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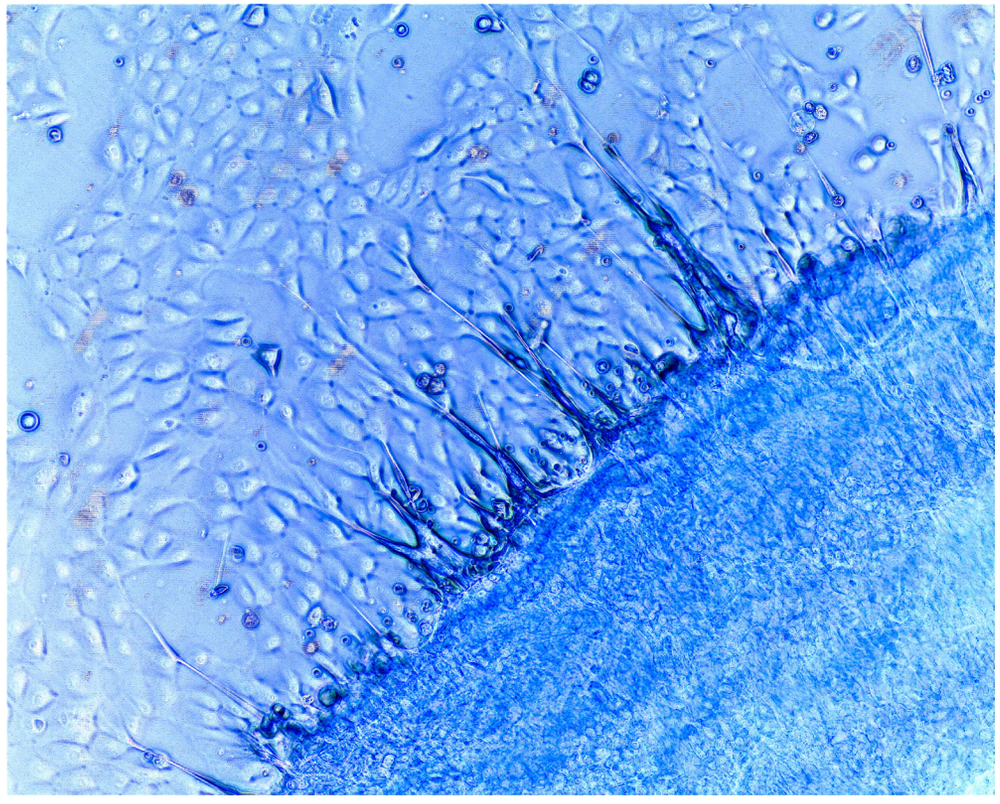

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IMMUNOREGULATORY EFFECTS OF PLACENTA-DERIVED DECIDUAL STROMAL CELLS

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THESIS FOR DOCTORAL DEGREE (PhD)

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ABSTRACT

Decidual stromal cells (DSCs) play a pivotal role in feto-maternal tolerance to prevent rejection of the fetus during pregnancy. This provides a rationale for immunomodulatory stromal cells from the placenta being isolated and used as cellular therapy for inflammatory conditions following hematopoietic stem cell transplantation (HSCT). The term placenta provides a ready source of cells, since this tissue is normally discarded after delivery.

Stromal cells were isolated from different parts of the term placenta, specifically chorionic villi, umbilical cord, and the fetal membranes. DSCs isolated from the fetal membranes had a consistent immunosuppressive capacity *in vitro* comparable to that of bone marrow-derived mesenchymal stromal cells (MSCs). This immune suppression was partly contact-dependent. Factors of importance in this process were found to include interferon-γ (IFN-γ), prostaglandin E_2, indoleamine-2,3-dioxygenase (IDO), and programmed death ligand 1 (PD-L1). In addition, IDO was found to play a role in the DSC-mediated induction of regulatory T cells (Tregs) *in vitro*. The addition of DSCs to the allogeneic setting *in vitro* also resulted in a reduction in the concentration of cytokines IFN-γ and interleukin (IL)-17, while the concentrations of IL-10 and IL-2 were elevated. There was also a correlation between increased IL-2 levels and reduced expression of the high-affinity IL-2 receptor on alloantigen-activated T cells. This was consistent with a reduced phosphorylation of STAT5 and reduced uptake of IL-2 in the cultures. The reduced sensitivity to IL-2 was not found to be correlated to an increased exhaustion state, based on expression of programmed death 1 (PD-1) and CD95.

Further characterization of DSCs showed that they have limited differentiation capacity, that they are of maternal origin, and that they have high expression of co-inhibitory markers and integrins that are of importance for migration to inflamed tissue. The expression of these markers was elevated in the presence of external IFN-γ. In contrast, addition of IFN-γ did not increase the antiproliferative effect of DSCs *in vitro*.

DSCs were expanded to high cell numbers at low passage number. These DSCs were then introduced as a treatment for severe graft-versus-host disease (GVHD), a common complication after HSCT with high mortality rates. In an initial pilot study, nine patients were treated with DSCs. In eight patients who could be evaluated, the overall response rate was 75% and three patients were alive six months after transplant. In a larger patient cohort, immune parameters were monitored up to four weeks after DSC intervention. The patients were divided into two groups, responders and non-responders, depending on GVHD status after DSC treatment. Increased plasma concentrations of IL-6, IL-8, and IP-10 distinctly differentiated the non-responders from the responders before DSC intervention. Although the expression of HLA-DR decreased over time in the CD4+ compartment of the responders, the same group had increasing expression of CCR9 in several cell subsets, including CD4+ T cells, B cells, and monocytes. The responders also had less naïve CD4+ T cells one week after DSC intervention.

Thus, DSCs can be isolated from term placentas and can be expanded to high cell numbers at low passage number. The DSCs have immunomodulatory functions, mediated by several

factors. DSCs may be used as a treatment for GVHD, and improvement in GVHD may be distinguished by a specific immune profile.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Immunsystemet spelar en avgörande roll i människans skydd mot infektioner och cancer. Störningar i immunsystemets funktion kan bland annat resultera i autoimmuna sjukdomar och infektionskänslighet. Om en patient har ett dysfunktionellt immunsystem, eller drabbas av leukemi, kan hen behöva en allogen (inte genetiskt identisk) stamcellstransplantation, tidigare kallat benmärgstransplantation. En sådan innebär att de blodbildande stamcellerna byts ut mot nya celler från en frisk donator.

Ett transplanterat organ, exempelvis en njure eller en lever, kan stötas bort av patientens immunsystem då transplantatet känns igen som icke kroppseget, allogent. Det kan även inträffa vid en stamcellstransplantation, men då kommer istället det nytransplanterade immunsystemet attackera patientens vävnader. Detta kallas för transplantat-kontra-värd sjukdom (eng. graft-versus-host disease, GVHD) och är en inflammatorisk komplikation efter transplantation som drivs av immunceller, främst T celler, från transplantatet. Akut GVHD drabbar främst organ såsom hud, tarm och lever. Vid svår GVHD är behandlingsalternativen få och dödligheten hög.

Under graviditeten har moderkakan flera uppgifter, inklusive att ge näring och syre, samt hindra moderns immunförsvar från att attackera det allogena fostret. Idén som ligger till grund för denna avhandling är att celler från moderkakan med påverkan på immunsystemet skulle kunna användas som behandling och minska inflammationen vid GVHD. Moderkakan slängs vanligtvis efter förlossningen, varför det är lätt att etiskt försvara användandet av vävnaden för forskning och behandling.

I en första studie isolerades stromaceller (bindvävsceller) från olika delar av moderkakan och cellernas påverkan på immunsystemet jämfördes i laboratoriemiljö (*in vitro)*. I analysen inkluderades även stromaceller från benmärg (MSCs) som tidigare använts som cellterapi för GVHD. Studien visade att deciduala stromaceller (DSCs) isolerade från fosterhinnorna konsekvent kunde minska allogent framkallad tillväxt av immunceller i odlingskulturer *in vitro*. DSCs inducerade även förekomst av lösliga faktorer med en anti-inflammatorisk profil. På cellytan av DSCs kunde vi identifiera ett uttryck av flera molekyler som är viktiga vid migration till inflammerad vävnad. Detta uttryck var högre om cellerna förbehandlades med signalmolekylen IFN-γ. Vidare kunde vi visa att DSCs hade begränsade stamcellsegenskaper. DSCs hade samma DNA som moderkaksdonatorn vilket bekräftade att de kom från deciduan, alltså den förändrade livmoderslemhinnan som utvecklas under graviditeten. Det var också lätt att snabbt odla upp stora mängder celler, vilket är en stor fördel om de ska användas vid behandling.

