From the Department of Laboratory Medicine
Karolinska Institutet, Stockholm, Sweden

MESENCHYMAL STROMAL CELL CROSSTALK WITH THE IMMUNE SYSTEM

Caroline Gavin

Karolinska Institutet

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Cover design by Gonçalo Brito. Illustration of a blood vessel with complement peptides, immune cells and MSCs that are being phagocytosed.

MESENCHYMAL STROMAL CELL CROSSTALK WITH THE IMMUNE SYSTEM

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Caroline Gavin

Principal Supervisor:
Professor Katarina Le Blanc
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Immunology and Transfusion Medicine

Co-supervisors:
Professor Gunnar Nilsson
Karolinska Institutet
Department of Medicine
Division of Immunology and Allergy

Dr. Nadir Kadri
Karolinska Institutet
Department of Medicine
Division of Centre of Infectious Disease

Opponent:
Professor Kristoffer Hellstrand
Gothenburg University
Department of Infectious Diseases
Institute of Biomedicine

Examination Board:
Associate Professor Anna Falk
Karolinska Institutet
Department of Neuroscience

Associate Professor Jaan Hong
Uppsala Universitet,
Department of Immunology, Genetics and Pathology
Division of Clinical Immunology

Associate Professor Andreas Lundqvist
Karolinska Institutet
Department of Oncology-Pathology

Till min Farfar Yngve
Du gav mig nyfikenheten att upptäcka världen

Till min mamma. Du har alltid funnits vid min sida,
stöttat mig och trott på mig!

"Nothing is impossible.
The impossible just takes a little longer"

Winston Churchill

ABSTRACT

Cell therapy is a promising treatment for several diseases. One of the most commonly used cell types are mesenchymal stromal cells (MSCs). MSCs are found in most connective tissues but for clinical use they are commonly harvested from bone marrow, adipose tissue and umbilical cord. MSCs have the ability to differentiate into connective tissues, such as adipocytes, chondrocytes and osteoblasts. Furthermore, they are known for their anti-inflammatory, immunosuppressive and regenerative properties. They release a large number of immunomodulatory factors, such as indoleamine-pyrrole 2,3-dioxygenase and prostaglandin E2, as part of their mechanisms of action. MSCs are safe to transplant and allogenic cells can be used without adverse reactions. Clinically, MSCs have been used to treat numerous diseases, including graft-versus-host disease (GvHD), type 1 diabetes (T1D) and multiple sclerosis.

A goal of this thesis was to study the interaction of MSCs with the blood compartment in an attempt to recapitulate the fate of the cells after intravenous infusion. By exploring MSC interactions with active plasma, containing complement proteins and immune cells, such as monocytes, we aimed to gain new insights into the mechanisms of action involved in MSC-mediated immunosuppression. We also studied whether MSC function was compromised in autoimmune diseases, using T1D as an example. Finally, to further decipher tissue microenvironment characteristics permissive for MSC responsiveness, we compared the immune profile in intestinal biopsies from patients receiving MSC transplantations to treat acute (a)GvHD.

In **paper I,** we investigated MSC interactions with components of peripheral blood. Using *in vitro* assays we demonstrated that proteins of the complement system (C3b/iC3b) bind to the cell surface of MSCs. We reported that MSCs survive complement binding and retain certain functional characteristics. Binding of complement proteins to the MSCs triggered enhanced phagocytosis by classical and intermediate subsets of monocytes. Monocyte-MSC interactions have been previously demonstrated to play a key role in MSC-mediated immunomodulation. These findings suggested that the therapeutic effects observed after intravenous delivery of MSCs may be mediated by interactions with the complement system. The subsequent skewing of monocytes, via phagocytosis, may also partly explain previous reports by our group regarding the fate of infused MSCs and their lack of tissue engraftment.

In **paper II,** we compared the genotypic and phenotypic profiles of MSCs isolated from T1D and healthy donors. Evaluation of bone marrow mononuclear counts, colony forming unit-fibroblasts and growth kinetics discerned no significant differences between the groups. Transcriptional comparisons, using microarray, indicated a number of differences between healthy and T1D donors, with respect to their expression of cytokines, immunomodulation and wound healing potential. However, these differences in gene expression did not reflect functional changes when tested in *in vitro* systems. We concluded that expanded MSCs from T1D donors were suitable for autologous therapy, thereby reducing risks associated with allogeneic treatments.

In **paper III,** we highlighted the importance of the gut mucosa immune cell profile of aGvHD patients, in the responsiveness to MSC treatment. Gut mucosal biopsies, obtained for aGvHD diagnoses were profiled using immunohistochemistry with a panel of innate and adaptive immune cell markers. Distinct baseline immune cell milieus were seen between patients who later responded to MSC therapy compared to those who did not respond. The responder group

presented with increased levels of CD8+ T cells and mast cells but decreased levels of CD4, CD56 and CD68. We concluded from this small pilot study that a pro-inflammatory profile within the gut at the point of MSC therapy may limit patient responsiveness. These findings need to be confirmed in larger patient cohorts but indicate that the patient's immunological milieu should be considered when evaluating potential responsiveness to MSC therapy.

In conclusion, this thesis ties together three major considerations for the development and efficacy of intravenous MSC therapy. Using a combination of basic science and clinical investigations we have demonstrated the importance of MSC interactions with the innate immune compartment, including both the complement system and peripheral monocytes. We further, report that MSCs from patients with immunological disorders, such as T1D, retain their therapeutic potential, as assessed *in vitro*, confirming that autologous therapies are an option for these patient cohorts. Finally, we evaluate how the patient's immune milieu may contribute to efficacy of MSC therapy and the need for further investigation of both the MSC biology in determining mode of therapeutic action, as well as, how the patient's status itself may impede or promote MSC responsiveness. We conclude that MSCs represent a cell source with strong therapeutic potential in a broad number of diseases. With further investigation in the highlighted areas, we hope to improve efficacy and to fully understand how these cells contribute to healing and tissue regeneration.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Kroppen är fantastisk, den försvarar oss mot infektioner orsakade av bakterier, virus och mikroorganismer. Den försvarar oss också mot våra egna celler som blivit defekta och kan skada oss om de inte elimineras, såsom cancerceller. Detta gör kroppen med hjälp av immunförsvarets olika delar. Först de yttre barriärerna: hud, saliv, tårar som stoppar infektioner och sedan immunceller som attackerar och dödar. Cellerna bildas från stamceller i den röda benmärgen (celler som kan dela på sig oändligt många gånger och mogna ut till olika celltyper i kroppen). Cellerna åker sedan ut i blodomloppet som mogna celler. Blodet utgör cirka en tiondel av vår kroppsvikt och består till ena hälften av blodplasma (klargul vätska bestående av bl.a. proteiner, molekyler, hormoner, näringsämnen, salter) och till andra hälften av blodkroppar/blodceller. Blodceller delas in i blodplättar samt röda- och vita blodkroppar. Blodplättarna koagulerar blodet vid skada, de röda blodkropparna levererar syre till kroppen. De vita blodkropparna utgör immunförsvaret och delas in i det *ospecifika försvaret*, som snabbt reagerar på infektioner och tar död på exempelvis bakterier, samt *det adaptiva försvaret* som genom sitt minne kan känna igen tidigare infektioner och då producera ett specifikt försvar mot dem. Monocyter och makrofager hör till det ospecifika immunförsvaret och kan således äta upp andra celler och bakterier. Immunförsvaret kan känna igen kroppens egna celler och hur de mår med hjälp av olika markörer på cellerna, så kallade receptorer eller antigen. Om en cell förändrats till en cancercell kan dess utseende förändras så att immunförsvaret känner igen den och t.ex. äter upp cellen eller aktiverar signaler i cellerna som tar död på dem, så kallad apoptos.

Om immunförsvaret inte fungerar som det ska kan det leda till sjukdom. Immunförsvaret kan bli överreaktivt och starta en reaktion mot individens egna vävnad och celler, så kallad autoimmun sjukdom (till exempel typ 1-diabetes och multipel skleros). Liknande reaktion kan ske efter en blodstamcellstransplantation men då är det donatorcellerna (det nya immunsystemet) som reagerar mot värden. En sådan reaktion kallas transplant-kontra-värd reaktion (GvH, engelsk förkortning). Detta är en svår sjukdom som bland annat manifesterar sig i hud, mun och tarmkanal och orsakar sår och diarréer. Tyvärr svarar en viss del av patienterna inte på behandling med steroider, den enda behandling som är visat effektiv mot GvHD. Som behandling av dessa tillstånd utvecklades en behandling med mesenkymala stromalceller (MSC). Den har visat bra resultat men tyvärr svarar inte alla patienter på denna behandlingen heller. I **studie III** undersökte vi därför om man vid rutindiagnostisering av GvHD sjukdomen även kan ta reda på om patienterna kommer att kunna svara på behandling med MSC. Vi frågade oss; hur ser immunförsvaret/immuncellerna ut i tarmen hos 8 patienter som svarade på behandling jämfört med 8 som inte svarar på behandlingen? Vi upptäckte att balansen av immunceller var skev i tarmkanalen. Det fanns fler mastceller (frigör histamin vid allergier) och CD8+ T celler (vita blodkroppar som försvarar oss mot infektioner) hos patienter som svarade på behandlingen.

Sedan MSC började användas i kliniska studier i början på 2000-talet har behandlingen visat sig framgångsrik inom flera olika områden. MSC har sedan dess använts i över 900 kliniska studier runt om i världen, p.g.a. deras vida behandlingsområden, regenerativ medicin, immunhämning vid inflammatoriska och degenerativa sjukdomar (GvH sjukdom, multipel

skleros, typ-1 diabetes, crohns sjukdom). Fördelen med MSC är att de bl.a. dämpar immunförsvaret vid överaktivitet och de har visat sig säkra att använda vid transplantation från tredje person. Cellerna kan isoleras från benmärgen hos friska volontärer, odlas utanför kroppen och sedan frysas ner tills de ska användas. Det finns vissa nackdelar med att använda celler från en annan person än patienten, bl.a. kan infektioner överföras via cellerna. Dock kan patientens egna celler ha försämrade funktion p.g.a. sjukdomsbilden, så i **studie II** studerade vi MSC från patienter med typ-1 diabetes i olika *in vitro* test för att se om de är defekta. Slutsatsen var att de är lika bra som de friska cellerna på att dämpa immunförsvaret och utsöndra olika proteiner som hjälper MSC att göra sitt jobb.

I **studie I** undersökte vi vad som händer med MSC när de injiceras in i blodet och kommer i kontakt med plasma och immunceller vilket är viktigt för att kunna optimera cellbehandlingen. Det vi fann var att cellerna binder till ett protein i blodet (komplementprotein) som då taggar cellerna så att en immuncell som känner igen proteinet äter upp MSC. Detta kan låta negativt, men detta gör att cellen som åt upp MSC nu blir aktiverad på ett sätt så att den stimulerar en inbromsning och lugnar ner immunförsvaret så att vävnader kan läka.

För att summera, MSC är en lovande behandling inom många olika områden, men fortsatt forskning behövs för att optimera deras användning och resultat.

LIST OF SCIENTIFIC PAPERS INCLUDED IN THE THESIS

I. **The Complement System is Essential for the Phagocytosis of Mesenchymal Stromal Cells by Monocytes**
C. Gavin, S. Meinke, N. Heldring, K. A. Heck, E. Iacobaeus, P. Höglund, B. Nilsson, K. Le Blanc*, N. Kadri*. *Frontiers in Immunology. 2019; 10:2249.*

II. **Type 1 Diabetes Mellitus Donor Mesenchymal Stromal Cells Exhibit Comparable Potency to Healthy Controls In Vitro**
L.C. Davies, J. J. Alm, N. Heldring, G. Moll, C. Gavin, I Batsis, H. Qian, M. Sigvardsson, B. Nilsson, L. E. Kyllonen, K. T. Salmela, P. Carlsson, O. Korsgren, K. Le Blanc. *Stem Cells Translational Medicine. 2016; 5:1–11.*

III. **Tissue Immune Profiles Supporting Response to Mesenchymal Stromal Cell Therapy in Acute Graft versus Host Disease – A Gut Feeling**
C. Gavin, E. Boberg, L. Von Bahr1, M. Bottai, A. Törnqvist Andrén, A. Wernerson, L. C. Davies, R. V. Sugars, and K. Le Blanc. *In Press Stem Cell Research & Therapy*

OTHER SCIENTIFIC PAPER NOT INCLUDED IN THE THESIS

I. **Phenotypic and Functional Alterations of Myeloid-Derived Suppressor Cells During the Disease Course of Multiple Sclerosis.**
E Iacobaeus, I Douagi, R Jitschin, M Marcusson-Ståhl, AT Andrén, C. Gavin, K Lefsihane, LC Davies, D Mougiakakos, N Kadri, K Le Blanc. *Immunol Cell Biol. 2018 Sep;96(8):820-830.*

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen-presenting cell
aGvHD	Acute graft-versus-host disease
BM	Bone marrow
C1q, C3, C3b, iC3b, C5, C6, C7, C8, C9	Complement component 1q-9
CCL2	Chemokine (C-C motif) ligand 2
CD4 -105	Cluster of differentiation molecule 4 -105
CFU-F	Colony forming unit-fibroblasts
cGvHD	Chronic graft-versus-host disease
COX2	Cyclooxygenase 2
CR1	Complement receptor type 1
CR3, CR4	Complement receptor (Macrophage-1 antigen)
CXCL9, 10, 12	Chemokine (C-X-C motif) ligand 9, 10, 12
CXCR4	Chemokine (C-X-C motif) receptor 4
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FCS	Fetal calf serum
FcγR	Fc gamma receptor
G-CSF	Granulocyte colony stimulating factor
GI	Gastrointestinal tract
GO	Gene ontology
GvHD	Graft-versus-host disease
GvT	Graft-vesus-tumor
HLA (DR / II)	Human leukocyte antigen
HSC	Hematopoietic stem cells
HSCT	Allogenic hematopoietic stem cells transplantation
IBMIR	Instant blood mediated inflammatory reaction

IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
Ig	Immunoglobulin (G, M and E)
IL (6 / 10)	Interleukin 6 / 10
ILC	Innate lymphoid cells
INF	Interferon
I.V.	Intravenous
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MHC	Major histocompatibility complex
miRNA	MicroRNA
MLC	Mixed lymphocyte culture, same as below
MLR	Mixed lymphocyte reaction
MMTT	Mixed meal tolerance test
MNC	Mononuclear cells
MRI	Magnetic resonance imaging
MSC	Mesenchymal stromal cell
NIH	National Institutes of Health
NK-cells	Natural killer cells
OSM	Oncostatin M
P	Passage
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBSC	Peripheral blood stem cells
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptors
PDL	Programmed death-ligand
PET	Positron emission tomography
PGE2	Prostaglandin E2
pHrodo	pH-sensitive fluorcent dye
Pkh26	Fluorecent dye

QDs	Quantum dots
RNA	Ribonucleic acid
qRT-PCR	Quantitative real time - polymerase chain reaction
SDF-1	Stromal cell-derived factor 1
SPECT	Single-photon emission computed tomography
SPIO	Superparamagnetic iron oxide
T1D	Type 1 diabetes mellitis
TGF-ß	Transforming Growth Factor-β
Th1, Th17, Th2	T helper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T-cell
VCAM-1	Vascular cell adhesion protein
VEGF	Vascular endothelial growth factor
7AAD	7-Aminoactinomycin D

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

The immune system is a complex host defence mechanism, protecting our body from pathogens, removing toxins, and eliminating tumour and infected cells. Comprised of two systems communicating with each other; the innate immune response is immediate, non-specific, and involves immune cells such as antigen presenting cells ((APCs) monocytes, macrophages and dendritic cells (DC)), Neutrophils, Eosinophils, Basophils, Mast cells Natural Killer (NK) cells, and NK T cells, and innate lymphoid cells 1-3, whereas the adaptive immune response is, in contrast, slow (days-weeks), highly specific, has clonal expansion capacity and an immunological memory (memory B and T cells). Stem cells are crucial cells providing the immune system with mature new cells. When functioning properly, the immune system is kept under control through several precise mechanisms, including cell death of over-reactive T cells. However, if the immune system is impaired, it can result in chronic inflammation or lead to autoimmune diseases where it attacks its own tissue.

