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HEMATOLOGY & REGENERATIVE MEDICINE (HERM)
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**ENGINEERING MORE EFFICIENT MULTIPOTENT
MESENCHYMAL STROMAL (STEM) CELLS FOR
SYSTEMIC DELIVERY AS CELLULAR THERAPY**

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Institutet**

Stockholm 2013

This PhD thesis was supported by grants from the Children's Cancer Foundation, the Cancer Society in Stockholm, the Juvenile Diabetes Research Foundation International, the Stockholm County Council, the Swedish Cancer Society, the Swedish Foundation for Strategic Research, the Swedish Medical Research Council, the Swedish Society of Medicine, the Tobias Foundation, VINNOVA and Karolinska Institutet. The thesis was partially supported by grants from the National Institutes of Health (NIH) in the USA.

Cover illustration by Cristina Pires depicting endothelial cells, lining the vascular lumen, and perivascular mesenchymal stromal cells, wrapping the outside of small blood vessels. *The cover illustrations is adapted from a picture in the following review by Bianco et al. ¹.*

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Published by Karolinska University Press, Box 200, SE-171 77 Stockholm, Sweden
Printed by Larserics Digital Print AB, Box 20082, SE-16102 Stockholm, Sweden

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ISBN 978-91-7549-259-9

**“We have learned to recognise stem cells,
Not necessarily by what they do in their dependent tissues within an organism,
But rather by what we can do with them in the laboratory”**

Pamela Gehron Robey²



To my family

ABSTRACT

Do mesenchymal progenitor cells naturally circulate *in vivo*? Are they fundamentally compatible with blood? What mechanism allows them to be in contact with blood? How do we make therapeutic cells with blood-compatible properties? Can we optimise their survival and therapeutic function upon systemic delivery? How should we best isolate and condition therapeutic multipotent mesenchymal stromal (stem) cells (MSCs) before infusion, to achieve an optimum and sustainable clinical response in patients? This thesis covers many aspects related to these questions. It describes how MSCs interact with the instant blood-mediated inflammatory reaction (IBMIR) upon infusion. The IBMIR was first documented and characterised after infusion of islet cells. More than a decade ago, clinicians observed a cascade of innate immune responses occurring after islet cell infusion into the portal vein of diabetic patients in an attempt to reverse insulin dependence. This response was characterised by the instant activation of the complement and coagulation systems, which was accompanied by platelet adhesion to the graft, effector cell infiltration, and rapid graft destruction. The reaction resulted in a massive cell loss; 80-90% of the infused cells were destroyed within hours of infusion. We wondered if similar events occur after systemic intravenous infusion of MSCs? Expression profiling showed that MSCs express typical hemostatic regulators, similar to those produced by endothelial cells, but display higher amounts of pro-thrombotic tissue / stromal factors on their surface, which trigger the IBMIR after blood exposure. This process was dependent on the cell dose, the choice of MSC donor, and particularly the cell passage number. Freshly harvested, short-term expanded MSCs triggered only weak blood responses *in vitro*, while cryostorage and freeze-thawing, extended culture, and co-culture with activated lymphocytes increased their pro-thrombotic properties. Particularly thawed cells, as used in many clinical applications, displayed impaired immunomodulatory and blood regulatory properties. Thawed cells showed reduced responsiveness to pro-inflammatory and impaired production of anti-inflammatory mediators, an increased triggering of the IBMIR, and a particularly strong activation of the complement cascade, which resulted in twice as efficient lysis after serum exposure. Triggering of IBMIR was augmented when the cells were washed and resuspended in human AB plasma before blood exposure, as done during clinical cell graft preparation. After infusion to patients, we found increased formation of blood activation markers, but no formation of hyperfibrinolysis marker D-dimer or acute phase reactants with the currently applied dose of $1-3 \times 10^6$ cells per kilogram, demonstrating product safety. Triggering of IBMIR could be reduced by culturing MSCs with human platelet lysate, or antagonised by cell surface heparin-modification and use of soluble anticoagulants. We conclude, that currently applied doses of low-passage clinical grade MSCs are safe and elicit only minor systemic effects, but higher cell doses, and particularly higher passage cells, should be handled with care. This deleterious reaction can compromise the survival, engraftment, and function of these therapeutic cells.

Key words: MSC, multipotent mesenchymal stromal / stem cell, immunomodulation, tissue repair, tissue engineering, cell therapy, cryopreservation, systemic cell delivery, innate immune response, instant blood-mediated inflammatory reaction (IBMIR), complement, coagulation, ABO blood group, xenoantigen

LIST OF PUBLICATIONS

This thesis is based on the following articles and manuscripts, which are referred to in the text by their Roman numerals (I-IV).

- I. **Mesenchymal stromal cells engage complement and complement receptor bearing innate effector cells to modulate immune responses.**
Guido Moll, Regina Jitschin, Lena von Bahr, Ida Rasmusson-Duprez, Berit Sundberg, Helena Lönnies, Graciela Elgue, Kristina Nilsson-Ekdahl, Dimitrios Mougiakakos, John D. Lambris, Olle Ringdén, Katarina Le Blanc, Bo Nilsson.
PLoS ONE. 2011 Jan; 6(7): e21703

- II. **Are therapeutic human mesenchymal stromal cells compatible with human blood?**
Guido Moll, Ida Rasmusson-Duprez, Lena von Bahr, Anne-Marie Connolly-Andersen, Graciela Elgue, Lillemor Funke, Osama A. Hamad, Helena Lönnies, Petra U. Magnusson, Javier Sanchez, Yuji Teramura, Kristina Nilsson-Ekdahl, Olle Ringdén, Olle Korsgren, Bo Nilsson, and Katarina Le Blanc.
Stem Cells. 2012 April; 30(7): 1565-74

- III. **Do cryopreserved mesenchymal stromal cells display impaired immunomodulatory and therapeutic properties?**
Guido Moll, Lena von Bahr, Jessica J. Alm, Lindsay C. Davies, Lillemor Stenbeck-Funke, Osama A. Hamad, Robin Hinsch, Lech Ignatowicz, Matthew Locke, Helena Lönnies, John D. Lambris, Yuji Teramura, Kristina Nilsson-Ekdahl, Bo Nilsson, and Katarina Le Blanc
Submitted

- IV. **Do ABO blood group antigens hamper the therapeutic efficacy of mesenchymal stromal cells?**
Guido Moll, Annika Hult, Lena von Bahr, Jessica J. Alm, Nina Heldring, Osama A. Hamad, Lillemor Stenbeck-Funke, Stella Larsson, Yuji Teramura, Helene Roelofs, Bo Nilsson, Willem E. Fibbe, Martin L. Olsson, and Katarina Le Blanc
Submitted

TABLE OF CONTENTS

1	INTRODUCTION	1
1.1	The concept of stem and progenitor cells	1
1.2	HSCT and supportive MSC immunotherapy	2
1.2.1	HSCT and recent experience with supportive MSC therapy	2
1.2.2	Regenerative and immunomodulatory properties of MSCs	3
1.2.3	Therapeutic efficacy of MSCs against immune ailments	4
1.2.4	Improving the clinical efficacy of therapeutic MSCs	5
1.2.5	Basic choices in preparation of therapeutic MSCs	6
1.3	Cell transplant incompatibility reactions	7
1.3.1	Are MSCs naturally in contact with and circulate within blood?	7
1.3.2	Fate of systemically infused MSCs in the microvasculature	9
1.3.3	The instant blood mediated inflammatory reaction	11
1.3.4	Allo- and xenoreactivity in MSC transplantation	13
1.3.5	How to prevent or antagonise cell graft failure?	14
2	AIMS OF THE THESIS	15
3	SUBJECTS, MATERIALS, AND METHODS	16
3.1	Ethical considerations	16
3.2	MSC recipients and therapeutic cells	16
3.3	Immunological and molecular studies	17
3.3.1	Blood preparations and complement inhibition (Study I to IV)	17
3.3.2	Cell culture and preparation of cells for experiments (Study I to IV)	17
3.3.3	Interaction of therapeutic cells with serum and blood (Study I to IV)	18
3.3.4	Mixed lymphocyte reactions and immunomodulation (Study I to III)	20
3.3.5	Expression analysis on properties of therapeutic MSCs (Study II & IV)	21
3.4	Data analysis and statistics	22

4	RESULTS AND DISCUSSION	23
4.1	Interaction of human MSC with human blood	23
4.1.1	Expression of pro- and anticoagulant factors by MSCs	23
4.1.2	MSCs triggering of IBMIR in the whole blood system	23
4.1.3	IBMIR response to freshly harvested or thawed MSCs	24
4.1.4	ABO antigen expression and IBMIR triggering by MSCs	24
4.2	Interaction of MSC with the complement system	25
4.2.1	Complement regulatory and activating properties after serum exposure	25
4.2.2	Complement activating properties after lepirudin-blood exposure	25
4.2.3	Complement activation and immunomodulation by MSCs	26
4.3	Evaluation of systemic MSC delivery to patients	27
4.3.1	Triggering of IBMIR by MSCs and its relevance for clinical use	27
4.3.2	Improving the therapeutic efficacy of systemically delivered MSCs	28
4.4	Strategies to prevent or antagonise IBMIR	29
4.4.1	Means to prevent IBMIR and improve lung passage	29
4.4.2	Anti-thrombotic strategies to antagonise IBMIR	30
5	CONCLUDING REMARKS	31
5.1	Summary of major findings	31
5.2	Epilogue: the perfect therapeutic mesenchymal	32
6	ACKNOWLEDGEMENTS	33
7	REFERENCES	35
8	PUBLICATIONS	43

LIST OF ABBREVIATIONS

ABS	Human blood type AB serum
ABP	Human blood type AB plasma
ADSC	Adipose derived stem cell
ALPC	Adult liver progenitor cell
ANOVA	Analysis of variance
AT	Antithrombin
BMMNC	Bone marrow mononuclear cell
BSA	Bovine serum albumin
C1q	Complement component 1 subcomponent q
C3	Complement component 3
C3a	Complement component 3 activation fragment a
C3b	Complement component 3 activation fragment b
C5a	Complement component 5 activation fragment a
C5b-9	Complement C5b to 9 complex
CD73	Cluster of differentiation number 73
CFU-F	Colony-forming units fibroblast
COL1	Collagen type 1
CPM	Counts per minute
DMEM	Dulbecco's modified Eagle's minimal essential medium
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EPC	Endothelial progenitor cell
FACS	Fluorescence-activated cell sorting
FVII – FXII	Factor VII to XII of the coagulation cascade
FCS	Fetal calf serum
FIB	Fibroblast
FMC	Fetal membrane cell
GCSF	Granulocyte colony-stimulating factor
GvHD	Graft-versus-Host Disease

HLA	Human leukocyte antigen
HMWK	High molecular weight kininogen
HRP	Horseradish peroxidase
HSA	Human serum albumin
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HPL	Human platelet lysate
IBMIR	Instant blood mediated inflammatory reaction
IL1a	Interleukin 1 alpha
INFg	Interferon gamma
ISCT	International Society for Cellular Therapy
iPSC	Induced pluripotent stem cell
LMWDS	Low molecular weight dextran sulfate
MAC	Membrane attack complex = C5b-9
MAPC	Multipotent adult progenitor cell
MBL	Mannose-binding lectin
MLR	Mixed lymphocyte reaction
MSC	Multipotent mesenchymal stromal (stem) cell
MSC1/2	MSC1 (pro-inflammatory) / MSC2 (anti-inflammatory)
NHS	Normal human serum (complement active, blood type AB)
NSC	Neural stem cell
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PMN	Polymorphonuclear leukocytes
PRP	Human platelet rich plasma
sC5b-9	Soluble C5b-9 complex
T1D	Type 1 diabetes
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
Th17	T helper cell type 17
TLR	Toll-like receptor
TNFa	Tumor necrosis factor alpha
tPA /uPA	Tissue- / urokinase-type plasminogen activator

1 INTRODUCTION

1.1 THE CONCEPT OF STEM AND PROGENITOR CELLS

In modern biology, the highest degree of “potency” – totipotent – is ascribed to the fertilised egg, which forms all other tissues in a complex cascade of differentiation. From the inner cell mass of the early embryo, it is possible to isolate the pluripotent embryonic stem cells (ESCs) ², which will differentiate into multipotent progenitors. The cells differentiate according to specific germ layers with specialised functionality. Yamanaka found that this process is fundamentally reversible (Figure 1) ^{3, 4}, and that committed cells also exhibit plasticity to differentiate into tissues of other germ layers, e.g. differentiated cells, such as fibroblasts, can be developmentally reprogrammed into induced pluripotent stem cells (iPSCs). But how are stem cells naturally maintained? Throughout adulthood, stem cells persist at particular sites within the human body, the so-called stem cell niches, which will allow stem cells to remain in a quiescent physical state of long-term self-renewal. One such site is the bone marrow. Haematopoietic and mesenchymal stem cells (HSCs and MSCs) are thought to reside in that reservoir ⁵. While HSCs have become a well-established entity since Weissman’s initial report ⁶, the true stem cell nature and exact phenotype of MSCs is more difficult to pin down ⁷, but efforts are ongoing to better characterise the self-renewing skeletal stem cells ^{1, 8}. Initial reports observed that bone marrow stroma contains cells capable of initiating clonal growth (colony-forming unit fibroblast, CFU-F) ⁹, which exhibit multilineage differentiation potential *in vitro* ¹⁰⁻¹². The isolated CFU-Fs contain a stem cell fraction capable of forming all the tissues within the skeletal segment upon transplantation: bone tissue, cartilage, adipocytes, fibroblasts and haematopoiesis-supporting stroma ¹. MSCs are often isolated via their ability to form CFU-Fs upon plastic adherence, and not all these adhering cells will exhibit true stem cell properties. Unsorted CFU-Fs will therefore constitute a cell mixture of stromal cells, often commonly referred to as: multipotent mesenchymal stromal cells (also abbreviated MSCs), a definition ¹³, which was established by the International Society for Cellular Therapy (ISCT) with the goal to establish some minimal criteria for therapeutic cell characterisation. The three characteristics for MSCs established in this paper are: fibroblastic morphology upon isolation in culture, expression of a set of typical stromal markers – demonstrating the absence of contaminating haematopoietic, myeloid, and endothelial cells, and to demonstrate multilineage differentiation potential into at least three lineages *in vitro*. The following paragraphs will refer to MSCs isolated according to these criteria.

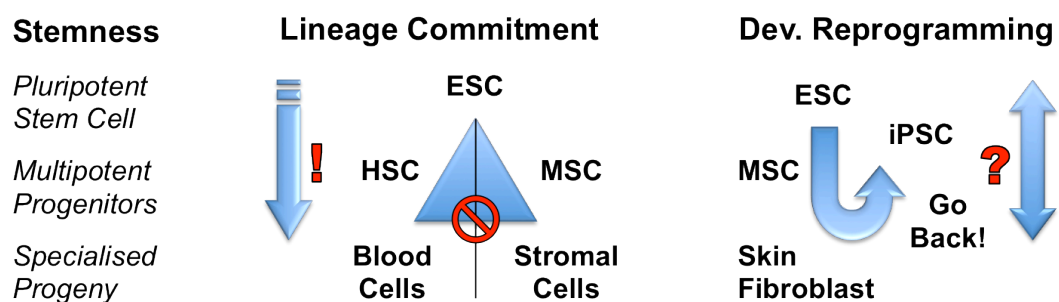


Figure 1: Differentiation potential, commitment, and developmental reprogramming of cells.

1.2 HSCT AND SUPPORTIVE MSC IMMUNOTHERAPY

1.2.1 HSCT and recent experience with supportive MSC therapy

The pioneering work for haematopoietic stem cell transplantation (HSCT) was conducted from the 1950s through the 1970s at the Fred Hutchinson Cancer Research Centre in Seattle and led by E. Donnall Thomas, later honoured with the Nobel Prize in Physiology and Medicine for his studies¹⁴. His work showed that intravenously infused bone marrow cells could repopulate the marrow and establish a new blood forming system in an immune-deficient recipient. His studies furthermore contributed to the understanding of donor-recipient allo-reactivity, the major cause for development of Graft-versus-Host Disease (GvHD)¹⁵. An outline on cell transplant incompatibility reactions is given in the last section of the introduction. Owing to the donor registries, HSCT is the most widely used cell therapy today, with >50.000 procedures conducted worldwide per year (2006)¹⁶, and further increases since then¹⁷. To undergo HSCT, the stem cell recipient undergoes a procedure called “conditioning” (e.g. reduced intensity or myeloablative conditioning), where his body is prepared for accepting the new stem cell graft¹⁵. To remove malignancy, chemo- and radiotherapy are employed, destroying the majority of malignant cells together with the old hematopoietic system. The conditioning is supported by “prophylaxis” against GvHD, infections, and tissue toxicity. The most common sources for stem cell transplants are the bone marrow, peripheral blood, and umbilical cord blood, with the later two being employed more frequently during the recent years. HSCT is used to cure life-threatening malignancy, such as haematopoietic disease, cancer, and genetic dysfunction. Until today, HSCT remains a risky procedure with many complications, which hamper a broader use¹⁵.