Ytterligare studier *in vitro* visade att DSCs delvis kräver direkt cellkontakt med immunceller för att verka. Vi identifierade ett flertal faktorer (IDO, IFN-γ, PGE_2, PD-L1) som var viktiga för denna immunologiska påverkan. Därtill fann vi att immunhämmande regulatoriska T celler ökar i proportion till övriga immunceller. Dessa fynd är i linje med andra studier som undersöker dessa fenomen i en liknande kontext.

En löslig faktor som leder till tillväxt av immunceller, specifikt T-celler, är cytokinen IL-2. En av våra fördjupade studier visade att om DSCs tillsattes i allogena cellkulturer så minskades uttrycket av receptorn för IL-2 på aktiverade T-celler. Vi tolkade denna observation som att DSCs först stimulerar till en hög produktion av IL-2 i immunceller. Den höga koncentrationen av IL-2 leder sedan till att dess receptor på cellytan minskar i uttryck. Cellerna får som en konsekvens en minskad förmåga att svara på stimuli från IL-2. Detta kunde bekräftas i experiment där den intracellulära signaleringen av IL-2 hämmades. Härmed identifierades ytterligare en möjlig immunologisk effekt som skulle kunna förklara varför DSCs minskar celltillväxt av immunceller *in vitro.*

Baserat på de studier där DSCs påverkan på immunsystemet undersökts *in vitro* användes dessa celler som behandling för allvarlig GVHD. I en första pilotstudie behandlades nio patienter. Av de åtta patienterna som kunde utvärderas hade sex stycken en initial förbättring av sina GVHD-symptom. Tre patienter levde vid halvårsuppföljningen. Dessa resultat är jämförbara med andra experimentella terapier för GVHD.

Slutligen gjordes en omfattande studie *ex vivo* där immunparametrar (lösliga faktorer och immuncellstyper) undersöktes i blodprover som tagits från 22 patienter som behandlats med DSCs för allvarlig GVHD. Målet var att undersöka hur immunsystemet påverkas av cellterapin, samt om det fanns faktorer som kunde förutsäga hur patienten skulle svara på behandlingen. Vi identifierade höga koncentrationer av tre lösliga faktorer, IL-6, IL-8 och IP-10, i blodet hos patienter som inte svarade på behandlingen. Patienter som svarade på behandlingen med DSCs hade ett minskat uttryck av aktiveringsmarkören HLA-DR på T celler. Samma patientgrupp hade även en minskad andel T celler med en naiv fenotyp jämfört med gruppen av patienter som inte förbättrade sin GVHD. Patienter som blev bättre efter behandling hade också en immunprofil som indikerade att immuncellerna i blodet hade en ökad förmåga att migrera till tarmen. Förändringar i immuncellers fenotyp på grund av tillsatta DSCs som kunde observeras *in vitro* kunde dock inte observeras *ex vivo*.

Sammanfattningsvis har vi i fem studier isolerat och expanderat deciduala stromaceller från moderkakan. Vi har bidragit till att kartlägga dessa cellers påverkan på immuncellers egenskaper *in vitro.* Vi använde cellerna som behandling för GVHD och undersökte immunologiska parametrar som visat sig vara viktiga i samband med GVHD. Ett flertal faktorer identifierades som kan vara vägledande för att prognosticera utfallet efter behandling. Randomiserade kliniska fas I/II studier, optimering av pre-kliniska och kliniska protokoll, och vidare studier där bindvävscellers immunreglerande egenskaper undersöks i detalj är centralt vid fortsatt forskning. Detta för att ge ytterligare insikt om hur cellterapi kan användas effektivt för att behandla svåra inflammatoriska tillstånd.

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V. **Erkers T,** Solders M., Verleng L., Nava S., Molldén P., Mattsson J., Ringdén O, Lundell A-C. and Kaipe H. *Ex Vivo* Immunological Analysis Following Decidual Stromal Cell Therapy in Patients with Acute Graft-versus-Host Disease. *Manuscript*

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CONTENTS

LIST OF ABBREVIATIONS

AE	Amniotic epithelia
APC	Antigen-presenting cell
ATG	Anti-thymocyte globulin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony forming unit
CSF	Colony stimulating factor
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CyA	Cyclosporine A
DCs	Dendritic cells
DSCs	Decidual stromal cells
FC	Flow cytometry
FMOs	Fluorescence minus-one controls
FOXP3	Forkhead box protein 3
GCs	Glukocorticoids
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSCT	Allogeneic hematopoietic stem cell transplantation
ICAM-1	Intercellular adhesion molecule 1
ICOS	Inducible T cell co-stimulator
ICs	Immune complexes
IDO	Indoleamine-2,3-dioxygenase
IFN-γ	Interferon-γ
IL	Interleukin
LPS	Lipopolysaccharide
MAC	Myeloablative conditioning
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MMF	Mycophenolate mofetil
MSCs	Mesenchymal stromal cells

NO	Nitric oxide
OPLS-DA	Orthogonal projection to latent structures by means of partial least-squares discriminant analysis
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1
PD-L1/2	Programmed cell death ligand 1/2
pSTAT5	Phosphorylated signal transducer and activator of transcription 5
PTLD	Post-transplant lymphoproliferative disease
PVSCs	Stromal cells from placental villi
RIC	Reduced-intensity conditioning
SRL	Sirolimus
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
T_{FH}	Follicular helper T cell
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
T_N	Naïve T cell
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
T_{TD}	Terminally differentiated T cell
UC	Umbilical cord
UCSCs	Stromal cells from umbilical cord

1 INTRODUCTION

The immune system is one of the most complicated and fascinating parts of human physiology. The immune system has evolved in a way that lets it defend the host in a safe and efficient manner. However, the immune system also has some important limitations. Disease development originates from a failure of the immune system to successfully complete a number of checkpoints. Firstly, immune cells will detect the presence of unknown antigens (e.g. from pathogens such as bacteria and viruses). Secondly, the immune recognition will lead to an immune effector function with the purpose of eradicating the unknown pathogen. This effector function must be regulated in order to prevent an overreaction, and also to promote a return to homeostasis after the infection has been cleared. Immune regulation is therefore vital in balancing the immune response. Lastly, the adaptive immune system will develop memory for any particular pathogen that is encountered, and mobilize a quick immune response following further encounter with the pathogen.

Just as the immune system must recognize a pathogen, mount an effector response, regulate it, and develop memory to prevent recurrent infectious disease, the same system is used by the immune system to eradicate cancer cells. In this respect, immune regulation plays a crucial role. The immune system must be able to detect and eradicate autologous cancer cells, but it cannot be too sensitive and activate a response to small natural variations—and induce autoimmunity. The immune system therefore has a key role in a wide range of diseases, including infections, cancer, and autoimmunity.

This thesis will focus on immune regulation following allogeneic hematopoietic stem cell transplantation (HSCT). HSCT is a potentially curative treatment for hematological malignancies, aplastic anemia and inborn errors of metabolism. However, the introduction of an allogeneic immune system in a patient fundamentally changes the concept of immune regulation. The transplanted immune system must initiate a response to microorganisms and residual cancer cells while minimizing any damage to healthy allogeneic tissue. Furthermore, the heavy conditioning regimen used before the transplant introduces abnormalities in several layers of immune homeostasis, which may trigger an allogeneic response. Immunosuppressive drugs are used to balance the allogeneic response in order to maintain an immune response against remaining cancer cells, while preventing the immune cells from attacking healthy tissue. This thesis will concentrate on immune regulation where there is a strong allogeneic reaction and I will present some circumstances in which stromal cells isolated from the placenta may influence alloreactive immune cells. The emphasis will be on T cells, which are key mediators of the alloreaction by the newly transplanted immune system. Clinically, this manifests as graft-versus-host disease (GVHD).

