbilical cord tissue was collected from Caesarean section deliveries by Tissue Solutions Ltd. (Glasgow, UK) 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 (MEM-α; Sigma-Aldrich, St Louis, MO, USA) containing 2 mM L-glutamine (Lonza, Verviers, Belgium), 1% penicillin/streptomycin solution (P/S; 100IU/ml penicillin, 100IU/ml streptomycin; Lonza) and supplemented with 15% fetal bovine serum (FBS; Lonza) and 1 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich) and kept at 37 °C, 5% CO_2 and air O_2. The medium was refreshed once a week and ucMSC were passaged using 0.05% trypsin-EDTA (Life technologies, Paisley, UK) at ~80-90% confluence. UcMSC were used in experiments between passage 3-6.

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 10min at 3000RPM 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 (Supplementary figure 1). 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 (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 / 200µl 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, USA, for imaging. At BioInVision 3D anatomical and molecular fluorescence videos were generated with CryoViz^{TM} 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/200ul PBS). 24 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 (0.5mg/ml, Life Technologies, Paisley, UK) for 30 minutes at 37 ºC 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, USA), CD45-Pe-Cy7, CX3CR1-PERCPCy5.5 (all Biolegend) and lung cells were stained in addition for CD68-PE (Biolegend) for 30 minutes at 4 ºC. 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 ºC. 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 1h, 4h and 24h in polypropylene tubes at 37 °C, 5% CO_2 and air O_2. 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 1h, 4h and 24h in polypropylene tubes in RPMI medium supplemented with 2 mM L-glutamine, 1% P/S and 10% heat-inactivated FBS at 37 °C, 5% CO_2 and air O_2.

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 ºC. Whole-blood samples were then fixed and red blood cells lysed for 10 minutes at 4 ºC 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 ºC. 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 min at 37 °C. The monocytes were cultured at 37 °C on gelatin-coated glass slides for 1h and 16h 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 (LAS AF) software, DPSS 561 nm lasers, using a 60 X (1.4 NA oil) objective. The microscope was equipped with a temperature-controlled incubator (incubator settings: 37 °C and 5% CO_2). Images were processed using ImageJ 1.48 (National Institutes of Health, Washington, USA).

Addition of ucMSC primed monocytes to mixed lymphocyte reaction

$CD14^+$ 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, USA) and MagniSort Streptavidin Positive Selection Beads (MSPB-6003, eBioscience, Affymetrix Inc, San Diego, CA, USA) and the EasySep™ Magnet (StemCell technologies, Germany). The obtained untouched primed monocytes showed a purity of >98% (Supplementary figure 2).

Primed and non-primed monocytes (10,000) were added to mixed lymphocyte reactions (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 min 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 500ng 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 <0.05 were considered significant.

Results

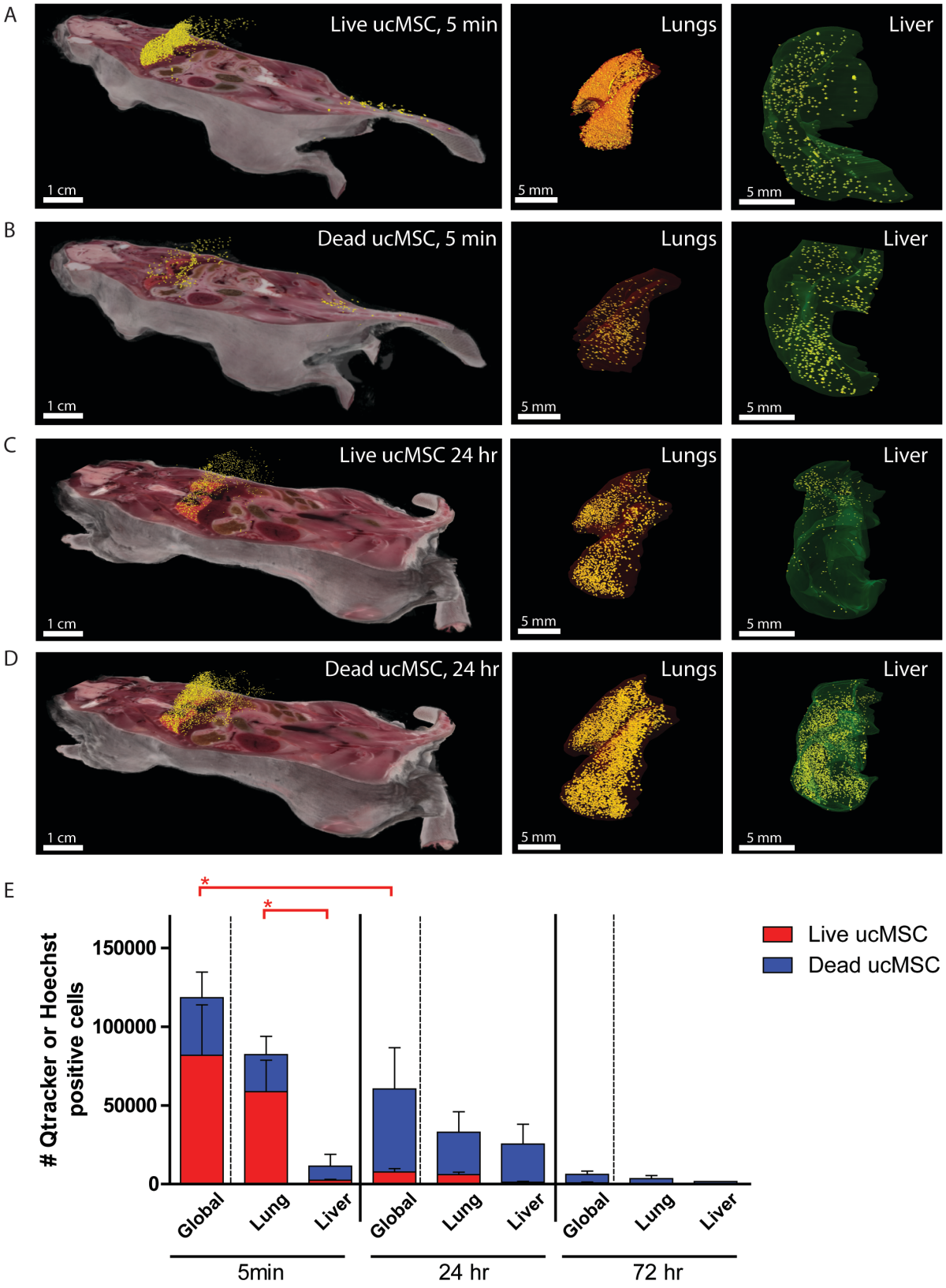
UcMSC accumulate in the lungs after intravenous infusion

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, E). In addition, few dead ucMSC were observed in the lungs and liver as detected by Hoechst33342 signal (Figure 1B, E).

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, E). 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-E). 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).

►**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. Scale bar in full body image of mouse (left), 1 cm; scale bar in image of lungs (middle), 5mm; scale bar in image of liver (right), 5 mm. (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 ($p<0.05$).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5 mm
B
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Liver
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C
Live ucMSC 24 hr
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D
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Qtracker or Hoechst positive cells
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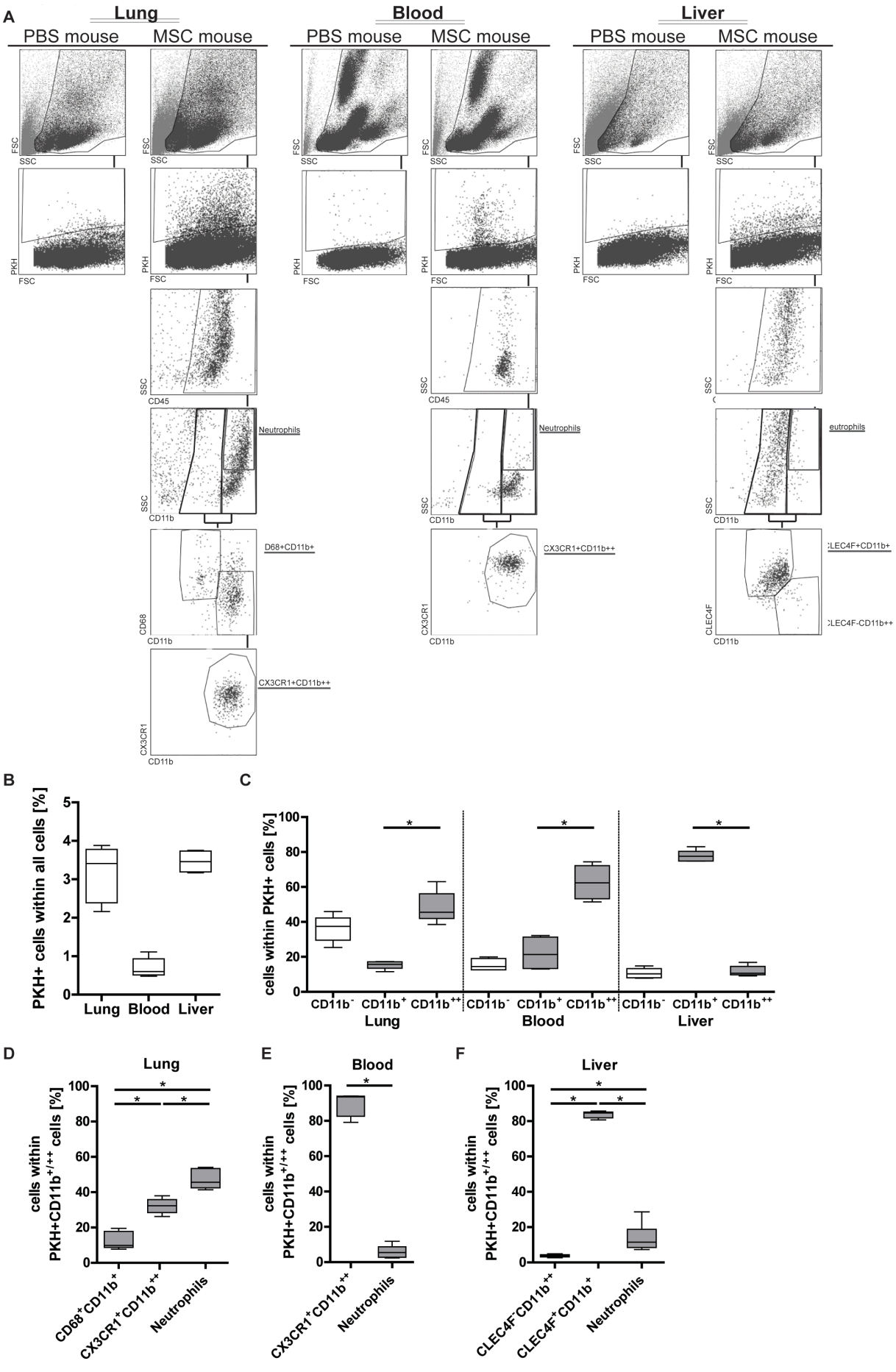
A
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CD68
D68+CD11b+
CX3CR1
CX3CR1+CD11b++
CLEC4F
CLEC4F+CD11b+
CLEC4F-CD11b++
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Blood
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Liver
cells within PKH+CD11b+/++ cells [%]
CD68+CD11b+
CX3CR1+CD11b++
Neutrophils
CX3CR1+CD11b++
Neutrophils
CLEC4F-CD11b++
CLEC4F+CD11b+
Neutrophils

◄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 (SSC^{high}CD11b$^+$) 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 ($p<0.05$).

UcMSC are phagocytosed and re-distributed 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-B). 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, C), 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, D). In the blood, 89.3±1.3% of PKH26$^+$ cells were CX3CR1$^+$CD11b^{++} monocytes and 5.7±0.7% were neutrophils (Figure 2A, E). 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, F).

Monocytes express a regulatory phenotype after phagocytosis of ucMSC

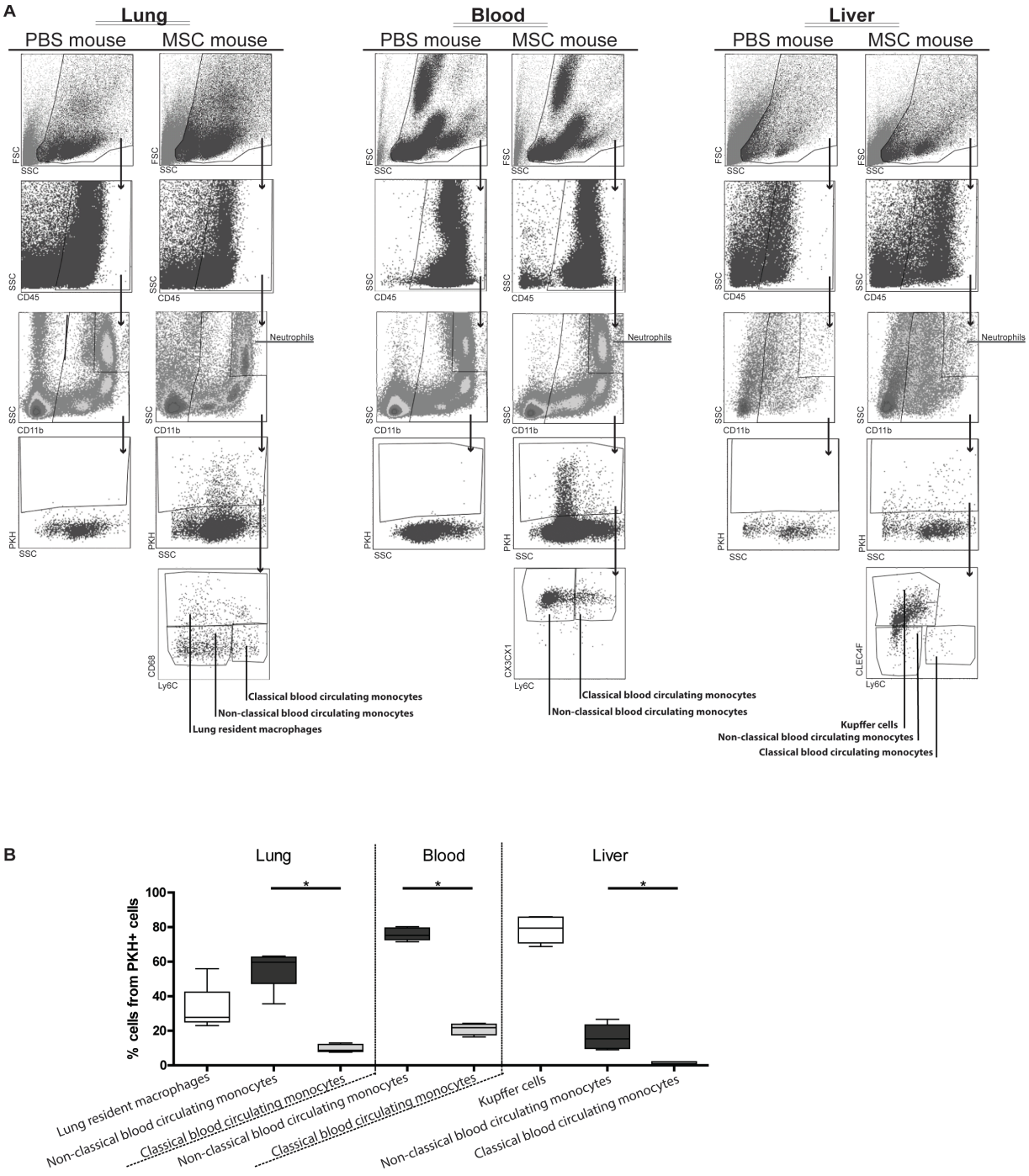
Thus, monocytes and neutrophils contribute to the clearing of infused ucMSC. In addition to their phagocytic activity, monocytes may play immune-activating as well

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 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*

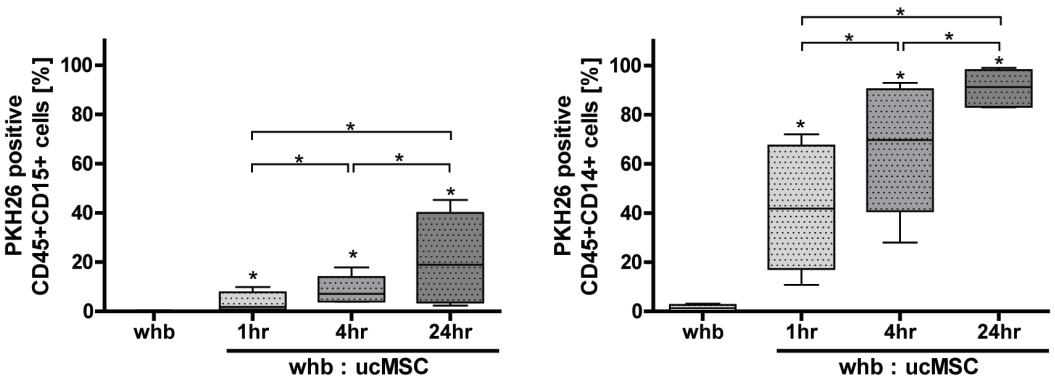
To further study the interaction of ucMSC with human innate immune cells, PKH26-labeled ucMSC were added to human whole blood *in vitro*. After 24h of incubation, 21±8% of $CD45^+CD15^+$ neutrophils and 91±3% of $CD45^+CD14^+$ monocytes had become positive for PKH26 (Figure 4A), thereby confirming the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

PKH26-labeled ucMSC were subsequently incubated with human blood-derived $CD14^+$ monocytes. Nearly all monocytes became positive for PKH26 within 24h as measured by flow cytometry (19±2% at 1h, 34±3% at 4h and 92±1% 24h) (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 towards ucMSC within 1h (Figure 4C). Monocytes with internalized fragments of PKH26-labeled ucMSC were observed 3h after the start of the co-cultures (Figure 4D). At 16h, the majority of monocytes contained PKH26-labeled ucMSC fragments (Figure 4E). In the control co-culture, which was left at 4 ºC for 16h, no phagocytosis of ucMSC by monocytes was observed (Figure 4F), demonstrating that phagocytosis of ucMSC by monocytes is an active process.