Our laboratory has contributed in pioneering studies on using therapeutic MSCs for HSCT-related complications, such as acute GvHD¹⁸, and severe tissue damage¹⁹, as well as to co-transplant MSCs with HSCs to promote engraftment of HSCT grafts²⁰, as reviewed by Tolar et al.²¹. The field is progressing quickly, and scientific interest in using MSCs for treatment of immune and inflammatory disorders is substantial, amounting to around 25% of published literature within the MSC field (in 2012)²². Although clinical translation of MSCs into widespread use is within close reach, major challenges remain²². These are mainly related to issues in MSC production, such as: cell product characterisation (functional cell component), cell potency and its loss with time in culture, and isolation / culture methods that maximise therapeutic cell efficacy. On the patient side, recent attention has focused on the mechanistic basis for rapid cell elimination after systemic infusion, anti-donor immune responses to allogeneic MSCs, the primary mechanism of action in different clinical situations, the need for disease specific assays to assess MSC potency, and the establishment of suitable animal models to develop *in vivo* pharmacokinetic profiling of MSCs. Multi-centre clinical networks are essential for increasing subject numbers and scientific output from clinical trials²², to best validate not yet solidly established regenerative / immunomodulatory therapies, as opposed to therapies relying on cell engraftment²³. A pressing question remains: What is the lowest effective MSC dose to achieve an optimum clinical response with minimal expanded MSCs? Response analysis in the Stockholm study group suggests that very early passage MSCs have higher therapeutic value²⁴⁻²⁶, potentially indicating higher potency. Use of short-term expanded MSCs may also reduce production costs.

1.2.2 Regenerative and immunomodulatory properties of MSCs

The regenerative and immunomodulatory properties of MSCs on innate and adaptive immune responses have already been reviewed extensively in the past ²⁷⁻³⁰. The cells display a hypo-immunogenic and anti-inflammatory profile, allowing their potential use across HLA-barriers ^{31, 32}. Their special properties allow MSCs on the one hand to evade host immune recognition for some time, and on the other hand to actively modulate or even suppress an ongoing inflammatory response. Naturally, these qualities of MSCs occur in a context dependent fashion ^{27, 28}, and are thus affected by a large number of parameters, such as the exact specifics of the therapeutic cell product, or the predominant inflammatory milieu encountered by the therapeutic cells *in vivo*, which all have to be considered for their optimal clinical use ^{28, 32}. In general, MSCs immunomodulatory properties are highly multi-factorial ^{27, 28, 33}, they target many different types of effector cells ²⁹, they are enhanced by cell contact ³⁴⁻³⁶, and they are triggered by various environmental cues, a process known as “licensing” (Figure 2) ²⁸. The cells have the capacity to secrete a broad array of soluble or surface-bound regenerative and anti-inflammatory mediators, which are usually even more effective when deposited or enriched in their surrounding tissue environment. The potency and outcome of MSCs integrated cellular response (e.g. chemotaxis, differentiation, immune-homeostasis or -modulation, and tissue damage regeneration), and in particular their pro- or anti-inflammatory phenotype, will be tuned by cues such as the physical environment (e.g. oxygen tension, tissue context, mechanical load) and the encounter of soluble mediators (e.g. immune cell mediators, such as cytokines and growth factors; blood activation products, such as complement anaphylatoxins; and Toll-like receptors, e.g. MSC1/2 polarization via TLR3/4, respectively) ²⁸. Taken together, MSCs may act like a catalyser – taking up pro-inflammatory mediators from their environment, which activates or augments the cells intrinsic production of anti-inflammatory mediators, with the potential to moderate local or systemic inflammation ^{28, 33, 37}.

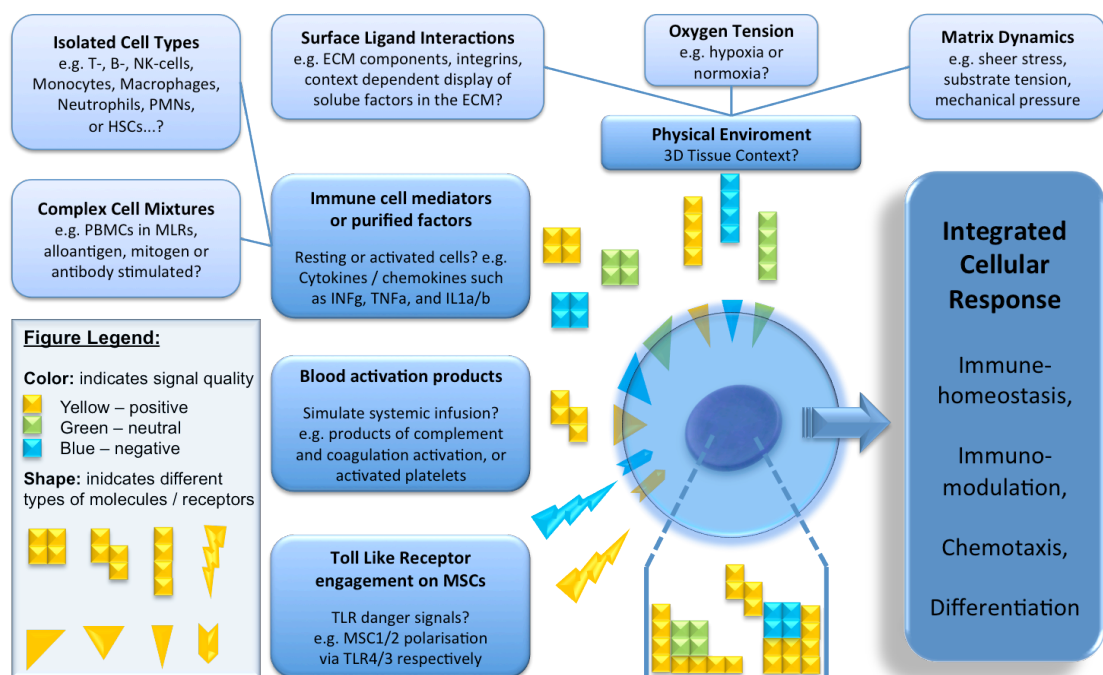


Figure 2: Licensing of MSCs by different stimuli tunes their integrated cellular response.
 (Adapted from Doorn J. and Moll G. et al. *Tissue Engineering Part B Reviews*, 2011)

1.2.3 Therapeutic efficacy of MSCs against immune ailments

The multitude of paracrine mechanisms and broad range of bioactive molecules that are employed by MSCs to exert their therapeutic function can be classified into six main actions: immunomodulation, anti-apoptosis, anti-scarring, angiogenesis, support of growth and differentiation of local stem and progenitor cells, and chemoattraction²⁷. In treatment of the complex systemic immune ailments often occurring after HSCT (such as acute GvHD and hemorrhagic cystitis), most of these actions would be highly beneficial for the patient, e.g. to restore immune-homeostasis, repair damaged tissues, or to reduce bleeding. However, the complex nature of HSCT-related pathologies, the great variation between patients and their supportive care, and even variations in therapeutic MSC preparation itself, makes every clinical evaluation very challenging. The two most basic indicators of treatment success would be a clear clinical response to the MSC treatment and an improved patient survival. More sophisticated indicators would document a desired bioactivity compared to a well-matched control group.

At its latest follow-up in our and collaborating centres¹⁸, 39 out of 55 patients with steroid-resistant, severe, acute GvHD responded to the MSC treatment (71%), with half of the patients achieving a complete and one fifth showing a partial response, with a better outcome in children than adults, and no observation of major toxicities. The 2-year survival in patients who responded to MSC treatment (52%) was higher than previously described for patients with a similar grade of GvHD (10%)¹⁸, and the 1-year survival was more prominent for early than late passage cells (75% vs. 21%)²⁵. We recently also published a report on alterations in the cellular immune compartment of patients treated with third-party MSCs following HSCT compared to placebo³⁷. This study came to the conclusion that MSCs are bioactive, as suggested by a reduction in epithelial cell death, and induce a tolerogenic shift in the patients' immune system. MSC treated patients showed increased levels of FOXP3+/IL10+ regulatory T-cells, reduced numbers of Th17-cells, and a skewing towards type-2 T-helper cell responses, without compromising the protective T-cell immunity. This is of particular importance, since T-cells represent the main mediator of graft versus host immune reactions, but are also essential for safeguarding the adaptive immunity against recurrent pathogens³⁷. Thus, we can conclude that all three basic indicators of treatment success are given, although further improvements in treatment response are desirable. Furthermore, great discrepancies exist between the experience at European centres and an industry sponsored phase III study in the United States, probably owing to potential variables in MSC production and their consequent therapeutic properties (Figure 3)²⁶.

In the past years, we conducted systematic follow up studies and retrospective patient analysis with the aim to eliminate confounding factors to treatment efficacy, in order to stepwise improve the patient response to MSC therapy. One focus was set on understanding the fate of MSCs after intravenous delivery and on optimising the therapeutic cell product for systemic delivery. Experience with islet cell infusion has shown that many therapeutic cells are damaged after systemic delivery by an innate immune attack – termed instant blood mediated inflammatory reaction (IBMIR)³⁸⁻⁴⁰, which compromises the engraftment and bioactivity of the islets cells in treatment of type 1 diabetes (T1D), therefore raising the need for infusion of multiple cell grafts⁴¹. It became clear during the last years^{39, 40}, that overcoming IBMIR related damage to the cell graft could prove to be a key factor in maximising the therapeutic efficacy of cellular therapies, as outlined in the following sections.

1.2.4 Improving the clinical efficacy of therapeutic MSCs

As outlined earlier, differences in outcome are reported for different centres, possibly owing to variables in MSC production and consequent therapeutic properties. In order to optimise our product, we first studied the interaction of the most commonly (systemically) applied therapeutic MSC product at our facility with human blood. We then identified major confounders (Figure 3), which may positively or negatively affect the biocompatibility of systemically delivered cells. Eventually, we correlated the cells IBMIR response with patient outcome, in order to find out if a higher biocompatibility leads to an improved treatment efficacy, which has been the case in developing HSCT, but is yet to be proven in therapeutic MSC transplantation. The two first issues arising in MSC therapy are the choice of cell donor and tissue origin. Functional characteristics of cells from bone marrow and other origins may differ, and need to be tested, e.g. for their triggering of IBMIR, if applied systemically. As outlined earlier, MSCs respond to environmental cues, potentially also present in donors with an underlying pathology (e.g. inflammatory conditions such as T1D, multiple sclerosis, or rheumatoid arthritis), which may modulate MSCs phenotype, and consequently affect their therapeutic value. It is also well established that the stem cell content in marrow decreases with age⁴². Non-pathological factors accumulating with donor age may affect MSCs efficacy⁴³, and cells from younger donors may therefore potentially be preferred for clinical use. The most evident factor affecting MSCs potency appears to be the time in culture. Potency reduces after expansion and particularly after repeated passaging^{24-26, 44-51}, resulting in a gradual loss of progenitor properties and tissue forming capacity^{44, 50}, reduced long-term engraftment⁴⁵, lower clinical response and survival benefit^{25, 48}, and increased triggering of IBMIR²⁴, which compromises cell engraftment and function. This may be affected by the choice of culture supplement (the experience above was made with cells grown with animal components, containing alpha-Gal-xenoantigen), and various other aspects of cell delivery (e.g. the use of fresh or thawed cells)²⁶.

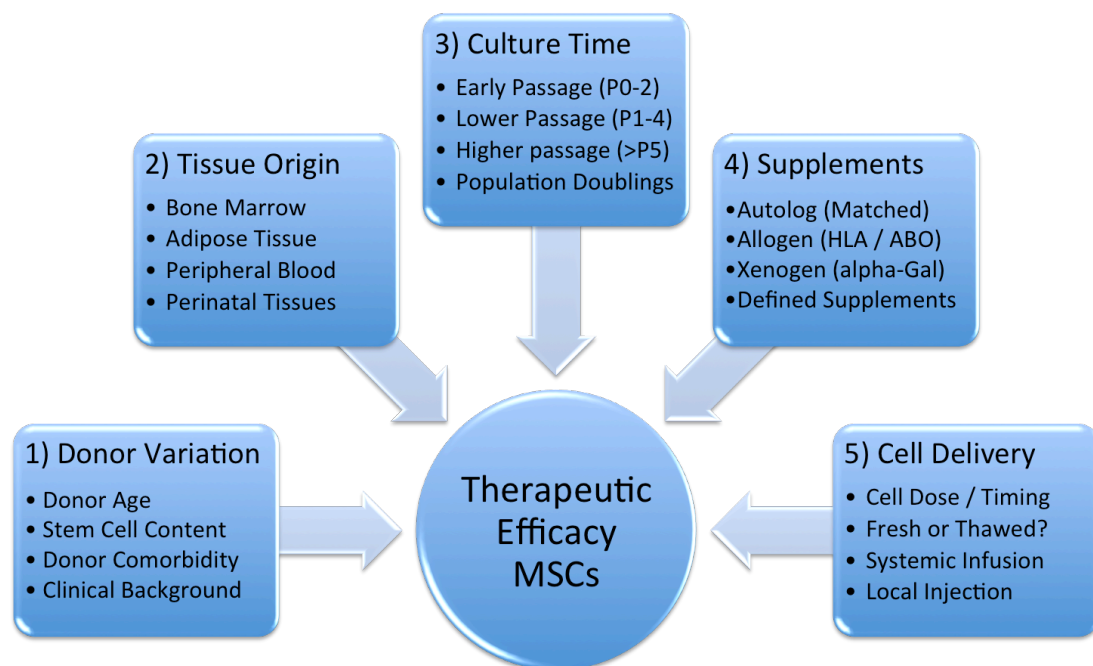


Figure 3: Potential parameters affecting the therapeutic efficacy of MSCs.

1.2.5 Basic choices in preparation of therapeutic MSCs

In summary, it can be said that isolated and culture expanded therapeutic MSCs show considerable differences compared to their native primary counterparts *in vivo*, creating unique therapeutic products with each specific isolation protocol used. All these cell products may differ to some degree in their therapeutic quality and effect, raising the absolute need for an exact clinical product definition and standardisation. Below, we have summarised some of the most essential considerations to be taken, e.g. when preparing MSCs for systemic delivery (Table 1). Most of the given parameters are related to product immunogenicity and to the maintenance of progenitor potential, based on the assumption that a transient cell engraftment is desirable / necessary, and that regenerative and immunomodulatory properties of MSCs are somehow related to their progenitor (stem cell) properties, which may not automatically be the case ²³. Indeed, it has been shown that the *in vitro* immunomodulatory properties of MSCs are a fundamental property shared by all stromal cells ⁵². However, cell engraftment and mesenchymal tissue formation capacity appear to be most effective with freshly isolated bone marrow derived cells, as opposed to cells derived from peripheral tissue ¹. When studying cell transplant incompatibility reactions, product variations in any of these parameters need to be kept in mind, since outcome may differ greatly.

Table 1: Potential parameters affecting the functionality of systemically delivered MSCs.