1.1 TRANSPLANTATION IMMUNOLOGY

1.1.1 Immunobiology

1.1.1.1 *A very brief introduction to the immune system*

The work performed for this thesis mostly involved studies of adaptive immunity, and specifically T cells. The introduction to the immune system in this thesis will therefore mainly cover important background information that is of relevance for discussion of the results presented in studies I–V. There are, however, excellent reviews in the literature that provide a thorough introduction to the immune system[1,2].

The immune system is not only composed of the immune cells that originate from the bone marrow. The first lines of defense against external pathogens are physical barriers such as skin and mucosa. Antimicrobial peptides, pH-variations, and commensal microbiota can further improve the effectiveness of physical barriers. The complement system is composed of a number of molecules that circulate in blood. It is an important part of the defense against infection and augments the functions of innate and adaptive immunity.

Hematopoietic stem cells (HSCs) mainly develop in the bone marrow. As the name suggests, HSCT is the procedure whereby the patient's HSCs are removed and replaced with HSCs from a donor. The new HSCs will proliferate and differentiate into cells that populate the blood, bone marrow, lymphatic organs, and other tissues. The hematopoiesis, with all the cell types that originate from HSCs, is illustrated in **Figure 1**.

Differentiation from HSCs can be seen as two specific lineages, the myeloid lineage and the lymphoid lineage. Functionally, the immune cells originating from the hematopoiesis can be involved in innate immunity or in adaptive immunity. The innate arm of the immune system includes the phagocyting neutrophils and macrophages (derived from monocytes), as well as eosinophils, basophils, mast cells and natural killer (NK) cells. Physical barriers and complement are also regarded as a part of the innate immune system. The adaptive immune system includes T cells and B cells. Dendritic cells are often referred to as being a bridge between innate immunity and adaptive immunity, as they use the products from the innate immune system to activate the adaptive immune system.

The innate immune system mounts a response to a pathogen more rapidly than the adaptive immune system. The cells in the innate immune system have the ability to respond to genetically conserved structures on pathogens (e.g. bacterial/viral DNA/RNA, lipopolysaccharides (LPS), and immune complexes (ICs)). A broad range of microbes express these conserved structures, which enables the innate immune system to quickly respond to most pathogens. The innate immune system is, however, restricted to responding to certain evolutionary conserved stimuli only, and it may need help from the adaptive immune system to eradicate pathogens.

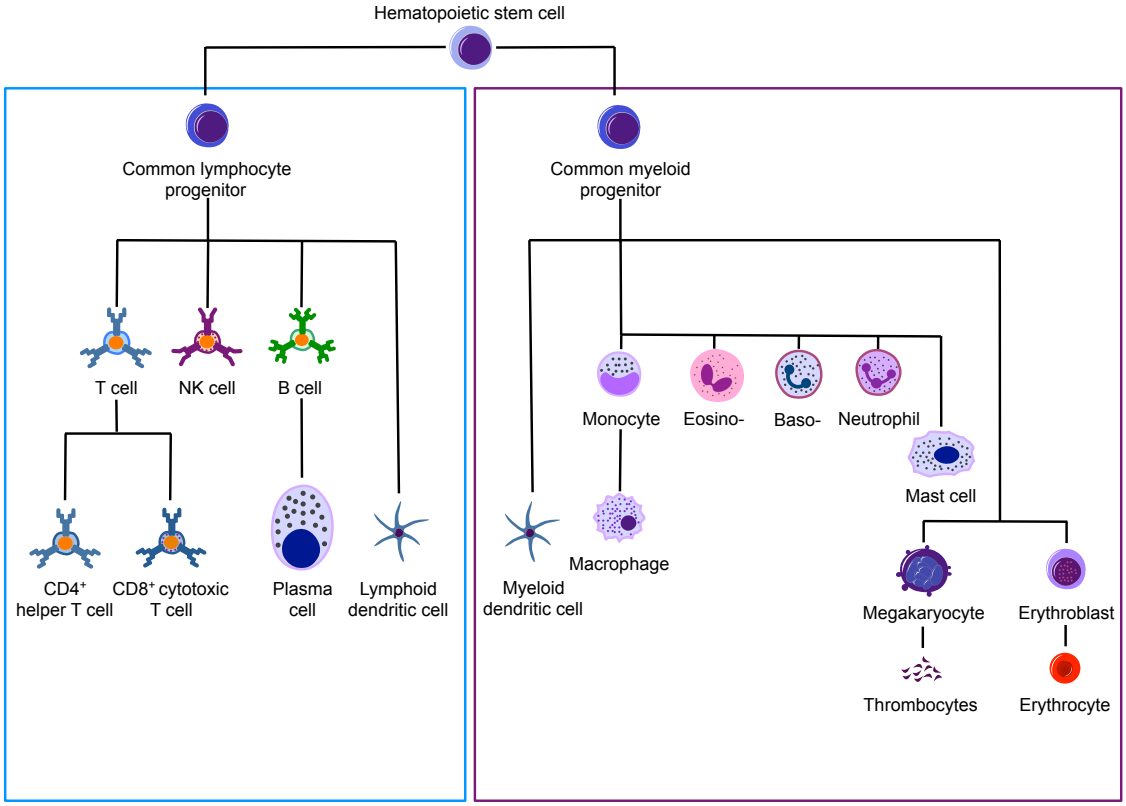


Figure 1. Simplified scheme showing the hematopoiesis.

Monocytes and macrophages are part of the innate immune system. The main task of macrophages is to phagocytose pathogens and debris such as dead cells during and after an immune response. Monocytes and macrophages also have the function of acting as antigen-presenting cells (APCs). This means that they are efficient in presenting peptides (foreign or self) to the adaptive immune system, thus working as a bridge between the innate immune system and the adaptive immune system. The subset of cells that specializes in antigen presentation is the dendritic cells[3]. Macrophages will be covered in more detail later.

The adaptive immune system is distinctly different from the innate immune system. The time taken for the adaptive immune system to deal with a pathogen is longer than the time taken by the innate arm of the immune system. However, the cell-mediated adaptive immune response is specific and very potent. As the name suggests, the adaptive immune system has the ability to recognize most structures, even though they have never been encountered before. Structures that are able to induce an immunological response are called antigens. The development of B and T cells includes a random recombination of their B and T cell receptors, making each B and T cell clone unique in its ability to recognize one specific antigen[4,5]. The entire T and B cell repertoire can therefore recognize a very diverse set of antigens. Antigens are mainly presented to T cells and to B cells in secondary lymphoid organs[6]. B cells can detect antigens in their native form, whereas the antigens have to be presented on major histocompatibility complexes (MHCs, in humans referred to as human leukocyte antigens, HLA) for T cells[7,8]. There are two main types of MHC, namely MHC class I and MHC class II. MHC class I is present on all nucleated cells in the body whereas

MHC class II is more restricted to certain types of cells in the immune system. For instance, MHC class II is constitutively expressed on dendritic cells, monocytes, and macrophages. MHC class II may also be expressed on B cells. Other non-immune cells such as epithelial cells or stromal cells may express MHC class II. If a certain T or B cell clone recognizes a specific antigen, the cell(s) will become activated, proliferate, and exert its/their effector function. After the pathogen has been cleared, most of the effector cells will go into apoptosis. Some cells will remain as memory cells. The next time the same antigen is encountered, these memory cells can become quickly activated and respond to the pathogen. This is acquired immunity.

1.1.1.2 *T cell development*

As briefly touched upon earlier, T cells must be tightly regulated in order to recognize foreign pathogens or detect malignant cells while not reacting to healthy tissue. The entire maturation process of the T cell during which these properties are acquired takes place in the thymus. Immature T cells, or thymocytes, are produced in the bone marrow and subsequently migrate to the thymus. When the thymocytes arrive in the thymus, they do not express the characteristic surface proteins that are used to identify mature T cells: cluster of differentiation (CD)3, the T cell receptor (TCR), CD4, or CD8. Since thymocytes do not express either CD4 nor CD8, they are referred to as double-negative thymocytes. After maturation, the thymocytes will comprise three functionally different types of immune cells: conventional αβT cells, γδT cells, and invariant NKT cells. The latter two types will not be described further since they are beyond the scope of this thesis. The maturation of a functional T cell from a thymocyte can be divided into seven distinct checkpoints. The first four developmental stages of double-negative thymocytes are designated DN1–DN4, and each stage can be identified by the variance in expression of CD44 and CD25[9]. The following two stages are when the thymocytes have a double-positive expression of CD4 and CD8. The last stage is the negative selection.