▲Figure 3. Monocytes that have phagocytosed 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 24h after PBS or ucMSC infusion. Results are shown as means ± SEM (n=3 PBS mice and n=5 ucMSC mice). * indicates significant difference ($p<0.05$).

A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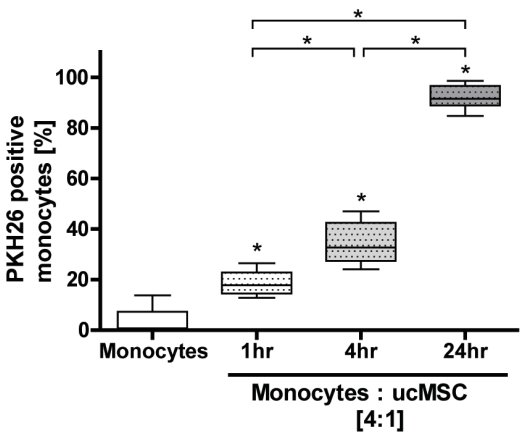
B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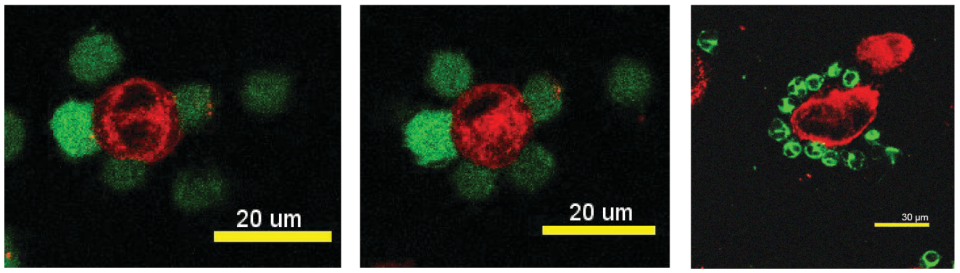
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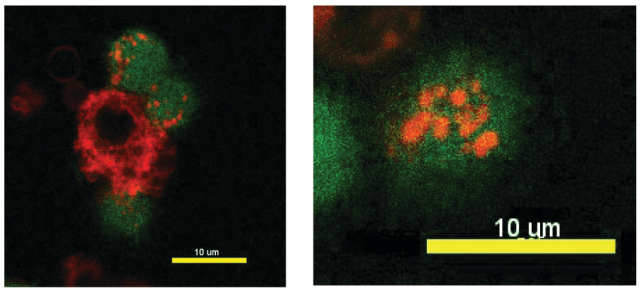
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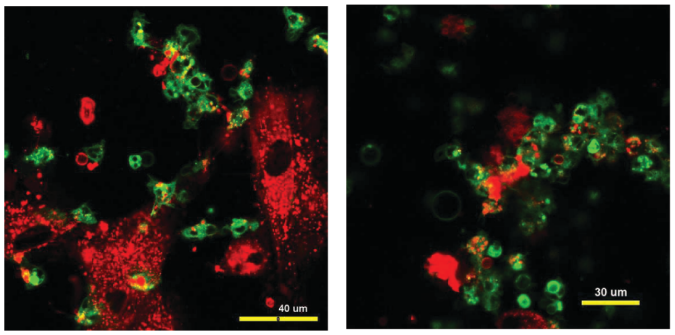
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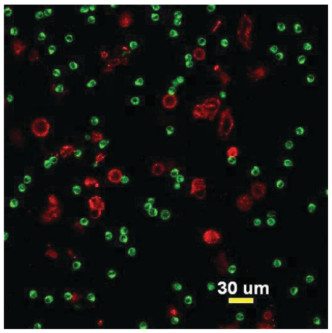
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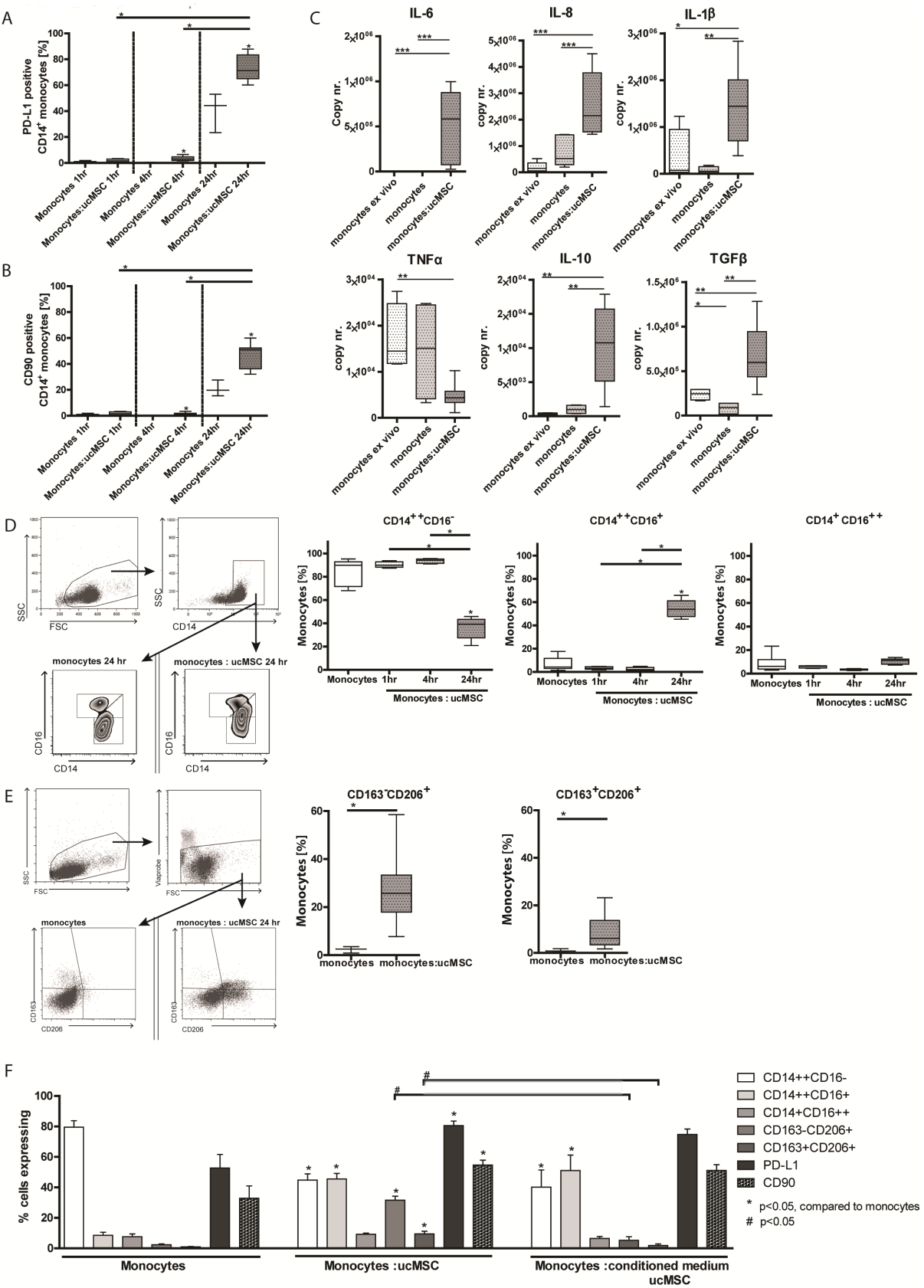
◄**Figure 4. UcMSC are phagocytosed by human monocytes *in vitro*.** (A) Frequency of PKH26$^+$ neutrophils (left) and monocytes (right) after addition of PKH26$^+$ ucMSC to human whole blood. An increase in PKH26$^+$ neutrophils and monocytes can be observed over time. (B) Percentage of PKH26$^+$ monocytes 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 ($p<0.05$).

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%, $p<0.05$) and CD90 (from 21±4% to 47±3%, $p<0.05$) after 24h of co-culturing with ucMSC (Figure 5A-B). Moreover, mRNA expression 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 24h, 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.


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.
copy nr.
monocytes ex vivo
monocytes
monocytes:ucMSC
D
SSC
FSC
CD14
CD16
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

◄Figure 5. 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 ($p<0.05$). (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 ($p<0.05$). (D) Representative flow cytometry plot demonstrating changes in monocyte subset composition based on CD14 and CD16 expression 24h after co-culture with ucMSC. During co-culture, the frequency of $CD14^{++}CD16^-$ monocytes decreased whereas $CD14^{++}CD16^+$ monocytes increased. * indicates significant difference ($p<0.05$). (E) Representative flow cytometry plot demonstrating increases in the frequency of $CD163^-CD206^+$ and $CD163^+CD206^+$ monocyte subsets after 24h of co-culture of monocytes with ucMSC. * indicates significant difference ($p<0.05$). (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 ($p<0.05$) and # indicates significant difference ($p<0.05$). Results are shown as means ± SEM (n=3).

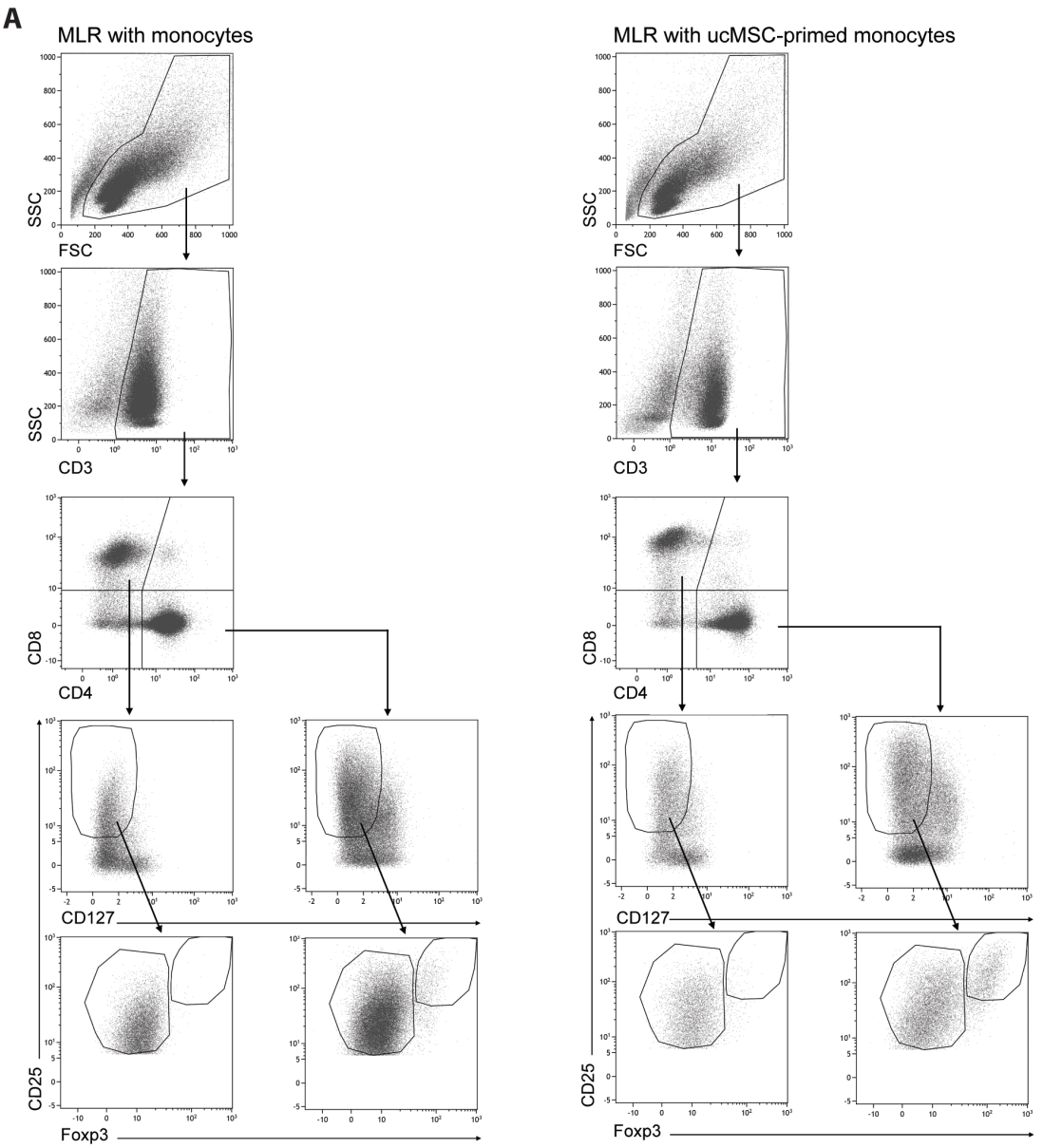
Skewing of monocytes by phagocytosis of ucMSC differs from skewing by ucMSC conditioned medium

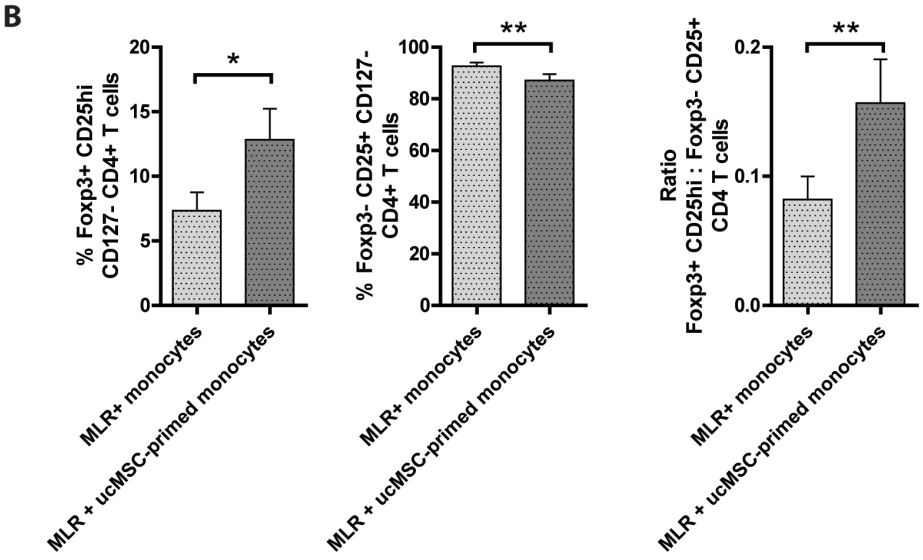
Monocytes that have phagocytosed ucMSC exhibit a different phenotype than prior to phagocytosis. To investigate whether this is caused by factors secreted by ucMSC or by phagocytosis of ucMSC, monocytes were cultured in ucMSC conditioned medium or co-cultured with ucMSC. After 3 days the phenotype of 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 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 $CD4^+CD25^{hi}CD127^-$ 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 ($Foxp3^-CD4^+CD25^{hi}CD127^-$). Finally, the ratio of $Foxp3^+$/$Foxp3^-$ $CD4^+CD25^{hi}CD127^-$ T cells increased from 0.1 to 0.2 upon addition of ucMSC primed monocytes (Figure 6B).