Parameter of interest	Potential advantage	Potential disadvantage
Progenitor cell source		
<i>Donor comorbidities</i>	Young and healthy donor	Old donor, systemic disease
<i>Donor recipient matching</i>	(Transplant antigen match)	(e.g. HLA mismatch)
<i>Autologous cell source</i>	No rejection, no pathogens	Price, time, availability?
<i>Third party cell source</i>	Readily available, higher dose, and repeated administration, Easy tracking of long-term cell function (X/Y chromosome)	Immunogenic - when given to immunocompetent patient, Long term function might be compromised (rejection risk)
Tissue of origin	(High stem cell content?)	(Low stem cell content?)
<i>Bone marrow</i>	High progenitor potential	Limited amount / invasive
<i>Adipose tissue</i>	Abundant / no expansion	Possibly lower potency
<i>Peripheral blood</i>	Easily accessible source	No / difficult mobilisation
<i>Perinatal tissues</i>	High potential / amount	Ethical concerns (donor)
Therapeutic cell preparation		
<i>Isolation procedure and ex vivo expansion time</i>	Direct isolation or short-term cell expansion (early passage)	Long-term cell expansion (higher passage cells)
<i>Maintenance of stemness, and differentiation potential</i>	Fetal supplements such as FCS that contain stemness factors	Adult donor derived additives may not promote progenitors
<i>Immunogenicity of culture medium supplements</i>	Antigen / antibody depleted or antigen neutral (autologous)	Poorly defined, can contain antigens (FCS, ABS, PRP)
<i>Immunogenicity of washing and cell infusion buffers</i>	Antigen / antibody depleted or human serum albumin (HSA)	Poorly defined immunogenic supplements (FCS or ABP)
<i>Cell harvesting, storage, and therapeutic delivery</i>	Freshly harvested or growing cells have optimal bioactivity	Cryopreserved / thawed cells have suboptimal bioactivity

1.3 CELL TRANSPLANT INCOMPATIBILITY REACTIONS

1.3.1 Are MSCs naturally in contact with and circulate within blood?

When studying interactions of culture expanded MSCs with blood, the question arises if native MSCs are at times in contact with and circulate within blood⁵³⁻⁵⁵? This actually seems to be the case in some exceptional situations, which would prove that MSCs have the intrinsic capacity to be in contact with blood, and furthermore, that tissue-resident MSCs have the fundamental capacity to extravasate through the vessel wall and circulate within blood for some period of time. Our results indicate, that isolated and culture expanded MSCs have a compromised haemocompatibility, but that it could possibly be preserved (e.g. if production parameters are adjusted for designing blood compatible therapeutic cells), or that it could be restored (e.g. by using suitable methods to promote the blood compatible phenotype before clinical use of the cells). Here, I will try to address some of these essential questions, before addressing the fate of conventional therapeutic MSCs after systemic – intravenous – delivery.

The closest relative to MSCs, which is highly adapted to be permanently in contact with blood, are endothelial cells (ECs), lining the interior of blood vessels, forming a blood-compatible interface between intra- / extra-vascular compartments⁵⁶. In contrast, MSCs are tissue cells, which reside in the perivascular compartment^{57, 58}, undergoing oxygen-dependent changes in phenotype according to *in situ* localisation⁵⁹. If the EC-interface is damaged, blood will come in contact with tissue components, which will then lead to an immediate thrombotic reaction. Interestingly, ECs and their progenitors (EPCs) permanently circulate in blood, to be recruited to sites of damage⁵⁶. Furthermore, circulating ECs and EPCs are phenotypically easily confused with MSCs, e.g. when isolated via plastic adherence or if an insufficient panel of surface markers is used for characterisation (e.g. both cells express high levels of frequently used CD105). But how exactly are ECs adapted to be in contact with blood, and how does their highly specialised profile compare to that of culture expanded therapeutic MSCs? To answer this question, we conducted EC-profiling on resting and cytokine-activated cultured MSCs and compared them to ECs, as summarised below (Figure 4)²⁴.

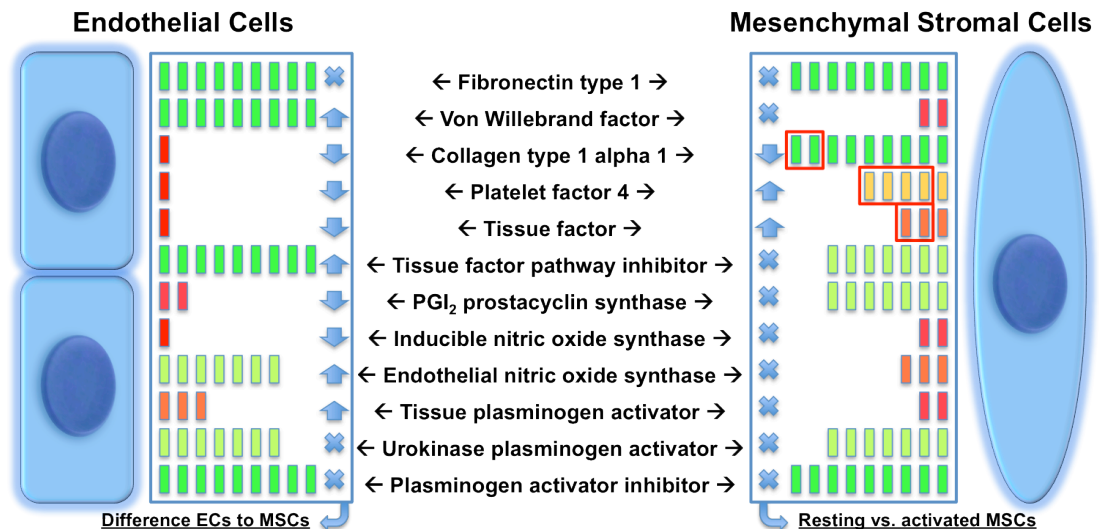


Figure 4: Expression intensity of typical factors governing blood-compatibility of ECs and MSCs. (Legend: red low-, yellow medium-, and green high-expression; red frame indicates degree of change)

ECs are the prototypic blood compatible cells, which can establish a non-thrombogenic luminal surface through a variety of specialised mechanisms⁶⁰. They present heparan sulphate on their luminal side, binding factor H and antithrombin, strong negative regulators of thrombotic events, a mechanism also employed by MSCs, when implanted on a carrier material *in vivo*, but lost after enzymatic treatment⁶¹. Both, ECs and MSCs express typical haemostatic regulators (Figure 4)²⁴, such as tissue factor pathway inhibitor, nitric oxide and prostaglandin producing enzymes, and similar amounts of tissue- and urokinase-type plasminogen activator and plasminogen activator inhibitor, suggesting fibrinolytic properties, as demonstrated for MSCs *in vitro*^{62, 63}. Particularly after engagement of activated platelets, MSCs secrete fibrinolytic enzymes and exert ECM remodeling activity⁶², which may promote damage repair *in vivo*. Culture-expanded MSCs express higher amounts of pro-thrombotic tissue factor and platelet agonist collagen²⁴, as typically found within the stromal compartment⁶⁴⁻⁶⁸, which are both increased after prolonged culture, and further strongly modulated upon inflammatory challenge²⁴. Thus, MSCs are equipped to infiltrate bleeding wounds and beneficial for wound closure^{19, 69-71}, but how do they react when infused systemically? Arnold Caplan and co-workers conducted the first study on the feasibility and safety of systemic infusion of *ex vivo* expanded MSCs⁷², and found no toxicity. They also tried to detect infused MSCs in venous patient blood in a subsequent study⁷³, and were able to detect circulating MSCs in some of the patients shortly after infusion, but not at later time points (see “first-pass effect”, discussed in the next section).

Caplan’s team also studied the presence of native circulating MSCs in healthy individuals, or in GCSF-mobilised patients undergoing peripheral blood stem cell collection. They were unable to detect any circulating native MSCs in either case⁷⁴. This goes in hand with recent reports that postnatal circulating stromal precursors are absent in the majority of human donors and extremely scarce in the remaining few⁷⁵. Although the issue of circulating MSCs in healthy adults remains controversial⁵³⁻⁵⁵, it appears certain that circulating MSCs are present in blood during the first trimester of human development⁷⁶, and also found in cord blood⁷⁷. In individuals with pathological conditions or subjected to strong insults, such as surgery or extensive trauma, cells with MSC-phenotype appear to be mobilised into the circulation and can be identified with flow cytometry or plastic adhesion assays⁷⁸⁻⁸⁰. At least in the later case of orthopaedic trauma, this can be related to a passive release of fat globules into the circulation⁵⁵, and may not be related to an coordinated – active egression of MSCs into the circulation. Various studies have shown a differential mobilisation of haematopoietic, endothelial, and stromal progenitor cells from the bone marrow depending on the predominant inflammatory milieu^{79, 81-84}. I would therefore like to conclude at this stage, that viable MSCs are found in blood under particular circumstances, and that MSCs have the capacity to be in contact with blood and possibly even to mobilise into the circulation (e.g. if activated by appropriate signals, such as inflammatory mediators, blood activation products, and complement anaphylatoxins formed upon injury or trauma)⁷⁹. This does not automatically apply to therapeutic cells, since culture expanded cells may change their phenotype and functionality (e.g. size increase leading to embolisation)⁴⁷. MSCs blood-compatibility may also be affected by various aspects of *ex vivo* handling and preparation for clinical use (e.g. passaging with trypsin, temperature / pH changes, cryostorage, immunogenicity derived from culture additives and washing buffers), but can possibly be increased if the therapeutic cell processing is adjusted accordingly.

1.3.2 Fate of systemically infused MSCs in the microvasculature

The most commonly used product at our facility is cryobanked, 3rd-party MSCs, cultured for 2-4 passages in medium containing FCS^{18, 24}, mainly used in strongly immunocompromised HSCT patients. More recently, also short-term (P1-2) expanded autologous MSCs cultured with platelet rich plasma (PRP) have been used. Both products may differ in their properties from industrial “universal donor” MSCs, which are presumably expanded extensively (>P5), to generate thousands of cell doses from a few cell donors for treating a multiplicity of subjects²⁶. Although great variation exists between MSC preparation protocols at different centres, most animal and clinical studies using intravenous or intraarterial delivery of culture expanded MSCs, observed that MSCs are predominantly embolized in the microvasculature of the lungs or tissue capillary beds, respectively, with a rapid demise of the detected signal⁵⁴. Two factors appear to be responsible for this effect: 1) the increase in MSCs size and other phenotypic changes occurring after cell expansion⁵⁴, and 2) the triggering of IBMIR, possibly augmenting embolisation and cell graft damage in the microvasculature²⁴.

Therapeutic MSCs are rapidly cleared from circulation, a phenomenon termed “first-pass effect”: the passive cell entrapment in small vessels as a function of cell size and deformability, presumably a consequence of MSC enlargement in culture^{47, 54, 85-94}. A first characterisation of MSC size was given by Koc et al.⁷³, who found a 2-3-times larger size for detached MSCs compared to neutrophils on cytopsin preparations. An often reported size of culture-expanded MSCs is around 10-30 μm ⁸⁷⁻⁹⁴, similar to early passage clinical MSCs (size range 10-30 μm , peak at 20-22 μm , Figure 5 and results), suggesting that a relevant portion of cells is small enough to pass the microvasculature (e.g. see Koc et al.⁷³). Size increase and other changes are continuously accumulated during *in vitro* culture on standard culture plastic⁵⁰, which makes it difficult to predict when MSCs acquire an unsuitable phenotype for systemic delivery. The chosen *in vitro* culture specifics (e.g. flasks vs. bioreactor^{95, 96}, culture surface topology and rigidity⁹⁷, cell density⁹⁸, medium supplementation⁹⁹, enzymatic detachment method¹⁰⁰, or use of thermo-responsive detachment¹⁰¹) may greatly affect cell product functionality, thus opening many new avenues for optimisation of systemically delivered MSC therapies.

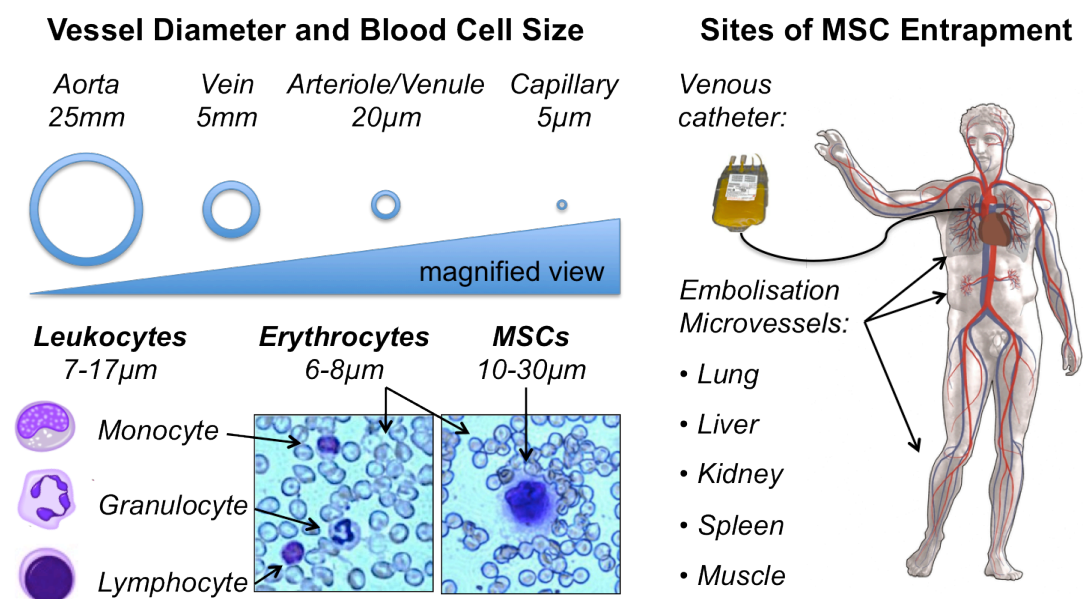


Figure 5: Therapeutic MSCs size and embolisation in the microvasculature upon systemic delivery.

Early experiments demonstrated that infused MSCs repopulate up to one-third of the MSCs in the marrow compartment of recipient animals (>1 month after infusion) that underwent marrow ablation to create space for long-term engraftment of MSCs⁷. Interestingly, none of the infused cells could be detected one week after infusion, thus, suggesting engraftment and growth of only few surviving cells after systemic delivery. MSCs (their progeny) appeared detectable in lung, liver, thymus, spleen, and marrow⁷. Similarly, engraftment of MSCs in humans was very limited (1-2% donor cells)^{45, 102}. It was suggested that higher engraftment (without marrow ablation) could be achieved, if larger cell numbers are infused, or if cells are infused in regular spaced intervals^{7, 32}. Our clinical experience suggests¹⁰³, that the engraftment efficacy of MSCs in HSCT patients is altogether fairly limited, with a positive signal for approximately 50% of the patients (8/15) and infusions (11/21). MSC donor DNA was detected in several tissues, such as lung (25% samples positive), kidney (27%), lymph node (27%), gut (22%), bladder (17%), liver (13%), marrow (10%), and spleen (7%), with no apparent homing tendency to typical tissue damage sites, and no correlation between MSC engraftment and clinical response to MSC therapy. We found no signs of ectopic tissue formation or malignant tumors of donor origin¹⁰³. Positive detection was furthermore negatively correlated with time from infusion to sample collection, with a stronger MSC signal the earlier the tissue was sampled¹⁰³. No stronger signal was observed when using cells from haploidentical donors. Despite the low level of donor cell long-term engraftment, infused MSCs exhibited bioactivity and a long-lasting beneficial modulation of several immune parameters in HSCT patients³⁷.

This may focus our attention on the early events directly after cell infusion. Initial studies on the bio distribution of MSCs after infusion suggested that large MSCs (diameter 20-24 μm) are instantly lodged in small-diameter capillaries (10-15 μm) of typical filtration organs such as lung, liver, kidney, and spleen, which could be partially counteracted by the vasodilator sodium nitroprusside (15% reduction in lung signal)⁹². Schrepfer et al.⁸⁸ achieved an even stronger reduction (50% reduction in lung signal), when using nitroprusside with smaller murine MSCs (diameter of 15-19 μm). Fischer also found better lung passage for smaller cells (MSC vs. MAPC, NSC, and BMMNCs: diameter 18, 16, 16, and 7 μm , respectively), with a 30-fold increase for BMMNCs, although in no case more than 1-5% of all infused cells passed the microvasculature, and blocking of cell surface adhesion receptors did not lead to major improvements⁸⁹. It thus appears that the large size of stromal cells is not the only factor leading to MSCs embolisation upon infusion. Improvements of lung passage could also be achieved with anticoagulant heparin¹⁰⁴, and pulmonary toxicity was greatly reduced when adding heparin to MSCs¹⁰⁵, avoiding cell aggregation and 95% of clinical symptoms after infusion to mice, suggesting an improved lung passage after the use of anticoagulants. Quimby et al.¹⁰⁶ compared infusions of fresh and cryopreserved MSCs in a cat-model, and found that low doses of allogeneic cryopreserved MSCs were well tolerated, while higher doses of cryopreserved, but not fresh MSCs, were associated with a higher incidence of adverse events (vomiting during infusion and increased respiratory rate). Therefore to summarise, engraftment of MSCs in HSCT patients is low to undetectable, and long-term persistence of therapeutic cells was not affected by the degree of HLA-disparity in these strongly immunocompromised individuals. Kinetics of therapeutic cell persistence indicate, that most cells are cleared rapidly after infusion, due to embolisation and graft damage in the microvasculature⁴⁶.