Cells in the DN1 stage express CD44. This expression is reduced during DN3, and thymocytes in DN4 do not express CD44. In DN2, the thymocytes start to express CD25. This expression is then lost during DN4. DN1 thymocytes also express c-kit[10], which is gradually lost during the later stages. Notch signaling is important during the entire selection process[11-13]. The diversity of the T cell repertoire arises from rearrangement of the TCR gene, which yields T cells with unique TCRs. Rearrangement of the TCRβ-chain occurs in DN2–3. If the rearrangement is successful, further rearrangement of the β-chain is inhibited and the chain is paired with CD3 and a surrogate α-chain. This pre-TCR complex is able to initiate ligand-independent signaling, leading to proliferation and expression of both CD4 and CD8[14]. Following expansion, the thymocyte starts rearrangement of the α-chain. If the TCR is then able to recognize an MHC complex with a self-peptide, the thymocyte successfully undergoes positive selection and will not face the same apoptotic fate as those thymocytes that fail to recognize the self-MHC:self-peptide complex[15,16]. The thymocytes now stop expressing both CD4 and CD8. Depending on the stimuli, the cells commit to either CD4 or CD8[17-21]. CD4 and CD8 will be associated with recognition of a specific type of MHC molecule. CD4-expressing cells will have a TCR that recognizes MHC class II, while CD8

cells will recognize MHC class I. In the negative selection, the thymocyte is again exposed to self-MHC:self-peptide. If the thymocyte becomes activated by the self-MHC:self-peptide, the thymocyte will fail negative selection and undergo apoptosis[22-24]. The negative selection prevents autologous reactivity to healthy tissue. The thymocytes that pass negative selection will move on and leave the thymus as naïve T cells.

1.1.1.3 *T cell activation and differentiation*

The primary sites *in vivo* where T cell activation occurs are the secondary lymphoid organs (e.g. the lymph nodes, spleen, Peyer's patches, tonsils, and adenoids), which are distributed over the entire body. There, peptides collected from the surrounding tissues will be presented to the T cells by APCs. Both APCs that have migrated from the tissues and APCs resident in lymph nodes are important in the priming of T cells[25]. Guided by stochastic forces and by chemokines, the T cell will scout the surrounding APCs for MHC ligands with peptides to which the T cell is destined to respond since its priming in the thymus. The interface between the T cell and the APC where the signaling occurs is referred to as the immunological synapse[26,27]. When the T cell encounters an activated APC that displays MHC-peptide complexes with high affinity for its TCR, the T cell can become activated, but it also requires other signals from the APC. First, there must be an interaction between the TCR (CD4/CD8) and the MHC molecule with peptide. The binding between the T cell and the APC is enhanced by integrins and their respective ligands. Examples of integrin-ligand interactions in the immunological synapse are LFA-1 and CD2 on T cells, and intracellular adhesion molecule (ICAM)-1 and CD48/CD59 on APCs. T cells are very sensitive to activation. As little as 100 specific MHC-peptide complexes or less on the APC is enough to activate the T cell[28,29]. There have also been reports showing that T cells can respond to as little as one single MHC class II peptide[30]. CD4 or CD8 is important to achieve this low degree of sensitivity[31]. Additionally, the T cell must receive co-stimulatory signals to become activated, proliferate, and survive. The most important co-stimulatory molecule on T cells is CD28[32,33]. CD80 and CD86 are expressed on APCs and binds to CD28[29]. Alteration of this co-stimulation is crucial for the regulation of T cell activation and survival. For instance, CD28 signaling is subject to feedback inhibition by reduction of CD28 synthesis[34]. There are also inhibitory molecules on T cells, such as PD-1[35,36] and cytotoxic T-lymphocyte-associated protein (CTLA)-4, that may regulate signaling and T cell activation[37]. Other co-stimulatory molecules present on T cells that may boost activation are inducible T cell co-stimulator (ICOS) (a member of the immunoglobulin superfamily, just as CD28) and members of the tumor necrosis factor (TNF) superfamily (e.g. CD40L and CD27[38]). Cytokines also play an important role in the activation, expansion, and differentiation of T cells. The most well recognized cytokine is interleukin-2 (IL-2), which is crucial for regulating conventional T cell differentiation and expansion[39-41]. IL-2 and the IL-2 receptor (IL-2R) will be covered in more detail later due to their specific relevance in **Paper IV**.

Once a T cell is activated, it will proliferate and differentiate into effector cells. However, there is a difference in activation threshold between helper T cells (from now on referred to

as CD4+ T cells) and cytotoxic T cells (from now on referred to as CD8+ T cells). CD8+ T cells have a higher threshold for activation than CD4+ T cells. In fact, one role of CD4+ T cells is to enhance activation signals in order to activate CD8+ T cells. For instance, signaling through CD40/CD40L[42] between an APC and a CD4+ T cell may enable priming of the CD8+ T cell. The reason for CD8+ T cells being more tightly regulated may be the destructive function of effector CD8+ T cells. The signature function of CD8+ T cells is their cytotoxic ability. There are two main types of cytotoxicity. The CD8+ T cell can release perforin[43] and granzymes to initiate apoptosis. This cell can also use the Fas lytic pathway to initiate apoptosis[44] . The targets of CD8+ T cells are cells that present MHC ligand:peptide complexes that binds to the TCR of the CD8. This allows the adaptive immune system to fight intracellular pathogens such as viruses and certain intracellular bacteria, but CD8+ T cells are also important in tumor surveillance[45]. NK cells also have cytotoxic ability, and complement T cells in the defense against tumors[46,47]. Although cytotoxicity is mostly used by CD8+ T cells and NK cells, CD4+ T cells have also been shown to be cytotoxic in some cases[48]. Antigen-specific cytotoxicity is one of the main mechanisms behind graft rejection (in organ transplantation) and GVHD in HSCT.

1.1.1.4 *CD4+ T cell subsets*

Different CD4+ T cell subsets were investigated throughout **Papers I**, **III-V**. Unlike CD8+T cells, which are regarded as one homogenous population in these papers, CD4+ T cells have been more rigorously divided into subsets. As the name suggests, the main function of helper T cells is to balance the immune response. This can, for example, be to augment CD8+ T cell and B cell activation. CD4+ T cells can also induce tolerance, or they can regulate the response of other immune cells as well. The main CD4+ subsets investigated in this thesis work and the markers that were used to identify them are presented in **Figure 2**.

A naïve T cell (T_N) that has not yet encountered its antigen roams the circulation and scans APCs for an antigen that binds to the TCR[49]. Since many of the professional APCs reside in the secondary lymphoid organs, the T cell must have access. By expressing the chemokine receptor CCR7, naïve T cells can enter secondary lymphoid organs[50]. T cells may also be differentiated further from each other based on their expression of CD45 isoforms. Naïve T cells express CD45 isoforms with high relative mass, which can be detected with anti-CD45RA antibodies[51]. Memory cells will express the CD45 isoform with a lower relative molecular mass; this can be detected with anti-CD45RO antibodies. After encountering its antigen, the T cell will gain effector function (and be defined as a CD45RA− effector memory T cell (T_{EM})), downregulate CCR7 expression, and increase expression of other integrins that are of importance for homing to the site of infection[52]. However, effector T cells defined as CD45RA− central memory T (T_{CM}) cells can express CCR7, which enables a quick response to its antigen on a second encounter. These three lineages have the ability to proliferate upon activation, and they show plasticity between the three of them. Lastly, a fourth subset with low proliferative ability and plasticity—but with high effector function—has also been detected[53]. Studies have shown that reactive T cells that are not naïve can indeed also be identified as CD45RA+[54]. These cells circulate in the periphery and are therefore CCR7−. This subset is defined as terminally differentiated T cells (T_{TD}). These are

the four stages of T cell maturation and the differentiation pattern of these four lineages is as follows: CD45RA+CCR7+ (T_N) → CD45RA−CCR7+ (T_{CM}) → CD45RA−CCR7− (T_{EM})→ CD45RA+CCR7− (T_{TD}). In this thesis, the maturation pattern presented is implemented on both CD8+ and CD4+ T cells.