►**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 $Foxp3^+CD25^{hi}CD127^-CD4^+$ regulatory T cells of CD4 T cells. Plots indicate means ± SEM (n≥4). (C) Median fluorescence intensity of Foxp3 within $Foxp3^+CD25^{hi}CD127^-CD4^+$ T cells. Plots indicate means ± SEM (n≥4). * indicates significant difference ($p<0.05$).


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
*
**
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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 two 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.

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]

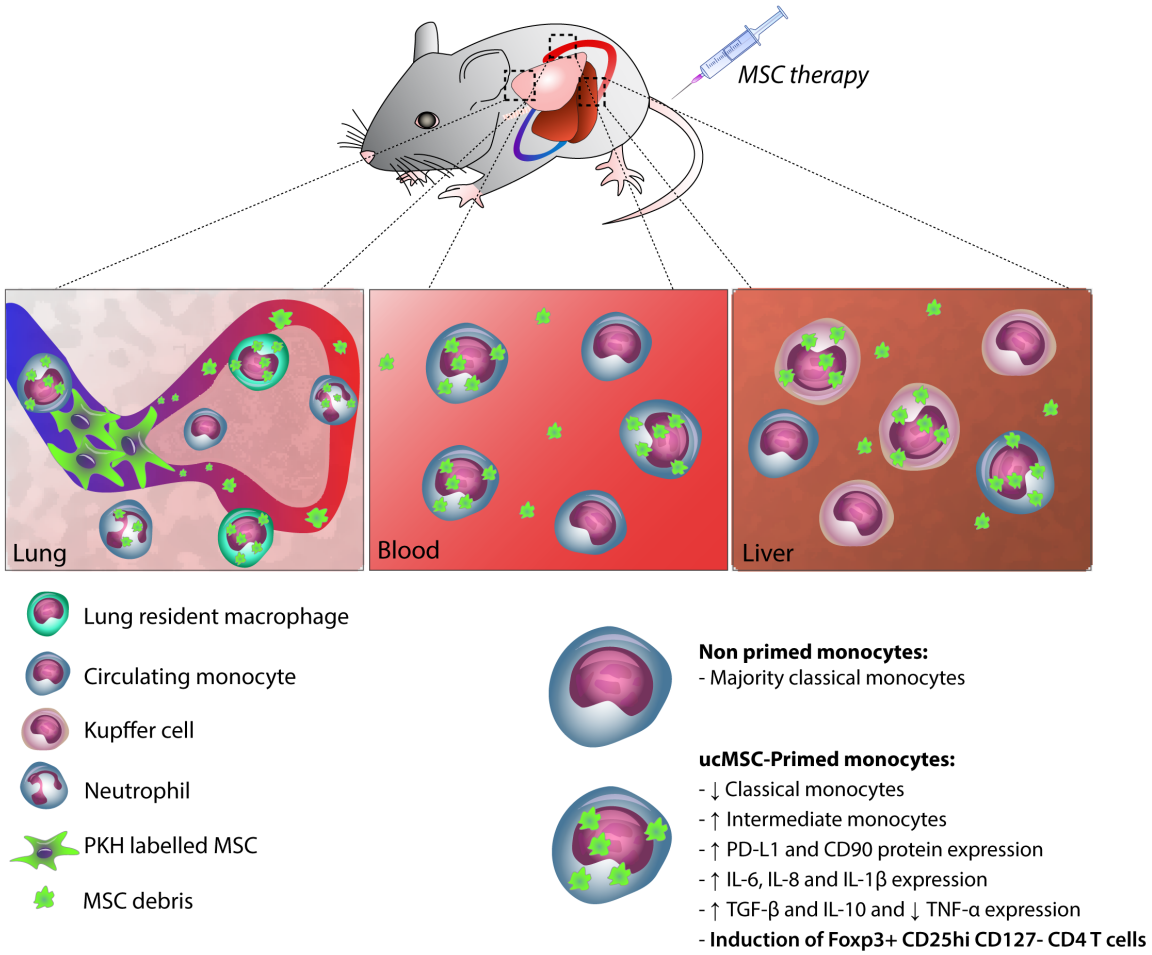
After phagocytosis of MSC, monocytes migrate to other body sites via the blood stream (summarized in Figure 7). In addition, some MSC may disintegrate and the remnants may be transported out of the lungs via the blood stream. We found accumulation of MSC remnants in the Kupffer cells of the liver. Kupffer 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 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 towards $CD14^{++}CD16^+$ 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 able to phagocytose ucMSC. These $CD206^+CD163^+$ co-expressing monocytes have been described in 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, i*n 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 $Foxp3^+CD25^{hi}CD127^-CD4^+$ regulatory T cells. Multiple studies have reported increased frequencies of regulatory T 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 regulatory T cells *in vitro.*[54] Our results give insight in how MSC driven polarization of monocytic cells may mediate increasing regulatory T cell numbers after MSC infusion

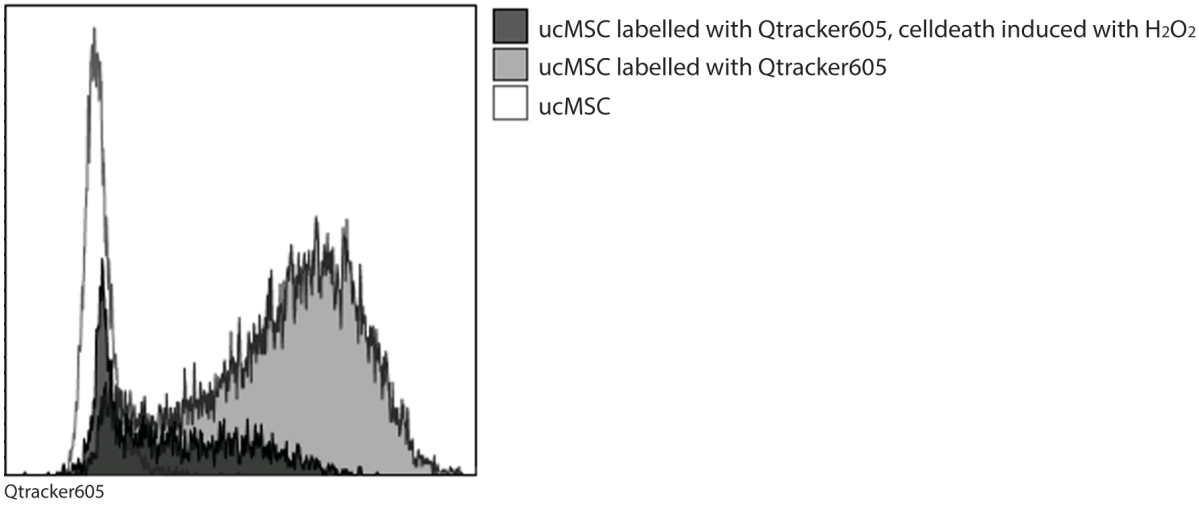


▲Figure 7. Overview of the interaction of monocytic cells with infused MSC. UcMSC get entrapped in the lungs after intravenous infusion and are rapidly cleared from the system through phagocytosis by neutrophils, lung resident macrophages and circulating monocytes. Monocytes containing ucMSC migrate 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 $Foxp3^{+}CD25^{hi}CD127^{-}CD4^{+}$ regulatory T cells

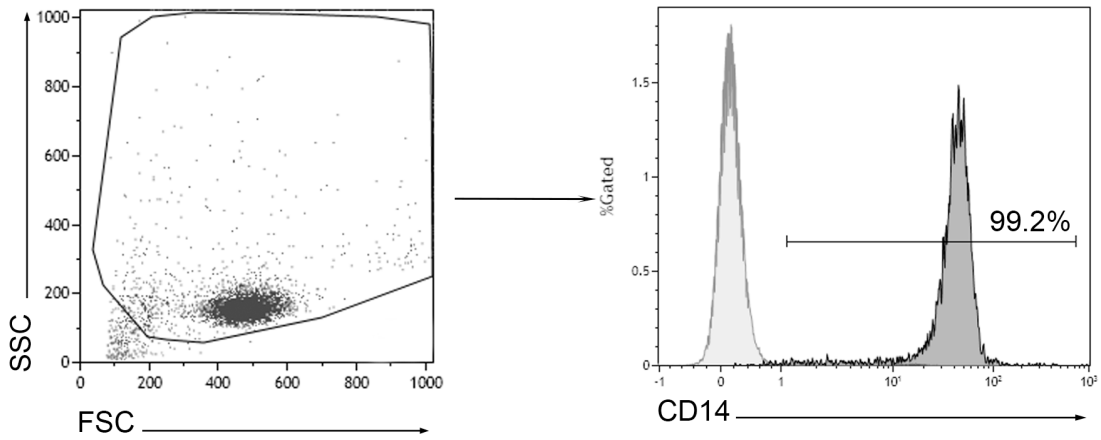
Conclusion

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.

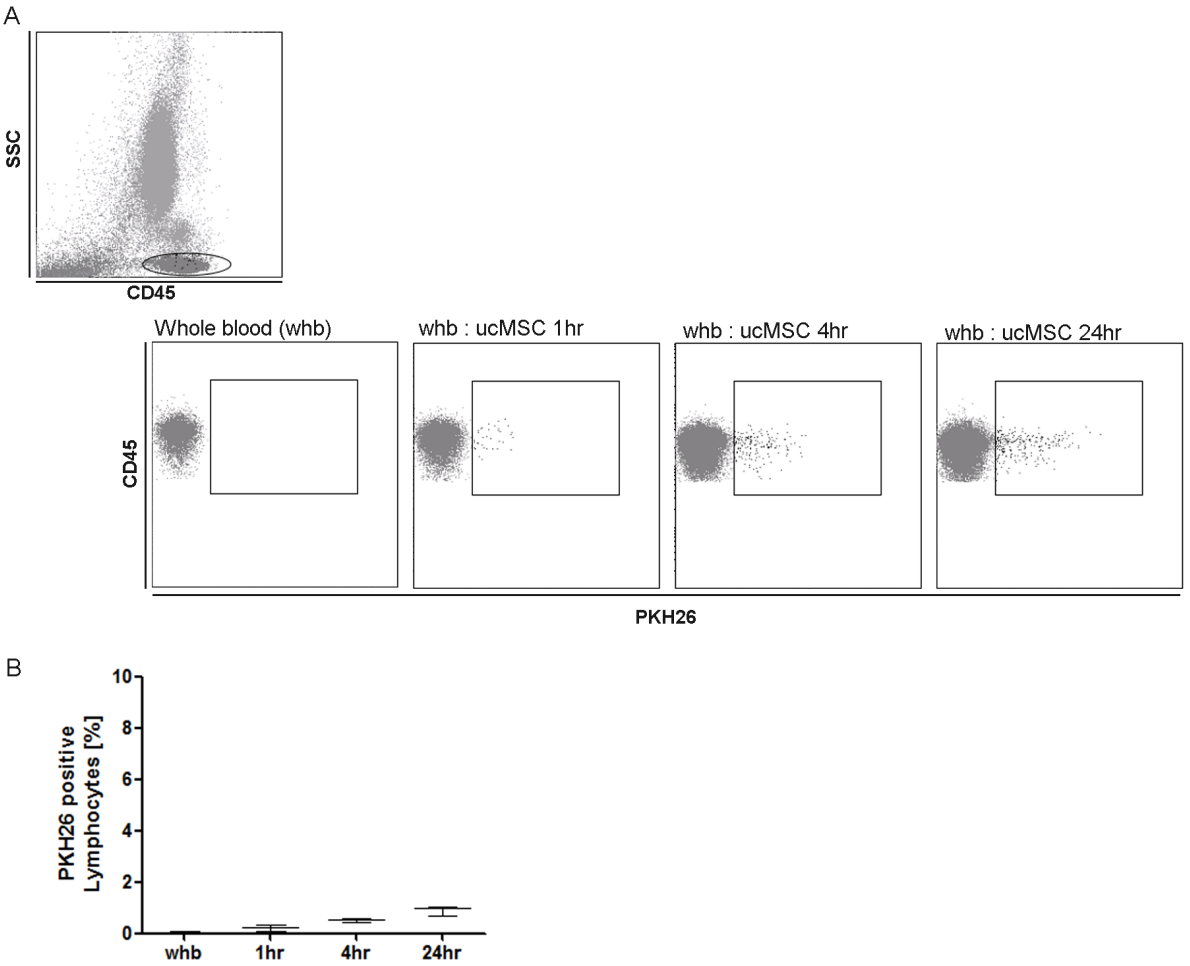
Supplementary materials



▲Supplementary Figure 1. ucMSC lose Qtracker beads after cell death. Flow cytometric plot showing unlabeled ucMSC (white), ucMSC labeled with Qtracker605 beads (light gray) and ucMSC labeled with Qtracker605 beads after induction of cell death by incubation with 50μM H_2O_2 (dark gray).



▲Supplementary Figure 2. Manual MACS separation of monocytes from MSC gives rise to a highly pure CD14$^+$ population. Monocytes were MACS separated from the ucMSC by negative selection of CD73. Flow cytometric plot showing the purity of the isolated monocytes (>98%).



▲Supplementary Figure 3. Phagocytosis of ucMSC by human lymphocytes *in vitro* is not significant. (A) Representative flow cytometry plot demonstrating of PKH26+ T-lymphocytes after addition of PKH26+ ucMSC to human whole blood. (B) Frequency of PKH26+ T-lymphocytes at t= 1, 4 and 24 hr after addition of PKH26+ labelled ucMSC to human whole blood. Plots indicate means ± SEM. (n=4)

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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 $1x10^4$ 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 IIflow 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 $2.5x10^3$ MSC/well. The next day, $5x10^4$ carboxyfluorescein succinimidyl ester (CFSE)-labeled healthy donor-derived peripheral blood mononuclear cells (PBMC) and $5x10^4$ 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 $0.3x10^6$ 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 $0.3x10^6$ 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, $0.3x10^6$ 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.

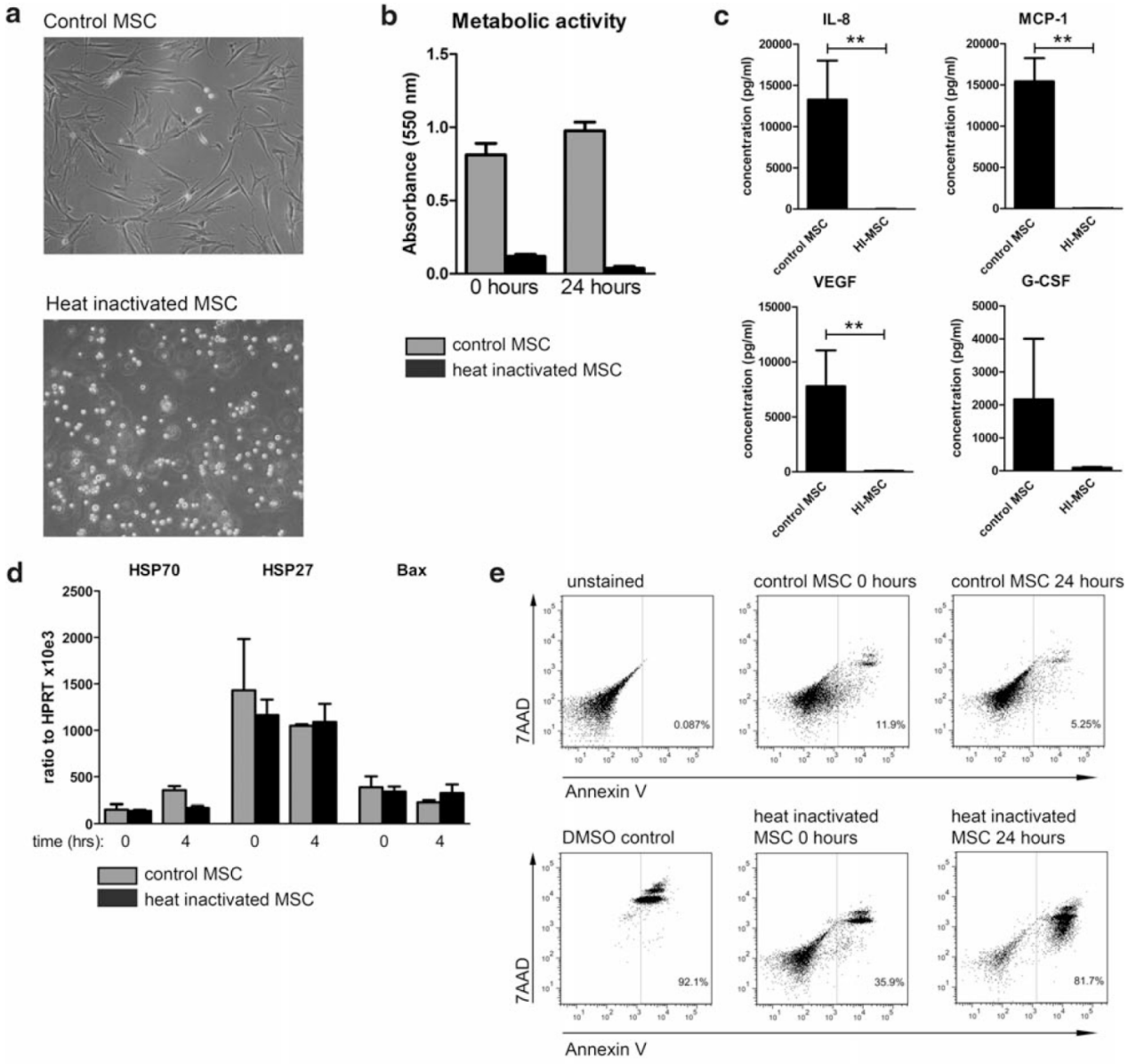
Statistical analysis

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 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.