Cellular therapy or immunotherapy has several advantages over traditional drug-based treatments as a promising tool to treat many diseases using different types of cells. A common aim of adoptive cell transfer is to support and stabilize the immune system or take advantage of its ability to boost an immune response (e.g. kill tumour cells) or reduce inflammation (e.g. type 1 diabetes (T1D)). The most successful and routinely used cell treatment is hematopoietic stem cell transplantation (HSCT), where hematological malignancies or pathologic immune systems can be replaced with a new healthy one.

Adoptive cell therapy can be either allogenic, where the cells are donated from another individual or autologous where the patient is both the donor and the recipient. Both have advantages and disadvantages. Allogeneic cells can be harvested, expanded and readily available off the shelf products, however they may initiate an immune reaction in the host. On the other hand, autologous cells do not activate an immune response but they may be impaired by the recipient's disease. Nevertheless, even if allogeneic HSCT donors and recipients are human leukocyte antigen (HLA) matched, minor differences exist and can be recognized by the immune system; eliciting an immune reaction. This is the pathogenesis behind Graft-versus-host disease (GvHD), where the transplanted immune system (the graft) initiates an immune reaction against the host's healthy tissues, commonly in the gastrointestinal tract (GI), liver, skin and mucosa. Unfortunately, treatment options for GvHD are limited, therefore adoptive cell treatment with mesenchymal stromal cells (MSCs) for steroid-refractory GvHD has emerged. MSCs are one of the most promising and successful cell therapies under clinical evaluation with over 800 registered clinical studies worldwide (www.clinicaltrials.gov.). MSC trials target a broad range of different diseases, including autoimmune inflammatory and degenerative diseases (Mastrolia, Foppiani et al. 2019) (www.clinicaltrials.gov.). Moreover, other cell therapies are advancing and evolving for example, restoration of immunological homeostasis with the transfer of regulatory T cells in acute (a)GvHD or chimeric antigen receptor T (CAR T) cell therapy treatment for cancer

(Mohanty, Chowdhury et al. 2019). In these immunotherapies, the cancer patient's own T cells are harvested, genetically engineered or expanded *ex vivo* and subsequently infused back into the patient.

Mesenchymal stromal cells are non-haematopoietic multipotent progenitor cells that reside in most parts of the body and have been isolated from different organs including adipose, bone marrow (BM) and umbilical cord (Jeon, Kim et al. 2016). Efficacy and important safety data have been mainly obtained from MSCs isolated from the BM (Le Blanc and Davies 2018, Caplan, Olson et al. 2019). In *ex vivo* cultures, MSCs are identified as plastic adherent cells, with self-renewal capacity and an ability to differentiate upon induction into mesodermal phenotypes including adipocytes, chondrocytes and osteoblasts (Horwitz, Le Blanc et al. 2005). Results from clinical trials suggest that MSCs repair or participate in the healing of injured tissues, including bones and cartilage (Caplan 2017). In addition, they are also involved in suppression of ongoing inflammatory processes (Shi, Wang et al. 2018). The mechanisms by which MSCs exert their action after *in vivo* infusion remains under debate but there is consensus regarding the involvement of many immunomodulatory cytokines, chemokines and growth factors that might contribute or explain MSCs' therapeutic success (Ankrum, Ong et al. 2014).

Intravenous (I.V.) injection is the most common route of administration for MSCs in both experimental animal models and in humans (Kurtz 2008). Although many studies have focused on the localization of MSCs after I.V. injection, little is known about the interaction of MSCs with blood compounds early after their infusions. In most cases, infused donor MSCs disappear within minutes after adoptive transfer (Zhang, Huang et al. 2015). Recent studies suggest that MSC immunomodulatory action is through their phagocytosis by monocytes (Braza, Dirou et al. 2016, Galleu, Riffo-Vasquez et al. 2017, de Witte, Luk et al. 2018, Cheung, Galleu et al. 2019). Nevertheless, the fate of MSCs and how they induce their therapeutic effect with their rapid disappearance from the blood circulation requires further investigation.

1.2 MESENCHYMAL STROMAL CELLS

1.2.1 Discovery and definition of MSC

MSC-like cells were first described by Friedenstein and colleagues in 1968 (Friedenstein, Petrakova et al. 1968). They discovered that BM in addition to haematopoietic stem cells (HSC) contained multipotent cells that could support the hematopoietic niche, differentiate into bone and form fibroblastoid colonies. Further characterisation of these cells and the term 'mesenchymal stem cells' was later proposed by Caplan and colleagues in 1991 (Caplan 1991). The *in vivo* differentiation capacity of MSCs into bone formation has been established, however their ability to meet general stem cell criteria, including the ability to self-renew and replenish as niche, has been questioned (Kuznetsov, Krebsbach et al. 1997, Horwitz and Keating 2000). The International Society for Cellular Therapy (ISCT) refined the nomenclature and urged the MSC community to define MSCs as 'multipotent mesenchymal stromal cells' instead of mesenchymal stem cells (Horwitz, Le Blanc et al. 2005). However, it is recognised that a few cells in a polyclonal MSC culture typically have full stem cell potential (Kuroda, Kitada et al. 2010, Kuroda, Wakao et al. 2013, Ghazanfari, Li et al. 2016). MSCs are a heterogenic cell population, without a unique marker to define them. Consequently, it has been hard to conclude that comparable cells are being used by different groups in clinical trials, which could be the reason for conflicting results in analogous clinical trials. The ISCT presented a minimal criteria guideline to define and characterise MSCs, including their adherence to plastic, expression of MSC surface markers CD73, CD90 and CD105, the absence of hematopoietic markers CD11b, CD14, CD34, CD45, CD19, CD79a, and low HLA-DR, and multi-lineage differentiation potential into adipocytes, chondrocytes and osteoblasts (fig1.2) (Dominici, Le Blanc et al. 2006). Controversy remains whether MSCs can trans-differentiate into non-mesenchymal tissues, such as cardiomyocytes and hepatocytes, which requires further investigation (Phinney and Prockop 2007). Current debates in the field touches the need for generalised functional assays defining the potency of MSCs used in clinical trials (Krampera, Galipeau et al. 2013).

1.2.2 The origin of MSCs

MSCs are harvested from supportive stroma tissue. Initially (1994 - 2009), BM MSCs were predominantly used in clinical trials (Pittenger, Mackay et al. 1999). However, with an increased use, the interest in MSCs from other tissue sources has dramatically increased. MSCs represent a small proportion; only 0.001-0.01 % of BM mononuclear fraction and is a limited source (Caplan 1991). In more recent years (2013-2018), MSCs derived from adipose tissue and perinatal tissue contribute to a higher proportion than BM MSCs (Moll, Ankrum et al. 2019). The benefit of using adipose tissue, as well as umbilical cord, is that these tissues are easily accessible waste products and there is no need for an invasive harvest procedure. With the increased number of tissue sources, it is important to take into consideration that MSCs from different organs may display similar morphology and phenotype but at the same time, have other organ-specific characteristics that could hamper both safety and clinical effect (Zuk, Zhu et al. 2002). This can be exemplified by the high levels of tissue factor in foetal membrane cells resulting in massive clotting when the cells come in contact with

blood, leading to a pronounced risk of thrombotic complications after I.V. infusion (Moll, Rasmusson-Duprez et al. 2012, Moll, Ignatowicz et al. 2015, Christy, Herzig et al. 2017, Le Blanc and Davies 2018). As a consequence, stromal cells from different tissue compartments need to be evaluated as distinct cell types and particularly the safety profile must be independently established for all stromal cell compartments (Le Blanc and Davies 2018). For future clinical trials, it will be important to address whether cells from different tissue sources are destined for a specific therapeutic indication or beneficial areas, such as regenerative medicine, wound-healing, immunomodulation and immunosuppression (Najar, Raicevic et al. 2010, Mattar and Bieback 2015). For example in the developmental disorder osteogenesis imperfecta, MSCs from perinatal tissues display increased regenerative potential and are a more suitable cell source (Chan and Gotherstrom 2014).

1.2.3 Characteristics and potency assays of MSCs

Due to the inherent MSC heterogeneity, numerous assays have been devised to accurately characterise and define the most suitable therapeutic subpopulation of MSCs. (Chinnadurai, Rajan et al. 2018, Chinnadurai, Rajakumar et al. 2019). However, this has led to contradictory or redundant data in some instances. Furthermore, these assays are dependent on the therapeutic properties required to treat a certain disease. Therefore, analysing a single effector pathway to measure MSC potency may be misleading since they possess a plurality of immune-modulatory and regenerative properties. The ISCT released a consensus statement to strengthen the reproducibility within the MSCs field (Krampera, Galipeau et al. 2013). The proposal included criteria to characterise MSCs, such as immune plasticity assay and the indoleamine 2,3-dioxygenase (IDO) response *in vitro* following interferon gamma (IFN-γ) licensing (Ren, Jin et al. 2011). Further, the statement stressed that conclusions should be drawn with caution when using xenorecipient animal models to predict clinical treatment efficacy and to monitor the fate of injected MSCs (Krampera, Galipeau et al. 2013). A frequently used functional assay to determine MSCs immunosuppressive characteristics is to assess their suppressive effect on T cell proliferation in co-cultures with activated T cells, a feature that is crucial for MSC anti-inflammatory applications. Additionally, new developing methods assess the suppressive capacity of MSCs, for example, a comparative phosphomatrix approach captures phosphorylation of the signal transducer and activator of transcription (STAT) in MSCs using Phosflow™ technology (BD Biosciences, Stockholm, Sweden, http://www.bd.com/se) (Chinnadurai, Rajakumar et al. 2019). In their assay, MSC phosphorylation of STAT1 and STAT3 correlated and predicted allogeneic T-cell suppression. Galipeau's group investigated a combinatorial assay matrix approach, combining molecular genetics and secretome analyses (Chinnadurai, Rajan et al. 2018). They showed that a upregulated expression of CXCL9 and CXCL10 by MSCs upon interaction with PBMCs or IFN-γ stimulation correlated with T cell suppression, and that VEGF and CCL2 predicts T cell suppression. Others have also suggested studying MSCs distinctive transcriptome or their secretome in response to licensing, where MSCs are primed with inflammatory stimuli such as IFN-γ and tumor necrosis factor alpha (TNF-α) (Ren, Jin et al. 2011). However, it is important to take into account that clinical outcome, function and characteristics of MSCs is affected by several parameters; passage (P) number, population doublings, culture and cryopreservation conditions, donor variations, tissue origin, delivery

route and the number of cells injected at delivery (von Bahr, Sundberg et al. 2012, Galipeau 2013). To date, no potency assays have been shown to have a predictive value on clinical outcome (von Bahr, Sundberg et al. 2012, Galipeau, Krampera et al. 2016, de Wolf, van de Bovenkamp et al. 2017).

1.2.4 Interactions of MSCs with humoral compounds, embracing the complement system

The first line of defence against foreign pathogens is the innate immune system. It can eliminate the intruder, recruit immune cells and interact with the adaptive immune system. Both immune systems include humoral (macromolecules such as complement proteins and antibodies) and cellular components.

The complement system is part of the early innate immune response. It prevents infection by the following three steps 1) opsonisation of pathogens and cells, causing engulfment by phagocytes that display receptors for complement (C3b, C3bi), 2) chemotaxis/recruitment of inflammatory cells via production of complement peptides, like anaphylatoxin (C3a, C5a), and 3) direct killing by creating the membrane attack complex (MAC) in the membrane of the target cell (C5b, C6, C7, C8, C9) (Rosales and Uribe-Querol 2017). The activation of the complement system involves a rapid protein cascade, including more than 30 plasma proteins that are mainly produced in the liver.

Complement can be activated via three different pathways: the classical, the lectin, and the alternative pathway (Nesargikar, Spiller et al. 2012). The classical pathway is primarily triggered by IgM or IgG antigen/antibody complexes binding to C1q, the first protein of this pathway. The lectin pathway is initiated by mannose-binding lectins or ficolins that identify sugar molecules on the surface of various pathogens. In contrast, the alternative pathway can be activated spontaneously via hydrolysis of C3 and is constitutively active at low levels. This pathway distinguishes self from non-self, an essential mechanism to consider in allogenic cell therapy. C3 is the key component in all three pathways and forms part of C3 convertase and subsequently C5 convertase that ultimately enables the formation of MAC (Merle, Noe et al. 2015). It has been established that the complement system can clear foreign pathogens but also apoptotic and activated cells. Furthermore, the complement system plays an important role in the rejection of allogenic and xenogeneic cells, further demonstrating its role in transplantation and allogenic cell therapy (Sacks and Zhou 2012).