CD4+ T cells that are of effector type (CD45RA−) have been divided further based on their expression of surface proteins. The two lineages that were first discovered were Th1 and Th2. From these two subsets, a theory was developed that the activation of a CD4+ T cell response could be classified as a Th1 response or a Th2 response[55]. A Th1 response is regarded as a proinflammatory response, with a high production of interferon-γ (IFN-γ), IL-2, and tumor necrosis factor-β (TNF-β). A Th2 response is associated with production of cytokines such as IL-4, IL-5, and IL-10. In the last decade, however, additional CD4+ T cell subsets have been identified, adding more complexity to the Th1/Th2 model. Apart from Th1 and Th2, the subsets investigated in papers III, IV, and V were Th17 and Tregs. Other CD4+ T cell subsets that have been identified are for instance T follicular helper cells[56] (T_{FH}) and Th9[57]. The most characteristic function of T_{FH} cells is their ability to enter B cell follicles and induce antibody production. Th9 cells are regarded as proinflammatory. They produce IL-9 and in humans they have been shown to be present mostly in the skin.

Th1 is recognized by secretion of IFN-γ and is associated with inflammation and tissue injury. Th1 cells and the cytokines associated with a Th1 response are regarded as being key factors in the pathophysiology of GVHD[58-60]. This is important for activation of macrophages and increased protection against intracellular pathogens. The transcription factor that is associated with Th1 cells is T-bet[61], and IL-12 is important in the induction of Th1 cells[62]. Th1 cells also express the chemokine receptor CXCR3[63], which was used to identify Th1 cells in **Paper V**.

Th2 cells are induced by IL-4[64], and one of the master regulators of Th2 cells is the transcription factor GATA-3[65,66]. Th2 cells also preferentially express CCR4[63]. The function of Th2 is to strengthen the body's defense against extracellular pathogens by production of the cytokines IL-4, IL-5, and IL-13. IL-4 is vital for the switching to IgE production in B cells[67] and IL-5 is important in the activation of eosinophils[68]. Interestingly, GATA-3 suppresses Th1 development[69].

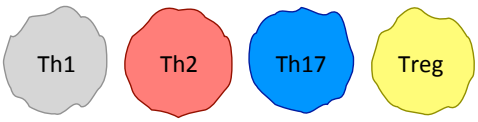
By stimulation with IL-6 and transforming growth factor-β (TGF-β), the IL-17-producing Th17 cell subset has been identified[70,71]. The need for TGF-β is debated, and studies have suggested that IL-1β is more important than TGF-β[72]. The transcription factor needed for Th17 cells is RORγt (the human ortholog is RORC). IL-17 is important in the defense against extracellular bacteria and fungi by recruitment of neutrophils to the site of infection[73] and induction of antimicrobial peptides[74]. Th17 cells have also received attention regarding the induction of autoimmune disease in several models[75]. The inter-regulation between the Th subsets is further suggested by studies showing that the Th17 phenotype is suppressed by the Th1-inducing cytokines IFN-γ and IL-12[76].

Regulatory T cells (Tregs) are important in order to control inflammation and restore homeostasis. The phenomenon of cellular immunity was mentioned in the seventies[77,78], and the presence of an inhibitory CD4+ T cell subset was identified almost 20 years ago[79,80], but

the Treg field exploded following the identification of Treg development through activation of the transcription factor forkhead box P3 (FOXP3)[81,82]. Phenotypically, Tregs are identified by their high expression of CD25, and an expression of FOXP3 and CTLA-4[83]. In **Papers IV** and **V**, we used dim and negative expression of CD127 as an alternative marker of FOXP3[84-86]. Tregs were the subset of T cells that received most attention in **Papers III**, **IV**, and **V**. This is due to the crucial role of Tregs in GVHD and pregnancy[87-91]. The Tregs can be divided into several categories depending on their origin and effector function (which has been excellently reviewed by Liston and Gray[92]). Briefly, Tregs can be generated directly in the thymus, whereas some of them are induced in the periphery under certain conditions. Functionally, Tregs can be divided into central Tregs and effector Tregs. The markers that can be used for identification of these subsets are thought to be the ones that are used for identification of conventional naïve and effector T cells. Activated Tregs have encountered their antigen and have less need for CCR7 and CD62L, while non-activated Tregs have higher expression of CCR7 and CD62L[92,93]. Upon TCR, CD28, and IL-2 stimulation, central Tregs will develop an effector phenotype and can have suppressive functions. Continuous TCR, CD28, and IL-2 stimulation is particularly important for Treg expansion and survival[94-96]. Compared to conventional T cells, which can produce IL-2 and use it in an autocrine fashion, FOXP3 represses production of IL-2 by Tregs themselves[97]. TGF-β can be used to induce Tregs[98], although whether TGF-β is absolutely required for homeostatic expansion of Tregs has been questioned[99]. There are several ways in which Tregs can reduce immune responses. IL-10 might perhaps be one of the most recognized ways for Tregs to exert immunosuppression, and it was one of the first to be identified[100,101]. IL-10 production does not occur exclusively in Tregs, but IL-10 production by Tregs is very important in maintaining tolerance[102]. Moreover, Tregs constitutively express CTLA-4 and this is crucial for their function[103]. Just like CD28, CTLA-4 binds to CD80/CD86 on APCs and through this interaction it can induce indoleamine-2,3-dioxygenase (IDO) in the APC[104,105]. Whether or not the binding to CTLA-4 on Tregs has an intrinsic effect on them is still under debate[106]. However, the effect on target cells appears to be enough for CTLA-4-mediated suppression. One mechanism of suppression that has received attention in recent years is the ability of CTLA-4 to reduce expression of the co-stimulatory molecules on APC[103,107] through transendocytosis[108]. Tregs may also suppress immune responses by several other mechanisms, including lymphocyte-activating gene (LAG)-3[109], CD39/CD73 expression[110,111], promotion of IL-10 and TGF-β production in dendritic cells[112], and production of TGF-β[113] or IL-35[114] by the Tregs themselves. Interestingly, while conventional T cells that respond to self-antigens are terminated during thymic selection, part of the naturally occurring Treg repertoire is to respond to self-antigens (generation of Tregs with self recognition is referred to as agonist selection)[115]. These Tregs are then dependent on continuous TCR activation to maintain functionality[116].

Following on from this introduction to the different T cell subsets investigated in **Papers I–V**, **Figure 2** shows the characterization pattern we used to identify the T cells. Tregs were identified from high expression of CD25 and from FOXP3 expression in *in vitro* studies, and from the low CD127 expression in *in vivo* studies. Th1, Th2, and Th17 cells were identified from the expression of the surface chemokine receptors CXCR3, CCR4, and CCR6[90,117,118]. The expression of these markers had previously been shown to be specific for CD4+ T cells with a cytokine profile associated with that specific T cell subset. In **Paper V**, we confirmed

that identification of the T cell subsets through expression of CXCR3, CCR4, and CCR6 will result in an enrichment of T cells with an expression of the transcription factor associated with each subset.



Transcrip. factor	T-bet	GATA-3	RORC	FOXP3
CD45RA	-	-	-	
CCR4	-	+	+	
CXCR3	+	-	-	
CCR6	-	-	+	
CD25				++
CD127				Dim/-

Figure 2. CD4+ T cell subsets and the markers used to identify them in this thesis. This classification is based on the work of Acosta-Rodriguez *et al.*[90]. Except for the profile presented in the figure, the cells should have a lymphocyte phenotype according to the forward-scatter area (FSC-A) and side-scatter area (SSC-A) filters in a flow cytometer. The cells must also show positive staining with anti-CD3 antibodies and anti-CD4 antibodies, and negative staining with the viability dye 7AAD.