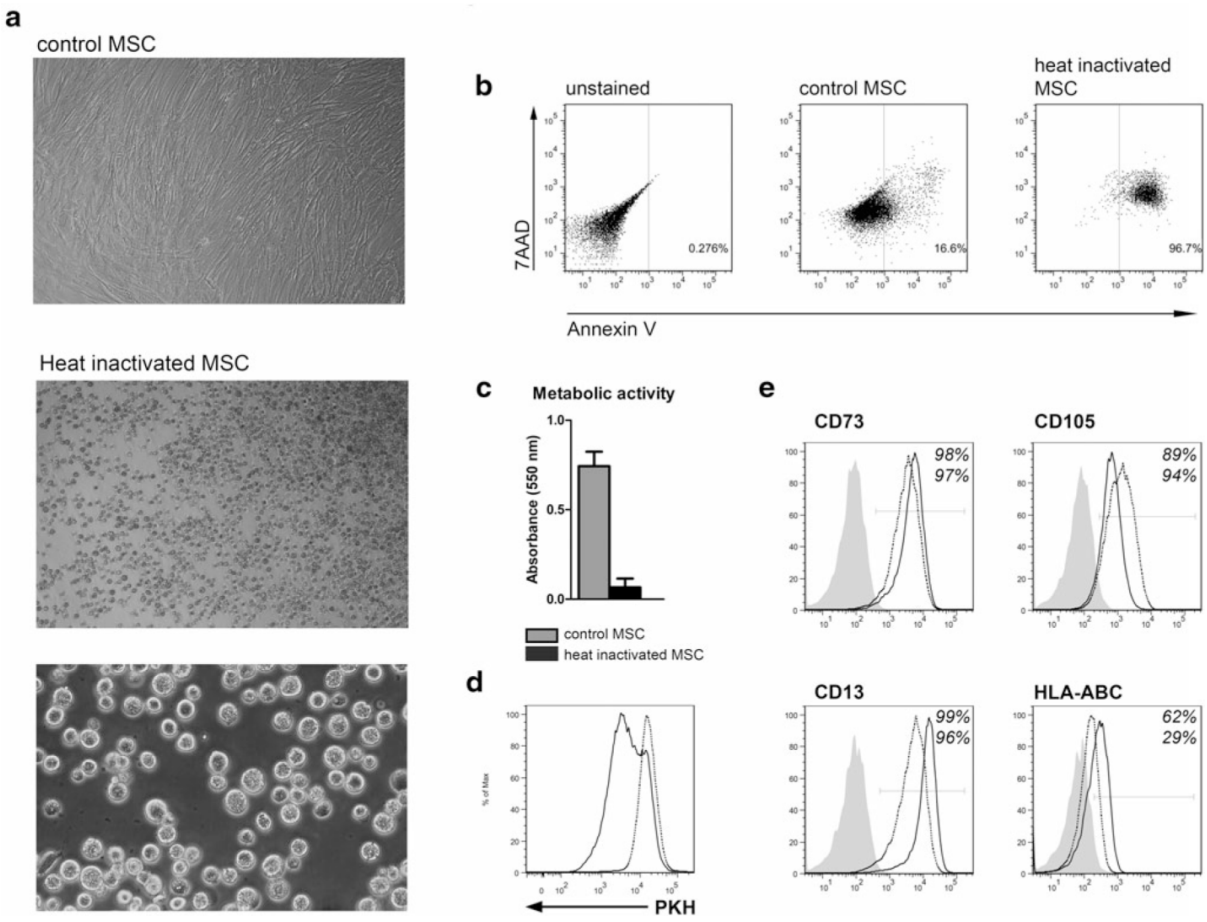

a
Control MSC
Heat inactivated MSC
b
Metabolic activity
Absorbance (550 nm)
1.5
1.0
0.5
0.0
0 hours
24 hours
control MSC
heat inactivated MSC
c
IL-8
MCP-1
VEGF
G-CSF
concentration (pg/ml)
**
control MSC
HI-MSC
d
HSP70
HSP27
Bax
ratio to HPRT x10e3
2500
2000
1500
1000
500
0
time (hrs): 0 4 0 4 0 4
control MSC
heat inactivated MSC
e
unstained
control MSC 0 hours
control MSC 24 hours
0.087%
11.9%
5.25%
7AAD
Annexin V
DMSO control
heat inactivated MSC 0 hours
heat inactivated MSC 24 hours
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%
CD13
99%
95%
CD73
99%
95%
CD31
0.5%
CD90
94%
87%
CD105
99%
96%
CD90
90%
68%
CD105
99%
94%

◄ **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

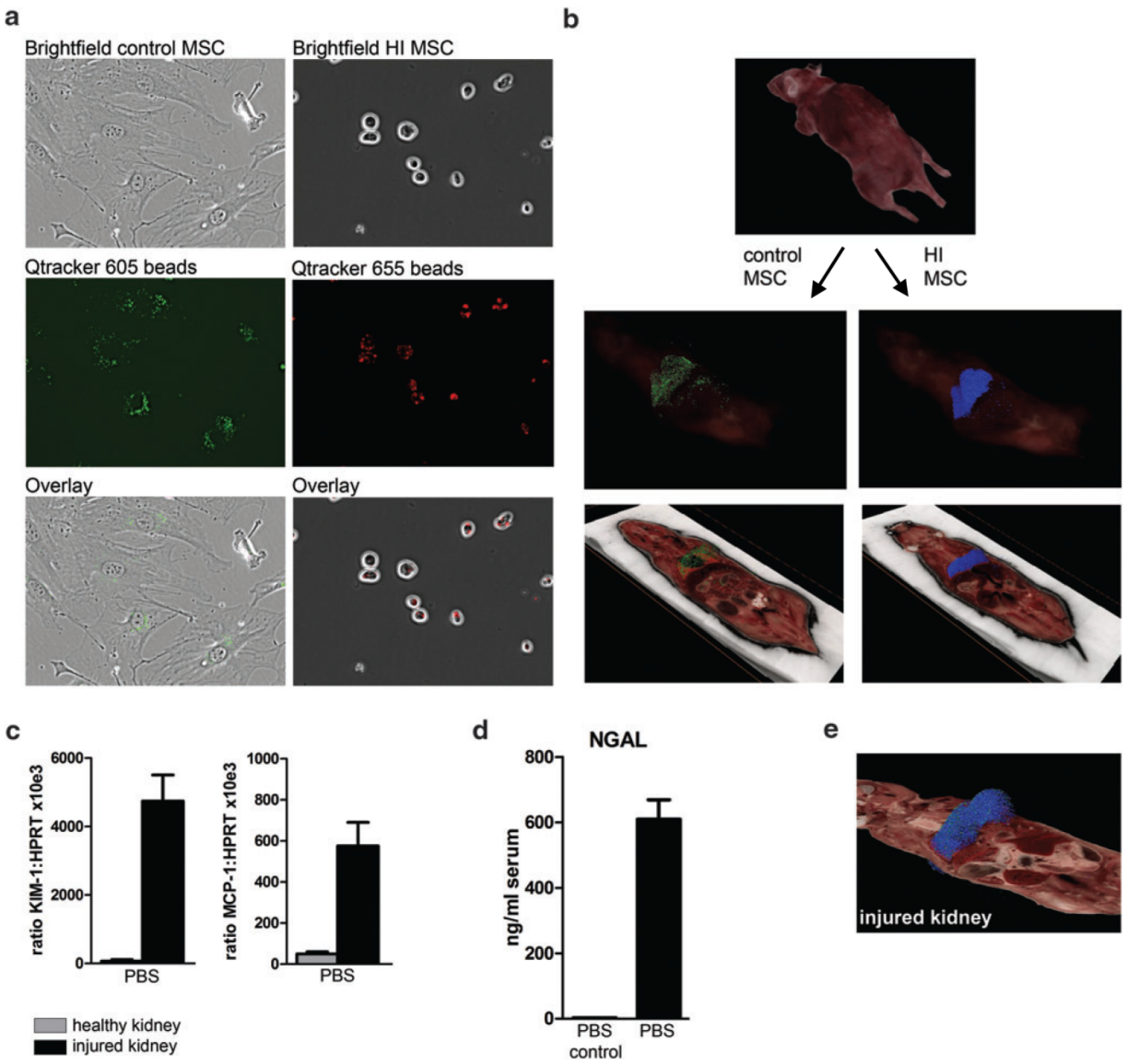
MSC do not recover from heat inactivation

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 (Fig. 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 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.



▲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.

Table 1. Heat-Inactivated Mesenchymal Stem Cells Show Similar Migration Properties as Control Mesenchymal Stem Cells

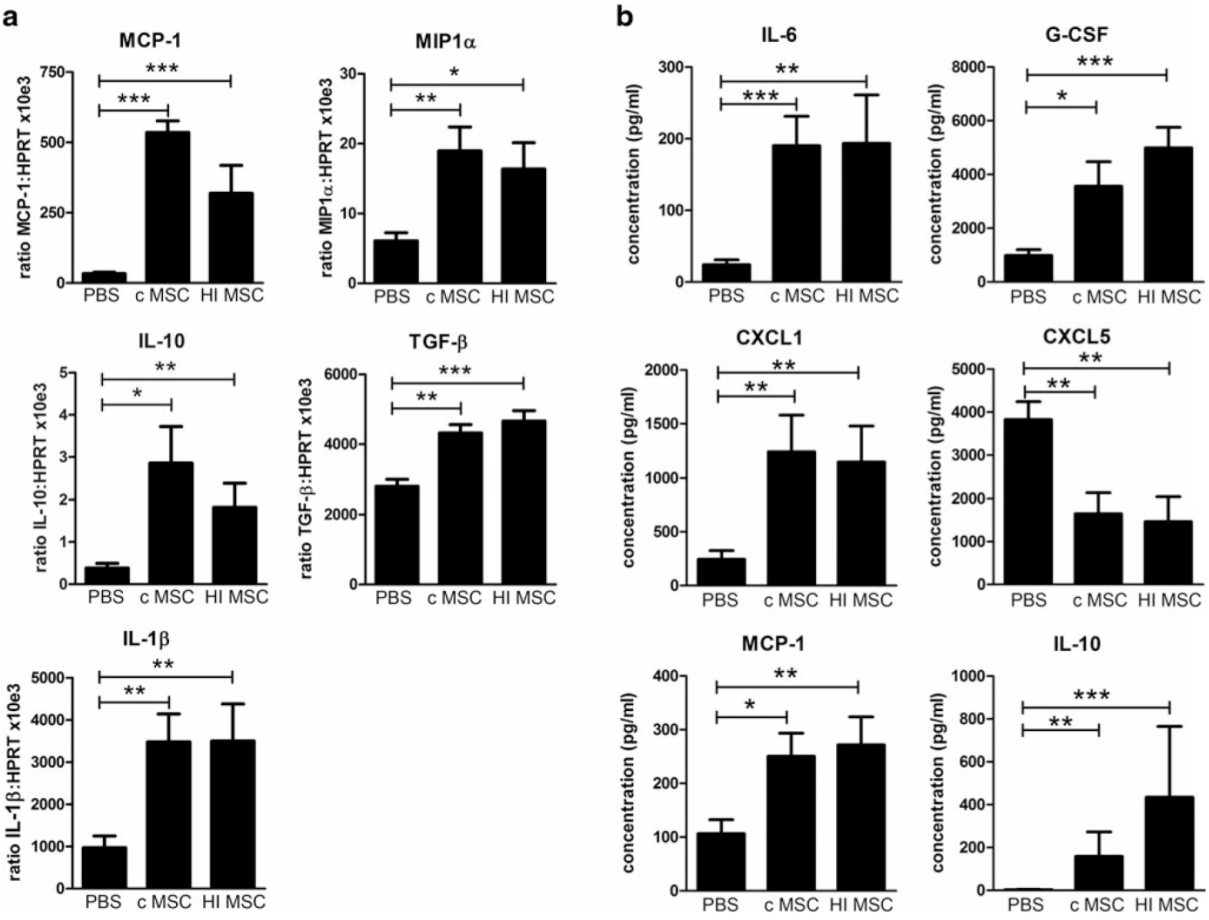
	Control MSC				*HI-MSC*			
Time point—treatment	*Injected*	*Recovered total*	*Injured kidney*	*Healthy kidney*	*Injected*	*Recovered total*	*Injured kidney*	*Healthy kidney*
2 h—control	150,000	47,186	—	52	150,000	82,082	—	10
24 h—control	150,000	210	—	0	150,000	959	—	0
2 h—kidney injury	150,000	36,801	126	129	150,000	137,723	17	13
24 h—kidney injury	150,000	3,134	11	17	150,000	11,320	0	2

▲Number of detected MSC recovered in whole animals and in the kidneys 2 and 24 h 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.

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 $0.3x10^6$ 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 control MSC *(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 $0.15x10^6$ labeled control MSC mixed with $0.15x10^6$ 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 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.



▲Figure 4 Control and HI-MSC induce the same immunomodulatory effect after infusion in healthy mice. Control MSC ($0.3x10^6$ cells), HI-MSC ($0.3x10^6$ 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.

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

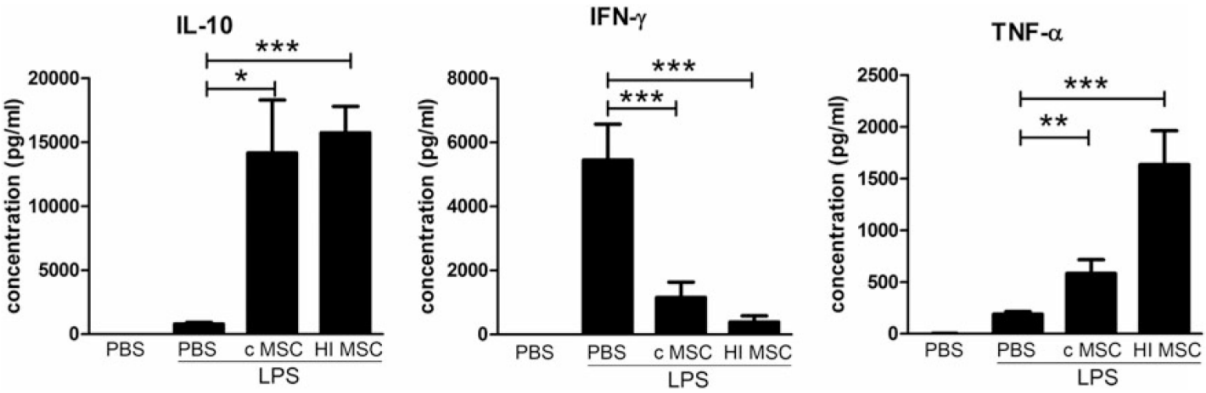
As described previously, MSC induce an immunomodulatory response after IV infusion in healthy mice that can be measured both locally in the lungs and systematically in the serum [34]. To investigate whether this response is dependent on the viability of MSC, we infused 300,000 syngeneic control MSC or 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.

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.



▲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.