Cells that are normally in contact with blood, such as endothelial cells, are protected against the complement system through the expression of membrane-bound complement regulators, such as complement receptor type 1 (CR1/CD35), C4b-binding protein (C4BP), membrane cofactor protein (CD46), decay accelerating factor (CD55) and protectin (CD59) (fig.1.1). CD35, CD46 and CD55 regulate the activation of C3/C5 convertase and CD59 inhibits MAC formation (Mamidi, Cinci et al. 2013). Additionally, plasma contains soluble complement inhibitors, including factor H and I (Ferreira, Pangburn et al. 2010). MSCs express all three complement regulators on their surface, suggesting their role in the protection against

complement attack (Moll, Jitschin et al. 2011, Li and Lin 2012, Davies, Alm et al. 2016). Further, MSCs secrete factor H (Tu, Li et al. 2010).

The ability of the complement system to kill unprotected cells before they reach their target tissue has an important impact on the outcome of cell therapy. For example, pancreatic islet cells or hepatocytes infused into the portal vein are attacked by complement, leading to thrombosis and destruction of the graft. With the hope of protecting cell grafts from clotting, MSCs have been used to coat the graft *ex vivo*, prior to infusion (Johansson, Rasmusson et al. 2008, Fransson, Brannstrom et al. 2015)

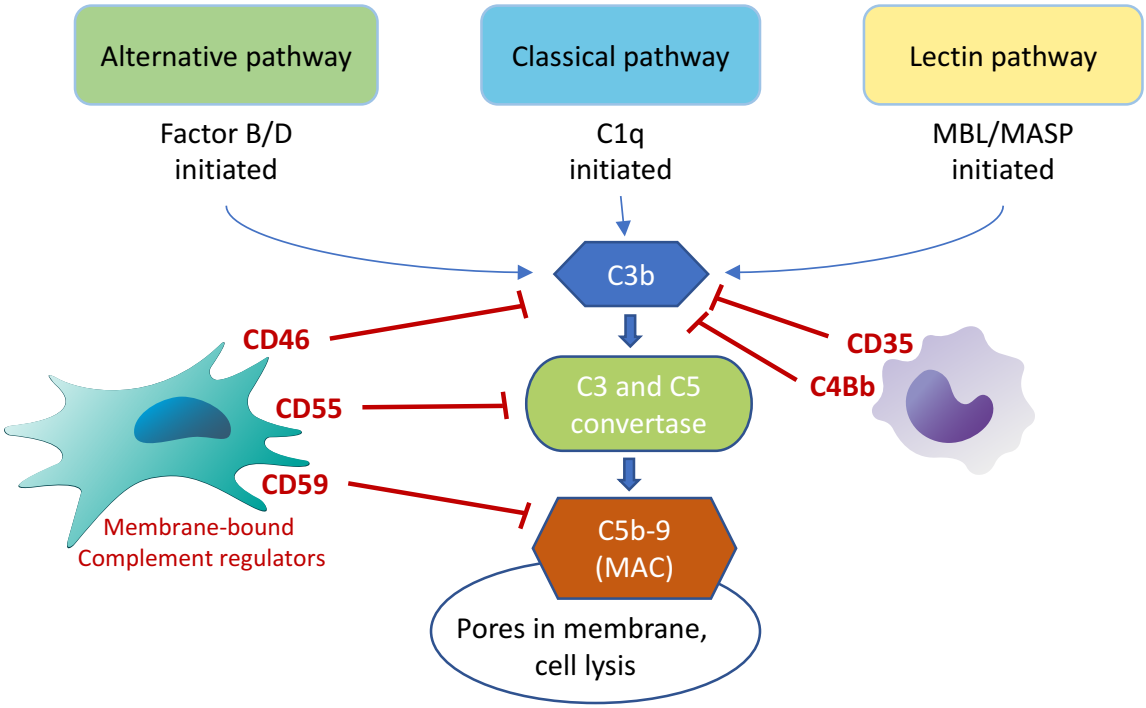

Figure 1.1. *Simplified version of the three pathways of the complement cascade: the alternative, classical and lectin pathway. The complement regulators detected on MSCs CD46, CD55 and CD59, and other complement regulators, CD35 (CR1) and C4BP on host cell not yet confirmed on MSCs, and their inhibition steps (Merle, Noe et al. 2015).*

It has been proposed that adoptively transferred MSCs are subjected to complement attack and injury at the time of infusion (Li and Lin 2012). The concept that MSCs are injured at injection does not explain the clinical success of allogenic MSCs seen in several clinical trials, including GvHD, arthritis and T1D (Le Blanc, Frassoni et al. 2008, Caplan 2017).

Low expression of HLA-II on MSCs and the absence of costimulatory molecules, such as CD40, CD80 and CD86, may also add to the protection of MSCs (Machado Cde, Telles et al. 2013). However, no donor-specific alloimmune reactions nor large differences in therapeutic outcome between autologous and allogenic MSCs were observed in a randomized comparison phase 1/2 study on therapy for ischemic cardiomyopathy (Hare, Fishman et al. 2012). The Le Blanc and other groups have published data displaying that multiple I.V.

injections of allogenic MSCs are safe and no adverse events were reported (Le Blanc, Frassoni et al. 2008, Alagesan and Griffin 2014). Taken together, they suggest that MSCs can potentially escape an immune attack by the complement system.

1.2.5 Interactions and immunomodulation potential of MSCs on immune cells

In murine experimental models, infused *ex vivo* expanded MSCs migrate to different organs including lung, spleen and liver, where they are in close proximity to immune cells, including resident macrophages. Fischer *et al*. showed that I.V. injection of MSCs led to their accumulation in the lung by interactions involving the adhesion molecule; vascular cell adhesion protein (VCAM)-1 (Fischer, Harting et al. 2009). Consequently, mitochondrial transfer from MSCs to macrophages, resulted in resident macrophage modulation, enhanced phagocytic activity and reduced lung inflammation (Jackson, Morrison et al. 2016). MSCs also regulate inflammatory processes indirectly by reprogramming monocytes into anti-inflammatory (M2) interleukin (IL) -10 producing macrophages, which induce regulatory T cells (Tregs) from CD4 naïve T-cells and T helper cell 17 (Th17) cells, resulting in the skewing of the immune response to an immunosuppressive milieu (Luz-Crawford, Kurte et al. 2013, Zheng, Ge et al. 2015). Thus, MSCs can interact with different players of cellular immunity.

1.2.5.1 MSC interactions and modulation of monocyte and macrophage

Monocytes circulate in the bloodstream after they egress from the BM and subsequently migrate into tissues where they differentiate into macrophages and DC. They are important to both the innate and adaptive immune systems by phagocytosing undesirable particles/cells (removal of pathogens, apoptotic, necrotic or foreign cells), antigen presentation and cytokine production. Phagocytosis requires a cascade of organized events, including membrane remodelling, generally receptor dependent activation, such as complement receptor 3/4 (CR3/CR4), Fc gamma receptor (FcγR), dectin, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and scavenger receptors, through recognition of opsonisation by complement, antibodies or via pattern-recognition receptors. Additionally, integrin clustering is required for adhesion, vesicle trafficking, phagosome formation, internalization and degradation in lysosomes (Rosales and Uribe-Querol 2017). This process plays a key role in tissue remodelling and homeostasis. There are different types of monocytes; the classical monocyte (CD14+ CD16-), the non-classical monocyte (CD14+CD16+) and the intermediate monocyte (CD14+CD16+) (Ziegler-Heitbrock 2007). In addition, numerous types of tissue-specific macrophages have been described. Many are found in the lungs, where intravenously infused MSCs accumulate, suggesting their potential to interact. Macrophages display plasticity by responding to outer stimuli and can skew towards a pro-inflammatory (M1), an anti-inflammatory (M2 – IL-10 producing) or an intermediate phenotype (M0) (Costantini, Viola et al. 2018).

It has long been recognized that MSCs mediate their effects through their secretome. Luk *et al.* recently generated an MSC unable to secrete immunomodulatory factors nor be able to

respond to inflammatory stimuli, nevertheless they observed an immunomodulatory effect on monocytes (Luk, de Witte et al. 2016). There was a decrease in monocyte TNF-α levels after co-culture with these MSCs. This suggests that MSCs potentially modulate cells on a secretome independent and subcellular level. Moreover, studies have shown that MSCs can be phagocytosed by monocytes, potentially leading to their skewing into an immuno-suppressive (M2) phenotype (Krasnodembskaya, Samarani et al. 2012, Braza, Dirou et al. 2016, de Witte, Luk et al. 2018). Several studies have shown that monocytes/macrophages participate in the clearance of infused MSCs (Braza, Dirou et al. 2016, Galleu, Riffo-Vasquez et al. 2017, de Witte, Luk et al. 2018, Cheung, Galleu et al. 2019). I.V. infused MSCs were phagocytosed by lung monocyte/macrophages in a mouse model of asthma (Braza, Dirou et al. 2016). Further, de Witte *et al*. also demonstrated that monocytes could be modulated after they have phagocytosed MSCs, and thereafter migrate throughout the body to have an effect on other organs. It was also suggested that remnants of MSCs were transported from the lungs via the blood steam to be cleared in the liver by Kupffer cells (liver resident macrophages) (de Witte, Luk et al. 2018).

The modulation of phagocytes after they have engulfed MSCs is not unique to immune cells. Cancer cells that phagocytose MSCs display altered behaviour and phenotype, supporting the concept that MSCs may modulate cells through phagocytosis (Bartosh, Ullah et al. 2016). Additionally, Galleu *et al*. has shown that MSCs killed by cytotoxic T cells, in a perforin-dependent manner, are phagocytosed by monocytes (Galleu, Riffo-Vasquez et al. 2017). Cells which phagocytose MSCs produced IDO, which immunosuppresses T cell proliferation, and was shown to be an essential step in MSCs-mediated immunosuppression in aGvHD mouse model. The authors suggested to use apoptotic MSCs in future clinical trials because of their enhanced activation of phagocytosis that resulted in the desired immunomodulation (Galleu, Riffo-Vasquez et al. 2017). Potentially, there are also other mechanisms of enhancing phagocytosis of live cells and there are additional phagocytes, including macrophages and neutrophils able to phagocyte MSCs. More research is needed to confirm this.

1.2.5.2 T cell modulation and suppression

T cells are one of the important players of the adaptive immune system, involved in many of the body's defence mechanisms. T cells originate from the BM, mature and proliferate in the thymus into cytotoxic (CD8+), helper (CD4+), memory and Tregs. Tregs have a crucial role in turning off immune reactions, suppresses autoreactive T cells and are important players in preventing autoimmune diseases. One important action of MSCs in immunomodulation and tolerance is the induction of newly generated CD4+ CD25+ FoxP3+ Tregs (Ge, Jiang et al. 2010).

Further, MSCs suppress the proliferation of CD4+ and CD8+ T cell subsets via multiple mechanisms, which can be cell contact dependent or via soluble factors. MSC suppression of T cell proliferation can be demonstrated in a mixed lymphocyte reaction (MLR) in a dose-dependent manner (Gieseke, Bohringer et al. 2010, Duffy, Ritter et al. 2011, Moll, Jitschin et al. 2011, Goncalves, Luk et al. 2017). MSC effects on T cell suppression have been reported to occur through both direct and indirect bystander mechanisms. The later,

principally depends on skewing of peripheral monocytes or tissue resident macrophages towards an anti-inflammatory, M2 like phenotype (Melief, Schrama et al. 2013). Furthermore, MSCs interact with DCs to skew the pro-inflammatory Th1 to the anti-inflammatory Th2 profile (Wang, Sun et al. 2008).

The role of cytotoxic CD8+ T lymphocytes (CTLs) is to lyse allogeneic cells after antigen recognition via major histocompatibility complex (MHC) class I molecules. MSCs can escape recognition and not be lysed by allogenic CTLs in MLR. If MSCs were added early in an MLR, they were cytotoxic but less effect was noted when MSCs were added later on in the cytotoxic phase of CTLs (Rasmusson, Ringden et al. 2003).The role of MSCs secretome in the modulation of T cells will be discussed in the next section.

MSCs can also promote immunomodulation, survival and expansion via their secretion of cytokines and other factors (Benvenuto, Ferrari et al. 2007, Najar, Rouas et al. 2009, Peng, Chen et al. 2015).

1.2.6 The secretome of MSCs

MSCs secrete a large number of chemokines and cytokines that play an important role in the regulation of hematopoietic and non-hematopoietic cells (fig. 1.2). MSC-derived IL-6, hepatocyte growth factor (HGF), IL-1 receptor antagonist (IL1-RA) and partially prostaglandin E2 (PGE2) can polarize monocytes/macrophages into the anti-inflammatory M2 phenotype (Chen, Zhang et al. 2007, Djouad, Charbonnier et al. 2007, Deng, Zhang et al. 2016, Luz-Crawford, Djouad et al. 2016). Such MSC-primed monocytes secrete large amounts of IL-10 and have lost their capacity to differentiate into DCs. In addition, these monocytes express increased levels of MHC class II, CD45R and CD11b, and can effectively suppress T cells functions. Interestingly, MSCs seem to exhibit different inhibitory actions. For example, MSCs upregulate IDO by IFN-γ stimulation. IDO catalyses tryptophan to kynunerine, with tryptophan being important for cell metabolism (Mbongue, Nicholas et al. 2015). Therefore, depletion of tryptophan via IDO is one of the mechanisms utilized by MSCs to inhibit T cell proliferation and function. Furthermore, IDO secretion supports differentiation of naïve CD4+ cells to FoxP3+ regulatory T cells, which are an important immune suppressor population (Munn and Mellor 2013). Other studies have implicated that transforming growth factor (TGF)-β1 or soluble programmed death-ligand 1 (PD-L1) are additional mediators of T cell inhibition by MSCs (English, Ryan et al. 2009, Melief, Schrama et al. 2013). Our group also recently showed that T cells can be inhibited via secretion of soluble PD-L2 by MSC (Davies, Heldring et al. 2017). Pro-inflammatory cytokines can activate MSCs or licence the cells to increase the secretion of specific molecules. Typical MSCs-licencing mediators are IFN-γ and TNF-α, which play an important role in combatting inflammation and exhibiting a strong immunosuppressive phenotype (Klinker, Marklein et al. 2017). Activated T-cells secrete IFN-γ and TNF-α, which can prime MSCs to secrete cytokines that subsequently inhibit T-cell proliferation and skews the T-cell repertoire towards an immunosuppressive state. Therefore, it could be speculated that feedback loops are triggered in an inflammatory milieu.