1.1.1.5 *Interleukin-2 and its receptor*

IL-2 has already been mentioned as an important cytokine for Tregs. IL-2 was first described in 1976[38], and was later shown to be a pivotal component of T cell growth and proliferation [39,119-123]. After TCR and co-stimulation through, for example CD28, IL-2 is regarded as one of the most important factors in T cell activation. IL-2 is important in regulating activation-induced cell death[124]. IL-2 is produced by CD4+ T cells[125,126] and to a lesser extent by CD8+ T cells, by NKT cells[127], and by dendritic cells[128]. IL-2 was the first cytokine to be isolated[129], produced[130,131], and used as immunotherapy against cancer[132].

The IL-2 receptor (IL-2R) consists of three subunits: IL-2Rα, IL-2Rβ, and IL-2Rγ_c. The IL-2R with all three subunits has a high affinity for IL-2. This high-affinity IL-2R is assembled upon initial binding of IL-2 to IL-2Rα[133,134]. This is followed by the interaction with the β and γ_c chains[135]. On the surface of the T cell, these three subunits are located in lipid rafts[136,137]. This enables assembly of the high-affinity complex after the initial binding. Once the high-affinity receptor with ligand is formed, there can be a heterodimerization of the cytoplasmic domains on the β and γ_c chains. Consequently, downstream signaling is initiated by the Janus family of tyrosine kinases, JAK1 and JAK3[138-140]. As a control mechanism, activation of IL-2R will subsequently lead to endocytosis of the receptor complex. The β and γ_c chains and IL-2 are degraded and IL-2Rα is recycled to the cell surface[141].

Signaling upon activation of the IL-2R will initiate several signaling pathways. They can be divided into three pathways that have been identified: the JAK/signal transducer and activator of transcription (STAT) pathway, the phosphoinositide 3-kinase (PI3K) pathway, and the RAS-mitogen-activated protein kinase (MAPK) pathway[142]. These pathways can in turn be regulated by TCR activation, for example[143]. In **Paper IV**, we determined the activation of T cells by measuring the phosphorylation of the main STAT molecules activated by IL-2, STAT5a, and STAT5b. However, STAT3 may also be phosphorylated by JAK1 and JAK3, which are present on the β and γ_c subunits, respectively[144,145]. The PI3K pathway is particularly interesting, since its downstream signaling can to some extent be suppressed by immunosuppressive drugs such as cyclosporine A (CyA)[146] and sirolimus (SRL)[147,148]. A

summary of these pathways with the aim to improve understanding of the work in **Paper IV** is presented in **Figure 3**. The full pathway networks regarding TCR and interleukin signaling can be seen on the National Cancer Institutes Pathway Interaction Database website (http://pid.nci.nih.gov).

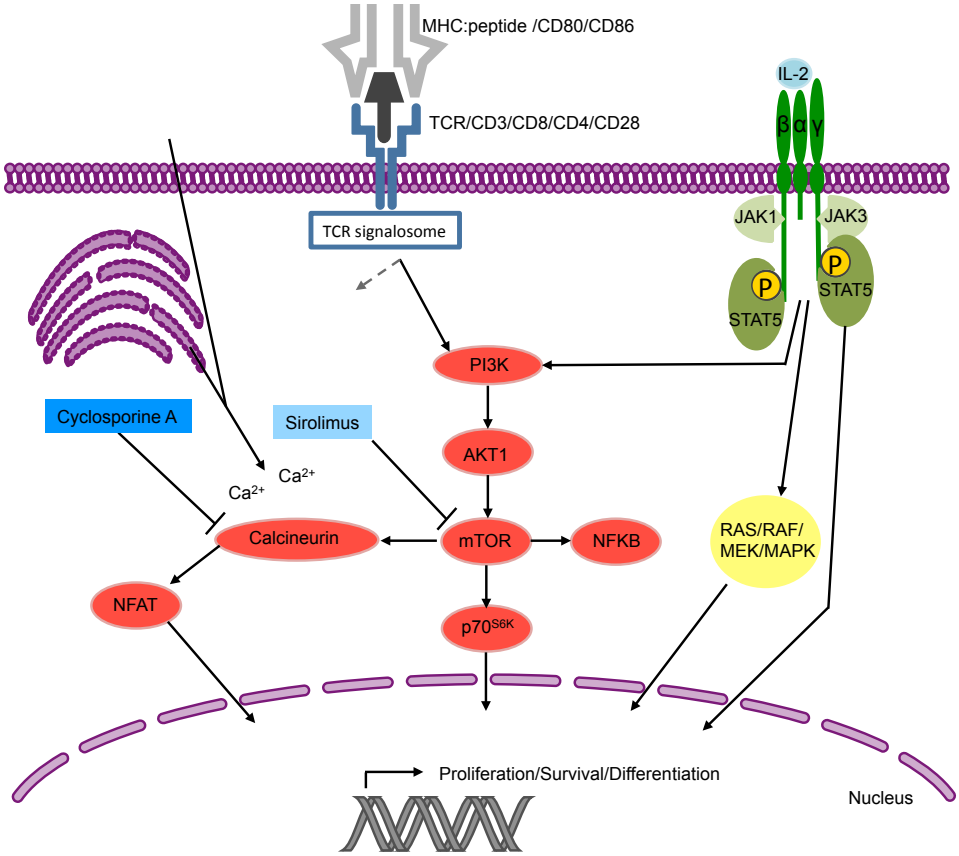


Figure 3. Schematic diagram of the major signaling checkpoints upon activation of the high-affinity IL-2R, in order to explain the molecules discussed in this thesis (linked with black arrows). Note that almost all of the checkpoints have several downstream and upstream regulators that are not illustrated (gray dashed arrows). For instance, Sirolimus does not block the entire downstream signaling upon TCR- and IL-2R-mediated activation. TCR activation initiates multiple signaling cascades, including PI3K, which is shared with and amplified by IL-2R signaling.

1.1.2 History and concept of HSCT

In the shadow of the atomic age, researchers began to develop strategies for counteracting the effects of radiation. Hematopoiesis is especially sensitive to radiation. The research initiated had the goal of replacing sick blood-forming cells with healthy hematopoietic stem cells[149-151]. Luckily, an atomic war has never started, and research regarding hematopoietic stem cell transplantation has saved hundreds of thousands of lives in patients with conditions such as leukemia, aplastic anemia and inborn errors of metabolism.

Before the concept of HSCT was established, induction of temporary remission in leukemia patients was achievable[152], but a diagnosis of leukemia was still regarded as a death sentence. Nowadays, complete remission in patients with leukemia is often achievable without transplantation, but allogeneic HSCT is still the only potentially curative treatment.

Autologous HSCT using the patients own HSCs was successfully achieved in the 1950s[153]. However, patients suffering from leukemia may need bone marrow from another individual to replace the sick bone marrow. Thus, the pioneer E. D. Thomas transplanted six leukemic patients with allogeneic bone marrow in 1957, after total body irradiation[154]. However, improvements were needed in order for the allogeneic transplant to be successful. One major obstacle in allogeneic transplantation compared to the autologous setting is the MHC incompatibility between different individuals. Appropriately for this thesis, the idea of an HLA system originated to some extent from the study of feto-maternal tolerance, where maternal antibodies to paternal antigens were detected[155]. Following identification of the HLA system[156], it was observed in dogs that matching of the donor and the recipient according to their MHC type before allogeneic HSCT improved the outcome[157]. But despite matching of the donor and the recipient, a graft-versus-host reaction still occurred whereby the immune cells of the donor attacked the tissues of the recipient. One way of managing GVHD was to use methotrexate[158]. It could then be shown that HSCT might be a successful treatment in patients with leukemia[159,160]. Survival rates following HSCT were increased further by the introduction of immunosuppressive drugs such as CyA[161,162].

Briefly, the procedure for modern allogeneic HSCT is as follows. First, the patient undergoes a conditioning regimen. The purpose of the conditioning regimen is to remove as many of the leukemic cells as possible, and to make physical and immunological space for the graft. The conditioning regimen usually consists of cytotoxic drugs and irradiation, but varies depending on the patient characteristics. There are two main types of conditioning: myeloablative conditioning (MAC)[163,164] and reduced-intensity conditioning (RIC)[165,166]. Myeloablative conditioning is defined as a conditioning where the patient's entire hematopoiesis is completely eradicated, without a transplant the patient will die. Reduced-intensity conditioning is, as it implies, a conditioning regimen where the patient (in theory) does not need a new transplant to survive. RIC was established in order to treat patient groups that would not survive MAC. Following the conditioning, the patient is neutropenic and very susceptible to infections. The next step is transplantation. An allogeneic graft is infused into the patient through an intravenous line. The graft is selected in advance to match the recipient as well as possible with regard to MHC genotype. Today, the alleles investigated for matching are HLA-A, -B, -C, -DP, -DQ, and -DR[167].