HI-MSC do not induce regulatory B-cell formation

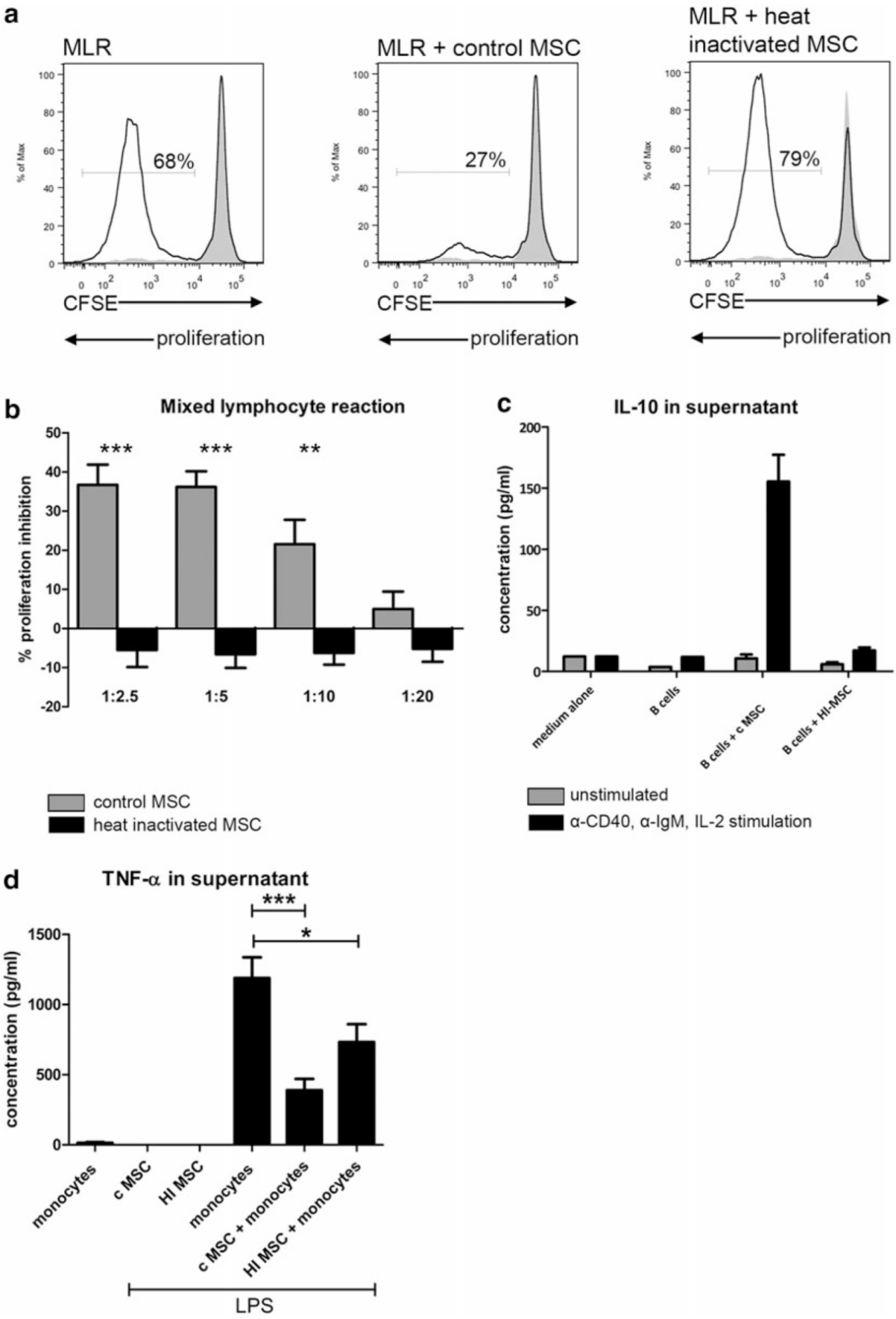
To examine whether HI-MSC are able to induce formation of IL-10-producing transitional B cells, as previously demonstrated for control MSC [39], control and HI-

MSC were cocultured with quiescent B cells obtained from human splenocytes. 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.

►**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 $P < 0.001$.


a
MLR
MLR + control MSC
MLR + heat inactivated MSC
% of Max
68%
27%
79%
CFSE
proliferation
b
Mixed lymphocyte reaction
% proliferation inhibition
***
***
**
1:2.5
1:5
1:10
1:20
control MSC
heat inactivated MSC
c
IL-10 in supernatant
concentration (pg/ml)
medium alone
B cells
B cells + c MSC
B cells + HI-MSC
unstimulated
α-CD40, α-IgM, IL-2 stimulation
d
TNF-α in supernatant
concentration (pg/ml)
***
*
monocytes
c MSC
HI MSC
monocytes
c MSC + monocytes
HI MSC + monocytes
LPS

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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9

Modulating MSC to alter phagocytosis by monocytes and their subsequent polarization

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In this thesis mesenchymal stromal cells (MSC) have been observed to be highly responsive to various priming protocols. Furthermore, inactivated MSC were observed to elicit similar immunomodulatory effects *in vivo* as control MSC. Hence, in an effort to combine all the above mentioned findings with the recent identification of monocytes as key players in the immunomodulatory mechanism of action of MSC, we investigated the effects of priming and inactivation of MSC on the polarization of monocytes. Human ucMSC were primed for three days with IFNγ, TGFβ, retinoic acid (RA) and a multi factor combination (MC; IFNγ, TGFβ and RA), labeled with PKH, inactivated and subsequently added for 24 hours to whole blood. Thereafter, PKH signal and CD14 and CD16 expression was analyzed in the monocyte population. Inactivated ucMSC were also cultured with enriched CD14+ monocytes for 24 hours and CD163 and CD206 expression on the monocytes was analyzed. We observed that priming of ucMSC with IFNy and MC affects the phagocytic behaviour of monocytes and steers their phenotype shift towards a more non-classical phenotype (CD14+CD16++). In comparison, inactivation of control as well as primed ucMSC resulted in a slightly bigger shift towards a more non-classical phenotype. In addition, inactivated ucMSC were able to skew monocytes post phagocytosis towards a more regulatory phenotype (CD163-CD206+ or CD163+CD206+). The results reveal that different treatments of MSC differentially affect monocyte behavior and these findings will help to further elucidatethe mechanism of action of MSC as well as help further development of MSC into effective immunotherapy.

Introduction

The immunomodulatory function of mesenchymal stromal cells (MSC) has been discussed in the preceding chapters of this thesis. In accordance with Galleu et al. [1], we revealed that cell death and phagocytosis are important for the immunomodulatory effect by therapeutic MSC. We observed in Chapter seven that phagocytosis of therapeutic MSC by monocytes induces polarization, relocation of monocytes and subsequently leads to immunomodulation [2]. With the help of these results we have identified that monocytes play a crucial mediator role in the immunomodulatory effect of MSC *in vivo*. Furthermore, we showed in Chapter eight that inactivated MSC, which lost both their ability to actively crosstalk with immune cells and secrete factors, actually maintain their ability to modulate inflammatory responses *in vivo* [3]. Hence, with these results we identified that the exterior of MSC and their recognition by host immune cells play a crucial role in the immunomodulatory effect of MSC. Application of inactivated MSC would reduce recurring concerns about the stability and safety of therapeutic MSC. In chapter five we showed that MSC can be primed with various stimuli to optimize their immunomodulatory and immunogenic properties [4]. Priming MSC with IFNγ, TGFβ, retinoic acid (RA) and the multi factor combination (MC; IFNγ, TGFβ and RA) led to changes in their surface marker expressions as well as their secretome. Priming MSC with TGFβ reduced their susceptibility to NK cell cytotoxicity and priming with IFNγ enhanced their T-cell inhibitory capacity. Furthermore, priming with a multi factor combination consisting out of IFNγ, TGFβ and retinoic acid (RA) resulted in reduced susceptibility to NK cell cytotoxicity, increased immunomodulatory capacity and enhanced their capacity to ameliorate liver inflammation in an *ex vivo* inflammatory liver model. Work in Chapter eight demonstrated that the interaction with monocytes is crucial for the therapeutic effect of MSC *in vivo*. The possibility that priming of MSC would steer the effect of their interaction with monocytes was however not addressed.

The present study aims to unify of the findings from previous chapters and provide more insight in the mechanisms of action and opportunities to specifically modify the immunomodulatory effects of MSC. Hence, the effect of inactivation and priming of MSC on the phagocytosis and phenotype shift of monocytic cells was analyzed here.

Materials and Method

Culture expansion of ucMSC

Human umbilical cord tissue was collected from Caesarean section deliveries by Tissue Solutions Ltd. (Glasgow, UK) from healthy donors (no 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. [4]. Post isolation, cells were counted, seeded for expansion and cryopreserved at passage 2. Cells were then shipped to Erasmus Medical Center. Here, ucMSC were cultured in minimum essential medium Eagle alpha modification (MEM-α; Sigma-Aldrich, St Louis, MO, USA) containing 2 mM L-glutamine (Lonza, Verviers, Belgium), 1% penicillin/streptomycin solution (P/S; 100IU/ml penicillin, 100IU/ml streptomycin; Lonza) and supplemented with 15% fetal bovine serum (FBS; Lonza) and 1 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich) and kept at 37 °C, 5% CO_2 and air O_2. The medium was refreshed once a week and ucMSC were passaged using 0.05% trypsin-EDTA (Life technologies, Paisley, UK) at ~80-90% confluence. UcMSC were used in experiments between passage 3-6.

Priming of ucMSC

MSC were stimulated with interferon gamma (IFNγ, 50 ng/ml; Life technologies), transforming growth factor beta 1 (TGFβ, 10ng/ml; R&D systems, MN, USA), retinoic acid (RA, 100 μM; Sigma) and a multi-factor combination (MC) of IFNγ, TGFβ and retinoic acid for 3 days.

PKH26 labelling of primed and non-primed ucMSC

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.

Inactivation of PKH labelled primed and non-primed ucMSC

UcMSC (primed and non-primed) were inactivated in suspension in PBS in parafilm-sealed tubes at 50ºC for 30 minutes in a temperature regulated water bath. After 30 minutes the inactivated (HI) MSC were washed twice and used for further experiments in MEM-α with 2 mM L-glutamine, P/S, 15% FBS and 1 ng/ml bFGF.

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 24h in polypropylene tubes at 37 °C, 5% CO_2 and air O_2. 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 24h in polypropylene tubes in RPMI medium supplemented with 2 mM L-glutamine, 1% P/S and 10% heat-inactivated FBS at 37 °C, 5% CO_2 and air O_2.

Whole-blood sampled incubated with ucMSC were stained for CD14-Pacific Blue (BD Biosciences), CD16-FITC (Bio-Rad, the Netherlands) and CD45-APC (BD Biosciences) for 30 minutes at 4 ºC. Samples were then fixed and red blood cells lysed for 10 minutes at 4 ºC with BD FACS Lysing solution (BD Biosciences). Samples were washed and measured on a FACSCanto II flow cytometer with FACSDiva software (BD Biosciences).

Isolated monocytes incubated with ucMSC were stained for CD206-Pacific Blue (BD Biosciences) and CD163-FITC (Bio-rad antibodies) respectively, for 30 minutes at 4 ºC. Samples were washed and measured on a FACSCanto II flow cytometer with FACSDiva software (BD Biosciences).

Results

Inactivation of ucMSC reduces phagocytosis by monocytes

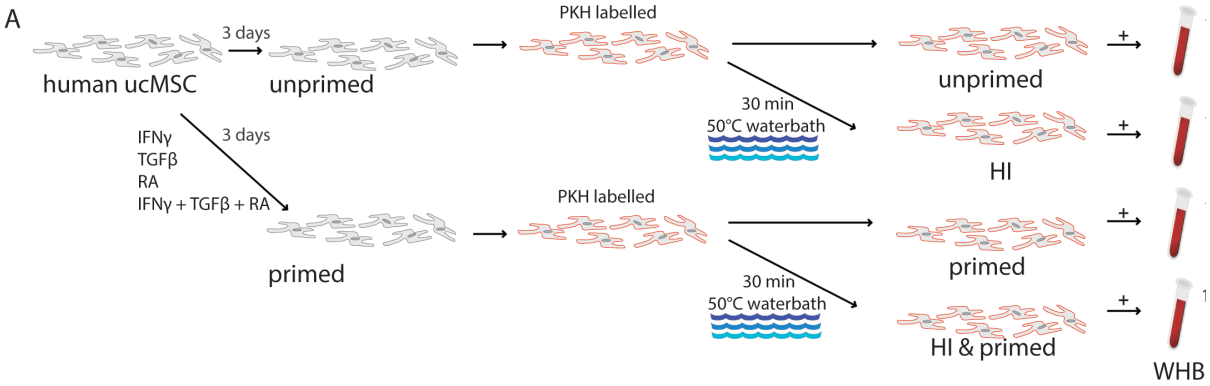
The phagocytosis of primed and/or inactivated ucMSC by monocytes was studied by adding PKH26-labelled ucMSC to whole blood for 24 hours (figure 1A). Figure 1B shows that PKH26 labelling is not affected by the procedure of inactivation of ucMSC. Nearly all monocytes became positive for PKH26 (mean±SEM: 90±3%, median=94%) after co-culture for 24 hours with unprimed ucMSC, indicative of phagocytosis of ucMSC by monocytes (figure 1C). No differences were observed in the percentage of phagocytosing monocytes when ucMSC were primed with IFNγ, TGFβ and RA. A small but significant increase was observed in PKH26 positive monocytes (mean±SEM: 96±1%, median= 97%) when ucMSC were primed with MC. Significantly less monocytes were positive for PKH26 when ucMSC were inactivated (mean±SEM: 64±6%, median= 64%) compared to control ucMSC. Compared to inactivated ucMSC, more monocytes phagocytosed inactivated ucMSC that were primed with MC (mean±SEM: 88±2%, median= 89%).

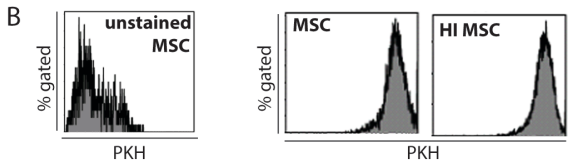
Phagocytosis of inactivated ucMSC differentially affects CD14 and CD16 expression on monocytes

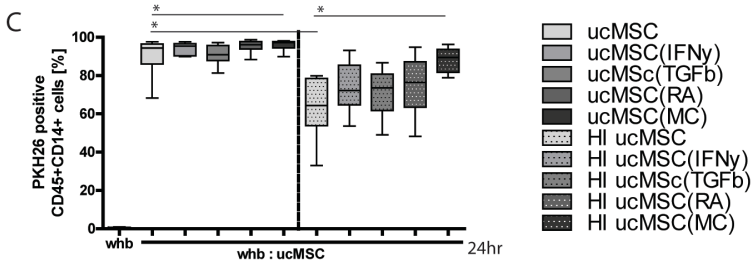
The phenotype of monocytes that phagocytosed ucMSC was examined by analyzing CD14 and CD16 expression of PKH26 positive monocytes, indicative of monocyte function. The percentage of monocytes expressing CD14++CD16-, so-called classical monocytes, was significantly lower when monocytes phagocytosed ucMSC that were primed with IFNy or inactivated (figure 1D). No differences were observed in the percentage of intermediate monocytes expressing CD14++CD16+ upon phagocytosis of primed or inactivated ucMSC (figure 1E). The percentage of non-classical monocytes expressing CD14+CD16++ was significantly higher when monocytes phagocytosed ucMSC that were primed with IFNy or MC or inactivated (figure 1F).

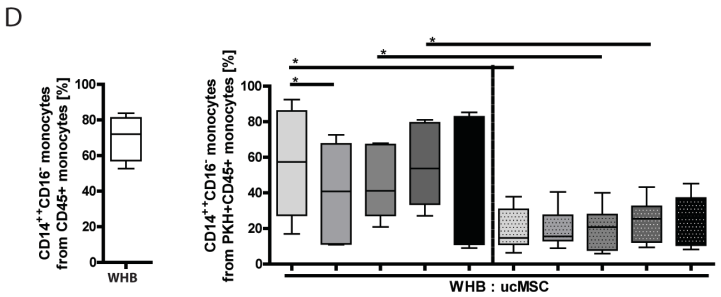
Different skewing of monocytes after phagocytosis of ucMSC and inactivated ucMSC

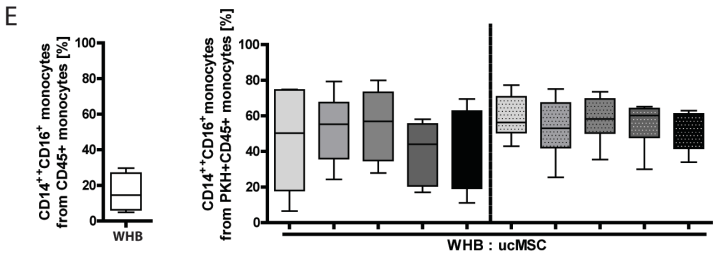
Priming of MSC had only minor effects on phagocytosis by monocytes and monocyte phenotype, whereas inactivation significantly affected monocytes. To further elucidate the effect of inactivation of MSC on monocyte polarization, ucMSC were added to CD14+ sorted monocytes for 24 hours after which their expression of CD163 and CD206 was analyzed (figure 2A). CD163 and CD206 are both alternative activation markers for monocytes and the differential expression of these markers (CD163-CD206+, CD163+CD206- and CD163+CD206+) denotes different anti-inflammatory monocyte subsets [5, 6]. The expression of CD163 and CD206 differed between monocytes that were cultured with ucMSC and inactivated ucMSC. Significantly more monocytes expressed CD163+CD206- (mean±SEM: 26±8%, median= 25%).) or CD163+CD206+ (mean±SEM: 12±4%, median= 7%) after phagocytosis of inactivated MSC, compared to monocytes that phagocytosed control MSC (respectively mean±SEM: 3±1% and 9±2% with medians: 4% and 8%)(figure 2B).