1.3.3 The instant blood mediated inflammatory reaction

The fast recognition, embolisation, and clearance of the infused MSCs suggests the involvement of the IBMIR³⁹, a sequence of incompatibility reactions of the innate immune system, observed after systemic introduction of cell therapies and biomaterials into the blood circulation³⁹. Particularly, the complement, coagulation, and contact activation systems are involved in the instant recognition, eliciting consecutive activation of platelets and leukocytes, leading to thrombotic and anaphylactic reactions, inflammation, and cell graft damage. These events may lead to adverse reactions, compromise treatment efficacy, lead to treatment failure (if too many cells are lost)³⁹, and can potentially provide an explanation for the mixed clinical results obtained with therapeutic MSCs⁹¹. The following sections will give a brief outline on documentation of IBMIR during systemic delivery of islets of Langerhans, hepatocytes, and various other types of stromal cell therapies.

A summary on innate immune responses to therapeutic cells can be found in Figure 6 (adapted from Figure 1 in the following review:³⁹). Upon exposure to blood, recognition molecules belonging to different innate cascade systems target altered-self and non-self structures on cells. Factor (F) VII, fibrinogen, and tissue factor (TF) are “recognition or trigger” molecules of the coagulation system, FXII and high molecular weight kinogen (HMWK) of the contact system, and C1q, mannose-binding lectin (MBL), and properdin of the complement system. The activation of each cascade system triggers amplification reactions. Activation of the coagulation cascade leads to the generation of prothrombin and thrombin. Further activation of the contact system elicits generation of the potent vasoactive peptide bradykinin from HMWK. In the complement cascade, there is a powerful amplification of C3 that initiates generation of the anaphylatoxins C3a and C5a, as well as the lytic C5b-9 complex. The activation products in turn trigger activation of platelets, polymorphonuclear leukocytes (PMNs), and monocytes / macrophages, which result in thrombotic and inflammatory reactions. These adverse events, together with complement-mediated cell lysis and coagulation-mediated sequestration may lead to rejection or serious damage to the cells.

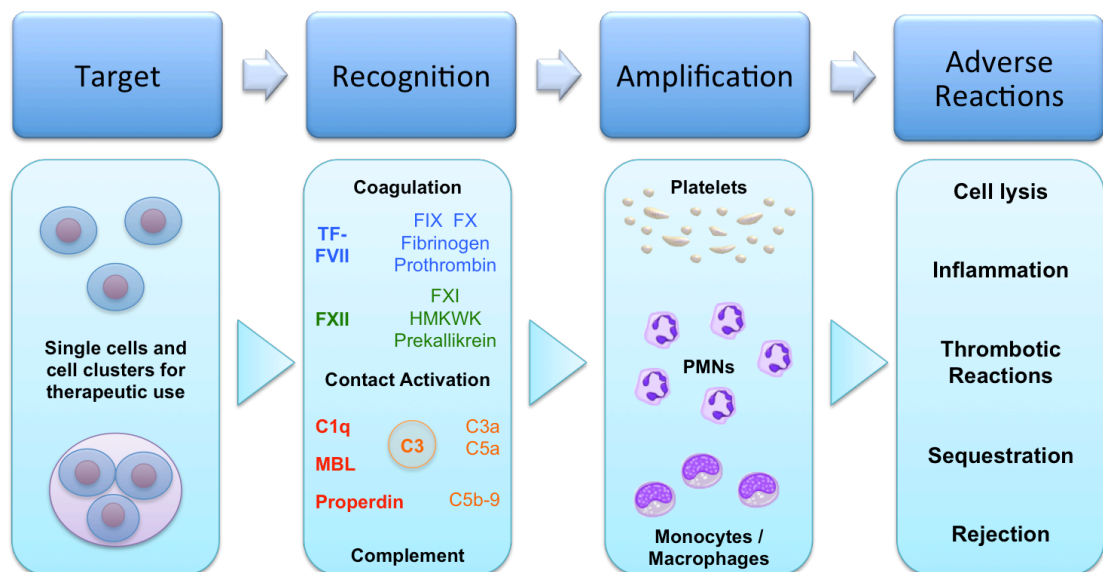


Figure 6: Incompatibility reactions triggered by innate immune responses to therapeutic cells.
(Adapted from Nilsson B. and Korsgren O. et al. *Trends in Immunology Reviews*, 2010)

The detrimental effects of IBMIR on cell therapies are already well documented in islet cell and hepatocyte transplantation (treatment of T1D and hepatic insufficiency, respectively) and should especially be considered in application of non-haematopoietic therapeutic cells, which are only poorly protected against the bloods cascade systems³⁹. The clinical infusion of purified islets has long been hampered by the fact that multiple cell grafts are necessary to achieve insulin independence⁴¹. Up to four grafts are necessary to restore 30% of normal functional capacity¹⁰⁷. Instant islet destruction is indicated by a sharp rise in C-peptide levels directly post islet infusion¹⁰⁸⁻¹¹⁰. Positron imaging reveals that up to 50% of the cells are destroyed instantly after infusion¹⁰⁸⁻¹¹⁰, attributed to innate immune responses and haemodynamic shear stress. Hot spots of islet activity within the portal branches indicate cell embolisation in clots, comprising islet cell dispersion and engraftment in the well-vascularised liver tissue. Innate immune reactions may indeed be a key factor in clinical islet graft destruction³⁹. Nilsson and co-workers identified TF, produced by the endocrine cells within the islets of Langerhans, as the trigger of the thrombotic reactions¹¹¹⁻¹¹³, accompanied by acute antibody-mediated complement activation and cell lysis¹¹⁴, which is amplified by the engagement of effector cells, such as platelets and PMNs¹¹⁵. Similar reactions occur after exposure of hepatocytes and MSCs to whole blood^{24, 116-118}. Stephenne et al. demonstrated that cells of mesenchymal ontogeny, such as bone marrow MSCs, hepatocytes, adult-liver progenitor cells (ALPCs), skin fibroblasts, and myofibroblasts demonstrate pronounced pro-coagulant activity compared to bone marrow HSCs¹¹⁷. While HSCs had a negligible effect, both MSCs and hepatocytes increased clotting by 50% in stark contrast to ALPCs and fibroblasts, which increased clotting by 80-90%. The pro-coagulant activity of different cell types correlated with their expression of TF, which was found to be absent in blood compatible ECs expressing high levels of TFPI. We also found a weak pro-coagulant activity of MSCs, but not ECs, which correlated with their expression of TF, and could be abrogated by blocking the TF-pathway²⁴. Recent findings by Tatsumi and co-workers found a strong TF-expression on adipose derived MSCs (ADSCs)¹¹⁹, which lead to a lethal pulmonary thromboembolism in a patient following systemic delivery, and a high mortality rate (85%) was documented following intravenous delivery of ADSCs in mice due to pulmonary embolism. This did not occur after infusion of uncultured adipose-derived cells, which did not display TF or pro-coagulant activity, but TF expression was induced after expansion in culture. Adverse effects to MSCs appeared to be more prominent with cryopreserved cells¹⁰⁶. Others and we have also reported on complement-activating properties of MSCs¹²⁰⁻¹²². MSCs were shown to activate all three pathways of the complement system¹²¹, despite expression of complement regulators: factor H, membrane cofactor protein (CD46), complement decay-accelerating factor (CD55), and protectin (CD59). Indeed, some of these factors showed a relative lack of expression compared to ECs¹²⁰, but increased CD59-expression resulted in reduced lysis¹²², and improved therapeutic efficacy¹²³. Thus, procoagulant activity of mesenchymal cells is affected by the site of isolation and degree of culture expansion: minimal expanded bone marrow MSCs and ECs elicit only minor procoagulant activity upon systemic delivery, while mesenchymal cells isolated from more distant sites or organs at risk for bleeding (e.g. liver or placenta), may display much stronger pro-thrombotic features. Despite basic expression of complement regulators by MSCs, a significant portion of therapeutic cells is damaged by complement activation upon systemic infusion.

1.3.4 Allo- and xenoreactivity in MSC transplantation

In the previous sections, we have described that MSCs mesenchymal ontogeny, the specific isolates anatomical location within the body, and the degree of expansion, affect the cells blood compatibility, presumably due to differences or changes within the gene expression profile (e.g. as demonstrated for the up-regulation of TF^{24, 119}), as often reported for stromal cells of different origins¹²⁴⁻¹²⁶ undergoing expansion^{50, 127}. Additionally, two major genetic barriers are embedded in every human, providing the genetic basis for transplant incompatibility and graft rejection when being ignored: 1) the xenogeneic-barrier between the different species, and 2) the allogeneic-barrier between different human individuals. Both aspects are frequently reviewed in the context of MSC transplantation, e.g. when discussing cell donor choice for clinical use, or the choice of supplements for cell culture and processing^{21, 22, 26, 28, 32, 91}. Thus, we will only briefly summarise some essential background on these important systems, as they may potentially affect the functionality of the therapeutic cells (please see table 1).

The xenogeneic-barrier comprises the phenomenon of inter-species reactivity, e.g. between humans and other mammals. A prominent example for xenoreactivity is the α -gal epitope, which is not expressed in humans, apes, and Old World monkeys (monkeys of Asia and Africa), due to inactivation of the α 1,3-galactosyltransferase¹²⁸. But α -gal is naturally expressed as millions of epitopes per cell in glycolipids and glycoproteins in all non-primate mammals, prosimians, and New World monkeys (monkeys of South and Central America). Humans express high titers of naturally occurring anti-Gal antibody, the most abundant natural human antibody, which constitutes ~1% of circulating immunoglobulin in plasma¹²⁸. Interestingly, the α -gal epitope shares a high degree of structural homology with ABO-antigens, and antibodies directed against A- and B-antigens also bind to α -gal¹²⁸. The ABO-blood type is another immune barrier hampering cell transplantation into immunocompetent hosts. Isolated MSCs do not appear to express ABO antigens^{129, 130}, but clinical cells could be contaminated with xeno- and ABO-antigens from culture supplements, and washing buffers used for cell infusion, such as fetal calf serum (FCS). Spees et al. found¹³¹ that 7-30 mg of FCS protein can be incorporated in a typical MSC dose (100 million cells), but antigen contamination can be reduced to less than 100 ng (100.000-fold reduction) by washing with human serum^{131, 132}. Alternatives, such as human AB serum (ABS), human platelet lysate (HPL), and human platelet rich plasma (PRP) have therefore been established in the past decade¹³³⁻¹³⁶. Nonetheless, also human donor substitutes must be handled with care, due to inter-individual differences, such as blood and HLA type, and prepared accordingly, to minimise adverse reactions¹³⁵. The allogeneic-barrier: MSCs display a hypo-immunogenic and anti-inflammatory profile, which allows their potential use across HLA-barriers^{31, 32}. Although MSCs fail to elicit immune responses in mixed lymphocyte reactions *in vitro*, and are frequently transplanted across HLA barriers without adverse reactions, the extent to which allogeneic MSCs evade immune reactions remains controversial³². Allogeneic MSCs show attenuated immunogenicity, but may be rejected over time, which limits their use in tissue replacement due to lack of long-term engraftment. However, in absence of instant acute rejection, 3rd-party cells may provide sufficient *in vivo* persistence for eliciting immunomodulatory effects, with a safety advantage of being only transiently present. Thus, efficient therapeutic MSCs could be understood as homing missiles, avoiding the instant immune response, effectively reaching their target to deliver a therapeutic effect, and then vanish.

1.3.5 How to prevent or antagonise cell graft failure?

Since the original description of IBMIR in islet transplantation 14 years ago³⁸, many strategies for cell modification and pharmacological intervention have been tested to counteract instant blood response and graft loss occurring after infusion^{39, 40}. Similarly, we have pursued several avenues to improve MSC survival and engraftment, in an attempt to increase their therapeutic efficacy, some of which are introduced in the thesis results section or outlined below (Table 2). The easiest method to minimise IBMIR is to use short-term expanded bone marrow MSCs, which exhibit only minimal TF expression and procoagulant activity. Another commonly used practice is to add heparin to therapeutic MSCs infusion buffer¹⁰⁵. Soluble heparin may not be sufficient to completely counteract strongly procoagulant activity of cells such as islets, ALPCs, and ADSCs^{38, 39, 117, 119}. Stephenne et al.¹¹⁷ therefore established a new protocol for combinatorial use of heparin with bivalirudin, to control ALPCs procoagulant activity in patients. Other drugs include thrombin inhibitor melagatran¹³⁷, TF-inhibitors^{24, 111}, activated protein C¹³⁸, and low molecular weight dextran sulphate (LMWDS)¹³⁹. Cells have also been subjected to conditioning with L-arginine, cyclosporine A, enalapril, or nicotinamide in order to change their inflammatory phenotype, and particularly nicotinamide had a favourable effect on reducing TF expression, leading to a reduction of IBMIR *in vitro*¹¹². Further, pre-infusion culture recovery of cryopreserved MSCs improved both their immunomodulatory¹⁴⁰ and blood regulatory properties *in vitro*¹⁴¹. The use of high doses of MSCs – freshly harvested from culture – lead to an reduced incidence of adverse events in an animal model, when compared to similarly high doses of cryopreserved cells¹⁰⁶. Other studies also suggest that blood compatibility and lung passage of MSCs can be improved by modification of culture supplements¹⁰⁵, washing buffers¹⁴², and enzymatic cell detachment method¹⁰⁰. Furthermore, strategies for non-invasive cell surface modification of therapeutic cells with macromolecular heparin-conjugate and PEG-lipid derivatives are being envisioned¹⁴³⁻¹⁴⁶.

Table 2: Methods to tackle triggering of IBMIR by therapeutic MSCs considering risk of bleeding.

Bleeding risk	Feasible types of intervention to tackle triggering of IBMIR by MSCs
None	Short-term expanded bone marrow MSCs exhibit only minimal TF expression and display minimal triggering of IBMIR compared to other cell sources ^{24, 119}
	Adaptions in culture medium / infusion buffer to increase blood compatibility, e.g. substitution of FCS / ABP by other supplements (e.g. ABS or PRP / HSA) ^{99, 142}
	Pre-infusion conditioning with substances that reduce procoagulant activity, e.g. nicotinamide reduces TF expression and triggering of IBMIR <i>in vitro</i> ¹¹²
	Pre-infusion culture recovery of MSCs improves their immunomodulatory ¹⁴⁰ and blood regulatory ¹⁴¹ properties and reduces adverse events in animal models ¹⁰⁶
Minimal	Soluble heparin in infusion buffer reduces cell aggregation, pulmonary toxicity, and dampens IBMIR after intravenous delivery of MSCs ^{24, 38, 105}
	Non-invasive cell surface modification with macromolecular heparin-conjugate or PEG-lipid derivatives reduces triggering of IBMIR by therapeutic cells ¹⁴³⁻¹⁴⁶
Substantial	Systemic anti-coagulation in patients with drugs such as melagatran, heparin, and bivalirudin may only be recommended in individuals without bleeding risk ^{117, 137}

2 AIMS OF THE THESIS

The overall aim of this PhD project was to increase our understanding on the fate and function of systemically delivered MSCs *in vivo* in an attempt to increase their therapeutic efficacy. A particular focus of this PhD thesis was therefore given to the instant innate immune response to therapeutic cells, commonly known as the IBMIR.

Specific aims:

Study I:

- To study the interaction of culture expanded human MSCs with the human complement system and to study the resulting effector cell responses in blood in order to optimise the efficacy of this immunomodulatory treatment.
- To study the complement compatibility of ECs and MSCs, and to elucidate the complement-mediated crosstalk between MSCs and blood-borne immune effector cells and the resulting immunomodulatory environment.

Study II:

- To investigate whether MSCs trigger the IBMIR after exposure to blood from healthy donors *in vitro* and after systemic – intravenous – infusion into strongly immunocompromised HSCT patients.
- To compare ECs and MSCs expression of pro- and antithrombotic factors under resting and activated conditions and their consecutive triggering of the IBMIR.
- To study the impact of typical therapeutic MSC production / clinical application related parameters on the triggering of IBMIR:
 - Variation between MSC donors / cell batches / cell lines
 - Effect of MSC passage number / culture time
 - Effect of cell dose / safety assessment

Study III:

- To compare the blood compatibility, immunomodulatory properties, and clinical efficacy of freshly harvested compared to freshly thawed cryopreserved MSCs in treatment of immune ailments in HSCT patients.
- And in particular, to study the triggering of IBMIR, the complement activating properties, the clinical response and long-term engraftment of freshly harvested compared to freshly thawed cryopreserved MSCs.