There are three different types of stem cell grafts: bone marrow (BM), peripheral blood stem cells (PBSCs; the stem cells are mobilized from bone marrow with granulocyte-colony stimulating factor[168,169]), and cord blood (CB). A graft from PBSCs or BM is preferred, but if there are difficulties in finding a well-matched BM or PBSC graft, a CB graft may be used. CB grafts require less MHC matching than BM and PBSC grafts due to the naïve nature of the graft[170], but the number of HSCs is generally lower. After the transplantation, the patient is still neutropenic and must be in isolation, either in the ward or at home[171]. Initially, people were treated in sterile environments, in so-called laminar airflow rooms[172]. Adoptive transfer of immune cells with the graft can help to some extent in fighting infections after transplantation. It will take approximately two to three weeks for the graft to re-populate the bone marrow and to start producing new immune cells. The myeloid lineage will reconstitute faster than the adaptive immune system[173,174]. Two years after HSCT, the adaptive immune

system has still not fully reconstituted[175]. A schematic diagram of the transplant procedure is presented in **Figure 4**.

There are several tasks that must be completed after HSCT in order for the patient to survive in the long term. The graft must populate the BM and restore hematopoiesis, infections must be controlled, and the remaining leukemic cells must be eradicated without letting the GVHD get out of hand. Despite conditioning, the graft may be rejected or undergo graft failure due to unsuccessful initial engraftment or loss of donor cells following initial engraftment. Recipient T cells, NK cells, or antibodies may cause this rejection. Increased mismatch of graft, UC grafts, and RIC are all major factors that increase the probability of graft failure and rejection[176]. If the graft is successfully engrafted, the patient may still undergo relapse later on. An allogeneic transplant, however, reduces the incidence of relapse significantly compared to an autologous transplant.

One of the great advantages of allogeneic HSCT is the graft-versus-leukemia effect (GVL)[177]. The concept of GVL is that allogeneic cells from the graft recognize the leukemic cells and eradicate them. The allogeneic immune cell from the graft will recognize both healthy recipient tissue and the leukemic cells, but hopefully the leukemic cells will be better at triggering an adaptive immune response than the healthy tissue, and the GVL effect will therefore be stronger than the GVHD. GVHD occurs when the adaptive immune cells from the donor attack the recipient's healthy tissues, and it will be discussed later in this thesis. But it is important to note here that to this date, GVHD and GVL cannot be differentiated from each other in clinical practice, and the balance between a potent GVL effect and not letting the GVHD get out of control is one of the most difficult parts of the post-transplant treatment of a patient. There have been reports of successful differentiation between GVHD and GVL using experimental animal models[178]. The conventional ways of managing GVHD and GVL are through the management of immunosuppressive drugs, graft composition, and the use of antibodies or additional transfer of immune cells. Using a graft that contains mature immune cells will be beneficial for management of infections and for maintenance of GVL, but it will cause more GVHD. It may differ between transplant centers whether or not the graft is depleted of immune cells before transplant[179]. Depletion of T cells is usually performed with anti-thymocyte globulin (ATG)[180].

The patient is closely monitored after transplantation in order to detect relapse. If the patient has increased blood levels of cells of recipient origin, which may be a sign of relapse, he or she may receive a donor lymphocyte infusion (DLI)[181]. A DLI contains graft and mature immune cells that can boost engraftment and mount an adaptive immune response against the recipient. The GVL and GVHD effect will therefore be enhanced. The possibility of giving DLI, if there appears to be a relapse, is a great advantage, and this is possible when BM or PBSCs are used as a source of graft. Due to the small quantity, CB grafts do not usually allow the possibility of DLI. As if handling of GVHD and prevention of relapse was not enough, one major problem after HSCT is infections. Since the patient suffers from a lack of cytotoxic lymphocytes following transplantation, even opportunistic infections are common complications. This includes viruses such as herpesviruses (cytomegalovirus, Epstein-Barr virus, and varicella-zoster virus)[182], but also fungal infections (e.g. *Candida* and *Asperigillus*)[183] and infections with bacteria from the commensal flora[184]. The occurrence of infections follows the immune reconstitution and immunosuppressive treatment of the

patient. Bacterial infections remain a problem until the physical barriers and the innate immune system have recovered. Fungal infections are common during the first six months after HCST. Clearance of viral infections is dependent on a fully functional adaptive immune system, and there can be recurrence even a year after transplantation. Compared to organ transplantation where immunosuppression is needed for the remainder of the patient's life, HSCT grafts often develop tolerance and immune suppression is generally discontinued within a year after tansplant[185].

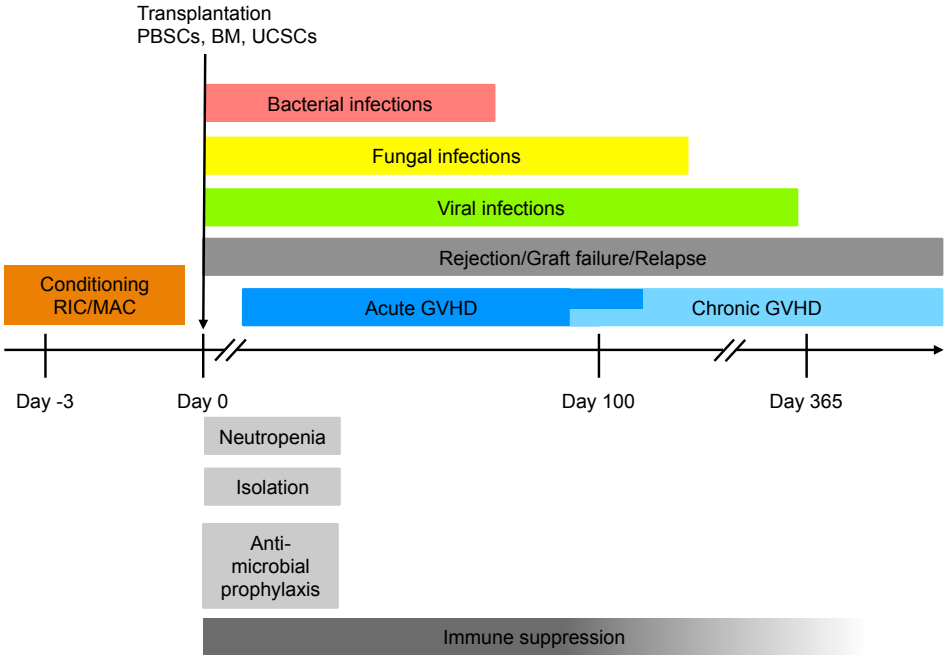


Figure 4. A schematic presentation of the main elements of allogeneic hematopoietic stem cell transplantation and common complications following transplant, including opportunistic infections, graft failure, and graft-versus-host disease (GVHD). Abbreviations: RIC, reduced-intensity conditioning; MAC, myeloablative conditioning; PBSCs, peripheral blood stem cells; BM, bone marrow; UCSCs, umbilical cord stem cells.

1.1.3 Graft-versus-host disease

As mentioned, the balance between GVHD and GVL is crucial for a successful HSCT. Since GVHD and GVL are currently not differentiable in the clinical setting, GVHD reduces the occurrence of relapse[186]. However, some studies have shown that DLI may induce the GVL effect without inducing GVHD[187,188]. Thus, mild GVHD may be desirable in order to reduce the incidence of relapse, while a more severe GVHD is a terrible complication and must be avoided. This is not easy, especially when GVHD becomes resistant to therapy.

GVHD was originally referred to as runt disease[189,190], and was described early in the field of transplantation. In fact, the early work regarding GVHD is still very important in daily

clinical work, as the conventional clinical grading of the acute manifestations of the disease originates from the work by Glucksberg *et al* in 1974[191]. Despite the efforts of the research community and technological advancements, GVHD is difficult to diagnose and there are no objective GVHD-specific biological parameters that can be used to assess the severity of disease. By going through the pathophysiology, we can gain an understanding of the complexity of the disease and the difficulty in diagnosis and treatment of GVHD.