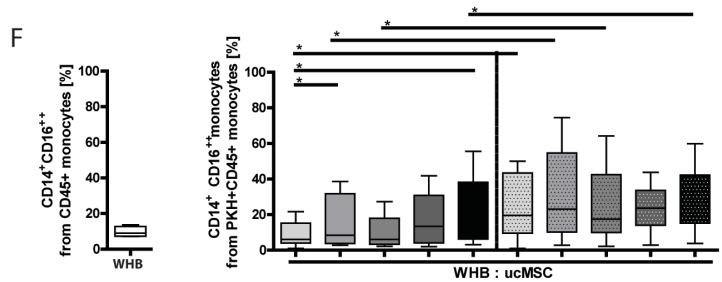

A
human ucMSC
3 days
unprimed
PKH labelled
unprimed
30 min
50°C waterbath
HI
IFNγ
TGFβ
RA
IFNγ + TGFβ + RA
3 days
primed
PKH labelled
primed
30 min
50°C waterbath
HI & primed
WHB
24hr


B
unstained MSC
% gated
PKH
MSC
HI MSC
% gated
PKH

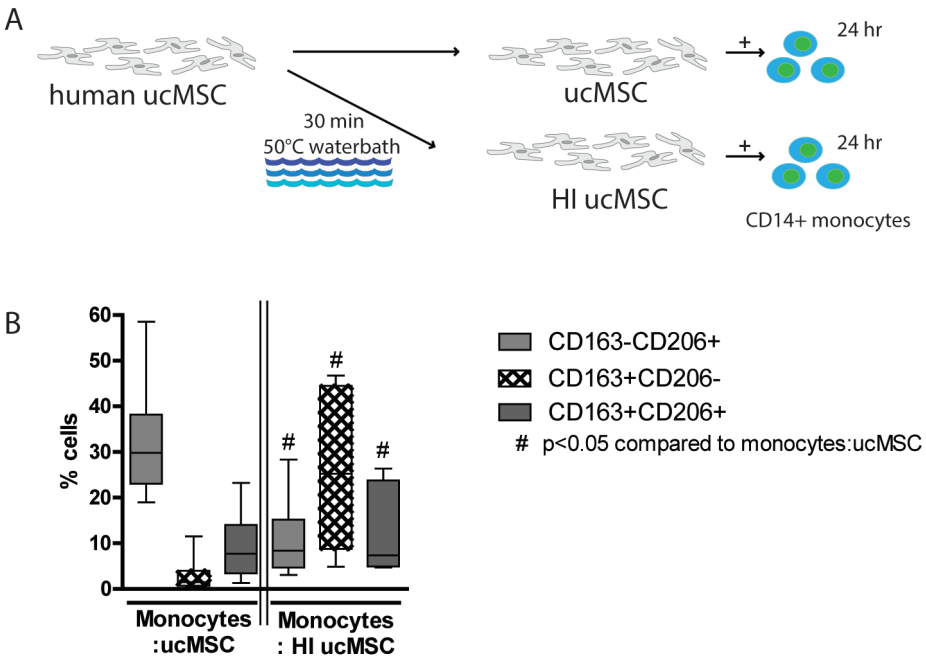

C
PKH26 positive CD45+CD14+ cells [%]
whb
whb : ucMSC
24hr
ucMSC
ucMSC(IFNγ)
ucMSc(TGFb)
ucMSC(RA)
ucMSC(MC)
HI ucMSC
HI ucMSC(IFNγ)
HI ucMSc(TGFb)
HI ucMSC(RA)
HI ucMSC(MC)


D
CD14++CD16- monocytes from CD45+ monocytes [%]
WHB
CD14++CD16- monocytes from PKH+CD45+ monocytes [%]
WHB : ucMSC


E
CD14++CD16+ monocytes from CD45+ monocytes [%]
WHB
CD14++CD16+ monocytes from PKH+CD45+ monocytes [%]
WHB : ucMSC


F
CD14+CD16++ from CD45+ monocytes [%]
WHB
CD14+ CD16++monocytes from PKH+CD45+ monocytes [%]
WHB : ucMSC

◄Figure 1 Phagocytosis of primed, inactivated or primed and subsequently inactivated (HI) MSC by monocytes. A) Experimental set-up. Human ucMSC were primed, labelled with PKH, inactivated (HI) and added for 24 hours to whole blood. After this, PKH signal and CD14 and CD16 expression was measured in the monocyte population. B) FACS plot of PKH26 staining of MSC and inactivated MSC. Inactivation of MSC does not affect PKH26 staining. C) Percentage of PKH positive monocytes (CD45+CD14+) 24 hours after addition of PKH26 labeled ucMSC. D-F) Changes in monocyte subset composition based on CD14 and CD16 expression 24h after addition of ucMSC that were either primed, inactivated or primed and subsequently inactivated. Percentage of monocytes expressing D) CD14++CD16-, E) CD14++CD16+ and F) CD14+CD16++. * for p< 0.05. Results are shown in boxplots with the center line indicating the median and the box limits at 25th and 75th percentiles (n=3).



▲Figure 2 Human monocytes adapt a different phenotype upon phagocytosis of inactivated (HI) ucMSC compared to phagocytosis of ucMSC *in vitro*. A) Experimental set-up. Human ucMSC were inactivated and added to CD14 positive sorted monocytes for 24 hours. After this CD163 and CD206 expression on the monocytes was analyzed. B) Percentage of CD14+ monocytes expressing CD163-CD206+, CD163+CD206- and CD163+CD206+ when monocytes phagocytosed ucMSC and when monocytes phagocytosed inactivated ucMSC. # indicates significant difference compared to monocytes that phagocytosed ucMSC (p<0.05). Results Results are shown in boxplots with the center line indicating the median and the box limits at 25th and 75th percentiles (n=3).

Discussion

MSC are able to elicit different responses by monocytes, depending on their treatment. We observed that inactivation of ucMSC had greater impact on the phagocytic behaviour of monocytes and their subsequent phenotype shift, than priming of ucMSC. These results lift a tip of the veil of how monocytes are prompted to phagocytose ucMSC and subsequently skew their phenotype. Identification of the key molecules that play a role in the differences observed in phagocytosis and polarization will give more insight in the mechanism behind the immunomodulatory action of MSC and their interaction with the monocytes.

Reduced phagocytic behavior of monocytes towards inactivated ucMSC suggests that monocytes are partially triggered to phagocytose ucMSC by active crosstalk between MSC and monocytes or by the secretome of MSC. In addition, monocytes that phagocytosed inactivated ucMSC were skewed into a different phenotype. Less monocytes were CD163-CD206+ while more monocytes were CD14+CD16++, CD163+CD206- and CD163+CD206+ positive and CD206+CD163+ Monocytes are known to be important cells for the generation of CD4+CD25hiFoxP3+ T-cells [7-9]. Previously, we observed that monocytes that had phagocytosed ucMSC indeed also increased their expression of CD206 and CD163 and these monocytes increased Foxp3+CD25hiCD127-CD4+ regulatory T-cells in mixed lymphocyte reactions (Chapter 8). Monocytes that have phagocytosed inactivated ucMSC showed a further increase in CD163 and CD206 expressing cells, suggesting that these monocytes may be even better inducers of Foxp3+CD25hiCD127-CD4+ regulatory T cells.

Compared to impact of inactivation of ucMSC on the monocyte phenotype, priming only had a minor impact on the phenotype shift of monocytes. Priming ucMSC with IFNγ and MC led to more monocytes expressing CD14+CD16++ upon phagocytosis ucMSC. In the future, additional analysis of the CD163 and CD206 expression and functionality assays of monocytes that phagocytosed primed ucMSC would to help tell more about their phenotype..

In conclusion, these results reveal the possibility to steer the response of monocytes by using modified MSC. Inactivated MSC were still susceptible to phagocytic activity of monocytes and able to skew the phenotype shift of monocytes. Identification of

which molecules are involved in this effect can help further development of effective immunotherapy with MSC.

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Summary and General Discussion

Summary

Conventional therapy for immunological disorders can cause severe side-effects and in many cases show insufficient effectiveness. There is therefore an interest in finding alternative immunoregulatory therapy. Mesenchymal stem or stromal cells (MSC) have been considered a promising alternative due to their easy accessibility, culture expansion possibilities, safety profile, and immunomodulatory properties (**chapter one**). Evidence of their efficacy as immunotherapy is slowly emerging, however their efficacy will benefit from improvement of their immunomodulatory capacity. This could be achieved by selection of MSC with potent immunomodulatory properties. The strength of these properties may vary intra- and inter- MSC population (within a MSC population and between MSC batches from different sources). However, this selection procedure is complicated and the isolation yield may be very low. Alternatively, immunomodulatory properties of MSC may be manually enhanced. The aim of this thesis is to optimize MSC for therapeutic purposes, by enhancement of their immunomodulatory properties and by modification of their immunogenicity, and furthermore by elucidating their mechanism of action post IV infusion. Here a summary is given of all the individual chapters (**chapters two** to **nine**) within this thesis:

In the review of **chapter two** various protocols, which have previously been developed for the generation of optimized immunomodulatory MSC, are described. These protocols consist of *in vitro* activation of the immunomodulatory properties of MSC via the addition of pro-inflammatory cytokines or Toll-like receptor (TLR) activators to modification of MSC culture medium or modification of culture conditions, such as using bioreactors or altering oxygen concentrations. In addition, desired immunomodulatory properties of therapeutic MSC are described. The requirements of customized MSC therapy may vary depending on the targeted disorder, for instance different requirements can be set on the bio distribution of administered MSC and the regulatory function of MSC on various host immune cells.

Safety and predictable functionality of therapeutic MSC should be guaranteed when using them in the clinic. Prior to their clinical application, MSC are culture expanded. This increases the yield of therapeutic MSC, yet MSC may change during culture expansion. Currently, low passage MSC are preferred as this keeps the risks for

culture-induced changes lower, such as changes that may compromise their therapeutic potential or safety. **Chapter three** shows that MSC are less efficient suppressors of T-cell proliferation when they are culture expanded for an extended period, meanwhile they are still genetically stable and their immunophenotype and susceptibility to NK cell cytotoxicity remain unaltered. These results reveal that whereas long-term culture expansion leads to a higher yield of therapeutic MSC, this may consequentially interfere with the efficacy of MSC therapy as we observed changes in their functionality.

Prior to their clinical application MSC can be cryopreserved after culture expansion and stored until use or they can be used directly from continuous culture. **Chapter four** aimed to firstly identify the effect of cryopreservation on MSC. MSC from continuous culture and cryopreserved MSC only minimally differ from one another in their gene expression and their induction of Instant Blood Mediated Inflammatory Reaction (IBMIR). No differences were observed in the cytokine and chemokine levels produced by the lung tissues following infusion of MSC from continuous culture or cryopreserved MSC. Furthermore, **chapter four** aimed to identify the effect of the lung microvasculature milieu on MSC, as MSC are trapped in the lungs post IV infusion. Major changes were observed in the transcriptional phenotype of MSC post infusion. Many genes were affiliated to pathways that affect the immunological function of MSC. The findings of this chapter are important for understanding the mechanisms of action of MSC therapy as it shows that MSC change their phenotype and potentially their functionality upon IV infusion.

In **chapter five** MSC were demonstrated to be responsive to various priming protocols, which aimed to improve their immunotherapeutic properties. Priming MSC with IFNγ, TGFβ and the multi factor combination (MC: IFNγ, TGFβ and RA) led to the most potent changes: IFNγ increased the immunosuppressive capacity of MSC, while priming with TGFβ decreased the susceptibility of MSC to NK cell cytotoxicity. Priming MSC with MC decreased their immunogenicity and increased their immunomodulatory capacity. Furthermore, priming with MC resulted in enhanced effectiveness to ameliorate liver inflammation in a liver tissue slice model, compared to unprimed MSC. These results indicate that *in vitro* priming of MSC with MC may make MSC more suitable as an effective immunotherapy, in this case to treat liver inflammation.

Subsequently, a more in-depth characterization of IFNγ, TGFβ and MC primed MSC and culture expanded MSC is provided in **chapter six**, by analysing epigenetic changes that arise upon priming and 14 days of culture expansion. DNA methylation was observed to undergo minor changes upon priming, while after culture expansion major changes were detected. The results of this chapter show that priming leaves no epigenetic imprint on MSC while during *in vitro* culture expansion MSC undergo extensive changes in DNA methylation profiles. The results stress the need for further elucidation of the impact of DNA methylation changes on the safety and efficacy of MSC therapy, and standardization and quality control of MSC therapy, whereby assessment of DNA methylation profiles could serve as a suitable quality control.

Chapter seven aimed to elucidate the fate and mechanism of action of MSC post IV infusion. We observed that MSC are phagocytosed by neutrophils and monocytes. In response to phagocytosis of MSC, monocytes were activated and changed their phenotype to a more regulatory phenotype. Via the bloodstream these monocytes migrate from the lungs towards other sites in the body. Furthermore, these monocytes were able to induce generation of $Foxp3^+$ regulatory T cells *in vitro*. These results demonstrate that MSC transfer their immunomodulatory effect to monocytic cells, which in turn continue to play a regulatory role after the disappearance of MSC.

Chapter eight continued to investigate interactions between MSC and immune cells and whether active crosstalk between immune cells and MSC is relevant for their immunomodulatory effect. IV infused MSC were shown not to actively migrate to inflamed sites and inactivated MSC were observed to elicit similar immunomodulatory effects *in vivo* as control MSC. Furthermore, the immunomodulatory effect by MSC was shown to be caused by passive interactions with host immune cells, such as monocytes, which subsequently can mediate the immunomodulatory effects. These results reveal that the exterior of MSC and their recognition by host cells are crucial for the clinical outcome.

Finally **chapter nine** examined the response of monocytes to MSC, which had undergone different treatments (priming with IFNγ, TGFβ, RA and MC or inactivation). As we discovered the key role of monocytes in the immunomodulatory

mechanism of action of MSC, we wanted to obtain a more representable view on the immunomodulatory behaviour of primed and inactivated MSC *in vivo*. Priming of MSC has a minor effect on the phagocytic behaviour of monocytes and their subsequent phenotype shift. In comparison, inactivation of MSC has a greater impact on the phagocytic behaviour of monocytes and their subsequent phenotype shift towards a more regulatory phenotype. The difference in response of monocytes towards control MSC and inactivated MSC shows that next to passive interactions there are also active interactions. These results showed that different treatments of MSC may affect monocyte behaviour and reveal that identification of the key molecules that play a role herein would be beneficial for the development of MSC into effective immunotherapy.

General Discussion

Mesenchymal stem or stromal cells (MSC) are considered as a promising cellular immunotherapy for diseases that currently have no sufficiently effective treatment. MSC are easily accessible, can be expanded in culture, are considered safe and possess proven immunomodulatory properties but whether MSC can be used as effective immunomodulatory medication for patients with autoimmune or inflammatory disorders (1-12) or after transplantation (13-15) is subject of a plethora of studies worldwide. The safety of MSC therapy has already been confirmed in several clinical trials (16-18), however, evidence of true efficacy of MSC immunotherapy is only slowly emerging (19). There is on-going research to find out how to use MSC to their full therapeutic potential. It is of great importance to understand the mechanism of action of the immunomodulatory property of MSC not only in a culture dish, but also in particular after administration to patients. The work described in this thesis was aimed at optimizing the therapeutic efficacy of MSC by enhancement of their immunomodulatory properties and elucidation of their mechanism of action.