Study IV:

- To investigate a possible harmful impact of highly immunogenic ABO blood group antigens at all given steps of MSC therapy in HSCT patients, from cell isolation and preparation for clinical use, to recipient response and outcome.
- To study ABO antigen expression in resting and activated clinical grade MSCs, ABO antigen adsorption from culture media supplements or washing buffers used for cell infusion, and subsequent whole blood and patient response.

3 SUBJECTS, MATERIALS, AND METHODS

3.1 ETHICAL CONSIDERATIONS

All the MSC recipients and donors gave informed consent and the Research Ethics Committee of Karolinska University Hospital approved all studies and experimental procedures in accordance with the policy on human care. The studies followed the principles for medical research according to the guidelines of the Declaration of Helsinki.

3.2 MSC RECIPIENTS AND THERAPEUTIC CELLS

A total of 200 MSC infusions, given for complications associated with HSCT at Karolinska University Hospital, Huddinge, Sweden, and Leiden University Hospital, The Netherlands, between 2002 and 2012, were evaluated in the different studies (I-IV: with 48 MSC recipients included in Stockholm, and 25 MSC recipients in Leiden). The patients received conditioning and routine GvHD prophylaxis according to previously published procedures¹⁸. The main indications for treatment were acute GvHD and hemorrhagic cystitis. Most of the MSCs were obtained from 3rd party unrelated donors, but also MSCs from HLA-identical siblings, and haploidentical donors were used. Response was classified as complete response, partial response, stable disease, and progressive disease, as defined previously^{24,18}, and patients were judged to have responded if they had a complete or partial response. Tissue samples taken at autopsy or colonoscopy from 15 of the patients were analysed for engraftment using PCR for MSC donor DNA³. Quantification of patient blood counts, D-dimer, fibrinogen, albumin and creatinine was performed by diagnostic routine methods.

Human MSCs were obtained from bone marrow aspirates of healthy volunteer donors, and isolated and characterised as described previously^{18,147}. To isolate MSCs, bone marrow aspirates of approximately 50 ml were taken from the iliac crest of healthy donors (n=50; median age, 37; range, 1 to 68 years). The expansion and characterisation of MSCs was performed according to the guidelines of the MSC Consortium of the European Blood and Marrow Transplantation Group (EBMT) and approved by the Swedish National Board of Health and Welfare^{18,20}. Briefly, bone marrow mononuclear cells were separated over a gradient of Redigrad (GE Health Care, Sweden), washed and resuspended in DMEM low-glucose medium (DMEM-LG; Invitrogen, USA); supplemented with 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 10% FCS (Hyclone, USA), and plated at 1.6×10^5 cells/cm². When the cultures neared confluence (>80%), the cells were detached by treatment with trypsin and EDTA (Invitrogen) and replated / passaged at a density of 4.0×10^3 cells/cm² for up to four passages, and given at a median dose of 1.7×10^6 cells/kg (range $0.7 - 4.2 \times 10^6$). For rapid availability, most of the cells were stored in liquid nitrogen and freshly thawed for IV infusion. Flow cytometry analysis indicated that the MSCs were positive for CD73, CD90, and CD105 but negative for CD14, CD31, CD34, and CD45. Adipogenic and osteogenic differentiation after induction was evaluated as previously described¹⁴⁷. The MSC suspensions were culture-negative for bacteria and fungi and PCR-negative for different strains of *Mycoplasma*^{18,20}.

3.3 IMMUNOLOGICAL AND MOLECULAR STUDIES

3.3.1 Blood preparations and complement inhibition (Study I to IV)

Blood and serum preparation: Fresh non-anti-coagulated human blood for the preparation of complement active serum, whole blood flow cytometry, and Chandler whole blood loop experiments was obtained from healthy volunteers who had given informed consent in accordance with the Helsinki Protocol and received no medication for at least 10 days. Complement active normal human AB serum (NHS) was processed within 1 hour of blood collection and stored at -70°C , to maintain complement activity. In all experiments that made use of human serum, the final concentration of NHS, or EDTA-inactivated serum (NHS/EDTA) was 50% (v/v). Whole blood flow cytometry was performed with lepirudin anti-coagulated blood (Refludan; 0.05 mg/ml, Hoechst, Germany). Lepirudin is a specific thrombin inhibitor that maintains complement function in blood¹⁴⁸. Chandler whole blood loops were conducted as outlined below.

Complement inhibition: Blood and serum were treated with cyclic compstatin Ac-I[CV(1MeW)QDWGAHRC]T (1628 Da)¹⁴⁹, or inactive linear control peptide Ac-IAVVQDWGHRAT (1532 Da), and C5aR antagonist AcF-[OPdChaWR] (896 Da)¹⁵⁰, or its inactive control peptide Phe-[Orn-Pro-dCha-Ala-D-Arg]. The inhibitors and the control peptides were produced in the laboratory of J. D. Lambris, situated at the University of Pennsylvania, USA.

3.3.2 Cell culture and preparation of cells for experiments (Study I to IV)

Cell culture: Isolation and culture of MSCs for all experimental studies was conducted as described above for the clinical MSCs. For comparison to conventional culture in medium, containing 10% FCS as supplement, MSCs from the same donor were also expanded in medium containing 5% human ABS or PRP. Human umbilical vein ECs (Promocell, Germany) were grown in EC growth medium, supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin, and replated at 10,000 cells/cm². In study IV, we included the adenocarcinoma cell line HPAF-II (blood type A Se/Se) as a positive control for ABO transcript expression, which was cultured in the same media as the MSCs (DMEM-LG, 10% FCS, plus Penicillin / Streptomycin).

Cell preparation: Cells used for blood/plasma/serum experiments were obtained either from frozen cryo-stocks (liquid nitrogen), or from sub-confluent cell layers that had been washed with PBS, to remove non-adherent or dead cells, and then detached with trypsin and EDTA. Briefly, to freeze MSCs, the cells were reconstituted in 4°C ABP containing 10% DMSO and allowed to cool down to -80°C in a rate controlled freezing vessel, and then transferred into the liquid nitrogen tank. For prospective, donor-matched comparison of fresh or thawed MSCs from the same batch, cells were adjusted to $1-2 \times 10^6$ cells/ml in PBS/EDTA containing 5% ABP, and split in two equal fractions. One fraction was kept at 4°C to simulate waiting time before infusion, the other fraction was reconstituted in 4°C cold ABP containing 10% DMSO, and allowed to freeze down at -80°C . Before addition to the blood/plasma/serum experiments, cryopreserved MSCs were thawed and washed twice with PBS containing 5% ABP to remove the toxic DMSO. In study IV, MSCs were briefly washed in 10% O plasma to remove DMSO, and then reconstituted and counted in fresh PBS containing 10% ABP, or alternatively 10% HSA, as outlined in study IV.

3.3.3 Interaction of therapeutic cells with serum and blood (Study I to IV)

Serum exposure of cells: Serum preparation and cell treatment was conducted as outlined in detail in study I and III. While in study I, NHS from individuals was used, in study III, a pool of 5 NHS donors was used, to obtain an averaged lysing activity, and a longer serum incubation time was chosen (60 min at 37°C, instead of 20 min). Briefly, the cells were mixed with an equal volume of serum in sterile polystyrene FACS tubes (Becton Dickinson, USA), and incubation was carried out at 37°C. Complement activity was stopped by adding EDTA to a final concentration 10 mM. As controls, non-serum treated cells and cells treated with NHS/EDTA were included¹²⁰. Supernatants were harvested after pelleting cells by centrifugation at 900g for 5 min, and frozen at -70°C, for ELISA analysis. In experiments requiring the presence of Ca²⁺ (detection of MBL, C1q, Ficolins, and Annexin-V), cells were prepared under non-chelating conditions with binding buffer (10 mM HEPES/NaOH, pH 7.4, with 140 mM NaCl and 2.5 mM CaCl₂) from BD¹⁵¹. Pellet fractions to be analysed with Western blot and flow cytometry were collected after three washes with PBS/EDTA, or binding buffer, or regular culture medium containing Ca²⁺ and Mg²⁺ ions.

Total cell number and cell viability in suspension was assessed with the automated electrical impedance-based Cell Counter and Analyser System Model TT (CASY-TT; Roche, Germany), or with a classical haemocytometer (trypan method). Apoptotic and dead cell analysis was conducted with flow cytometry, according to the manufacturers instructions (FITC Annexin-V Apoptosis Detection Kit II, BD)¹⁵¹. Viable cells are FITC Annexin V (AV) and Propidium iodide (PI) negative; cells that are in early apoptosis are FITC AV positive and PI negative; and cells that are in late apoptosis or dead are both FITC AV and PI positive.

Complement activation and binding: SDS-PAGE was performed in a Mini-Protean 3 electrophoresis apparatus according to the supplier (Bio-Rad, USA). The cells were prepared as described above. To remove unbound proteins, cell pellets were washed three times with 10 mM PBS/EDTA, resuspended with protease inhibitors (Sigma-Aldrich, Sweden) and incubated with 0.1 M methylamine (pH 9.0) for 1 hour at 37°C, to disrupt the covalent linkage of C3 fragments to cells. Proteins were solubilised with lysing buffer (1% SDS, 10 mM Tris-HCl pH 7.4, and protease inhibitors) and cell debris was pelleted at 13,000g. Equal amounts of protein were separated on a 10% SDS-PAGE, electro blotted onto a PVDF membrane (Perkin-Elmer, USA), and probed with a 1:8000 dilution of a horseradish peroxidase (HRP)-labelled rabbit anti-human C3d-HRP and anti-C3c-HRP antibodies (Dako, Denmark). Blots were developed using enhanced chemo-luminescence detection kit (Western Lightning; Perkin-Elmer, USA). Purified C3b, iC3b, and C3d (1 µg/lane) were used as positive controls for immune detection. Flow cytometry was conducted on extensively washed cell suspensions, labelled with respective antibodies, fixed with 1% paraformaldehyde, and analysed on a LSR-II Fortessa (BD). Acquisition was performed in a forward / sideward scatter dot plot. Excluded debris (%), and signals of 2,000 – 10,000 gated events was quantified, with detection of median fluorescence intensity, and analysed with Summit (Dako). The cells were labelled with the following mouse-anti-human antibodies (all from BD): isotype IgG1-FITC, and IgG2a-PE; negative controls CD45-FITC, and CD14-PE; positive controls CD73-PE, CD90-FITC, and CD105-PE (Ancell, USA); and a panel of antibodies against different complement regulatory proteins and complement initiators (see overview table in manuscript III).

Time lapse microscopy imaging: Freshly detached and thawed MSCs (P2-4) were seeded at a density of 1×10^6 cells/ml in 24-well flat bottom plates modified with an Ultra-Low Attachment surface (Costar; Corning, USA), and exposed to either active or inactivated serum as outlined above, and described in detail in manuscript III. Imaging was performed at 37°C on a Leica DMI6000 wide field microscope with an EM-CCD 16-bit camera (Evolve; Andor Technology PLC, Northern Ireland), with an exposure time of 10 milliseconds. Transmitted light images were obtained every 2 min for the duration of 1 hour with a 20 x extra long working distance objective. Images were processed (AVI video) and visible cell counts were quantified with Image-J.

Whole blood flow cytometry was performed according to Mollnes et al.¹⁴⁸. Blood was distributed in surface-heparinised FACS tubes (Corline Systems, Sweden), and treated with PBS, EDTA, antagonists, or corresponding control peptides. The final concentrations of inhibitors in blood were as follows: EDTA, 10 mM; compstatin or its control peptide, 60 μ M; and C5aRA or its control peptide, 10 μ M. The blood was split equally into two tubes for each condition and either MSCs or a similar volume of PBS was added (100 μ l/ml). Different doses of MSCs were tested ($0.1-1.0 \times 10^6$ cells/ml). Samples were incubated at 37°C, and remaining complement activity was stopped after 40 min by the addition of EDTA. Blood sample aliquots of 100 μ l were collected after gentle mixing, labelled for 20 min with 5 μ l of antibody (anti-C3c-FITC, or CD11b-FITC and respective isotype controls), and lysed for 5 min by adding 2 ml FACS lysing solution (BD). The lysed samples were centrifuged at 900g for 5 min, the supernatants discarded and the cells were washed once again with 3 ml of PBS; 50.000 events were analysed. The remaining blood volume was diluted in an equal volume of 10 mM PBS/EDTA and centrifuged for 5 min at 2000g to yield plasma supernatants for use in ELISA analysis. These supernatants were stored at -80°C until later use.

Cell surface heparin modification: MSCs were first biotinylated by incubating $1-2 \times 10^6$ cells/ml for 30 min in PBS containing 1 mg/ml SNL biotin (EZ-Link Sulfo-NHS-LC-Biotin; Pierce Biotechnology, UK), followed by washing with PBS, then incubated for 5 min with 1 mg/ml Avidin (Pierce), washed with PBS, and then labelled for 30 min with 1 mg/ml macromolecular heparin-conjugate (Corline System, Sweden).

Chandler whole blood loops: Different cell types (ECs vs. MSCs) and MSCs prepared in different ways (resting vs. MLR-activated cells, fresh vs. thawed cells, MSCs resuspended in 10% ABP or HSA, and heparin-modified vs. native MSCs) were exposed to blood by using the Chandler whole-blood loop system, consisting of plastic tubing with a heparinised inner surface (Corline Systems), as described previously¹⁵². Briefly, pieces of plastic tubing containing 7 ml of human blood were prepared and supplemented with 100 μ l PBS containing 10% ABP or HSA +/- either resting vs. MLR-activated MSCs, freshly harvested vs. freeze-thawed MSCs, or heparin-modified vs. native MSCs, and compared to resting ECs as controls. To determine the time course of the reaction between blood and cells, 1-ml samples from each blood tube were collected before cell addition and at 5, 15, 30, and 60 min after cell addition. Reactions were stopped by addition of 10 mM EDTA (pH 7.4). Platelet and cell counts were obtained for each sample by using a cell counter (Beckman Coulter, USA). Remaining volume was centrifuged at 3000g for 20 min at 4°C, plasma collected, and stored at -80°C. Formation of the blood activation markers thrombin-anti-thrombin complex (TAT), activated factor VII anti-thrombin complex (FVIIa-AT), FXIa-AT, FXIIa-AT, and complement activation makers C3a and sC5b-9, in plasma were measured by ELISA, according to previously described methods¹⁵³⁻¹⁵⁵.

3.3.4 Mixed lymphocyte reactions and immunomodulation (Study I to III)

Mixed lymphocyte reactions (MLRs) were performed as described earlier¹⁴⁷. PBMCs were prepared by centrifugation of heparinised blood on Ficoll-Isopaque (Lymphoprep, Norway) and cultured in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM; Invitrogen Ltd, UK) and 10% ABS (PAA Laboratories Ltd, UK). Responder PBMCs were stimulated with PHA-mitogen, or alloantigen-stimulated with a pool of allogeneic donors (n=5). Freeze-thawed or fresh third-party MSCs (P2-4) were irradiated (20 Gy) and added at 1:10 ratio to PBMCs. MSC-mediated suppression of PBMC proliferation was assessed with ³H-thymidine incorporation (18 hours) as counts per minute (cpm), either at day 3 to 4 (PHA-mitogen), or at day 5 to 6 (alloantigen). Following antagonists were added to the MLRs: linear compstatin, 20 µM; cyclic compstatin, 20 µM; and cyclic C5aR-antagonist, 5 µM. MACS-depletion was used to remove the CD14/CD11b-high fraction from PBMCs (Miltenyi Biotech, Germany); additionally blocking experiments of complement receptor 3 (CD11b/CD18) were performed with anti-CD11b monoclonal (5 µg/ml; Acris Biotechnology, Germany) or respective isotype control IgG (5 µg/ml).

Cells for gene expression (Study II): ECs, resting and MLR-activated MSCs, were stimulated by co-culture with activated PBMCs for 5 days in trans-well MLRs, and compared to unstimulated MSCs or ECs. Responder PBMCs were stimulated with a pool of allogeneic donors, and placed together in the top well of the culture plate; third-party MSCs were placed in the bottom well, at a ratio of 1:10 to PBMCs.