MSCs are known to secrete extracellular vesicles and exosomes (Baglio, Rooijers et al. 2015). Therefore, MSC-derived exosomes and conditioned media are currently under investigation as alternative cell-free therapy. With compelling advantages over MSCs, such as lower immunogenicity, they represent a ready-made biological source (Konala, Mamidi et al. 2016). There is increasing evidence in the literature suggesting the possibility that the effect of MSCs can be mediated through secretion of exosomes (Phinney and Pittenger 2017). Interestingly, Kordelas *et al*. recently published promising results from a clinical trial, where refractory GvHD patients injected with MSC-derived exosomes showed an improvement up to five months after infusion (Kordelas, Rebmann et al. 2014). Thus, secretome-based approaches implying MSC-derived exosomes may represent a promising therapeutic tool.

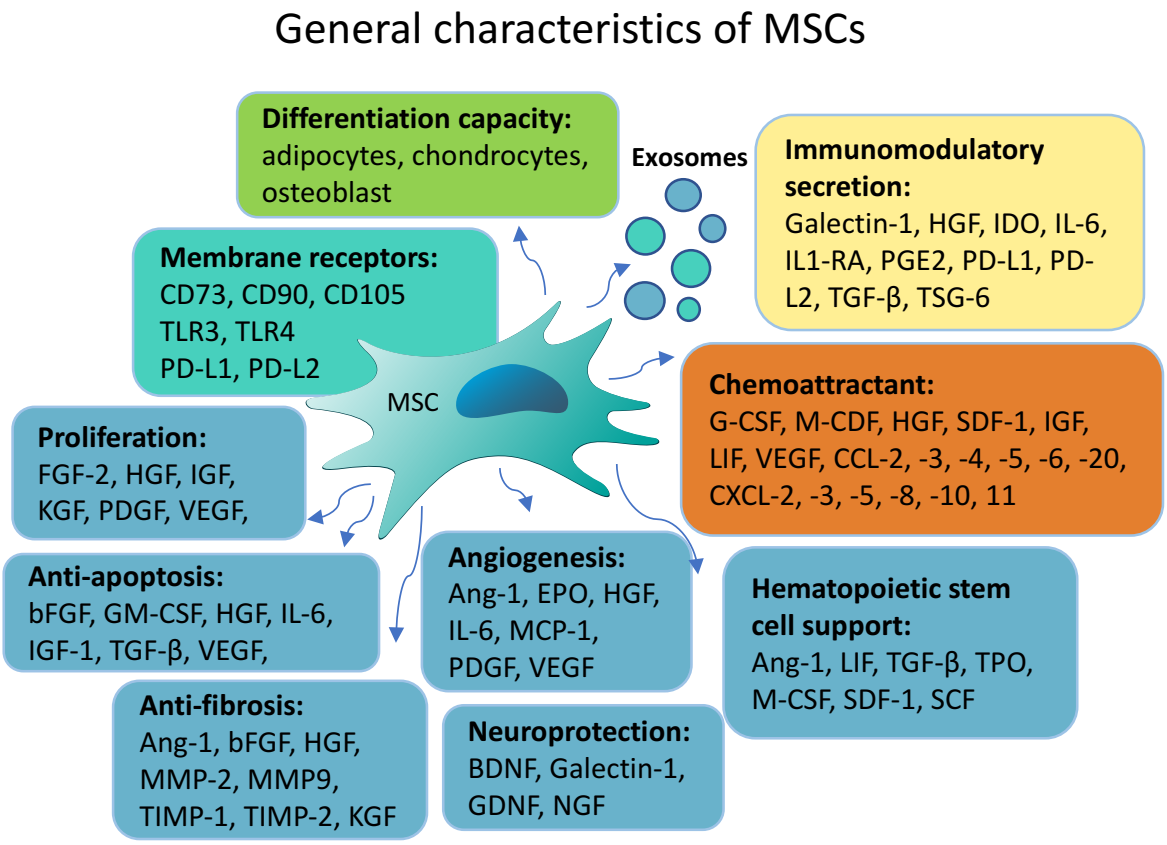

Figure 1.2. *Simplified overview of MSCs general characteristics and secretome, including cytokines, chemokines and exosomes, divided into different biological areas.*

1.2.6.1 The paradigm of MSC1 and MSC2

Several classes of immune cells have been demonstrated to exhibit plasticity, allowing them to skew towards a pro-inflammatory or anti-inflammatory phenotype depending on the cytokine and chemokine stimuli they receive from the surrounding environment. This has also been considered for MSCs. Waterman and colleagues published a new paradigm for the MSC field, based on the knowledge that MSCs have several Toll-like receptors (TLRs) and their specific stimulation results in different immunomodulatory effects (Waterman, Tomchuck et al. 2010, Waterman, Henkle et al. 2012). TLR4-primed MSCs (MSC1) showed a pro-inflammatory phenotype, whilst TLR3-primed MSCs (MSC2) presented with a more

immunosuppressive phenotype. These results potentially contribute to the explanation of conflicting MSC data, with regards to their different secretomes and phenotypes. Additionally, their immunomodulatory potential was shown by co-culturing MSC1 or MSC2 with peripheral blood mononuclear cells (PBMCs), which resulted in permissive or suppressed T-lymphocyte activation (Waterman, Tomchuck et al. 2010, Waterman, Henkle et al. 2012). However, this concept has not been fully adopted into the field. The anti-inflammatory phenotype has been most widely studies. We and other groups have tried to establish a pro-inflammatory (MSC1) and anti-inflammatory (MSC2) phenotype using protocols similar to the ones published by the Waterman group but we were unable to reproduce the findings and have therefore used the standard licensing procedure, using TNF-α and IFN-γ for experiments requiring primed MSCs.

1.2.7 Tracking, tracing and labelling of MSC

The increased interest and number of clinical trials using adoptive cellular transfer augment the need to trace the cells *in vivo,* along with being able to determine the fate of transplanted cells, engraftment potential, viability, migration and safety. Consequently, development of novel molecular imaging techniques for sensitive, non-invasive, safe technologies are required. Currently, there are several sensitive methods available for *in vitro* studies and animal models *in vivo*, however there is no ideal method available to trace MSCs in clinical settings (Nguyen, Riegler et al. 2014).

1.2.7.1 Tracking of cells in clinical setting

Tracking MSCs *in vivo* is vital to understand their mechanism of action and fate in the clinical setting. The current hurdles are safety and sensitivity of the methods used to track MSCs, such as toxicity and radioactivity for the patient (Duffy, Weitz et al. 2014). Direct labelling techniques can be used to track cell delivery, localisation and homing. Cells are labelled before transplantation and this involves the following methods: 1) Magnetic particle labelling, which is a non-invasive detection of iron-labelled donor cells with for example superparamagnetic iron oxide (SPIO) for magnetic resonance imaging (MRI) (Li, Suzuki et al. 2008). However, it is not easy to distinguish the cells from the background, including hemorrhage, necrosis and macrophages. Nevertheless, it is the preferred method with the least safety issues for clinical trials (Karussis, Karageorgiou et al. 2010, McClelland, Wauthier et al. 2011). 2) Radio nuclide labelling, which has a higher intrinsic sensitivity due to the use of radiolabelled markers, like ^{III}Oxine for single-photon emission computed tomography (SPECT) or ^{64}Cu for positron emission tomography (PET). However, a significant disadvantage is the risk of transferring the radiotracer and adverse effects on therapeutic cells (Acton and Zhou 2005). 3) Nanoparticle labelling, which uses fluorescent quantum dots (QDs). This method has an undefined effect on cells and is therefore not used clinically yet (Nguyen, Riegler et al. 2014). Unfortunately, a general limitation of direct labelling techniques is that the signal declines over time as the imaging agent inevitably gets broken down (Duffy, Weitz et al. 2014).

1.2.7.2 Studies of cells in vitro and in animal models in vivo

Transgenic animals are widely used to trace MSCs *in vivo*, specifically for linage tracing and differentiation patterns (Park, Spencer et al. 2012). Animal models are of great significance to understand and investigate the fate of MSC and mode of action, however, not all human diseases can be recapitulated by animal models. Reporter gene labelling can be used to detect viability and biology of cells. Luciferase for bioluminescence imaging is most commonly used in small animals but is not deemed to be safe in human trials.

Despite the clinical barriers, nuclear imaging of radio-labelled cells, and other methods show promising results in animal studies. One benefit with animal studies is the possibility to track cells with sensitive techniques, not safe in human (Leibacher and Henschler 2016). It is vital to use the right labelling technique for a certain hypothesis. Additionally, comparisons between studies can be restricted if different methods have been applied. One such example is assessing cell membrane integrity using fluorescent calcein dye versus bis-carboxyethyl-carboxyfluorescein (BCECF)), which may be less suitable since it can leak spontaneously from the cytoplasm (Lichtenfels, Biddison et al. 1994).

1.3 THERAPEUTIC USE OF MSC IN IMMUNE DISORDERS

MSCs have numerous characteristics that make them suitable for adoptive cell transfer and facilitates their prompt use. This includes a large secretome, immunomodulatory and immunosuppressive potential, their ability to be transplanted into third-party unmatched recipients, a high safety profile and their expansion and cryopreservation potential *in vitro* (Conget and Minguell 1999, Kotobuki, Hirose et al. 2005). The first phase I trial of MSCs was conducted in 1995 with autologous, culture expanded cells from 15 patients with hematologic malignancies in complete remission. No adverse reactions were observed (Lazarus, Haynesworth et al. 1995). Subsequently, over 800 registered clinical studies have been conducted worldwide, across a number of regenerative therapies, to support hematological recovery after HSCT, GvHD, autoimmune diseases and cardiac diseases, amongst others (Bernardo, Cometa et al. 2012) (www.clinicaltrials.gov.). The first case of MSC treatment in a patient with severe aGvHD was conducted in 2002 in a 9-year-old child (Le Blanc, Rasmusson et al. 2004). It was followed by a multicentre clinical trial of MSC treatment for steroid-refractory GvHD on 55 patients, with partly positive outcomes (Le Blanc, Frassoni et al. 2008). Meta-analyses of both immunocompetent and immunocompromised patients confirmed that BM MSCs were safe to transplant (Ringden, Uzunel et al. 2006, Lalu, McIntyre et al. 2012). Nevertheless, contradictive data and unanswered questions have arisen following clinical studies regarding, MSC survival time, biodistribution, engraftment and differentiation potential *in vivo.*

A crucial safety concern for stem cell therapy in general is whether the cells can form ectopic tissue or malignant tumours after infusion. von Bahr and colleagues evaluated biopsies from autopsy material from several organs and tissues and observed no ectopic nor malignant tumours of MSC donor origin (von Bahr, Batsis et al. 2012). In post mortem samples, MSCs were mainly localised in lung tissue, lymph nodes, and intestine. As expected, the number of MSCs detected decreased with time from MSC treatment. There was no correlation between MSC engraftment and response. Together these results suggest that the potential MSC therapeutic effect was either through mediator release and/or cell interactions. Furthermore, studies show that MSCs home to injured tissue in experimental models. Once there, they assist in wounding healing through their ability to secrete paracrine factors and recruit cells, such as endothelial cells, immune cells and activating tissue-resident progenitor cells to the injury site (Chen, Tredget et al. 2008, Sasaki, Abe et al. 2008, Karp and Leng Teo 2009, Meirelles Lda, Fontes et al. 2009). Additional, MSC positive effects have been reported in models where BM MSCs were rapidly cleared after being trapped in the lung microvasculature (Lee, Pulin et al. 2009). Data from a liver cirrhosis patient study confirmed several animal studies showing that MSCs first accumulate in the lungs and thereafter in liver and spleen up to ten days post-infusion (Kurtz 2008, Gholamrezanezhad, Mirpour et al. 2011, Eggenhofer, Benseler et al. 2012). Interestingly, tracing radioactive ^{111}In-oxine labelled MSCs were found to be highest within the spleen after ten days, whereas MSCs in the lung decreased from 35 % to around 2 %. Several independent studies show MSC clearance via phagocytosis by monocytes or macrophages (Braza, Dirou et al. 2016, Galleu, Riffo-Vasquez et al. 2017, de Witte, Luk et al. 2018, Cheung, Galleu et al. 2019). There may be factors that enhance this process and more research is needed to understand MSCs function after injection in human trials.

1.3.1 Clinical effects with regards to expansion processes and administration

1.3.1.1 Production of MSC for clinical use

MSC are traditionally expanded as adherent cultures. Mononuclear cells (MNC) are isolated from BM using Ficoll™ and seeded into flasks. After 10-14 days, colony-forming unit fibroblasts (CFU-Fs) can be harvested and the adherent cells re-seeded for additional expansion. Manual expansion is time-consuming and labour-intensive with bioreactors being the future strategic approach to generate MSCs in sufficient amounts to fulfil demand. However, it must be considered that changes in culture processes may affect the function of the MSCs. MSC production for clinical use must show compliance with good manufacture practices (GMP), with established methods and must pass the pre-defined product specification according to European Standard Criteria (Sensebe, Bourin et al. 2011). Nevertheless, as already highlighted, MSC batches are heterogenic and therapeutic efficacy still differs considerably between laboratories; dependent on donor age, health, passage or doubling numbers but also on the patient being treated. An example of this discrepancy can be observed in two different clinical trials that used BM MSCs to treat aGvHD (Le Blanc, Frassoni et al. 2008, Galipeau 2013). An American phase III trial used MSCs expanded to very high passage numbers from just one donor compared to the European phase II trial, where low passage numbers were used and a better patient outcome was observed (Le Blanc, Frassoni et al. 2008, Galipeau 2013). However, these data should be interpreted cautiously because the recipient patients were naturally different.

1.3.1.2 Administration of MSCs

MSCs have been administrated through different methods depending on the disease and clinical trial. The delivery route is often chosen based on the indication, with the intention to maximize engraftment and effect of the cells. The most common delivery route is I.V., although other alternatives such as intra-arterial (IA), local injection, for example intraspinal, intrathecal, intracardiac, and sometimes with a scaffold, such as a hydrogel (Borden, Yockman et al. 2010, Molendijk, Bonsing et al. 2015, Thompson, Matsiko et al. 2016). The time of administration, number of repeated injections and number of cells can be crucial for the clinical outcome. Zappia *et al.* reported in an experimental autoimmune encephalomyelitis (EAE) model that administration of MSCs at early stage or even prior to disease progression showed improvement of EAE, whereas administration after disease development had no effect (Zappia, Casazza et al. 2005). Moreover, MSCs effect on Th-cell subsets and local joint disorder was found to be dependent on the timing of administration in an experimental arthritis model (Bouffi, Bony et al. 2010, Tso, Law et al. 2010, Moll, Ankrum et al. 2019). However, in humans, it is hard to control the disease course and ideal time of administration. Moreover, HLA alloimmunization can occur after consecutive transfusions. The immunogenicity can be both intrinsic and acquired from xeno-supplemented culture media and effect the response to MSCs (Haque, Kasim et al. 2015).