In contrast to conventional immunomodulatory drugs, which are defined by a molecular formula, MSC are a changeable immunosuppressant. They can be obtained from various tissues (e.g. bone marrow, fat, or umbilical cord) and isolated from donors of different age, gender, health status or genetic background, which will result in MSC cultures that differ in certain aspects. MSC can be used directly after harvesting from short-term or long-term continuous culture or after cryopreservation, which further diverse therapeutic MSC. Furthermore, MSC cultures are heterogeneous and particular subsets possess more potent immunomodulatory properties than others and variations in the relative contribution of subsets in MSC populations may affect therapeutic efficacy. Although there are reports that different MSC demonstrate functional differences (20-28), the significance of differences between MSC preparations on therapeutic efficacy after administration to diseased animals or human is far from clear. As shown in **chapter two** to **six**, MSC respond to treatment with cytokines, growth factors, cryopreservation and culture confluency levels by changing gene expression, immunophenotype, secretome and interaction with T lymphocytes *in vitro*. However, these phenotypic and functional changes resulted in marginal enhancements in the ability to modulate liver inflammation and

did not influence bio-distribution and survival in a preclinical model of liver inflammation. Hence, the use of optimised MSC was not beneficial for the treatment of liver inflammation. One possible explanation is that different optimising methodologies are required to generate therapeutic MSC with enhanced efficacy. Alternatively, the adapted phenotype and function of optimised MSC is irrelevant in the mechanism of action of MSC immunotherapy. To explore which phenotypic characteristics of MSC are of importance for their therapeutic efficacy, it is crucial to improve our understanding of the mechanisms of action of MSC.

It is generally assumed that T cells are the key players in the immunomodulatory mechanism of action of MSC and control their survival *in vivo*. However, our work provides evidence that not T cells, but monocytes are at the centre of the immunomodulatory effect of MSC after intravenous infusion and that the nature of the interaction of MSC with monocytes is very different from the interaction of MSC with T cells (**chapter seven, chapter eight** and **chapter nine**). The identification of monocytes as key players in the effect of MSC gives an explanation for the limited success of generating optimized MSC. Optimisation protocols were aimed at enhancing their immunomodulatory capacity by elevating the expression of molecules such as indolamine 2,3-dioxygenase (IDO), programmed death ligand-1 (PD-L1) and secretion of anti-inflammatory molecules to the improve their capacity to inhibit T cell proliferation and to lower their immunogenicity by modulating their HLA expression to decrease their recognition by T cells and their susceptibility to NK cytotoxicity to prolong their survival *in vivo*. However, for the interaction between monocytes and MSC these factors are of less importance. Instead, recognition and phagocytosis of MSC by monocytes and subsequent phenotype shifts determine the effect of MSC. Optimisation of MSC should therefore be aimed at generating MSC that induce more potent types of immunoregulatory monocytes through improved recognition and phagocytosis of MSC by monocytes.

The generation of optimised MSC will only be possible when we know which phenotypic traits and molecules on MSC play roles in their immunomodulatory effect so that optimising can be specifically directed at modifying those traits and molecules. We showed that administration of inactive MSC (these MSC are metabolically inactive and lost the capacity to secrete factors, but preserved cell membrane protein expression) leads to an immune response in a sepsis model with

a similar efficacy as to when control MSC are administered (**chapter 8**). Henceforth, if even the viability of MSC is of low importance for their immunomodulatory effect, MSC from various tissue sources, donors of different age and underlying disease, different subsets (that admittedly show phenotypic differences) may initiate similar immunomodulatory responses, as their phenotypic differences are irrelevant for their interaction with monocytes. When key molecules that determine the effect of MSC on monocytes are identified, MSC subtypes with potentially superior capacity to enhance the immunomodulatory function of monocytes can be selected. Noteworthy, monocytes are not the only cells that interact with infused MSC. There are indications that MSC interact with neutrophils (29, 30). We observed that neutrophils phagocytose infused MSC. The short lifetime of neutrophils make these cells hard to study and the relative contribution of neutrophils to the clearance of MSC and the share of these cells in the immunomodulatory effect of MSC is currently unknown.

The lungs are a known barrier for large particles in the bloodstream. Culture expanded MSC accumulate in the lungs due to size restrictions and potential adherence to the pulmonary endothelial cells (31, 32). The use of smaller or less adherent MSC may bypass this phenomena. Optimizing protocols may thus be directed at generating smaller and/or less adherent MSC. The use of selected components of MSC, such as small-sized membrane particles or extracellular vesicles that can pass the lungs may be an alternative (33-35). The question is, however, whether bypassing the lung barrier would enhance the efficacy of MSC therapy. The studies in **chapter 7** show that monocytes upon phagocytosis of MSC adapt an immune regulatory phenotype and subsequently travel via the bloodstream to other sites, thereby distributing the immunomodulatory effect of MSC from the lungs. This observation shows that to achieve systemic immunomodulation, bio-distribution of IV infused MSC is not as an important determinant as previously thought.

Studies have been directed at improving the survival time of MSC post administration. It is generally assumed that a longer survival time will go hand in hand with improved therapeutic efficacy. However, the significance of survival time of MSC for their therapeutic efficacy is questionable. Galleu et al. demonstrated that the apoptotic cell death of MSC is crucial for their immunosuppressive effect in graft versus host disease after infusion (36). Furthermore, this thesis demonstrated that

the viability of MSC is not correlated with their therapeutic efficacy in a sepsis model (**chapter 8**). Thus the importance of pro-longed detainment of MSC after infusion for their immunomodulatory effect is questionable.

Although there is currently no hard evidence that MSC, of different tissue origins or donors of different age, gender, health status or race, have different immunogenicitiy or therapeutic immunomodulatory efficacy, for legislation purposes the use of a well-defined MSC population for clinical application is preferred. The use of defined subset (e.g. a subset that expresses a certain marker or exhibits a similar epigenetic profile) is preferred for safety reasons as well as to ensure consistency between studies. Epigenetic screening can be used as a tool to exclude divergent MSC cultures as an addition to the current set of tests (karyotyping, viability and phenotype). The use of this and similar tools will lead to more standardized MSC products and help protect the safety and therapeutic efficacy of MSC immunotherapy.

Future perspectives

The work in this thesis has brought us a small step closer to effective MSC immunotherapy by elucidating a part of the immunomodulatory mechanism of action of IV infused MSC. From here, further elucidation of their mechanism of action as well as identification of key molecules in the immunomodulatory mechanism of action of MSC via monocytes is advised. In addition, due to our discovery regarding the mechanism of action of MSC therapy it is anticipated that parameters such as the recognition of MSC by monocytes, their subsequent phenotype shift and functionality will be used more to monitor the immunotherapeutic effects of MSC treatment.
In the foreseeable future, the application of MSC therapy may take a turn. Currently, a common type of MSC has been investigated for a whole spectrum of immunological disorders. However, each immunological disorder demands different properties of MSC and this will eventually lead to the generation of customized MSC immunotherapy. This may for instance include immunotherapy with MSC with specific regulatory properties, immunotherapy with MSC products (such as membrane particles or extracellular vesicles) or immunotherapy through administration of MSC via different routes.

Conclusions

- The work in this thesis contributed to the elucidation of the immunomodulatory mechanism of action of IV infused MSC
- IV infused MSC indirectly modulate the immune system via their phagocytosis by monocytes
- Phagocytosis of MSC induces a regulatory phenotype in monocytes and promotes the generation of regulatory T cells
- MSC-induced regulatory monocytes distribute via the bloodstream throughout the body, which leads to spreading of the immunomodulatory effect
- This thesis provided new insights on the impact of priming, inactivation and culture expansion of MSC
- Age, cryopreservation and viability are of limited importance for the immunomodulatory effect of IV infused MSC
- The level of confluency of MSC cultures affects their epigenetic profile
- Priming MSC to increase their survival *in vivo,* after IV infusion, by reducing their susceptibility for T and NK cytotoxicity, is of limited importance for their immunomodulatory effect
- Priming MSC to improve their capacity to inhibit T cell proliferation is of limited importance for their immunomodulatory effect after IV infusion

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Dutch summary (Samenvatting)

De behandeling van patiënten met een immunologische aandoening of patiënten die een orgaantransplantatie hebben ondergaan bestaat uit langdurig gebruik van medicijnen die het immuunsysteem onderdrukken. Deze medicijnen hebben naast hun therapeutische werking ook veel bijwerkingen of de therapeutische werking is niet volledig effectief. Hierdoor is er behoefte aan een nieuwe behandelingsmogelijkheid met minder of, idealiter, geen bijwerkingen. Het gebruik van mesenchymale stam of stromale cellen (MSC) biedt hierbij uitkomst. Het gebruik van deze cellen als celtherapie is veelbelovend door de verscheidene eigenschappen die deze cellen bezitten om het immuunsysteem te reguleren. Oftewel MSC hebben immuunregulerende eigenschappen. Het doel van dit proefschrift was om de immuuntherapeutische eigenschappen van MSC te optimaliseren, door hun immuunregulerende eigenschappen te versterken en hun immunogeniciteit te veranderen, en om daarnaast ook hun werkingsmechanisme uit te zoeken.

MSC bevinden zich overal in het lichaam. Het zijn voorlopercellen en ze kunnen zich differentiëren in onder andere bot-, kraakbeen- en vetcellen. Daarnaast kunnen MSC ook communiceren met de cellen van het immuunsysteem. Verscheidene studies hebben al getracht de immuunregulerende eigenschappen van MSC te activeren om zo de functie van MSC te verbeteren, met als einddoel tot betere therapeutische uitkomsten te komen. In **hoofdstuk twee** van dit proefschrift is een overzicht gepresenteerd van de huidige kennis met betrekking tot de immuunregulerende eigenschappen van MSC en hoe deze geactiveerd kunnen worden.

Voordat MSC gekweekt en/of bewerkt kunnen worden in het laboratorium moeten ze eerst uit het weefsel geïsoleerd worden. Ondanks het feit dat isolatie uit weefsel makkelijk is, is hun opbrengst laag. MSC worden daarom na isolatie een tijd gekweekt in het laboratorium om zo aan genoeg cellen te komen voor de behandeling. Hoewel dit een noodzakelijk fenomeen is, brengt dit risico's met zich mee. MSC kunnen namelijk tijdens het kweken veranderingen ondergaan. Deze kunnen nadelige gevolgen hebben voor de veiligheid en effectiviteit van de therapie. In **hoofdstuk drie** is het effect van langdurige kweek op het fenotype en de functionaliteit van MSC onderzocht. Er is aangetoond dat MSC minder goed zijn in het remmen van de deling van T-cellen na langdurige kweek, terwijl hun uiterlijke

kenmerken, genetische stabiliteit en hun gevoeligheid om geëlimineerd te worden door cytotoxische immuuncellen wel stabiel blijft. Dit betekent dat wanneer remming van de T-cel proliferatie van MSC wordt verwacht de voorkeur zal gaan naar het gebruik van MSC uit korte kweken.

MSC kunnen direct na het kweken gebruikt worden of ze kunnen worden ingevroren (ook wel cryopreservatie genoemd). Cryopreservatie en de daarop volgende ontdooiing kunnen de eigenschappen van MSC ook beïnvloeden. **Hoofdstuk vier** laat zien dat MSC direct uit kweek (vers) en MSC welke ontdooid zijn na cryopreservatie weinig verschillen van elkaar en eenzelfde reactie initiëren in de longen na toediening. Dit laat zien dat zowel verse MSC als gecryopreserveerde MSC gebruikt kunnen worden voor therapeutische doeleinden.

Wanneer MSC via intraveneuze route worden toegediend lopen ze, vanwege hun grootte, vast in de microvasculatuur van de longen. Hier initiëren ze een ontstekingsreactie welke een bekend onderdeel is van hun werkingsmechanisme. Maar heeft de microvasculatuur van de longen invloed op de MSC? Dit is ook onderzocht in **hoofdstuk vier**. Hier werd vervolgens inderdaad aangetoond dat MSC door de long microvasculatuur beïnvloed worden.

In **hoofdstuk vijf** werden MSC in het laboratorium behandeld met verschillende cytokines, groeifactoren en kweekomstandigheden met als doel om de therapeutische eigenschappen van MSC te activeren en te optimaliseren. Aangetoond werd dat het mogelijk is om MSC te modificeren door stimulatie: IFNγ, TGFβ en een multifactor combinatie (MC: IFNγ, TGFβ en retinoic acid (RA)) stimulatie resulteerde in veranderingen met betrekking tot hun immuunregulerende eigenschappen en gevoeligheid om geëlimineerd te worden door cytotoxische immuuncellen. Bovendien waren MSC gestimuleerd met MC ook iets beter in het verhelpen van lever inflammatie in een model waar dunne leverplakjes werden gebruikt, in vergelijking met ongestimuleerde MSC. Bij gestimuleerde MSC was er geen verbetering te zien in hun overleving en verdeling over het lichaam na infusie. De kennis dat MSC gemodificeerd kunnen worden kan vervolgens benut worden om op maat gemaakte immuuntherapie met MSC te genereren in de toekomst.

Voor de verdere ontwikkeling van MSC in een effectief cellulair immuuntherapie is het belangrijk om een volledig beeld te krijgen omtrent de veranderingen die MSC ondergaan na stimulatie met IFNγ, TGFβ en MC of gedurende het kweken. Epigenetische veranderingen spelen een grote rol in het reguleren van de functie van een cel doordat deze de genexpressie beïnvloeden. Deze veranderingen kunnen zowel erfelijk zijn als tot stand komen door invloeden van buitenaf. Door te kijken naar epigenetische veranderingen, zoals veranderingen in DNA methylatie, welke optreden in MSC na stimulatie of kweek kan er een meer gedetailleerd beeld gecreëerd worden van het therapeutische product. In **hoofdstuk zes** werd aangetoond dat MSC vrijwel geen DNA methylatie veranderingen ondergaan na stimulatie met IFNγ, TGFβ en MC. Daarentegen zorgde het kweken van MSC wel voor grote veranderingen in hun DNA methylatie profiel. De impact van deze epigenetische veranderingen is nog grotendeels onbekend, maar ze kunnen gevolgen hebben voor de veiligheid en functionaliteit. Het beoordelen en valideren van het epigenoom van therapeutische MSC ten opzichte van een vastgestelde standaard naast de huidige kwaliteitscontroles wordt vanwege deze resultaten daarom aangeraden.

Het werkingsmechanisme van intraveneus toegediende MSC is in **hoofdstuk zeven** onderzocht. Monocyten werden hier aangewezen als de belangrijkste schakel in het werkingsmechanisme van MSC. Aangetoond werd dat intraveneus toegediende MSC opgemerkt en gefagocyteerd worden door monocyten, nadat ze vast komen te zitten in de longen. MSC zijn dus niet zo laag immunogeen al we voorheen dachten, omdat ze herkent worden door cellen van het “innate” afweersysteem. Deze monocyten nemen vervolgens een meer regulatoir fenotype aan, waarna ze zich via de bloedbaan door het hele lichaam verplaatsen. Ook is er aangetoond dat deze monocyten de productie van Foxp3^{+} regulatoire T cellen *in vitro* (in een kweekbakje) aanzet. Dit is gunstig, omdat deze cellen een rol spelen in tolerantie en directe interacties aangaan met cellen van het immuun systeem. Deze resultaten samen laten dus eigenlijk zien dat MSC alleen een snelle trigger zijn voor monocyten waarna hun immuunregulerende functie door monocyten wordt voortgezet.

Hoofdstuk acht laat vervolgens zien dat het immuunregulerende werkingsmechanisme van MSC niet afhangt van actieve interacties met immuuncellen, maar juist aan de herkenning van MSC door immuuncellen, zoals

monocyten. Het is dus de buitenkant van MSC en vervolgens hun herkenning door monocyten van de ontvanger dat van groot belang is voor het therapeutische effect van MSC.

Vervolgens werd in **hoofdstuk negen** naar de herkenning en respons van monocyten op MSC, welke geïnactiveerd (MSC met intacte buitenkant, maar niet in staat om actieve interacties aan te gaan) of gestimuleerd waren met IFNγ, TGFβ, RA of MC, gekeken. Het fagocyterende gedrag van monocyten en hun hierop volgende fenotype switch werd beïnvloed door het stimuleren van MSC met IFNγ, TGFβ, RA of MC. Ook verschilde de respons van monocyten op geïnactiveerde MSC erg met hun respons op controle MSC. Zo namen de monocyten nadat zij in aanraking waren gekomen met geïnactiveerde MSC een meer regulerend fenotype aan. Deze bevinding laat zien dat naast passieve interacties er waarschijnlijk ook nog invloed is van het secretoom van MSC of dat er ook nog actieve interacties tussen monocyten en MSC plaatsvinden.