IDO-expression and activity (Study III): MSCs were cultured +/- 100 U/ml recombinant human interferon gamma (INFg; Sigma Aldrich, UK) for 1-7 days. Conditioned media and cell lysates were harvested after 24 hours and 7 days of INFg. The indoleamine 2,3-dioxygenase (IDO) protein expression within lysates of resting or INFg-activated MSCs was quantified with Western blot. Proteins were solubilised in lysis buffer supplemented with protease inhibitors (Sigma Aldrich)¹²⁰. Lysates were sonicated before addition of Laemmli buffer, boiled, and to ensure equal loading, protein concentration was determined by BCA-protein assay (Pierce). Ten microgram of total protein was loaded onto a 10% SDS-PAGE, electro-blotted onto a PVDF membrane (Perkin-Elmer), blocked with 5% non-fat milk, and probed with primary IDO antibody (H-110; Santa Cruz Biotechnology, Inc., USA; 2 µg/ml) or beta-tubulin loading control antibody (TUB2.1; Sigma, 2.6 µg/ml), followed by washing, and incubation with peroxidase conjugated secondary antibody (IDO 1:2000 goat-anti-rabbit antibody and beta-tubulin 1:3000 goat-anti-mouse; both antibodies from Dako). All antibodies were diluted in 2% non-fat milk for incubations. Blots were washed, and developed by using ECL-kit (Perkin-Elmer), and positive signal was quantified with densitometry after subtraction of background. The enzymatic activity of IDO within conditioned MSC media was determined by measuring the concentration of tryptophan metabolite, L-kynurenine, as outlined previously¹⁵⁶. Briefly 150 µl of conditioned media was combined with 75 µl of 30% trichloroacetic acid and centrifuged at 8000g for 5 min at room temperature, and 75 µl of the supernatant was combined with an equal volume of Ehrlich's reagent in a 96-well plate. Absorbance was read at 492 nm using a micro plate reader. Concentrations of L-kynurenine in the samples were calculated using a standard curve of defined L-kynurenine concentrations (0-100 µM).

3.3.5 Expression analysis on properties of therapeutic MSCs (Study II & IV)

EC biology QRT-PCR array and confocal microscopy: Cell lysates of ECs, resting and MLR-activated MSCs were harvested with RLT buffer (Qiagen, Germany), RNA extracted by using the Qiagen RNeasy minikit, and then stored in RNase-free water at -80°C . RNA concentration and purity were estimated by reading absorbance at 260 / 280 nm with a spectrophotometer (Nanodrop; Thermo Fisher Scientific, USA). The cDNA samples used for PCR analysis were obtained with the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) or the RT² PCR Array First Strand kit (SuperArray Bioscience, USA). QRT-PCR assays were performed using the human EC biology RT² ProfilerTM PCR Array on an ABI PRISM 7900 HT Fast Block (data analysis available on website: <http://www.superarray.com/pcr/arrayanalysis.php>). QRT-PCRs were performed with the Applied Biosystems 7900 HT sequence detection system. Primers were designed for TF, collagen type-1 subunit A1 (COL1A1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression levels of GAPDH and Beta-Actin was used as an internal standard: $\text{Ct} = \text{Ct}_{(\text{Gene})} - \text{Ct}_{(\text{GAPDH})}$, with Ct being the cycle threshold of GAPDH or the gene of interest. Results were calibrated against a negative control and further analysed by the $2^{-\Delta\text{Ct}}$ method¹⁵⁷. Sub-confluent cells were detached with trypsin, allowed to adhere to microscope slides, fixed with 70% ethanol containing 30% acetone, and labelled with antibodies reconstituted in PBS with 2% goat serum and 1% BSA. The cells were first labelled with mouse control IgG (Dako), or primary mouse anti-human monoclonals directed against endoglin (BD), TF (Calbiochem, USA), collagen type-1 or fibronectin 1 (both Sigma), and visualised with a secondary AlexaFluor488-conjugated goat anti-mouse antibody (Molecular Probes, USA). Cell-surface-immobilized heparin-conjugate was visualised with TexasRed-conjugated avidin (1 mg/ml, Pierce), and functional antithrombin binding capacity was quantified with AlexaFluor488-conjugated antithrombin (1 mg/ml, Corline)^{146, 158}. Hoechst 33342 dye (Sigma) was used to detect cell nuclei. Images were acquired at 63x magnification with a confocal microscope (Zeiss LSM510 Meta; Carl Zeiss, Germany). To obtain 3D projections, several 20- μM Z-stacks were acquired and then visualized with IMARIS imaging software (v.6.3.1, Bitplane AG, Switzerland).

ABO and FUT2 genotyping: Two methods for determination of ABO genotype were performed on DNA prepared from thawed clinical grade MSCs at the University Hospital Blood Centre in Lund. These included an allele-specific primer (ASP) PCR¹⁵⁹, and a restriction fragment length polymorphism (RFLP) PCR¹⁶⁰, and FUT2 genotyping was performed with an unpublished method developed in house. ABO grouping including detection of anti-A/B titers in blood was done by using automated serological testing based on a micro-column technique blood grouping system (OrthoAutoVue, Sweden) and by agglutination of erythrocytes of known blood type.

ABO promoter methylation of clinical grade MSCs (n=4 donors) of known blood and secretor phenotype was analysed with MagMeDIP kit (Diagenode, Belgium) according to user manual, as outlined in detail in manuscript IV. Briefly, DNA from MSCs (n=4) was tested. Methylated DNA positive control and unmethylated DNA negative control were spiked into one sample in order to control for the assay. The samples were analysed in a SYBR green Q-PCR assay using the following ABO promoter region specific primers: FW; CCCTTGACACCCTGTCTCC REV; AGCTTCACGGGTTTCGTCTC. The primers for the methylated promoter (TSH2B) and the unmethylated promoter (GAPDH) controls were provided in the kit.

ABO transcript analysis: To study mRNA transcripts in resting or stimulated cells MSCs were subjected to standard differentiation or pro-inflammatory mediators, as outlined above. Adipogenic and osteogenic differentiation was induced¹⁴⁷, and confirmed by lipid vacuole formation and upregulation of gene aP2, and matrix mineralisation, respectively. To activate MSCs with cytokines, the cells were exposed for 5 days either to 100 U/ml IFN γ (Sigma Aldrich) or to pro-inflammatory mediators secreted by activated PBMCs in trans-well MLRs, and harvested for PCR analysis as described earlier²⁴. Cell lysates were harvested with RLT buffer, RNA extracted, its concentration and purity tested, and cDNA generated, as outlined above. Expression of beta-actin served as standard: $Ct = Ct_{(Gene)} - Ct_{(Actin)}$, with Ct being cycle threshold of beta-actin or gene of interest. Results are calibrated against negative control and analysed by the $2^{-\Delta Ct}$ method.

ABO antigen expression: A highly sensitive flow cytometry assay for detection of low levels of ABH histo-blood group antigens, previously developed in Lund for quality control of blood group O converted A, B and AB red blood cells (RBCs) was applied¹⁶¹. Its sensitivity was confirmed by detection of A-antigen on weak AX subgroup RBCs and detection of the minute levels of A-antigens on group B RBCs (data not shown in this report). Paragloboside carbohydrate antigen served as positive control for secondary antibody binding, blood group O MSCs served as negative controls for anti-A and -B detection, and MSCs labelled with secondary antibody only were used as baseline values. To detect antigen adsorption from culture supplements, cells were isolated and expanded in the presence of 10% FCS, 5% ABS, or 5% PRP, as described above. Additionally, adsorption of blood group A/B-antigen from clinical grade AB-plasma, which is used as supplement for washing, reconstitution, and clinical infusion of MSCs was tested. Clinical MSC preparations were thawed, briefly washed in 10% O plasma to remove toxic DMSO, resuspended in a small volume of PBS, and aliquoted in FACS tubes for incubation with different types of fresh plasmas for either 1 or 3 hours. As positive control for A/B-antigen adsorption, MSCs were incubated with 100% A1B, and A2B plasma, and as a negative control with 100% O non-secretor plasma, and 200,000 MSCs were incubated with either 0.5ml of undiluted plasma or with 2ml PBS containing 10% of respective plasmas. Anti-A/B labelling and detection was performed on washed cells as described above. Additionally, cells were labelled with the PE-conjugated isotype (IgG2a), positive (CD105-PE) and negative (CD14-PE) detection control antibodies. All antibodies are summarised in manuscript IV.

3.4 DATA ANALYSIS AND STATISTICS

Statistical analysis was performed using ANOVA 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 confidence intervals, 95%; $P < 0.05$ was considered statistically significant; Prism 5.0; Graphpad Software). For clinical response, differences between responders and non-responders were evaluated using Fisher's exact test, or when appropriate, Chi2-test.

4 RESULTS AND DISCUSSION

MSCs have entered an accelerated rate of clinical trial activity during the past years. Key challenges remain in translation to widespread clinical use, such as the incomplete understanding of the cells fate after systemic infusion^{22, 91}. This thesis aims to answer some of these questions, based on work presented in articles I-IV:^{24, 120, 141, 142}.

4.1 INTERACTION OF HUMAN MSC WITH HUMAN BLOOD

4.1.1 Expression of pro- and anticoagulant factors by MSCs

ECs are prototypic blood compatible cells¹⁶². It was not clear whether marrow MSCs have similar properties. We found, that both ECs and resting or MLR-activated (low passage) clinical grade MSCs expressed typical hemostatic regulators²⁴, including tissue factor pathway inhibitor, tissue- and urokinase-type plasminogen activator, and prostacyclin synthase. Endothelial nitric oxide synthase was more prominent in ECs, but its inducible form predominated in MSCs. More importantly, MSCs differed in expression of pro-coagulant tissue and stromal factors. While TF and collagen type 1 were absent in ECs, they were both expressed in MSCs. Early passage MSCs (P3) displayed only weak TF-expression, but it increased either after prolonged culture (P6) or upon pro-inflammatory challenge in MLRs. Collagen was already expressed at high levels in low passage MSCs, and its expression further increased after extended culture, but was weakly down-regulated after challenge in MLRs. Thus, extended culture and pro-inflammatory challenge shifted MSCs neutral profile to a pro-coagulant phenotype. This is in agreement with Tatsumi et al.¹¹⁹, who also observed up-regulation of TF after culture of adipose derived MSCs, initially only expressed at very low levels in freshly isolated cells. Similarly, Stephenne et al.¹¹⁷ found strong variation in expression of TF and blood regulatory capacity in various cell types of mesenchymal ontogeny.

4.1.2 MSCs triggering of IBMIR in the whole blood system

We next used the Chandler whole blood loop system, in order to study the interaction of different types of cell products with non-anticoagulated human blood²⁴. Blood exposure of MSCs, but not ECs, lead to initiation of the coagulation cascade, as indicated by increased formation of thrombin and other clotting factors. The number of free platelets strongly decreased compared to control blood, and visible clots formed, with a concomitant decrease in granulocytes and monocytes, infiltrating the fibrin clot. Only minute amounts of complement activation products C3a and sC5b-9 could be detected at the used dose (15,000 cells/ml), restricted mostly to higher passage MSCs. Triggering of IBMIR correlated with MSCs expression of TF, could be abrogated by blocking agents against TF, displayed strong donor variation, and was dependent on the cell dose and passage number. Low passage MSCs (P1-4) elicited less IBMIR than MSCs harvested at higher passage (P5-8). MLR-stimulated MSCs showed stronger triggering of IBMIR than resting cells, going in hand with up-regulation of TF. This is in agreement with other investigators^{117, 119}, who found that the pro-coagulant activity of mesenchymal cells is correlated with their expression of TF, varies between cells of different mesenchymal ontogeny and increases after extended *in vitro* culture.

4.1.3 IBMIR response to freshly harvested or thawed MSCs

Recent attention has been attributed to the fact that many MSC-therapeutics are cryobanked for immediate “off-the-shelf” availability in the clinic^{26, 106, 140}, suggesting that cryopreserved / freeze-thawed cells have impaired therapeutic properties compared to cultured cells, used in many pre-clinical studies to demonstrate efficacy^{106, 140}. We thus thawed low passage MSCs, washed them according to preparation for clinical use, and exposed them to human whole blood in the Chandler loop model. Interestingly, we observed strongly augmented triggering of IBMIR¹⁴¹ compared to our prior experience with freshly harvested low passage MSCs²⁴. We found formation of C3a and sC5b-9, indicating activation of the complement cascade in response to cryopreserved cells¹⁴¹. This was further substantiated by observations of increased complement lysis after exposure of cryopreserved cells to complement active normal human serum, and is in agreement with observations by Li et al.¹²¹ that MSCs are injured after serum contact. Triggering of IBMIR, and complement activation, were reduced for culture-recovered MSCs handled in parallel, which also showed superior immunomodulatory activity, although patient analysis did not reveal improved long-term engraftment of fresh cells; Not to our surprise, since complement lysis was also substantial with fresh MSCs¹⁴¹. These findings go in hand with observations by Quimby et al.¹⁰⁶, that systemic delivery of cryopreserved, but not cultured MSCs, was associated with an increased incidence of adverse effects in an animal model, attributed to the triggering of IBMIR.

4.1.4 ABO antigen expression and IBMIR triggering by MSCs

The ABO blood type is one of the major immunogenic barriers¹²⁸. We studied the possible impact of immunogenic ABO antigens on the outcome of MSC therapy, from *ex vivo* preparation, to cell infusion, and consecutive patient response evaluation. In agreement with earlier observations by Sundin and Schäfer^{129, 130}, we found that clinical MSCs do not inherently express or upregulate ABO blood group antigens after inflammatory challenge or differentiation, and did not adsorb antigens with reactivity to ABO antibodies upon culture with various supplements, such as FCS, ABS, or PRP¹⁴². However, MSCs adsorbed small amounts of ABO-antigen from the fresh human AB plasma (ABP) used for washing and clinical cell infusion at our centre, which was dependent on the antigen concentration and adsorption time. But is the antigen amount sufficient to cause ABO-antibody mediated graft rejection^{128, 163}? It is known from solid organ transplantation, that predominantly adult blood type O recipients are at risk for early allograft rejection, due to the higher levels of anti-A/B IgG antibodies¹⁶⁴. Compared to cells washed with non-immunogenic human serum albumin (HSA), MSCs washed with ABP elicited stronger IBMIR responses after exposure to blood from healthy O-donors, containing high titers of ABO-antibodies¹⁴². Analysis of HSCT recipients found only low titers of ABO antibodies at the time of MSC treatment, accountable to HSCT conditioning, GvHD prophylaxis, and transfusion requirements. Patient analysis revealed a trend for lower response in Swedish blood type O recipients, displaying higher amounts of circulating ABO antibodies, although overall titers were low in the strongly immunocompromised patients. We concluded, that native clinical grade MSCs are ABO-neutral, but the ABP used for washing and infusion of MSCs, contaminates cells with immunogenic ABO-substance, and should be substituted by GMP-compliant HSA, particularly when given to immunocompetent individuals.

4.2 INTERACTION OF MSC WITH THE COMPLEMENT SYSTEM

4.2.1 Complement regulatory and activating properties after serum exposure

We found a weak expression of complement regulatory molecules membrane cofactor protein (MCP, CD46) and decay accelerating factor (DAF, CD55), and fairly strong expression of protectin (CD59) on trypsin detached MSCs¹²⁰. Comparison to ECs harvested in a similar fashion suggested a relative lack of expression for MCP and DAF on MSCs, while CD59 is expressed to similarly strong levels, therefore providing some degree of protection from complement lysis to MSCs. Serum exposure of MSCs lead to complement activation and consecutive binding of complement component C3-fragments C3b and iC3b to the surface of MSCs and the anaphylatoxin C3a could be detected in the supernatants, which did not occur with ECs or EDTA-inactivated serum. Flow cytometry suggested a fairly similar expression of all three regulators on fresh and thawed MSCs (DAF slightly lower on thawed cells)¹⁴¹. Freshly harvested MSCs bound only very few immunoglobulin's and classical or lectin complement pathway initiators C1q or MBL from complement active normal human AB serum (NHS)¹²⁰. Binding of pattern recognition molecules C1q and MBL was increased after NHS exposure of thawed cells, while only very small binding of Ficolins was detected¹⁴¹. Particularly freshly thawed MSCs (P2-4), as often used in the clinic, were prone to complement activation and lysis, as suggested by a 80% reduction in viable counts after 1h of NHS-exposure, while culture-derived cells were more resistant (50% lysis)¹⁴¹. This is in agreement with other investigators who also documented presence of MCP, DAF, and protectin on MSCs with flow cytometry and other methods^{121, 122, 165}. Similarly to our recent findings¹⁴¹, studies by Li and Soland concluded^{119, 120}, that although complement regulators are present on MSCs, the cells get overwhelmed and lysed by complement, and up-regulating the expression of complement regulators by various means was shown to antagonise complement lysis^{121, 122}. Adoptive transfer of MSCs to complement-deficient or depleted mice resulted in reduced cellular injury, as compared to wild type mice, indicating complement-mediated cell injury after infusion, which was further substantially reduced when autologous MSCs were used¹²¹.