Commonly, MSCs are expanded, frozen and thawed on the day of treatment. The use of fresh cells is not necessary for MSC therapy, as the cells survive freeze-thaw cycles well but studies have indicated that fresh MSCs are more potent than frozen (Moll, Rasmusson-Duprez et al. 2012). This is a benefit since it allows for quality control of the cells whilst in cryo-storage. With a standardised and careful freezing and thawing processes, the viability of MSCs are over 90 %. Frozen cells can recover from freeze thawing damage after only 24 hours in culture (Francois, Copland et al. 2012).

1.3.2 Autologous versus allogenic MSC cell therapy

The advantage of using autologous MSCs is the limited risk for transmission of donor-derived infections, prions and diseases, as well as HLA mismatches that can lead to rejection and immune activation (Carlsson, Schwarcz et al. 2015). Nevertheless, it is important to take into account that different diseases can cause alterations in the stromal cell compartment, impairing autologous MSCs. This may be true for multiple sclerosis as an example, where autologous MSCs are under investigation (Uccelli, Laroni et al. 2019). Several studies propose no differences exist in therapeutic outcome between autologous and allogenic MSC sources (Planka, Gal et al. 2008, Wolf, Reinhard et al. 2009, Davies, Alm et al. 2016). In *in vitro* assays, autologous MSCs from T1D patients showed similar phenotypical and functional characteristics, including cytokine and chemokine secretion, and suppression of T-cell proliferation, compared to BM MSCs from healthy donors although some gene expression differences were observed (Davies, Alm et al. 2016). Autologous cells cannot be used in fulminant diseases, since the urgency of the situation requires immediate availability of cells and the time for expansion is restricted. This is a major advantage of allogenic MSCs, since they can be harvested from a healthy donor beforehand, expanded, tested and stored. Immunogenicity of the graft is a challenge in all transplantations, albeit MSCs exhibit lower immunogenic potential than other allogenic cells (Eliopoulos, Stagg et al. 2005, Ankrum, Ong et al. 2014). MSCs lack the expression of co-stimulatory molecules and express low levels of MHC class II proteins. Nonetheless, they are not completely invisible to the recipient's immune system. They express MHC class I molecules and can therefore theoretically trigger an allogeneic immune response (Gazdic, Volarevic et al. 2015). Fortunately, at least the immune suppressive property of MSCs is MHC-independent and non-antigen specific (Le Blanc, Tammik et al. 2003, Duffy, Ritter et al. 2011, Galleu, Riffo-Vasquez et al. 2017).

However, unlike other types of transplantation, allogenic MSC are not HLA matched to the recipient in order to prevent an allo-rejection. Right- or wrongfully, MSCs are considered immune privileged and clinical trials have not yet suggested HLA matching to impact on MSC response. On the other hand, the presence of allo-antibodies after MSC infusion have been reported in both human and animal studies (Isakova, Lanclos et al. 2014, Owens, Kol et al. 2016). Thus, allo-antibodies produced against MSCs in the host might induce a comparable cascade of immune cell activation to the one engaged when antibodies are produced against pathogens. As a result, complement will be activated, Fc receptor will bind and activation of monocytes, neutrophils, and NK cells will occur. MSCs are protected, despite MHC class II activation of allo-reactive T cells, due to the absence of co-stimulatory

molecules and the secondary signal will not be activated, leading to T cell anergy (Klyushnenkova, Mosca et al. 2005, Machado Cde, Telles et al. 2013). Regardless of whether the MSCs are autologous or allogeneic, their unique ability to inhibit T cell allo-responses is present, and appears to be independent of the MHC (Le Blanc, Tammik et al. 2003).

1.3.3 The role of the patient opposed to MSCs in therapeutic success

The concept of "good" versus "bad" MSCs has long been discussed in the MSC field, referring to MSCs batches, often from different donors, with or without a clinical response in the recipient. However, it is possible that patient factors rather than the properties of the cell batch decide patient responsiveness. Nevertheless, no *in vitro* potency test is available that correlates with a positive response to treatment. von Bahr *et al*. investigated *in vitro* immunosuppressive capacity of MSCs used to treat the patient as a predictor of the patient response to MSC therapy (von Bahr, Sundberg et al. 2012). Using an *in vitro* allogeneic MLC assay, no correlation between MSC responders and T-cell suppressive potential of MSCs was observed. Moreover, unpublished data from the Le Blanc group on chronic GvHD patients, observed characteristics in the immune profile in the peripheral blood of responder patients, including an increased level of CD8+ T cells. This data suggests that MSCs may need to be primed/triggered to accomplish their therapeutic effect in the host. This concept fits well with recently published studies in mice, showing the primordial role of host CTL CD8+ cells in response to MSCs (Galleu, Riffo-Vasquez et al. 2017). This indicates the importance of evaluating the patient's immune profile at the time of injection and its correlations to response to MSC therapy. There are limited studies available on the immune cell profile of either whole blood or tissues before adoptive cell transfer.

1.3.4 MSCs in type 1 diabetes mellitus

T1D is a metabolic disorder characterized by excessive glucose levels due to insulin resistance in peripheral tissues or insufficient production of insulin by the pancreas. Insulin-producing beta (β)-cells in the pancreas are gradually destroyed by autoimmune attacks by autoantibody-producing B cells and autoreactive CD4+ and CD8+ T cells (Carlsson, Schwarcz et al. 2015). A very small fraction of the β-cells remains when the patient begins to show symptoms (Klinke 2008). Optimized glucose control and a delayed or reduced need for exogenous insulin by preventing further destruction of the remaining β-cells is believed to particularly reduce long-term complications of the disease. There are no clinically available treatments to halt β-cell deterioration(Carlsson, Korsgren et al. 2015). Several characteristics of MSCs make them attractive for T1D therapy. First, their immunosuppressive effect on activated T-cells that is presumed to mediate β-cell destruction, that could rescue some β-cell function and slow down T1D progression (Lee, Seo et al. 2006, Urban, Kiss et al. 2008, Ho, Tseng et al. 2012, Gabr, Zakaria et al. 2013). Second, MSCs stimulate angiogenesis, which can support pancreatic islet recovery (Gamble, Pawlick et al. 2018). Previously, in collaboration with Uppsala University, the Le Blanc group showed that infusion of autologous MSCs to newly diagnosed T1D patients was safe and could preserve

β-cell function, which was shown by maintained endogenous insulin-response to a mixed-meal tolerance test (MMTT) (Carlsson, Schwarcz et al. 2015). However, many cell types isolated from T1D patients show intrinsic abnormalities (Drexhage, Dik et al. 2016).

Chronic exposure to hyperglycemia causes cellular dysfunction that has the potential to become irreversible over time. Damage of glucose toxicity can be seen in many organ complications caused by diabetes, such as vascular degeneration, retinopathy, neuropathy, ulcers and kidney disease that all feature a reduced capacity for wound healing (Forbes and Cooper 2013). Albierto *et al*. have shown that the BM is also changed in T1D, in that patients have a reduced capacity to mobilize CD34+ HSCs (Albiero, Poncina et al. 2015). These changes affected the levels and polarization of macrophages in the circulation in patients with type 1 and type 2 diabetes, as well as T1D responsiveness to the CD34+ HSCs mobilizing agent granulocyte colony stimulating factor (G-CSF) (Fadini, Albiero et al. 2013, Albiero, Poncina et al. 2015). A murine T1D model (streptozotocin-treated animals), demonstrated that soluble-factor oncostatin M (OSM) induced expression of C-X-C motif chemokine receptor (CXCLR)1 on macrophages, which in turn potentially increased the homing of CD34+ cells to the BM and reduced the response to G-CSF.

Human cell *in vitro* experiments showed that treatment of BM MSC with conditioned medium from different macrophage populations increased C-X-C motif chemokine (CXCL)12 (stromal cell-derived factor 1(SDF-1)) levels in MSC cultures treated with pro-inflammatory (M1) but not intermediate macrophages (M0) or anti-inflammatory (M2) conditioned medium. CXCL12/CXCLR4 interactions are important in the retention of HSCs and their progenitors in the BM, a site characterized by high CXCL12 expression (Liekens, Schols et al. 2010).

Autologous MSC therapy has been evaluated in clinical trials to treat non-healing wounds in diabetics (Cao, Gang et al. 2017). The beneficial results indicate that although, there may be differences at a transcription level, the functionality and the safety profile of T1D indicate the same effects as BM MSC derived from healthy donors.

1.3.5 MSCs in acute GvHD

GvHD causes treatment-related morbidity in patients after allogeneic HSCT, despite the use of immunosuppressive prophylaxis (Amorin, Alegretti et al. 2014). Non-self-antigens in the host are recognized by immune cells in the graft, resulting in an immune response against the host tissue, mediated mainly by cytotoxic T-cells. The acute form of GvHD presents with a strong inflammatory response whereas chronic GvHD shows a more autoimmune-like syndrome and fibrotic tissue response. aGvHD typically manifests in skin, liver and GI. The overall severity of aGvHD is often graded by an accumulated scoring of severity and number of organs involved, the so-called Glucksberg criteria (Przepiorka, Weisdorf et al. 1995).

Grade 1 aGVHD is considered to be mild, grade 2 moderate, grade 3 severe and grade 4 very severe. Of all HSCT patients 30 to 50 % have aGVHD (grades 1–4) with 14 % presenting with severe aGVHD (grades 3–4) (Zeiser and Blazar 2017).

Pathologists use a specific histological GI classification from I-IV, depending on immune cell infiltration and damage to the tissue for GI aGvHD diagnostics (Przepiorka, Weisdorf et al. 1995, Shulman, Kleiner et al. 2006). Steroids are the first line treatment but unfortunately not all patients respond. Steroid-refractory aGvHD is defined as resistance to treatment with no overall improvement in GvHD grade or disease progression (Le Blanc, Frassoni et al. 2008). There are few established second line options available for steroid-refractory patients. MSC therapy emerged because of their immunosuppressive properties and their ability to promote tissue healing (Amorin, Alegretti et al. 2014). Le Blanc *et al*. reported the successful infusion of MSCs to aGvHD patients in a phase II study that resulted in a 55 % complete and 16 % partial response rates (Le Blanc, Frassoni et al. 2008).

A question remains as to why some patients responded to MSC treatment and others not. MSCs interact with innate immune cells and one such cross-talk is with mast cells. They play an important role in allergies and various autoimmune diseases (Amin 2012, Kolb 2013). Mast cells secrete various pro-inflammatory mediators, such as histamine, tryptase and several other cytokines. MSCs are shown to suppress mast cell functions, *in vitro* and *in vivo* through the cyclooxygenase2 (COX2)/PGE2 pathway (Brown, Nemeth et al. 2011). Interestingly, post-HSCT increased levels of immunoglobulin (Ig)E have been reported at the time of aGvHD onset, suggesting a role for mast cells in the induction of GvHD (Kolb 2013). One possibility suggests that mast cells may accumulate during onset in the gut of aGvHD patients and their interaction with infused MSCs might change them from a pro-inflammatory to an anti-inflammatory phenotype.

To summarize, understanding MSC fate after infusion including, resistance to complement attack, distribution, and how MSCs interact with immune cells, specifically the innate immune system, in the local tissue where diseases manifests, is fundamental for the development of efficient targeted therapies in autoimmune and inflammatory disorders.

2 AIMS AND HYPOTHESES OF THE THESIS

The overall aim of this thesis was to increase the understanding of MSC interactions with the innate immune cells and with blood compounds at treatment, both in the presence or absence of inflammation.

Specific aims for each study

I. To study the effect of complement interactions with MSCs with respect to survival and functionality.

II. To compare MSCs from healthy donors to autologous MSCs from T1D patients, to ensure they have normal functionality and potential use in therapy.

III. To understand and determine the role of the GI mucosa local immune cell profile in influencing responsiveness of aGvHD patients to MSC treatment.

Hypotheses

I. MSCs are targeted by phagocytic cells via opsonisation by complement proteins on the MSC surface. Therefore, MSCs ‘’disappear’’ from the circulation after infusion. Thus, this results in MSC-mediated immunosuppressive modulation of the immune cell that leads to reduced inflammation.

II. T1D does not compromise MSC function and therefore allows adoptive transfer of autologous cells.

III. The immune cell profile in aGvHD gut biopsies correlates with the level of response to MSC-treatment, which in turn will predict those patients that will respond to MSCs treatment.

3 METHODOLOGICAL APPROACHES AND CONSIDERATIONS

This section will discuss general considerations and key methods employed in the studies. For detailed materials and method of each paper, please see the respective articles.

3.1 ETHICAL CONSIDERATIONS

Ethical Review Boards in Sweden approved all projects before any work was conducted. In this thesis, only human samples have been used but we recognise the importance of testing new drugs and therapies in animal models before human trials. Great care was taken in planning before conducting any experiments, to minimize any errors and limit the use of human samples, thus avoiding the unnecessary cost of repeated experiments.

The main cell type used in these studies were BM MSCs taken from iliac crest aspirates of healthy volunteers or TID patients, by trained physicians. The procedure was routine and relatively complication-free. All safety parameters were considered. Written consent was obtained from all donors, where they approved the use of their cells for research. All ethical numbers were included in the corresponding manuscripts.

Fresh whole blood and plasma were required to investigate the role of complement proteins after MSC exposure *in vitro*. Donors were informed of the study and its aim, and joined on a voluntary basis. Peripheral blood (9-18 ml) was taken in a routine procedure by medical research staff of Karolinska Institutet /University Hospital from the arm. This volume was considered small and should not be at any risk or affect the donor. The general recommendations for blood donation in Sweden is 450 ml blood three (women) – or four (man) times per year (Geblod.nu).

aGvHD biopsies were taken from patients for routine diagnostic purposes to evaluate whether they had aGvHD. Ethical permission was approved to use these biopsies for research purposes. It is considered ethically appropriate to use these ‘’archived patient biopsies’’ taken for diagnostic purpose in scientific research, since further knowledge of disease development and how to best treat patients will be of benefit to both current and future patients.

3.2 PATIENTS, DONORS AND CELLS

3.2.1 Cell preparation

Human BM-derived MSCs were isolated and characterised as described previously (Caplan 1991, Le Blanc, Frassoni et al. 2008). BM MNCs were separated by Percoll® density gradient centrifugation. Cells were expanded in growth medium, detached and passaged at a minimum cell confluence of 70 %, and cultured until a sufficient number of cells was obtained. At harvest, the cells were frozen in 10 % dimethyl sulphoxide (DMSO) / Dulbecco's

modified eagle medium (DMEM) complete medium until further use. Due to the heterogeneous nature of MSCs, and the absence of a definitive marker, flow cytometric analyses were used to confirm >95 % expression of CD73, CD90, CD105, HLA-ABC and <5 % expression of CD14, CD31, CD34, CD45 and HLA II cell surface marker in each MSC culture.