Beschouwing

MSC kunnen in verschillende soorten en maten voorkomen. Ze kunnen namelijk uit verschillende weefsels (bijvoorbeeld beenmerg, vet of navelstreng) geïsoleerd worden of komen van donors met verschillende karakteristieken (denk hierbij aan leeftijd, geslacht, gezondheidsstatus of genetische achtergrond) afkomen. Ook kunnen ze gebruikt worden na kortstondige of langdurige kweek of na cryopreservatie. Al het bovengenoemde zorgt ervoor dat MSC in sommige aspecten verschillen. Er zijn studies die aantonen dat verschillende MSC in functionaliteit kunnen variëren, maar het is nog onduidelijk of de diversiteit in MSC tot een daadwerkelijk verschil in therapeutische uitkomst zal leiden na hun toediening. In **hoofdstuk twee** tot en met **zes** wordt aangetoond dat MSC reageren op een behandeling met verschillende factoren, cryopreservatie en de celdichtheid (ook wel confluentie genoemd) van de kweek. Dit doen ze door hun uiterlijke kenmerken te veranderen, maar ook door veranderingen aan te brengen op DNA niveau. Deze veranderingen resulteren echter in ons geval in marginale verbeteringen in het vermogen van MSC om lever inflammatie te behandelen. Een mogelijke verklaring hiervoor is dat er andere methodes nodig zijn om MSC te produceren met een verbeterde therapeutische werkzaamheid. Een andere verklaring is dat het fenotype en de functionaliteit van deze MSC niet relevant is in het werkingsmechanisme van MSC. Het is dus van belang om eerst ons inzicht in het werkingsmechanisme van MSC te verbeteren en te onderzoeken welke kenmerken van MSC van belang zijn voor hun therapeutische werkzaamheid zodat we daarna die kenmerken kunnen versterken.

Er wordt algemeen aangenomen dat T cellen de hoofdrolspelers zijn in het immuuunmodulerende werkingsmechanisme van MSC en hun overleving. Wij tonen echter aan in **hoofdstuk zeven**, **acht** en **negen,** dat monocyten de hoofdrolspelers zijn. Deze bevinding laat zien waarom wij eerder niet succesvol waren in het genereren van geoptimaliseerde MSC. Wij keken namelijk naar parameters welke geassocieerd zijn met het immuunmodulerende effect van MSC ten opzichte van T cellen en hun immunogeniciteit in relatie tot de respons van T cellen (en NK cellen). Onze huidige bevinding laat juist zien dat deze parameters van beperkt belang zijn voor het werkingsmechanisme van MSC. In het vervolg moet er dus bij het optimaliseren van MSC gekeken worden naar het genereren van MSC die

verbeterde immuunregulerende monocyten induceren door de herkenning en fagocytose van MSC door monocyten.

Voor de ontwikkeling van geoptimaliseerde MSC is het belangrijk om te weten welke kenmerken en moleculen van belang zijn voor het werkingsmechanisme van MSC. Hierdoor kan de optimalisatie van MSC specifiek gericht worden op het modificeren van deze kenmerken en moleculen. In **hoofdstuk acht** laten we zien dat de levensvatbaarheid van MSC maar van beperkt belang is voor hun werkingsmechanisme, doordat geïnactiveerde MSC eenzelfde immuunrespons initiëren als controle MSC. Als zelfs de levensvatbaarheid van MSC van beperkt belang is voor het effect, kunnen we speculeren dat MSC van verschillende weefseltypes, donors of verschillende subtypes ook eenzelfde immuunmodulerende reactie zullen genereren, omdat hun fenotypische verschillen waarschijnlijk minder van belang zijn voor hun interacties met monocyten. De identificatie van belangrijke moleculen in het werkingsmechanisme van MSC zal leiden tot de selectie van MSC met een superieur versterkingsvermogen voor de immuunmodulerende functie van monocyten.

MSC lopen na intraveneuze toediening vast in de longen. Dit komt ten eerste doordat de diameter van MSC groter is dan de diameter van de bloedvaten en ten tweede doordat ze mogelijk vast kleven aan cellen in de longen. Gebruik van kleinere en minder klevende MSC of componenten van MSC, zoals kleine membraan deeltjes, zal hiervoor uitkomst bieden. Het is alleen onzeker of de werkzaamheid van MSC inderdaad verbeterd zal worden wanneer MSC verder komen dan de longen. In **hoofdstuk zeven** laten we namelijk zien dat monocyten die MSC gefagocyteerd hebben en een immuunregulerend fenotype hebben aangenomen zich via de bloedbaan verspreiden over het gehele lichaam en zo het immuunmoduerende effect verspreiden. Dit laat zien dat de verdeling van MSC in het lichaam na intraveneuze toediening van beperkt belang is voor hun immuunmodulerende werkzaamheid.

Een andere algemene aanname is dat een langere overleving van MSC na toediening voor een verbeterde werkzaamheid van de therapie zal zorgen. Wij en andere onderzoekers hebben aangetoond dat de dood van MSC echter van belang is voor hun immuunmodulerende effect (**hoofdstuk zeven**). Daarnaast hebben we

laten zien in **hoofdstuk acht** dat levensvatbaarheid van MSC van beperkte toegevoegde waarde is op hun werkingsmechanisme. Het is daarom betwijfelijk of een langere overleving na toediening van MSC zal zorgen voor een betere werkzaamheid van de therapie.

Ten slotte, desondanks dat er nog geen hard bewijs is of verschillen in MSC, door verschillende donors, weefsels waaruit ze geïsoleerd zijn, van belang zijn voor hun werkingsmechanisme is het voor de wetgeving wel van belang om een zo goed mogelijk gedefinieerd en consistent mogelijk MSC product te gebruiken. Men kan hierbij denken aan het gebruik van een specifieke subset die een bepaalde marker heeft of een bepaald epigenetisch profiel heeft.

Concluderend,

- Het onderzoek beschreven in dit proefschrift beschrijft de rol van monocyten in het immuunregulerende werkingsmechanisme van intraveneus toegediende MSC
- Fagocytose van MSC induceert een regulatoir fenotype in monocyten, welke de productie van regulatoire T cellen bevorderd
- Door MSC-geïnduceerde regulatoire monocyten verdelen zich via de bloedbaan over het gehele lichaam. Dit zorgt voor verdeling van het immuunmodulerende effect
- Het onderzoek toont nieuwe inzichten omtrent de impact van voorbehandeling, inactivatie en kweken op MSC
- Leeftijd, cryopreservatie en levensvatbaarheid van MSC zijn van beperkt belang voor het immuunmodulerende effect van IV toegediende MSC
- De mate van confluentie van een MSC kweek heeft effect op hun epigenetische profiel
- Voorbehandeling van MSC, met als doel hun overleving *in vivo* te verlengen door middel van het verlagen van hun immunogeniciteit ten opzichte van T en NK cellen, is van beperkt belang voor het immuunmodulerende effect na intraveneuze infusie
- Voorbehandeling van MSC, met als doel om hun immuunmodulerende capaciteit ten opzichte van T cel proliferatie te verbeteren, is van beperkt belang voor het immuunmodulerende effect na intraveneuze infusie

Appendices

List of publications

PhD portfolio

Curriculum Vitae

Acknowledgements (Dankwoord)

List of publications

Epigenetic changes in umbilical cord mesenchymal stromal cells upon stimulation and culture expansion

S.F.H. de Witte, F.S. Peters, A.M. Merino, S.S. Korevaar, S.J. Elliman, P.N. Newsome, J.B.J. van Meurs, K. Boer, C.C. Baan and M.J. Hoogduijn. *Cytotherapy Jun 20 2018. pii: S1465-3249(18)30516-4.*

Immunomodulation by therapeutic mesenchymal stromal cells (MSC) is triggered through phagocytosis of MSC by monocytic cells

S.F.H. de Witte, F. Luk, J.M. Sierra Parraga, M. Gargesha, A.M. Merino, S. S. Korevaar, AS. Shankar, Lisa O'Flynn, S.J. Elliman, D. Roy, M.G.H. Betjes, P.N. Newsome, C.C. Baan and M.J. Hoogduijn*. Stem Cells 2018 Apr;36(4):602-615*

Inflammatory conditions dictate the effect of MSC on B cell function

F. Luk, L. Carreras-Planella, S.S Korevaar, **S.F.H. de Witte**, F.E. Borras, M.G.H. Betjes, C.C. Baan, M.J. Hoogduijn and M. Franquesa*. Frontiers in Immunology, August 2017: 8:1042*

Ageing of bone marrow and umbilical cord derived MSC during expansion

S.F.H. de Witte, E.E. Lambert, A.M. Merino, T. Strini, H.J.C.W. Douben, L. O'Flynn, S.J. Elliman, A.J.E.M.M. de Klein, P.N. Newsome, C.C. Baan and M.J. Hoogduijn. *Cytotherapy. 2017 July;19(7):798-807*

Cytokine treatment optimises the immunotherapeutic effects of umbilical cord derived MSC for treatment of inflammatory liver disease

S.F.H. de Witte, A.M. Merino, M. Franquesa, T. Strini, J.A.A. van Zoggel, S.S. Korevaar, F. Luk, M. Gargesha, L. O'Flynn, D.h Roy, S.J. Elliman, P.N. Newsome, C.C. Baan and M.J. Hoogduijn. *Stem Cell Research and Therapy. June 2017; 8: 140.*

Inactivated Mesenchymal Stem Cells Maintain Immunomodulatory Capacity

F. Luk, **S.F.H. de Witte**, S.S. Korevaar, M. Roemeling-van Rhijn, M. Franquesa, T. Strini, S. van den Engel, M. Gargesha , D. Roy, F.J. Dor, E.M. Horwitz, R.W. de Bruin, M.G.H. Betjes, C.C. Baan and M.J. Hoogduijn. *Stem Cells and Development 2016 September 15;25(18):1342-54.*

Biomaterials Influence Macrophage–Mesenchymal Stem Cell Interaction In Vitro

N. Grotenhuis, **S.F.H. de Witte**, G.J.V.M. van Osch, Y. Bayon, J.F. Lange and Y.M. Bastiaansen-Jenniskens. *Tissue Engineering Part A. September 2016, 22(17-18): 1098-1107.*

Effects of freeze–thawing and intravenous infusion on mesenchymal stromal cell gene expression

M.J. Hoogduijn, **S.F.H. de Witte**, F. Luk, M.C. van den Hout-van Vroonhove, L. Ignatowicz, R. Catar, T. Strini, S.S. Korevaar, W.F. van IJcken, M.G.H. Betjes, M.Franquesa, G.Moll and C.C. Baan. *Stem Cells and Development. 2016 April 15;25(8):586-97*

Towards development of iMesenchymal Stem Cells for Immunomodulatory Therapy

S.F.H. de Witte, M. Franquesa C.C. Baan and M.J. Hoogduijn. *Frontiers in Immunology, 2015; 6:648.*

Efficacy of immunotherapy with mesenchymal stem cells in man: a systematic review

F. Luk, **S.F.H de Witte**, W.M. Bramer, C.C. Baan and M.J. Hoogduijn. *Expert Review of Clinical Immunology. Early online, 1–20 (2015)*

PhD portfolio

Name PhD student: Samantha Francina Huixian de Witte

Erasmus MC department: Internal Medicine, Section Nephrology and Transplantation

PhD Period: February 2014 – February 2018

Research School: Postgraduate School Molecular Medicine

Promotor: Prof. dr. Carla C. Baan

Co-Promotor: Dr. Martin J. Hoogduijn

PhD training

Courses and workshops		**Workload (ECTS)**
2014	Laboratory animal competance (article 9), MolMed	**4**
2015	Advanced Immunology, MolMed	**4**
2015	NIHES Statistics Course	**3**
2016	Scientific Integrity	**0.3**
2017	Adobe Indesign Course, MolMed	**0.3**

Attended meetings		
2014-2018	Lab meetings internal medicine department, transplantation lab, Erasmus MC, Rotterdam.	**2**
2014-2018	Journal Club internal medicine department, Erasmus MC, Rotterdam.	**1**
2014-2018	MERLIN consortium meetings	**0.5**

Attended seminars / conferences		
2014	Annual Meeting NTV (Boot congres), Leiden	**1**
2014	MolMed day, Rotterdam	**1**
2016	Emerging techniques in the analysis of extracellular vesicles, Erasmus MC, Rotterdam *	**0.3**

2016	Young Professionals Netwerkdag van Nederlandse transplantatie vereniging, Utrecht.	**0.3**
2017	Mini Symposium: Monocytes: origins, destinations, functions and diagnostic targets, Erasmus MC, Rotterdam *	**0.2**
2017	Bio-techne Symposium, Amsterdam	**0.5**

(Inter)national conferences-poster presentations

2014	Dutch Society For Immunology (NVVI), Kaatsheuvel	**1**
2015	Science days, Dept. of Internal medicine, Antwerp	**1**
2015	MolMed day, Rotterdam	**1**
2015	Annual Europe Regional Meeting ISCT, Sevilla	**1**
2016	Science days, Dept. of Internal medicine, Antwerp	**0.5**
2016	MolMed day, Rotterdam	**0.5**
2017	Science days, Dept. of Internal medicine, Antwerp	**0.5**
2017	American Transplant Congress (ATC), Chicago *presented by colleague*	**0.1**
2017	MolMed day, Rotterdam *two poster presentations*	**1**
2018	Science days, Dept. of Internal medicine, Antwerp	**1**

(Inter)national conferences-podium presentations

2015	Joint BTS/NTV Congress, Bournemouth *two podium presentations*	**1**
2016	Annual Meeting NTV (Boot congres), Groningen	**1**
2016	21st Nantes Actualites Transplantation (NAT), Nantes	**1**
2016	International Congress of The Transplantation Society (TTS)	**1**
2017	Annual Meeting NTV (Boot congres), Zeist *two podium presentations*	**1**
2017	European Society for Organ Transplantation (ESOT), Barcelona *two podium presentations*	**1**
2017	European Hematology Association (EHA) – Scientific Working Groups (SWG) scientific meeting, Amsterdam	**1**

Teaching activities: Supervising	Workload (ECTS)
Infection and Immunology master student:	**8**
Eleonora E. Lambert (2016, 40 weeks)	
JMS students:	**1**
Pim den Boon & Shabnam Babakry (20 July - 14 August 2015)	
JMS students:	**1**
Pascal Gunsch & Willem Jan de Voogd (4 August - 29 August 2014)	

Miscellenous		
2015	Organization Labday Internal medicine, Erasmus MC	**0.5**

Total workload 44.4 ECTS

Memberships

2014-2018 Dutch Transplantation Society (Nederlandse Transplantatie Vereniging, NTV)

2017-present European Society for Organ Transplantation

Travel grants

2016 Trustfonds financiële ondersteuning

2017 Nederlandse transplantatie vereniging Scholingsbeurs

Awards

2018 International transplantation science mentee/mentor award

Abstract: Monocytic cells phagocytose therapeutic mesenchymal stem cells, which induces polarization, relocation and immune regulation.

Curriculum Vitae

Samantha Francina Huixian de Witte was born on 28 September 1989 in Rotterdam, the Netherlands. In 2007 she completed her secondary school studies at Emmauscollege in Rotterdam. Hereafter, she started studying Biomedical Engineering at the Technical University in Eindhoven. During her studies, she became interested in the field of stem cells and went abroad for a six month internship at the Tissue Modulation Lab at the National University of Singapore under the supervision of prof. dr. Carlijn Bouten and prof. dr. Michael Raghunath. She subsequently conducted an one-year internship during her Master degree at the department of Orthopaedics at the Erasmus MC under the supervision of prof. dr. Carlijn Bouten, prof. dr. Gerjo van Osch and dr. Yvonne Bastiaansen-Jenniskens. In October 2013 she obtained her master degree in Biomedical Engineering. She started her PhD project in February 2014 at the Transplantation Laboratory of the Internal Medicine Department, Division of Nephrology and Transplantation, at the Erasmus MC, under supervision of prof. dr. Carla Baan and dr. Martin Hoogduijn. This research is presented in this thesis. In February 2018 Samantha started her training as a Clinical Embryologist at the Amsterdam UMC, location VUmc in Amsterdam.

Acknowledgements (Dankwoord)