4.2.2 Complement activating properties after lepirudin-blood exposure

In order to study the signalling role of complement factors between complement activating MSCs and different types of effector cells circulating in blood, we employed lepirudin anti-coagulated whole blood, which does not impair complement function¹⁴⁸. To obtain a sufficient resolution with the assay, we used a higher MSC-dose in blood (0.1-1.0 x 10⁶ cells/ml) compared to the blood loops and clinical dose (15,000 cells/ml), corresponding to a MSC:PBMC ratio of 1:10 in FACS, as typically used in MLRs¹⁴⁷. We found complement and effector cell activation after addition of MSCs to blood, leading to C3-fragment deposition on MSCs and anaphylatoxin generation in plasma, which was abrogated in the presence of Compstatin or EDTA. Compstatin and C5aR antagonist successfully prevented up-regulation of CD11b on PMNs and monocytes, which is in agreement with findings by Mollnes et al.¹⁴⁸. In contrast to the MSC-lysis studies with complement active serum, we did not observe a reduction in gated MSCs after exposure to complement active blood, as compared to EDTA-inactivated blood, interpreted as resistance to lysis, although viability of the gated cells was not tested.

4.2.3 Complement activation and immunomodulation by MSCs

We found that both the degree of complement activation elicited by MSCs, and the subsequent intensity of CD11b-mediated effector cell priming, were correlated with the capacity of these cells to suppress the proliferation of PBMCs in MLRs¹²⁰. Inhibition of complement with compstatin and removal of CD14/CD11b-high myeloid cells (monocytes) strongly impaired the immunosuppressive function of MSCs *in vitro*, going in hand with studies indicating that MSCs engage myeloid cells to elicit their immunosuppressive effects^{29, 166-168}. This suggests a role of complement activation and innate effector cell polarisation in MSCs immunomodulatory activity (Figure 7).

Triggering of complement activation leads to C3 convertase-mediated cleavage of C3 into its active fragments C3a and C3b. The covalently bound C3b is degraded to iC3b by factor I. C3b and its degradation products mediate phagocytosis and may trigger immune responses via complement receptors, such as CD11b/CD18 on host cells. Accumulation of C3b leads to assembly of C5 convertase, activating C5 to C5a and C5b, which may lead to formation of the lytic membrane attack complex (MAC). Cell lysis can be prevented by the regulatory function of inhibitors such as CD59. C3a and C5a may induce cell activation and chemotactic responses by binding to receptors C3aR and C5aR on host cells or MSCs, potentially leading to a synergistic production of an anti-inflammatory microenvironment composed of many factors. To clarify if the highly suppressive MSC phenotype is actually associated with a beneficial clinical response in treatment of immune ailments, we quantified the average suppressive activity of MSCs in MLRs and correlated this value with the resulting clinical response to individual MSCs after treatment of acute GvHD and hemorrhagic cystitis. Highly suppressive MSCs did not elicit a better clinical response than average suppressors¹²⁰.

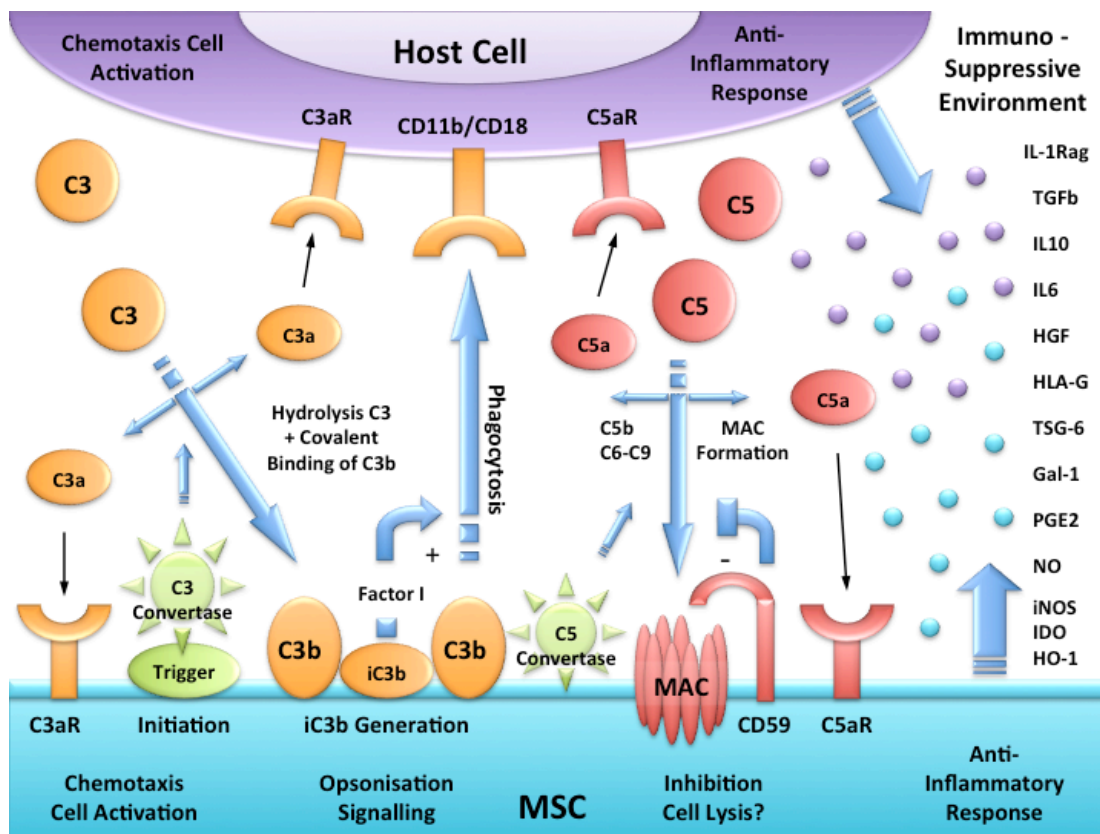


Figure 7: Complement crosstalk with innate effectors mediates MSCs immunomodulatory effect.
(Adapted from Moll G. and Jitschin R. et al. PLOSone - Supplement, 2011)

4.3 EVALUATION OF SYSTEMIC MSC DELIVERY TO PATIENTS

4.3.1 Triggering of IBMIR by MSCs and its relevance for clinical use

During the past decade, more than 200 infusions of MSCs were given at centres in Stockholm and Leiden for treatment of life-threatening complications to HSCT such as acute GvHD and hemorrhagic cystitis. No major adverse events were observed either during or after MSC infusion^{18, 24}, with thousands of patients in phase I studies^{21, 22, 91}. We performed a retrospective patient analysis and found a weak drop in platelet counts after MSC infusion (15%), but counts of leukocytes, neutrophils, lymphocytes, and monocytes did not show any acute changes within 24 hours. Analysis of soluble blood activation markers in patients' plasma revealed a mean five-fold increase in coagulation marker TAT and complement activation marker C3a, whereas haemoglobin and hyperfibrinolysis marker D-dimer were not significantly changed after MSC infusion, and no significant changes in fibrinogen, albumin, or creatinine were seen, indicating altogether that the occurrence of thrombotic events is rather limited on a systemic level, at the currently applied cell dose. To date, no thrombotic events have been reported after systemic delivery of bone marrow MSCs, although reports of potentially lethal microvascular plugging and ectopic tissue formation in lungs of experimental animals have warned clinicians of possible risks associated with this type of delivery^{87, 94, 98, 123}. MSCs used in experimental settings are often cultured to higher passages. We found that higher passage MSCs (P5-8) initiated significantly more IBMIR in the loop model. The combination of late-passage MSCs infused at high doses may potentially contribute to the lethal thrombotic complications reported in animal studies. The MSCs used in our patients were all harvested in low passage, not exceeding passage 4, and very early-passage MSCs (P1-2) also appeared to yield a higher therapeutic benefit²⁵. Recent reports from Stephenne and Tatsumi^{117, 119}, also indicated that MSCs from sources other than marrow might behave very differently, particularly after expansion for several passages. Early passage bone marrow MSCs, with limited pro-thrombotic effects, may therefore generally be recommended for most clinical applications. An exception is the treatment for hemorrhagic cystitis or major hemorrhages, where a localised pro-thrombotic activity of MSCs could be desirable, to stop the bleeding at sites of vascular damage^{19, 71}. Of interest, MSCs upregulate pro-thrombotic factors such as TF and PF4 after encounter of activated immune cells in MLRs. Thus, triggering of IBMIR is potentially augmented at inflammatory sites and after embolisation in the microvasculature, where MSCs may encounter activated immune cells, such as macrophages and monocytes. Animal studies indicated^{89, 90, 94, 169}, that embolized MSCs are activated to release biologically active substances, mediating tissue repair by limiting stress responses and apoptosis^{94, 169-171}. Upon embolisation, MSCs were shown to modulate immuneresponses by recruiting and educating immune and reparative cells, which lead to a systemic shift in cytokine production¹⁶⁸. Most interestingly, chemotaxis and immunomodulatory properties of MSCs can also be triggered by complement activation products^{28, 120, 172}, which were found to be formed in blood after systemic infusion of MSCs. After contact with activated platelets, MSCs secrete fibrinolytic enzymes and exert ECM remodeling activity⁶², which may contribute to repair of tissue damage. Thus, triggering of IBMIR after systemic infusion of MSCs may potentially contribute to both wound healing and immunomodulation.

4.3.2 Improving the therapeutic efficacy of systemically delivered MSCs

In order to improve the efficacy of our MSCs, we conducted retrospective analysis of patient data, to identify any negative impactors to treatment efficacy^{141, 142}. Based on findings by Galipeau and co-workers^{26, 140}, that storage and reconstitution procedure could potentially affect the therapeutic efficacy of MSCs, we specifically tested this parameter within our established assays and analysed our patient cohort¹⁴¹. Clinical response to fresh MSCs (n=9) was compared to that of thawed MSCs (n=35) in a comparable patient cohort matched for treatment indication and application time. Effects of confounding variables (MSC passage, cell dose, HLA-match, patient age), which could possibly influence the response to MSC therapy, were also considered in an attempt to separate individual effects. As the number of fresh infusions is limited, no multivariate analysis could be performed. No individual factor was shown to be significantly associated with an improved outcome, but we found a trend towards better response in patients receiving early passage (P1-2) and fresh MSCs. Clinical response appeared to be better in children than adults, but neither HLA-match, nor cell dose, showed any correlation to response, when corrected for the passage effect. Patients infused with fresh MSCs of early passage showed a 100% response rate, in comparison to a 50% response rate in comparable patients receiving thawed MSCs at passage 3-4. This difference was statistically significant, indicating potential additive positive effects for both of these factors, regarding the therapeutic value of the cells. Our limited analysis thus indicates a potentially favourable therapeutic value for freshly harvested cells, in addition to the previously reported advantage for very early passage cells^{25, 45}. This potentially improved outcome for giving fresh cells at very early passage is an important finding, which needs further investigation. We further show that an improved clinical response can already be achieved by using cryopreserved cells of very early passage (P1-2). Both, fresh and thawed MSCs had similar viability before infusion, and viability did not differ between responders and non-responders. We also revisited the retrospective evaluation for long-term engraftment in MSC-treated patients previously published¹⁰³, and re-evaluated the data comparing the use of freshly harvested to cryopreserved cells. Six of the 22 analysed infusions were with fresh MSCs, but no major difference in engraftment could be observed between the two groups. For both, freshly harvested and thawed MSCs, approximately half of the patients showed a positive signal for MSC donor DNA in one or more tissues, with higher positivity for earlier sampling¹⁰³. The only difference between fresh and thawed cells was the lungs, where 4/13 samples were positive for thawed MSCs, but none of the 4 samples for fresh cells. Analysis of MSC donor DNA in patient tissue showed limited engraftment for fresh and frozen MSCs alike. The absence of positive findings for fresh MSCs in the lungs might potentially indicate improved cell passage through the lung capillaries, in consistency with our *in vitro* findings of generally improved blood compatibility for freshly harvested cells. Sophisticated studies on the biodistribution of freshly harvested and freeze-thawed cells after systemic infusion could therefore be of interest for prospective studies. Also preconditioning, licensing, and activation of MSCs are important aspects to be considered for designing future MSC products. Importantly, any form of licensing protocols for cell therapeutics should be designed with consideration to the anticipated delivery method, e.g. if systemic delivery is used and cell engraftment is desired, studies of cell interaction with blood should be conducted.

4.4 STRATEGIES TO PREVENT OR ANTAGONISE IBMIR

4.4.1 Means to prevent IBMIR and improve lung passage

We explored simple variations in culture method for marrow MSCs, together with adjustments in preparations for clinical use, such as comparison of fresh and thawed cells (manuscript III) ¹⁴¹, and different infusion buffers (manuscript IV) ¹⁴². MSCs grown with different supplements such as 10% FCS, and 5% ABS or PRP, showed plastic adherent fibroblastic morphology, although cells grown with 5% PRP grew faster, appeared to be smaller, and were more abundant on a similar surface area. Platelet residue could easily be removed by washing the cells (Figure 8A). The smaller cell size of PRP-cultured cells could indeed be confirmed with CASY measurements (Figure 8B; peak size MSC-FCS: 22 μm , MSC-ABS: 21 μm , and MSC-PRP: 20 μm). Many PRP-cultures reached an average peak diameter of only 15 μm , suggesting better suitability of MSC-PRP for microvascular passage after systemic delivery. We thus compared the cells triggering of IBMIR (Figure 9C). Preliminary data suggest that both fresh and thawed MSCs grown with PRP (with platelet residue from medium removed) exhibit substantially reduced triggering of IBMIR, as indicated by lower clot formation, compared to cells grown with FCS or ABS. However, triggering of IBMIR could also be reduced for cells grown in FCS or ABS, when washing and suspending the cells in buffer containing 10% HSA instead of 10% ABP, or using freshly harvested cells obtained from culture instead of cryopreserved cells. It appeared to us, that cells grown in FCS are harder to detach from culture plastic (trypsin 8min) and may thus be more prone to activate IBMIR, while cells expanded with ABS are often very fragile and appeared to become senescent and disintegrate after a few passages in culture (>P5).

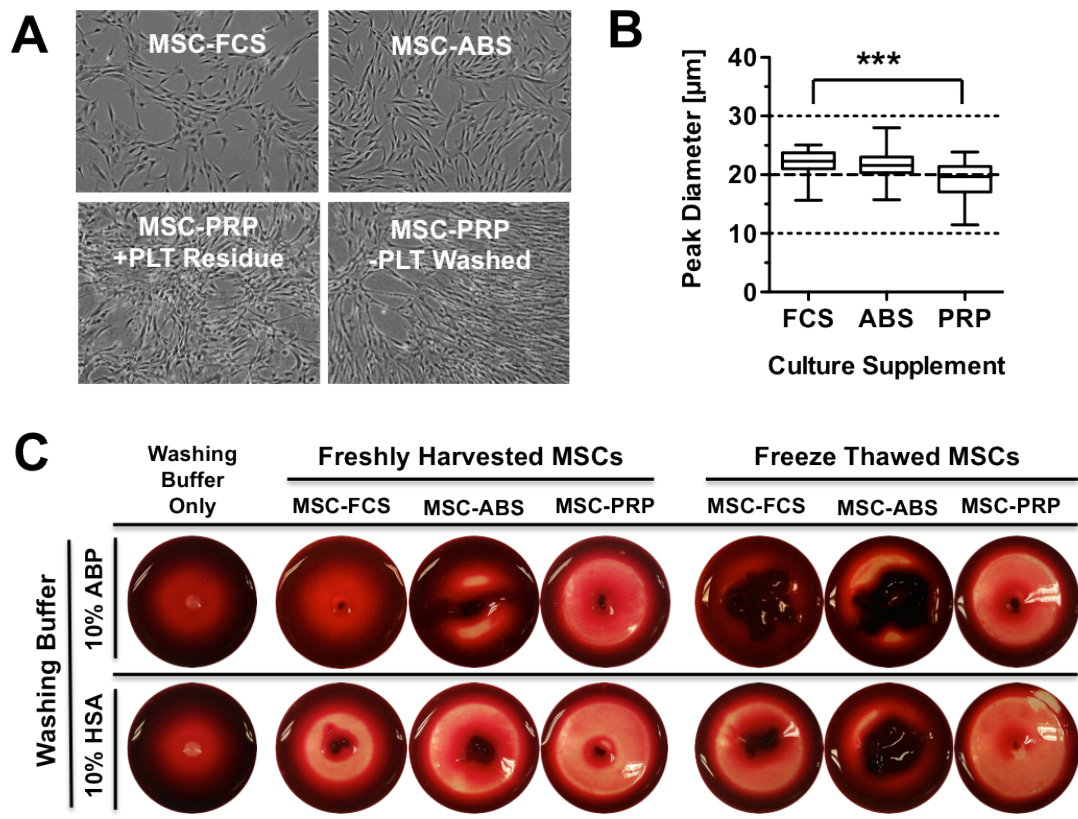


Figure 8: Triggering of IBMIR according to supplement, washing buffer, and fresh vs. thawed MSCs.