All *in vitro* experiments included MSCs cultured at low passage (P2-4), which corresponded to the same passage used in our clinical projects. Additionally, cells were thawed on the day of experiments in study I to mimic the clinical setting. However, in study II fresh cells were used and most *in vitro* studies were performed on freshly cultured MSCs.

3.2.2 Plasma preparation

About half of the blood of the human body consists of plasma/serum. In our study, plasma was obtained by directly adding an anticoagulant and centrifuging at 2000 g for 10 minutes at 4 °C. The plasma was kept at 4 °C until use. Serum, on the other hand, was prepared by allowing clotting at room temperature to remove fibrinogens. All proteins involved in blood clotting were removed by centrifugation at 2000 g for 10 minutes at 4 °C. The advantage with plasma is that it is a controlled removal of the clotting proteins and has an equal process every time. The thrombin inhibitor used was Lepirudin, which does not affect the complement system (Gosselin, Dager et al. 2004). For negative controls ethylenediaminetetraacetic acid (EDTA) (10 mM), was used to stop the complement system and additionally heat inactivated plasma was used to cause protein aggregation.

3.3 METHODOLOGICAL CONSIDERATIONS

3.3.1 Licensing of MSCs

To mimic the disease state where MSCs are surrounded by an inflammatory milieu, a common protocol for *in vitro* response was applied, where the cells were exposed to pro-inflammatory cytokines (Le Blanc, Tammik et al. 2003). IFN-γ has an effect on MSC suppressive potency and upregulation of MHC class II expression, although the response depends on the dosage and duration (Hemeda, Jakob et al. 2010). The dosages used in this thesis were based on previous observations on HLA upregulation in MSCs (Le Blanc, Tammik et al. 2003). Additionally, MSCs were exposed to plasma prior to licensing. Briefly, MSCs were exposed to 50 % plasma +/- 10 mM tri-potassium (K3) EDTA, heat inactivated plasma or DMEM complete medium for 1 hour. Cells were washed, replated and thereafter licensed with 10 ng/ml TNF-α and 100 U/ml IFN-γ for 72 hours. For the detection of intracellular IL-6 and IDO, GolgiPlug™ (BD Biosciences) was added 5 hours prior to the end of the experiment. Analysis was done by flow cytometry (BD LSR Fortessa, BD Biosciences). For analysis of IDO activity supernatant was collected and the tryptophan metabolite L-kynurenine was quantified as previously described (Davies, Lonnies et al.

2012). IL-6 was measured in the supernatant using enzyme-linked immunosorbent assay (ELISA) according to the supplier's instructions (R&D Systems, Minneapolis, MN).

3.3.2 Survival assays

To detect if MSCs died after exposure to plasma, we used a recently established protocol for platelet survival, and they were used as positive control (fig.3.1) (Meinke, Sandgren et al. 2016). MSCs were loaded with calcein red-orange acetoxymethyl ester (calcein RO AM) (Molecular Probes, Eugene, OR) at a concentration of 2.5 µg/mL in phosphate-buffered saline (PBS), and incubated for 10 min at 37 °C, prior to culture. This dye was used because it remains contained within the cytoplasm until the cell membrane is disrupted. Briefly, MSCs were thawed and labelled with calcein. They were exposed to 50 % plasma, or control media (50 % EDTA-plasma, 50 % heat inactivated plasma, or media only) for 1 hour at 37°C. Subsequently washed and stained for flow cytometry analysis. Anti-human C3c antibody was used to confirm that the plasma was active and that complement peptides (C3b/iC3b) were deposited on the surface of MSCs (Moll, Jitschin et al. 2011).

Li and colleagues assessed MSC injury in the presence or absence of active complement *in vitro* through the release of the fluorescent dye bis-carboxyethyl-carboxyfluorescein by MSCs. However, this dye can leak from cells spontaneously, therefore we used the fluorescent dye calcein to confirm the data (Lichtenfels, Biddison et al. 1994).

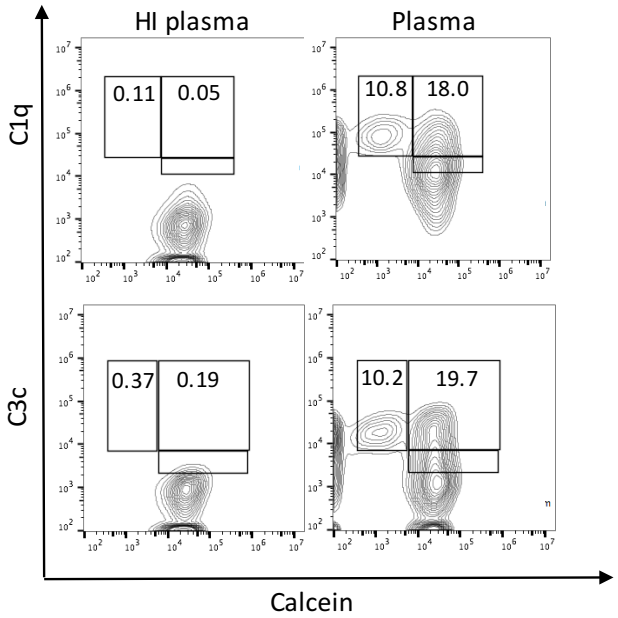

Figure 3.1. *Cell survival determined by using calcein leakage assay. Method setup by using platelets as controls, incubated with active plasma for 10 minutes. Deposition of C3b/iC3B (C3c-FITC antibody) and C1q (C1q-FITC antibody) was evaluated by flow cytometry with the gating strategy shown in these plots.*

3.3.3 Phagocytosis assay

Phagocytosis was determined by the detection of engulfed cell fragments inside the phagocyte. In this study, we used pHrodo because of its advantage of only being fluorescent at acidic conditions, which is the case inside the lysosome (~pH 4) but not in the cytoplasm (~pH 7) nor outside of the cell. Phagocytosis was detected inside the lysosome after 2 hours incubation of MSCs with pHrodo bright fluorescence by flow cytometry (BD Fortessa LSR-II). Briefly, MSCs were labelled with 51 nmol/L pHrodo succinimidyl ester (Molecular Probes) in PBS for 10 minutes at room temperature, thereafter exposed to active plasma or control conditions. Subsequently the negative control fraction was incubated with 10 µg/mL Cytochalasin D (Sigma-Aldrich, http://www.sigmaaldrich.com), to inhibit actin polymerization and thereby block phagocytosis. Co-cultures of MSCs with PBMCs or the macrophage cell line (THP-1) were performed for 2 hours. Experiments were stopped, centrifuged and stained with flow cytometry antibodies for evaluation.

3.3.4 Blood chamber assay

To better understand the effect of blood components on MSCs, we used a whole blood chamber technique developed at Uppsala University (Nordling, Nilsson et al. 2014). This system has chambers attached to glass slides and are pre-treated with heparin to prevent complement activation by the plastic surfaces. Briefly, fresh whole blood was drawn from a healthy volunteer, and slowly fed into a pre-heparinised tube, to not activate the complement system, on the day of the experiment. Lepuridine was added to block thrombin and consequently the coagulation pathway. Whole blood was transferred into chambers attached to glass slides. MSCs were prepared beforehand and ready to add into the blood chambers. Chambers were incubated on a rotator at 37 °C for 5 minutes, 30 minutes, 1 hour and 3 hours. The reaction was stopped by adding 10 mM K_3EDTA, which was also used as a negative control. C3b/iC3b deposition (C3c-FITC antibody), CD73-APC and 7-Aminoactinomycin D (7AAD) was measured on FlowSight® Imaging Flow Cytometer (Merck Millipore, Billerica, MA). The advantage of this technique over traditional flow cytometry was that every single cell could be visualised in the bright field.

3.3.5 T1D Study

In this project, general differentiation protocols for adipocyte and osteoblast assays were used. We looked at both early (P2) and late (P6) passages, to confirm that there was no impairment during the time of culture or if MSC expansion was limited.

Flow cytometry was used to identify any differences in expression pattern, including complement regulators, such as CD46, CD55 and CD59. These were assessed to establish MSCs potential protection against the complement system.

We performed microarray data on MSCs from healthy and T1D patients. We wanted to confirm differentially expressed genes from microarray analyses using quantitative real time

(qRT)-polymerase chain reaction (PCR) methods. We choose the genes of interest with the help of the gene ontology (GO) term analysis program, which performs enrichment analysis on gene sets. This was applied on the microarray data to identify, which biological pathways involved in the differentially expressed genes between T1D and healthy MSCs.

3.3.6 Acute GvHD study

To determine if the immune cell profile of steroid-refractory aGvHD patients segregated with clinical responses following MSC treatment, GI mucosa biopsy specimens were collected in collaboration with the Pathology Department at Karolinska University Hospital. Based on availability of biopsy material, eight responders and eight non-responder patients were included in the study. Biopsies were taken for diagnostic purposes to assess patients with aGvHD-like symptoms, including diarrhoea, prior to MSCs transfer.

Biopsies were stained using the automatic stainer at the Pathology Department with 3, 3 - diaminobenzidine; DAB, to identify antibodies targeted against CD4, CD8, Foxp3, CD56, CD68 and β-tryptase (mast cell marker). Markers were selected on the basis of their involvement in aGvHD. Biopsies were digitalised and immunohistochemical staining quantified on images using the open-source software CellProfiler version 2 (https://cellprofiler.org/). All images were 40x magnification on 1366x768 screen size = 683x706 dpi/image. Within CellProfiler each marker could be optimally localised based on the positive chromogenic DAB staining, meaning that separate workflows were established for each respective antibody (Iacobaeus, Sugars et al. 2017, Tollemar, Tudzarovski et al. 2018). Data were normalised with total pixel area of DAB stain divided on total area of pixels of the image to provide relative distribution of the marker staining. Statistical assessment was made in collaboration with the Unit of Biostatistics at Karolinska Institutet. Statistical analyses were performed using generalized estimated equations with Poisson family in Stata version 14 (StatCorp LLC, TX, USA).

4 RESULTS AND DISCUSSION

MSCs have been widely studied in both clinical, pre-clinical and *in vitro* settings, and discoveries through the years have increased our understanding of MSC immunomodulatory and regenerative mechanisms. In this thesis, we have focused on MSC cell therapy and presented new findings on their fate after injection, the function of autologous T1D MSCs, and examined the optimal gut immune cell profile for aGvHD patients to support response to MSC treatment. The main results of studies I-III are presented and discussed. Detailed figures, results and discussions for each paper can be found in the respective articles.

4.1 THE COMPLEMENT SYSTEM IS ESSENTIAL FOR PHAGOCYTOSIS OF MSCS BY MONOCYTES (STUDY I)

The main goal of this study was to evaluate the interactions of MSCs after contact with blood components, plasma and immune cells, and more specifically monocytes. Previous literature has shown MSCs to be rapidly cleared from the circulation after I.V. infusion, despite this they still elicit a therapeutic effect. Therefore, we sought to reveal their mechanism of action.

4.1.1 Results

Blood chamber experiments and cell cultures containing 50 % plasma were employed to challenge MSCs at different time points to evaluate any impairment to the cells after exposure to plasma.

The key findings are the following:

- MSCs bound C3b/iC3b on their surface without causing cell death.
- Deposition of C3 fragments on MSCs did not effect cell function, including T cell supression, response to licensing (IDO and IL-6 expression) and differentiation.
- MSCs might be protected from complement lysis by CD59, a complement regulator of the MAC.
- C3 fragment deposition on the surface of MSCs enhanced MSC phagocytosis by classical and intermediate monocytes.

The knowledge obtained from this study is of major significance to the field of MSC therapy. The enhanced uptake of MSCs by monocytes due to the deposition of the C3 fragment onto their surface, means that most of the observed effects of MSCs are driven through monocytes. To determine whether MSCs survived and functioned upon contact with blood components, and active plasma was addressed by incubating MSCs with plasma or blood for different time periods. The highest C3b/iC3b deposition was observed after one hour of incubation (fig. 4.1). The deposition of C3b/iC3b onto the MSCs varied (10-60 %) amongst donors but viability was not affected after plasma exposure. This outcome was further supported using a fixed concentration of purified C3 protein. It was concluded that after contact with plasma, MSCs survived and preserved functionality, such as their immunosuppressive properties,

cytokine production in response to high concentrations of IFN-γ and TNF-α licensing, and differentiation capacity into adipocytes and osteoblast (fig. 4.2).

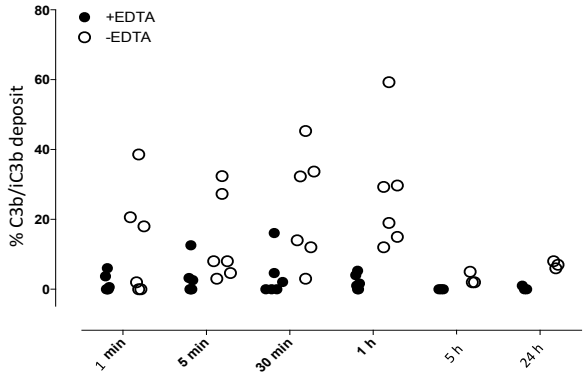

Figure 4.1. *MSCs exposed to complement through active or inactivated plasma for 1, 5, 30 minutes and 1, 5, 24 hours. The percentage of C3-FITC antibody deposition was measured by flow cytometry. The complement peptides have probably been processed or internalized after 5 and 24 hours and can therefore no longer be detected.*

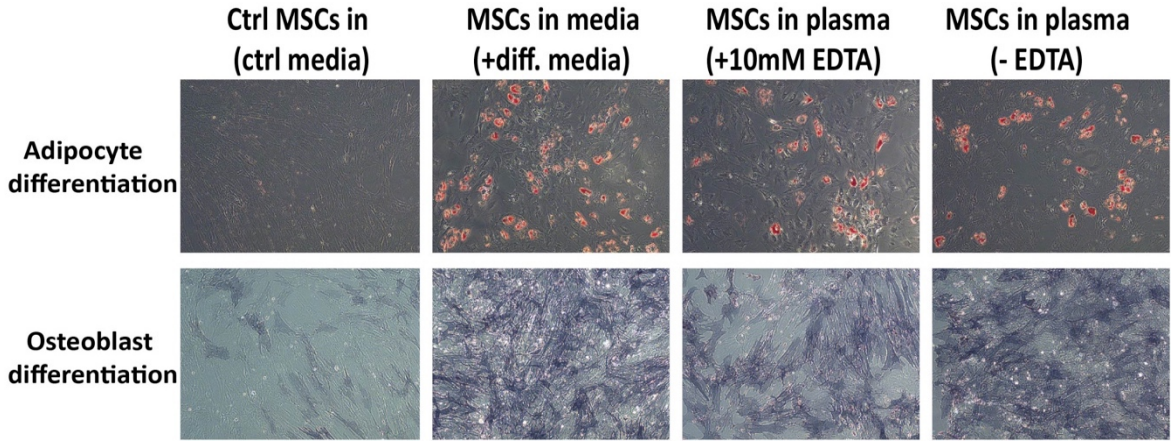

Figure 4.2. *MSCs maintain similar potential to differentiate into adipocytes and osteoblasts after contact with plasma for one hour and thereafter cultured in differentiation media compared to control.*

Survival and C3b/iC3b deposition was evaluated in a whole blood chamber assay for 5 minutes, 30 minutes, 1 hour and 3 hours (Nordling, Nilsson et al. 2014). All data showed similar results at all time points. MSCs survived and deposited C3b/iC3b onto their surfaces and interestingly the deposition of C3b/iC3b was polarized on the cell surfaces (fig. 4.3).

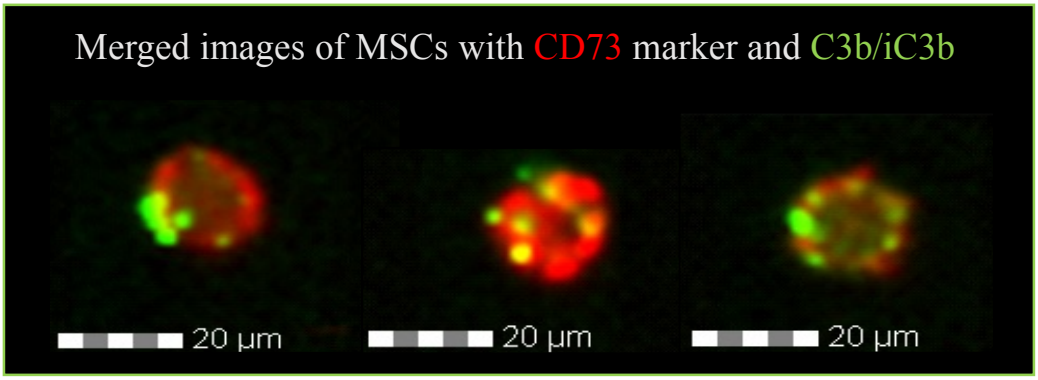

Figure 4.3. *MSCs exposed to whole blood, with additional Lepuridin, a thrombin inhibitor. The C3b/iC3b deposition was visualized by C3c-FITC antibody and MSCs stained with CD73-APC antibody on FlowSight® Imaging Flow Cytometer (Merckmillipore).*

To ascertain what protects MSCs against a complement attack, the complement regulators, CD46, CD55 and CD59 were located on the MSCs surface. Their expression remained unchanged after MSCs exposure to plasma compared to controls. Antibodies against CD46, CD55 and CD59 were used as inhibitors in a survival/leakage assay. MSCs were loaded with the cytosolic dye calcein that leaks from the cell if the MAC is inserted into the plasma membrane. By blocking CD59, C3b/iC3b deposition accumulated on the surface of MSCs prior to calcein leakage and resulted in a change in cell shape and granularity.

We hypothesised that MSCs were rapidly cleared from the blood circulation after I.V. injection by phagocytes. This was supported by the rapid deposition of C3 fragments on MSCs after contact with blood, acting as opsonisation agents for phagocytosis. Phagocytosis was assessed using pHrodo, as described above in section 3.3.3 (Miksa, Komura et al. 2009). Approximately 50 % of MSCs were phagocytosed by monocytes (gated on CD14+) *in vitro*, after pre-treatment with plasma. Interestingly, in the absence of complement peptides 15-25 % of MSCs were also phagocytosed by monocytes, suggesting other factors beyond complement binding may also mediate phagocytosis.

Upon examination of which monocyte fraction phagocytosed the MSCs, it was apparent that classical ($CD14^+$ $CD16^-$) and intermediate ($CD14^+$ $CD16^+$) monocytes were involved, rather than non-classical ($CD14^-$ $CD16^+$) monocytes. A positive correlation was found between C3b/iC3b deposition on the MSC surface and the percentage of pHrodo bright monocytes detected. Through the use of C3 and C5 inhibitors, a significant decrease in phagocytosis was observed when a C3 inhibitor was added.

4.1.2 Discussion

A general consensus in the MSC field is that the cells rapidly disappear from the circulation after adoptive transfer (Eggenhofer, Benseler et al. 2012, Eggenhofer, Luk et al. 2014). The complement system is essential in the innate immune system to trigger phagocytosis of injured cells or pathogens and to puncture the plasma membrane with the MAC (Merle, Noe

et al. 2015). Therefore, many groups have postulated that the "MSC effect" occurs via the release of extracellular vesicles or exosomes after interaction with complement.

This current study focused only on the complement system and did not take into consideration coagulation, thereby excluding the instant blood mediated inflammatory reaction (IBMIR). Complement and coagulation interact and are closely linked *in vivo* but to avoid complexity in our experimental system, we used the thrombin inhibitor, Lepuridin to block the coagulation pathway and concentrated on the complement system. Thus, MSCs were found to be fully functional and to survive contact with complement active plasma and blood.

Some cell types exist that are hemacompatible, such as endothelial cells. However, some cells used in patients that are not compatible are destroyed at I.V. injection, like pancreatic islets (Bennet, Sundberg et al. 1999). To prevent complement activation, these cells have complement regulators on their surfaces, such as CD46, CD55 and CD59 (Zipfel and Skerka 2009). To confirm the role of these regulators on MSCs, we used inhibitors against them and confirmed a protective role of CD59 in active complement plasma. These data corroborate findings by Lin *et al*. who showed that CD59 protects against complement-mediated cytotoxicity (Li and Lin 2012). Additionally, we saw an increase in C3b/iC3b deposition on the CD59 inhibited cells. We cannot conclude that CD59 regulates C3b/iC3b deposition without confirming our findings using on more CD59 inhibitor antibody or using MSCs deficient in CD59. The anti-CD59 antibody used in this study was a murine IgG2a, which can itself activate human complement through the classical pathway. Of note, the other inhibitors used were IgG subtypes. This also limits comparability of the different inhibitory molecules.

Next, to study whether MSCs are injured after contact with active complement, we used a different fluorescent dye (calcein) to detect cytoplasmic leakage than the one used by Lin *et al* (BCECF) (Li and Lin 2012). This may also contribute to the discrepancies between the results of the two studies. However, our findings are supported by mouse and human *in vivo* tracing experiments that have identified MSCs in different organs after infusion (von Bahr, Batsis et al. 2012, Vaegler, Maerz et al. 2014, K, P et al. 2015, Braza, Dirou et al. 2016).

Dazzi's group reported on increased phagocytosis of killed MSCs *in vivo*. We showed *in vitro*, that there was an additional mechanism of enhancing phagocytosis of live MSCs by complement deposition. However further studies are needed to confirm these data *in vivo*. Interestingly, phagocytosis was also seen control media and heat inactivated plasma but at a lesser degree, possibly attributable to MSC expression of adhesion molecules that allow monocytes to adhere and trigger this process (Rosales and Uribe-Querol 2017, Rubtsov, Goryunov capital Ka et al. 2017). Monocytes express receptors for different C3 derivative fragments, including CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18) and CR4 (CD11c/CD18), which are all involved in phagocytosis (Jongstra-Bilen, Harrison et al. 2003). Further experiments are needed.

Whether MSCs can be phagocytosed by other cells or only by monocytes remains an open question. Indeed, a number of phagocytes exist in the body, including macrophages and neutrophils. These sub-populations can display differential expression of phagocytic

receptors, including pathogen-associated molecular patterns receptors, apoptotic receptors, patterns of endogenous danger receptors and the opsono-phagocytic receptors. Therefore, it is logical to speculate that different mechanisms might be involved in the phagocytosis of MSCs by other cells.

In our study, mainly classical and intermediate monocytes phagocytosed MSCs. It would be of interest to study the fate of these monocytes post-phagocytosis of MSCs. de Witte *et al.* found umbilical MSC fragments on monocytes in the lungs 24 hours after injection and three hours after co-cultures *in vitro* (de Witte, Luk et al. 2018). They could see a shift of monocytes towards an anti-inflammatory phenotype, although this needs to be confirmed in future studies. Moreover, in a murine asthma model, Braza *et al.* found injected labelled MSC were engulfed by lung macrophages within 24 hours following I.V. injection (Braza, Dirou et al. 2016). Both these studies used the lipophilic membrane dye, PKH26, which can incorporate through a cell-to-cell membrane transfer process called trogocytosis that can give rise to false positive signals (Wallace, Tario et al. 2008, Jambou, Combes et al. 2010).

Thus, this current study provides new insights into the fate of MSCs and for the first time proposes a mechanism of interaction between MSCs and phagocytic cells that could in turn be used to improve approaches of adoptive MSC cellular therapy.

4.2 TYPE 1 DIABETES MELLITUS DONOR MSCS EXHIBIT COMPARABLE POTENCY TO HEALTHY CONTROLS IN VITRO (STUDY II)

The aim of this study was to evaluate if MSCs from T1D patients were impaired using functional and phenotypic characterisation of the cells. Our group has previously demonstrated that autologous MSCs benefit the treatment of T1D (Carlsson, Schwarcz et al. 2015). As mentioned above, autologous cells have certain advantages to allogenic cells, such as avoiding rejection, immunisation and transmission of diseases, as long as their function is not impaired by the disease.

4.2.1 Results

MSCs from healthy controls and T1D patients showed similar functional potential in a selected set of *in vitro* assays, based on immunotherapy potential measured *in vitro*. T1D MSCs were compared to healthy controls in regard to growth kinetics, CFU-F capacity, cytokine secretion, immunomodulatory activity and wound healing potential.

The key findings are the following:

- Comparable *ex vivo* expansion capacity between T1D MSCs and MSCs from healthy controls.
- No difference in immunomodulatory phenotype, suppressing T-cell proliferation.
- No difference in preserved hemocompatibility.

Differentially expressed genes between healthy and late-stage T1D MSCs were identified by microarray analysis but phenotypic and functional experiments showed no significant differences between the two cell populations. Thirty transcripts were more than 1.5-fold expressed in MSCs from early-stage T1D donors and 211 out of 47,000 differentially expressed in late-stage T1D donors compared to healthy controls. A majority of these genes were upregulated in late-stage renal failure T1D. The GO term analysis involved pathways of growth factor activity, multicellular organism development and processing, response to external stimuli, stress and wounding, adenylate kinase and the TGF-β-signalling pathway.

Unpredictably, the *in vitro* scratch wound assay showed no difference in time-dependent scratch wound closure between healthy and late-stage T1D MSCs. Meanwhile in the resting unscratched state, the GO term of "response to wounding" from the microarray analysis was confirmed by qRT-PCR. However, at 24 hours following scratch wounding, there were no differences between late-stage T1D and healthy MSCs, in expression levels of any of the nine genes analysed. Therefore, these data suggest similar wound closure capacity between the cell types.

The CFU-F capacity was reduced in the early T1D MSCs compared to healthy controls, suggesting a reduced number of cells in the BM MSCs progenitor pool of T1D patients. However, MSCs cumulative population doubling (P6) and population doubling time at P1 showed no difference in growth rate, indicating similar expansion times during *in vitro* culture. We further evaluated if the differentiation capacity of adipogenic and osteogenic lineages *in vitro* were impaired by the diabetic environment but comparable results were observed between the groups.

To evaluate the immunomodulatory phenotype of MSCs, we compared the constitutive secretion of IL-6, VEGF, TGFβ and SDF1-α between the groups and found them to be similar. Moreover, MSC immunosuppressive activity was assessed by co-culturing MSCs with alloantigen-stimulated PBMCs in a MLR. T1D MSCs showed comparable suppressive capacity on T cell proliferation at P6, and at P2 and P4 they were more efficient than healthy MSCs.

As highlighted in **study I**, it is important that cells do not trigger coagulation cascades and IBMIR at infusion. As cells are I.V. infused and since hemocompatibility is known to vary between MSCs derived from different tissues (Moll, Ankrum et al. 2019), clotting formation was tested in the Chandler loop system (Bennet, Sundberg et al. 1999). Whole blood and MSCs from healthy and T1D donors were run through pre-heparinised tubes. T1D donors showed similar or even less complement activation of C3a and sC5b-9 (platelet consumption and thrombin formation) compared to healthy MSCs.

4.2.2 Discussion

The phenotype and potential of MSCs from individual patients should be evaluated before adoptive cell transfer, to assure that the stromal compartment is not compromised by the patient's disease. Interestingly, the cells investigated in this study were used in a clinical

study to treat newly diagnosed TID patients (Carlsson, Schwarcz et al. 2015). This successful study showed no adverse effects of the MSC therapy, and improved C-peptide response to a test of MMTT. Considering the overall similarities in phenotypes and *in vitro* properties, such as long-term growth kinetics and immunomodulatory capacity of T1D MSCs compared to healthy MSCs, we proposed that MSCs from early-stage and late-stage T1D could be used in autologous cell therapy. This is in spite of the fact that MSCs of late-stage T1D patients may have a disease imprint, which we observed at the gene expression level compared to healthy. Other studies have suggested disease memory, meaning a retained phenotype in BM MSCs after exposure to a diabetic environment (Madhira, Challa et al. 2012).

Microarray analysis and qRT-PCR data showed differences in gene expression in the wound healing pathway, however these were not translated into our *in vitro* functional scratch wound healing assay. A limitation in our approach is the lack of other cells, signalling molecules and cytokine pathways present in an *in vivo* wound healing process. An additional example of normal function of T1D MSCs was the four-fold increase in IL-6 gene expression in late-stage T1D compared to controls, which was not seen in IL-6 protein secretion, where the result was similar. No other pathways were found to be enriched in early-stage T1D, except for chromosome Y, due to skewed distribution of sex of the donors, which highlights the importance of matched groups on different confounding factors.

Studies have shown the effect of glycation products in diabetic conditions on the differentiation potential, including inhibition of adipogenic, chondrogenic and osteogenic differentiation (Kume, Kato et al. 2005). Besides, glucose metabolism may have a crucial effect on autologous MSCs and high glucose exposure of diabetic patients may have irreversibly affected the MSCs. However, other studies and the current investigation showed no difference in basic differentiation assays of T1D MSCs compared to healthy controls (Bautch 2011, Kramann, Couson et al. 2011, Noh, Yu et al. 2012, Reinders, de Fijter et al. 2013, Reinders, Roemeling-van Rhijn et al. 2013).