4.4.2 Anti-thrombotic strategies to antagonise IBMIR

Any pharmacological approach or cell surface modification to antagonise IBMIR has to be evaluated with consideration of bleeding risk. Thus, non-invasive cell surface modification with macromolecular heparin-conjugate or PEG-lipid derivatives, which have the capacity to reduce the triggering of IBMIR by therapeutic cells¹⁴³⁻¹⁴⁶, may be preferred, although translation into GMP-compliant procedures is challenging. Alternatively, supplementation of anticoagulants to cells, such as low-dose heparin, hirudin, or low molecular weight dextran sulphate, is effective in reducing IBMIR³⁹. We found that MSCs can be effectively surface-modified with heparin-conjugate in a three-step procedure (in collaboration with Corline Systems, Sweden, Figure 9A), based on cell labelling with biotin and binding of macromolecular heparin-conjugate to therapeutic cells, by using an avidin-linker. Non-labelled cells displayed minimal binding of antithrombin in confocal microscopy, while labelling with heparin-conjugate lead to a uniform surface distribution of avidin and antithrombin binding to cells, which could also be confirmed with flow cytometry (Figure 9B). Blood exposure of low passage MSCs lead to a weak triggering of IBMIR, as demonstrated by a reduction in free platelets and increase in thrombin formation (Figure 9C), which could be reduced to background levels (dotted line) after surface modification with heparin-conjugate, or by supplementation of blood with soluble anticoagulants, such as low-dose heparin. Pre-clinical tests to translate this method into clinical use are ongoing.

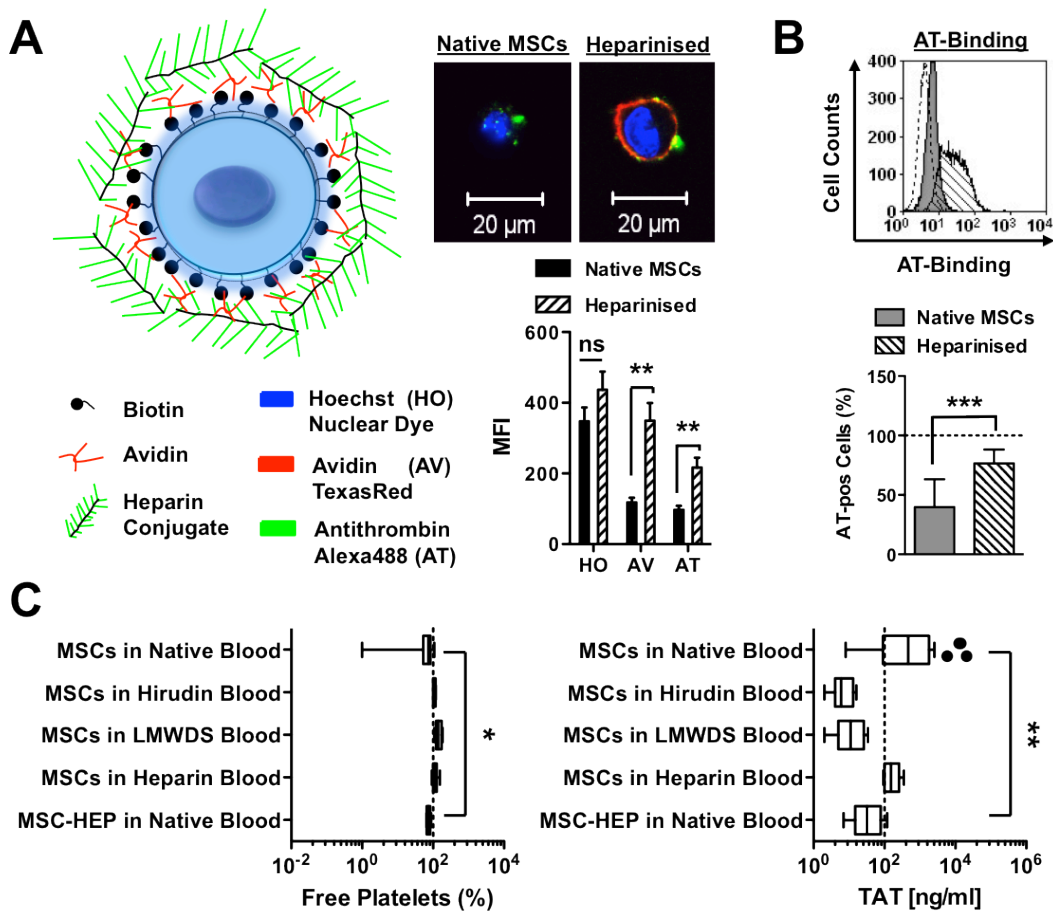


Figure 9: Cell surface heparin modification and soluble anticoagulants antagonize IBMIR.

5 CONCLUDING REMARKS

5.1 SUMMARY OF MAJOR FINDINGS

Therapeutic MSCs, as opposed to ECs, elicit the triggering of IBMIR after exposure to human blood *in vitro* and after systemic MSC infusion into patients.

MSCs express similar hemostatic regulators as ECs, but display higher amounts of pro-thrombotic tissue / stromal factors on their surface, which trigger activation of IBMIR.

Triggering of the IBMIR *in vitro* is characterised by activation of the complement and coagulation cascades, platelet activation, clot formation, and effector cell infiltration.

In patients, the occurrence of thrombotic reactions to MSCs is minimal, but IBMIR may promote graft embolization and consecutive damage in the microvasculature.

Triggering of IBMIR by therapeutic MSCs:

- 1) Shows considerable cell donor variation, which appears to be the strongest single confounding factor when comparing different types of MSC isolates.
- 2) Generally increases after prolonged ex vivo expansion. Early-passage MSCs (P1-4) elicit a weaker trigger of IBMIR than higher passage cells (P5-8).
- 3) Is augmented for cryopreserved / freeze-thawed cells, as opposed to fresh cells, and thawed cells also display impaired immunomodulatory activity.
- 4) Is weakly augmented for cells resuspended in fresh human donor AB plasma, as opposed to cells resuspended in GMP-compliant human serum albumin.
- 5) Is dose dependent; regular doses of low passage cells are safe in patients, but higher doses and particularly higher passage cells should be handled with care.

In conclusion, *in vitro* exposure of MSCs to human blood elicits triggering of IBMIR, which is affected by their degree of expansion, and is stronger for freeze-thawed cells resuspended in AB plasma (clinical use), potentially compromising cell graft function.

This seems to be paired by clinical observations, that particularly early passage cells show the most beneficial clinical effect, with further indications for a better outcome when using fresh cells and eliminating the AB plasma in washing and infusion buffers.

Apart from compromising cell graft survival and function, activation of IBMIR may also lead to formation of blood activation products, such as anaphylatoxins, which were shown to trigger the chemotactic and immunomodulatory properties of these cells.

Triggering of IBMIR, tissue injury, and acute inflammation may therefore be crucial for promoting the context dependent repair functions of surviving therapeutic cells, once the critical phase is passed and MSCs have adapted to their new environment.

5.2 EPILOGUE: THE PERFECT THERAPEUTIC MESENCHYMAL

MSCs are tested for a large variety of clinical treatment indications. A frequently asked question would be: What kind of general properties should our therapeutic MSCs have? Is the triggering of IBMIR generally a good or a bad thing for MSC therapy?

Two ideas seem to predominate:

1) The transplanted MSCs engraft and form tissue, and 2) MSCs are “magic bullets”, eliciting a certain clinical response, not necessarily requiring long-term engraftment³².

Engraftment of expanded cells may not always be desirable simply for safety reasons. The preferred level and time span of therapeutic cells persistence *in vivo* may in fact be depended on the intended long-term function in specific patient groups.

Long-term engraftment and tissue formation of culture-expanded infused MSCs is low, presumably due to the poor homing efficacy to suitable target and engraftment sites, with donor chimerism rates of 1-5% in experimental animal models³².

When talking about tissue replacement, cell engraftment is indeed essential. However, tissue repair, regeneration, and modulation of immune responses may also occur without therapeutic cell engraftment. Would it be augmented if MSCs survive longer?

If therapeutic cell function would be dependent on a transient level of engraftment, the first issue to consider would be the general mode of cell delivery. The cells could either be injected locally or delivery systemically, e.g. by intravenous or intraarterial infusion.

If MSCs are applied systemically, and medium to long-term engraftment is desired, they should fundamentally be compatible with human blood, and furthermore they should not be recognised as “foreign cells” by the recipient immune system.

Accordingly, a better therapeutic mesenchymal would display the following properties:

It is shortly expanded in culture (early passage) with minimum epigenetic alterations.
It does not trigger the IBMIR (e.g. does not activate complement and clotting system).
It does not express or display any incompatibility / foreign epitopes on its cell surface.
It does not bind any type of natural antibodies and does not trigger effector cell lysis.
It efficiently homes to the desired target site / tissues *in vivo* (e.g. tissue damage site).
It transiently engrafts at the target sites and forms / regenerates the appropriate tissue.
It elicits strong and long-lasting modulation of immune effector cell function *in vivo*.

Can this hypothetical scenario ever be achieved? I think that detailed studies, and consequent stepwise improvements in cell production and their mode of application, combined with an improved patient supportive care, will eventually achieve this goal.

I would like to close here with the words of Bakunin: “By striving to do the impossible, man has always achieved what is possible.”

6 ACKNOWLEDGEMENTS

We have gone a long way during the past 6 years in Sweden and all those stopovers at other collaborating centres. I have met so many kind and passionate people on the way, I really don't know how to thank you all. But I will try my best here... Big thanks, to all my friends and supporters in Berlin, Helsinki, Leiden, Lund, Stockholm, and Uppsala. I learned a lot during the first years at **Clinical Immunology** in Huddinge and Uppsala, but one should never get tired and strive for new horizons. So I got the chance to start all over once more. I made a lot of new friends at the end of my PhD when our group moved from the hospital to the new **HERM** (Hematology and Regenerative Medicine) at NOVUM. There, I also got to know the great tissue-engineers from **ACTREM** (Advanced Centre for Tissue Engineering and Regenerative Medicine) and **COB** (Centre for Oral Biology). I got to witness the birth of **TIM** (Therapeutic Immunology) back at the hospital, and eventually I got to know the molecular engineers from **DBRM** (Developmental Biology and Regenerative Medicine) in Solna. It was a little confusing at times, but in the end, I think, we all learned a lot from one another. I really enjoyed interacting with all of you and trying to bring everyone together on one table. At first, I would like to thank my supervisors and closest colleagues:

Katarina Le Blanc, my principle supervisor, thank you for inviting me to join your team in Stockholm, for all the generous support over the past years, and for giving me the unique opportunity to get to know translational research / medicine at KI at its best. **Bo Nilsson** and **Kristina Nilsson-Ekdahl** in Uppsala, for being the kindest supervisors and mentors one can possibly have. All of you took much time to listen and discuss, and it has been a real pleasure to share your thoughts and learn from you.

Cecilia Götherström and **Ida Rasmusson-Duprez**, for accompanying my path with Katarina and Bo from start to end. Cecilia, I truly admire your organisation skills, and thank you so much for organising the T&M Network and the Regenerative Medicine Doctoral Program. Ida, your humour is always appreciated and you never let me down. **Lena von Bahr**, thank you for great discussions and patience with clinical evaluation. **Lena, Berit, Kerstin, Kristina, Lili,** and **Monika**, I am really indebted to you for your enormous support with all the big and little things. You all deserve a big hug ☺

Javier Sanchez and **Graciela Elgue**, thanks for being such great companions during the three hardest years of my PhD thesis and for helping me to get started at Rudbeck. **Elisabeth, Lillemor, Peetra, Jaan, Osama, Olle, Rolf,** and **Yuji** for being there whenever help was needed, for doing this extra work, and for sharing your experience. Without you, it would have been impossible, and you made me feel at home at C3/C5.

Olle Ringdén and **Mark Maeurer** at TIM, thank you for the good collaboration over the past years and for being great teachers in stem cell transplantation and immunology. **Philipp, Evren, Nina,** and **Jessica**, it is so sad that we only met at the end of my PhD. All of you really enriched and inspired me. Thank you for the good discussions, for illuminating a lot of new ideas, for opening my eyes to see the bigger picture, and for pushing me to be a little bit more brave than I used to be before I met you ☺

A great thank you, to all my former and present colleagues at KI and other places, who contributed in so many ways. I have made many good friends at KI, and unfortunately there is not room enough to thank everyone individually on this page, but I will try.

Anne-Marie Connolly-Anderson, you were one of the first friends I have made at KI and my best collaborator at SMI/MTC. It has been a real pleasure to work with you. I really admired your never-ending enthusiasm. Science with you was always fun ☺

Lech and Gregory, thanks for being always positive, easy-going and cheering me up. You kept my humour alive during those dark and long winters. I owe you so much for your great help at the end of my thesis. I will never forget this. You are true friends!

Lalit, Srinivas, and Sridha, we met late, but I am glad it happened after all. All three of you have a great heart, you always reminded me to stay true to myself and to stay on the respectful path. You have great karma and I wish you all the best for the future ☺

Adil, Alf, Asa, Carolina, Caroline, Christian, Deepika, Dina, Elisa, Ellen, Eva, Gabriel, German, Hani, Hong, Iyadh, Julian, Leonie, Magnus, Mari, Maritha, Michael, Mohsen, Nadir, Petter I/II, Pingnan, Rachel, Regina, Robert, Simona, Stellan, Stephanie, Chaz, Teresa, Thuy, Valentina, and Yaser at the new HERM lab. Particular gratitude to **Stephan Meinke** and **Tolga Sutlu** for helping out so many times and to **Lilian Walther-Jallow** for sacrificing many weekends to run Luminex with us. I really appreciated your enthusiasm and the time with you at HERM. All other places: **André, Anki, Anna, Ausi, Behnam, Brigitta, Catalin, Chaniya, Dan, Darius, Davide, Dimitry, Eva, Filip, Frank, Fredrik, Graham, Giovanni, Greg, Hamid, Helen, Hero, Ioannis, Ion, Isabelle, Ivana, Jan, Jens, Joel, Johannes, John, Jola, Kalle, Karl-Henrik, Karin, Laure, Lennart, Lisbeth, Makoto, Mantas, Maria, Marie, Marijke, Marita, Maryam, Marzia, Mats I/II, Mehmet, Meiling, Michael, Mikael, Mohammed, Naradja, Nina, Olga, Oscar, Pierre, Rado, Rainer, Rami, Randi, Rebecca, Reka, Rósi, Rozina, Sandra, Sebastian, Shawn, Shawon, Silvia, Sofia, Stefan, Sulaiman, Sylvia, Taichi, Tom, Tomas, Ulrika, Vasili, and Ylva.**

Anastasia Felker, Anna Welsapar, Cecilia Haglind, Jianri Lim, and Robin Hinsch, it was great to have you in the lab as interns, and all of you did really good ☺

Erja, Saara, and Matti at **FRCBS in Helsinki**, it was a real pleasure to work with you. **Annika** and **Martin**, it was interesting to visit you in **Lund** and get introduced to ABO. **Erika** and **Frank**, thanks for giving me the chance to visit **MD Anderson in Houston**. **Helene** and **Wim**, thanks for being such great and supportive collaborators in **Leiden**. **Lindsay**, we both came and went frequently. Thanks for your collaboration in **Cardiff**. **Jacques**, thank you for asking all those good questions, it really improved my work ☺

Professor Mark Pittenger, thank you for accepting Katarina's invitation to come this long way to Sweden to discuss the findings of this thesis. Also thanks to the committee, **Benedict Chambers, Mark Maeurer, and Matti Korhonen**, to accept our invitation.

Martina Seifert, Hans-Dieter Volk and Roland Lauster at **BCRT and TU in Berlin**, a big thank you from me and my family for being so supportive when needed the most.

Finally, a big hug to **Cristina**, my son **August**, my loving **family** and all my **friends** ☺

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