We investigated and compared both early (P2) and late (P6) passages of T1D and healthy MSCs, to study whether stromal function deteriorated with disease duration. We observed an age-dependent decline of BM MNCs in all groups. Interestingly, we showed that MSCs from early-stage T1D (minimal systemic damage) had lower CFU-F efficacy, suggesting that the BM "stem cell niche" was affected possibly by high glucose levels, already within the first months of T1D diagnosis. This is supported by data showing diabetes-induced cellular damage on the BM microenvironment, including hematopoietic progenitor cells in the endosteal and vascular niches (Mangialardi, Oikawa et al. 2012).

Overall, MSCs are important to T1D patients, because they give hope of disease improvement without side effects, when no successful clinically available treatment exists to halt the progressive loss of insulin-producing β-cells (Carlsson, Korsgren et al. 2015). Subsequently advanced research on MSCs for adoptive cell transfer in T1D is strongly encouraged.

4.3 TISSUE IMMUNE PROFILES SUPPORTING RESPONSE TO MSC THERAPY IN AGVHD – A GUT FEELING (STUDY III)

Successful MSC treatment is dependent on many factors, including patients and disease characteristics, donor variability, donor-recipient match, passage number, and culture conditions. Here we focus on the recipient. In this study, we aimed to evaluate the immune cell profile of the gut mucosa in patients diagnosed with aGvHD prior to MSC infusion. More specifically, to establish differences in baseline cellular immune composition between responders and non-responders to identify a "cell profile" that predicts MSC responsiveness.

4.3.1 Results

This is a novel approach to look at the patient's immune cell profile and the findings are highly significant. It is retrospective study with 16 patients; including eight responders and eight non-responders to MSCs therapy. All patients had severe aGvHD (grade III-IV) according to the aGvHD global clinical classification (Glucksberg, Storb et al. 1974, Harris, Young et al. 2016). Responder patients exhibited resolution of GvHD symptoms after MSC infusion, whereas non-responders demonstrated no clinically evaluable response. We found significantly different levels of immune cells in gut biopsy specimens obtained at diagnosis of aGvHD. These differences could be used to predict treatment response to MSC therapy. This study addressed the importance of further studies in finding the immune cell profile of patients responding versus non-responding to MSC therapy, despite the sample number being relatively small.

The key findings are the following:

- Patients who later responded to MSCs therapy exhibited significantly higher CD8+, Foxp3+ and β-tryptase (mast cell marker) levels in intestinal mucosa compared to non-responders.
- Responders showed significantly lower levels of CD4+, CD56+ and CD68+ compared to non-responders. This suggests that a pro-inflammatory immune profile within the gut at the point of MSC treatment may hamper the cells therapeutic potential.

4.3.2 Discussion

CD8+ T cells participate in the pathophysiology of aGvHD (Blazar, Murphy et al. 2012). Quantitative analysis of tissue biopsies from gut mucosa indicated significantly higher levels of CD8+ T cells in the responder group compared to non-responders. The presence of CD8+ T cells at the time point of MSC therapy may provide a niche environment for the induction of CD8+ CD28- Tregs (Liu, Zheng et al. 2015). This immune subset correlated to clinical efficacy in chronic GvHD trials with MSCs, and promotion of allograft tolerance. Donor T cells interact with activated APCs, causing their proliferation and differentiation. A lost balance between suppressive Tregs cells and effector CD4+ T cells can damage tissues (Matthews, Lim et al. 2009). MSC infusion triggers a cascade of anti-inflammatory events,

starting with skewing of monocytes towards an anti-inflammatory phenotype and the formation of CD8+ Tregs. These cells down-regulate APC function, resulting in decreased proliferation of CD4+ T cells that are linked to allograft rejection (Mou, Espinosa et al. 2014, Le Blanc and Davies 2015). FoxP3 is a transcription factor related to anti-inflammatory immunomodulation and Treg maturation, and this marker had a significantly increased expression in the mucosa of responders compared to non-responders (McMurchy, Gillies et al. 2013).

Mast cells are thought to have a significant role in GvHD. They recruit immune cells and are located in the mucosa of GI tract, skin and liver where GvHD typically manifests. Additionally, MSCs have been shown to have a role in reduction of gut GvHD in murine models through an IL-10 mediated suppression of conventional T cells proliferation, independent of Tregs (Leveson-Gower, Sega et al. 2013). Levels of mast cell ß-tryptase were significantly higher in the responder group, indicating their involvement in the resolution of GvHD following MSC therapy. It would have been of interest to determine if immune cells are patient or host derived. Unfortunately, that was not possible in this study because of limited biopsy material. The mast cells present are most likely of host origin, since mast cells survive irradiation and chemotherapy. One study showed that at 88 and 126 days after HSCT, mast cells (CD117+, CD34-) were of host origin, and that donor mast cells could be found through PCR only after as long as 198 and 494 days later (Fodinger, Fritsch et al. 1994).

The patients in the current study had received myelosuppressive and immunosuppressive treatment that depleted most immune cells, but at the time of MSC infusion, their total leukocyte counts in the peripheral blood were within the normal range. The leukocyte counts were similar between the two groups, supporting the hypothesis that the response to MSC treatment was dependent on the local tissues' cell profile, rather than the blood compartment. Improvement of biomarkers indicating responsiveness to MSC treatment is of crucial importance for optimal patient treatment.

As mentioned above, this was a retrospective study and a number of parameters could confound the analysis. Factors such as the timing of the gut biopsy, the site in the intestine where the biopsy was taken all may have importance since mucosal involvement can vary in different parts of the intestine. It is essential to note that the histological grade does not always correlate with the clinical stage, indicating the importance of both a global grading system as well as specific grading of the gut, liver, and/or skin in GvHD. According to suggested guidelines, biopsies from a minimum of four sites in the GI tract are recommended to span different segments (Naymagon, Naymagon et al. 2017). A limitation of this report is the low number of biopsies available for assessment. Although the clinical diagnosis of GvHD was based on several biopsies taken from different locations in the intestine, we only had access to one biopsy per patient in our quantitative analysis of the immune repertoire. There can be significant differences in single patients. However, in patients with mucosal damage, the colonoscopy procedure and taking biopsies involve risks for the patients, limiting access to tissue samples.

Other factors, yet to be revealed, may also influence MSC responsiveness. For example, recent studies indicate how conditioning regimens and tissue damage to the gut epithelium can change the microbiome, influencing GvHD pathogenesis. (Teshima, Maeda et al. 2011).

To conclude, the number of mast cells and CD8+ cells were significantly higher in the group responding to MSC treatment, suggesting a putative role of them in response. The mechanisms by which MSCs resolve GvHD is not fully understood. Future investigations need focus on understanding the host immune microenvironment that is permissive for MSC responsiveness and which cells need to be present in order to trigger the therapeutic effect.

From a clinical point of view, it is of interest to be able to predict which patients will respond to MSCs treatment based on biopsies.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The main conclusions drawn in this thesis are:

I. Complement opsomisation of MSCs plays a central role in the fate of MSCs and their therapuetic potential after I.V. infusion. MSCs are protected against complement attack by CD59 expression. MSCs are phagocytosed by classical and intermediate CD14+ monocytes.

II. There is a comparable *ex vivo* expansion capacity between T1D MSCs and MSCs from healthy controls, with no difference in immunomodulatory phenotype, T-cell suppression, nor differences in preserved hemocompatibility.

III. The immune cell profile of mucosa GI tract of aGVHD patients prior to MSC cell therapy can give insights into which patients will benefit from the treatment.

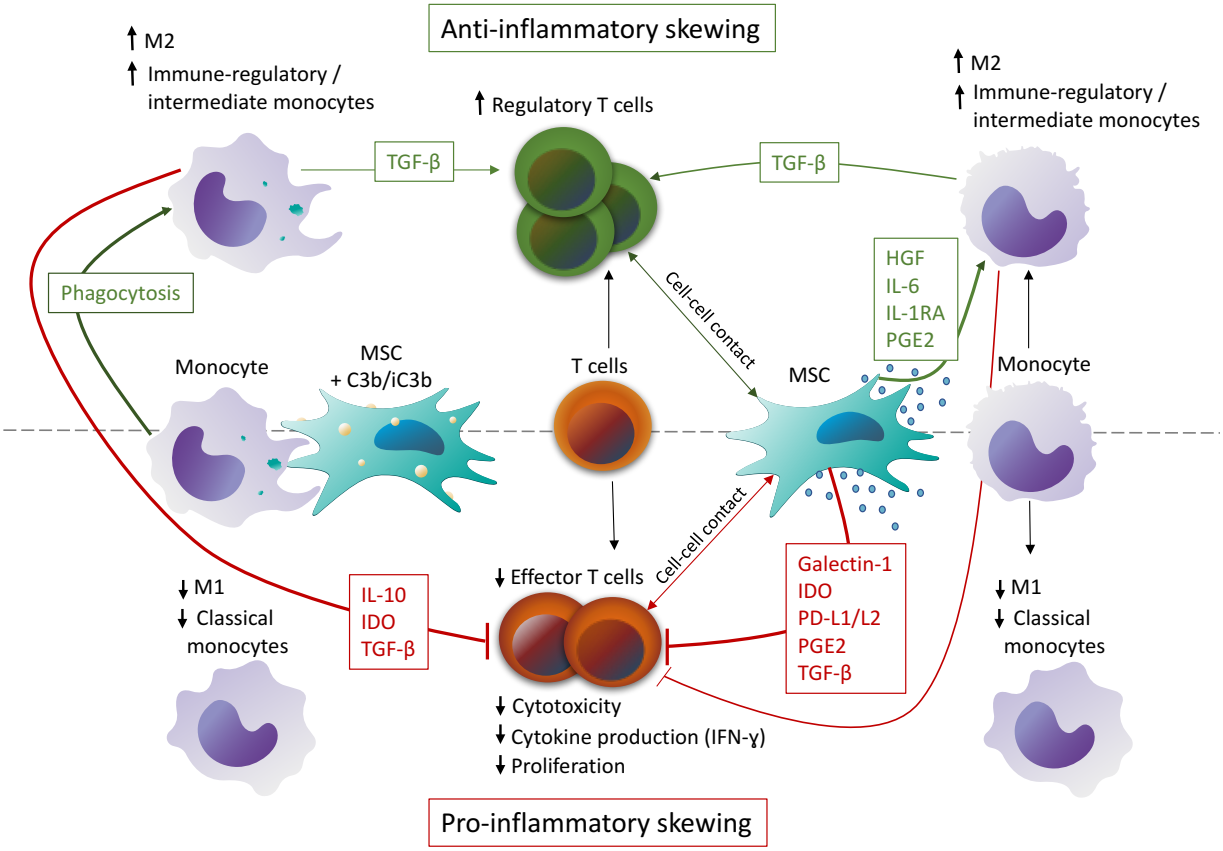

Figure 5.1. *Possible model of MSCs mechanism of action after I.V. injection. One fraction of MSCs are opsonised by complement and phagocytosed by monocytes. Subsequently, monocytes are immunomodulated into an anti-inflammatory phenotype, thereafter effecting T cells. The other fraction of MSCs are triggered to release cytokines, chemokines and exosomes which effect both monocytes, T cells and other immune cells.*

Future prospective and general considerations during this thesis:

The main factors to take into consideration when optimising MSCs therapy include but not exhaustively; donor variation, donor cell source, MSC expansion culture conditions, cell administration, optimal MSC dosage, and the most suitable time point frame (serial infusions) for delivery. The large number of MSC clinical trials performed to date vary considerably in all the strategies highlighted above, which reinforces the diverse clinical results. Therefore, collaborations between centres across the world with investigations standardising MSC delivery and treatment approaches would ameliorate clinical trial outcomes.

Most *in vitro* research is done on fresh cells, directly from culture, however a large number of clinical trials use MSCs thawed on the day of injection. Furthermore, experiments are most commonly performed on cells only in culture media, nevertheless the exposure of MSCs to plasma may have a large influence on MSCs function. Hence, more experimental studies on frozen cells after direct thawing and with plasma exposure as a step in the process prior to experimental assays are desired to mimic the clinical process. The question remains whether fresh or thawed MSCs are to be preferred, and how to establish a way to produce MSCs in an efficient way to be able to use fresh cells if it is found to be more favourable.

The use of allogenic versus autologous MSCs in the clinic remains an open issue. Moreover, few assays are currently available to determine "good" versus "bad" MSCs batches, and find the right donor to recipient match. Today, the suppression of T-cells is one of the few methods *in vitro* but there is a significant need for more specific methods. Moreover, it is crucial to examine the biological status of the patient in more depth, and maybe it will even be possible to find a "biomarker" for a "responder patient" in the future? The immune status and immune cells of blood can give important clues. Unpublished data from our group suggest that an active immune response is beneficial for MSCs cell therapy response. Furthermore, to determine and find the immune cell profile for MSC patient response, larger cohort studies are needed.

Understanding how MSCs survive, migrate, distribute in tissues of interest can make it more efficient, and finding out the key molecules/proteins mediate their therapeutic effect is fundamental for the development of efficient targeted therapies in autoimmune and inflammatory disorders. It is especially important to further understand the cross talk between MSCs and the innate immune system. We here studied monocytes but there are a number of phagocytes in the body, including neutrophils and DCs that warrant additional investigation. The interactions with the adaptive immune system is much more studied. The interactions between MSCs and mast cells would also be of great interest, referring to my study III.

It is of interest to identify how complement peptides drive phagocytosis, in order to fully understand interactions between MSC, the complement system and phagocytes. Moreover, it remains unclear how phagocytosis of MSCs skews immunosuppressive characteristics in the phagocytes that engulf them. Moreover, it is important to determine where MSC-phagocytosed immune cells migrate to, and which cells they consequently interact with.

Significance of this thesis:

Cellular therapy with MSCs is a promising therapeutic approach for several autoimmune and inflammatory conditions, including TID and GvHD, nevertheless to further improve their therapeutic potential a deeper understanding of MSC fate and interactions with blood components and immune cells were needed. We herein gave new insights in this important area. Increasing evidence suggests that in addition to their direct effects, MSCs initiate a strong indirect effect via secreted factors, such as exosomes and cytokines, and the modulation of other cell types, including mast cells. Improving our understanding of how MSCs mediate immunomodulation may allow for the further development of MSC cell-based therapeutics.

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