GVHD can be broadly divided into two distinct pathophysiologies, acute GVHD (aGVHD) and chronic GVHD (cGVHD). This thesis focuses on acute GVHD. After this section, acute GVHD is the condition being referred to if not stated otherwise.

Chronic GVHD usually occurs later than 100 days after transplantation. Although cGVHD reduces the risk of relapse, cGVHD is associated with morbidity and mortality[192]. The pathophysiology of cGVHD resembles that of autoimmune disorders[193-195] such as sicca, scleroderma, primary biliary cirrhosis, wasting, and bronchiolitis obliterans.

Acute GVHD commonly arises within 100 days of HSCT. The organs most commonly affected by aGVHD are the skin, intestine, and liver. aGVHD can be divided into four grades of severity: I–IV, where grade IV is the most severe form and is associated with a very high mortality rate[196]. Grade I only includes skin involvement, while higher grades include gastrointestinal (GI) GVHD and/or liver GVHD[191].

The pathophysiology of GVHD can be divided into three important steps. This is theorized in a review by Ferrara[59] with inspiration from "the danger model" presented earlier by Matzinger[197]. Briefly, the danger model discusses self and non-self recognition in the presence of a highly inflammatory environment, and proposes that stimuli associated with damage are more important than actual recognition of self and non-self. This concept is especially applicable to the field of autoimmunity.

The first step of GVHD is initiated by the conditioning regimen. Chemotherapy and irradiation are blunt weapons to counter cancer cells, and will disturb nucleotide production and induce DNA damage in all cells in the body. The highly proliferative cells are hit hardest (e.g. epithelium of skin and GI tract). The tissue damage induced by the conditioning regimen will cause release of proinflammatory cytokines such as TNF-α[198] and IL-1[59]. Additionally, the reduced integrity of physical barriers will increase the presence of pathogen-associated molecular patterns[199] (e.g. lipopolysaccharides from bacteria). All these factors contribute to the activation of APCs. This is a recipe for GVHD. Activated APCs will migrate from the tissue to a nearby secondary lymphoid organ and present self-peptides. After HSCT, donor T cells from the graft will scout the surface of the host-activated APCs. Following immune reconstitution, activated donor APCs will also present recipient peptides. This leads to the second step in GVHD pathophysiology; a direct or indirect allorecognition by the donor T cell. The proinflammatory milieu described above will augment this, and all the co-stimulatory factors needed for a potent T cell response are present. Upon allorecognition, the T cells will expand and differentiate to effector lineages. It is mainly towards this step that the

prophylaxis against GVHD is targeted. For the last decades, the gold standard for immunosuppression in HSCT has been CyA combined with methotrexate[200]. Also, T cell depletion *in vivo* (ATG) or *ex vivo* can be used to prevent GVHD[201]. It is important to use drugs that target the IL-2 pathway prophylactically since if the cells are allowed to expand, they may still be able to perform effector functions later. There have been studies performed where the use of drugs that target IL-2 has been questioned regarding their impairment of Tregs, which are especially dependent on IL-2. In mice, Tregs have been shown to reduce GVHD while preserving the GVL effect[202]. Moreover, one study suggested that TNF-α and not IL-1 was of importance for the GVL effect in an experimental model[203]. In another study, the same group showed that CyA but not SRL inhibited Tregs that are important in the reduction of GVHD[204]. There is also increasing evidence in clinical trials that SRL used as GVHD prophylaxis is associated with more Tregs[205]. Moreover, one approach to prevention of GVHD is an adoptive transfer of Tregs; this was described in 2011. Even though GVHD was reduced in the group of patients who received Tregs with their UC transplant, it had no impact on survival[206]. Apart from IL-2, other cytokines have been reported to be elevated in GVHD, for instance IFN-γ[207], IL-6[208], IL-8[209], and IP-10[210].

The last step in GVHD pathophysiology is the effector function of CD8+ T cells, causing further tissue damage[211]. Also, NK cells have a role in GVHD as well as in GVL[212]. The potency of the response will depend on the tissue damage, the disparity in major and minor histocompatibility antigens, and the use of immunosuppressive drugs/T cell depletion to prevent priming of an adaptive effector response.

As stated earlier, a clinical evaluation using the Glucksberg criteria is still the conventional method for diagnosis of GVHD, but finding objective biological parameters in order to improve diagnosis and prediction of GVHD is the subject of intensive research. There is a wide range of different biological factors whose expression has been shown to be elevated or downregulated in GVHD. These are involved in all categories of GVHD pathophysiology, including tissue damage and repair[213], cell trafficking[214,215], pro- and anti-inflammatory cytokines[215], T cell function, and clonality[216]. The molecules that have received most attention in recent years are regenerating islet-derived-3-α (Reg3α, GI-GVHD)[217,218], suppression of tumorgenicity 2 (ST2)[219], and elafin (skin GVHD)[220]. Some of these soluble factors have been implemented in a model with the aim of correctly predicting the severity of GVHD[221]. These findings are limited, however, since the studies came from the same group and the reproducibility has so far been low (Uzunel, unpublished data) or debatable[222].

Despite efforts to prevent severe GVHD, it is still a common complication following HSCT. The conventional treatment for GVHD is corticosteroids[223,224]. However, treatment with steroids is not always sufficient, especially when the GVHD becomes refractory[225]. Unfortunately, there are no other conventional treatments for steroid-refractory GVHD. Immunosuppressive drugs such as CyA and SRL have been shown to be successful in prevention of GVHD, but they have limited efficacy for reduction of GVHD. The many different secondary treatments reflect the complexity of the disease, as each one targets a

wide variety of known GVHD symptoms. Some of the therapies that have been suggested or performed with some success include Psoralen and irradiation with ultraviolet A (Puva) [226,227], intravenous immunoglobulin[228], adoptive NKT or Treg therapy[229], mesenchymal stromal cells[230-232], decidual stromal cells[233], and a variety of checkpoint inhibitors[234] (e.g. SRL, MMF, ATG, and Ruxolitinib[235]).

Recent progress regarding innate lymphoid cell (ILC) biology in GVHD has also shown that ILCs of subtype 3 (RORC as master regulator) that produce IL-22 may be of importance for the prevention of GVHD[236,237]. IL-22 has been shown to increase the function of gut epithelial stem cells, speeding up the regeneration of the intestine following conditioning[236]. This is an interesting approach, since it builds on removal of danger signals, which have been theorized as the first part of GVHD pathophysiology. There is a clinical trial aimed at investigation of whether therapy with IL-22 can reduce severe GVHD (ClinicalTrials.gov identifier number: NCT02406651).

1.2 THE FETO-MATERNAL INTERFACE AND FETO-MATERNAL TOLERANCE

Reduction of maternal immune responses to fetal tissues is key to a successful pregnancy[238]. In this section, I will briefly discuss some of the mechanisms that may be of importance for feto-maternal tolerance.

The concept of acquired immunological tolerance during pregnancy was first described by Medawar in the early 1950s[239]. Although much research has been performed in the field, the mechanisms known today are still insufficient to give a complete picture. Also, most of the research has been performed in experimental models—which do not necessarily reflect feto-maternal tolerance in man[240].

Placentation will not be covered since it is outside the scope of this thesis (but for those interested, see review by Moffett[241]), and we will instead move to the structure of the placenta and adjacent tissues at the end of gestation. The sites at which the fetal and maternal tissues and cells are in contact are referred to as the feto-maternal interface. The feto-maternal interface can be divided into two compartments. First, there is the contact between the maternal decidua, which is derived from the endometrium, and the outer layers of the placenta. The outer layers of the placenta are the chorionic plate and the chorionic membrane. Depending on whether the decidua are in contact with the site of implantation or the fetal membranes, its is referred to as the decidua basalis or the decidua parietalis, respectively. The second feto-maternal interface is where the maternal blood infiltrates the vascularized placental body and interacts with the fetal trophoblasts that reside there. A schematic diagram of the structure of the feto-maternal interface is presented in **Figure 5**.

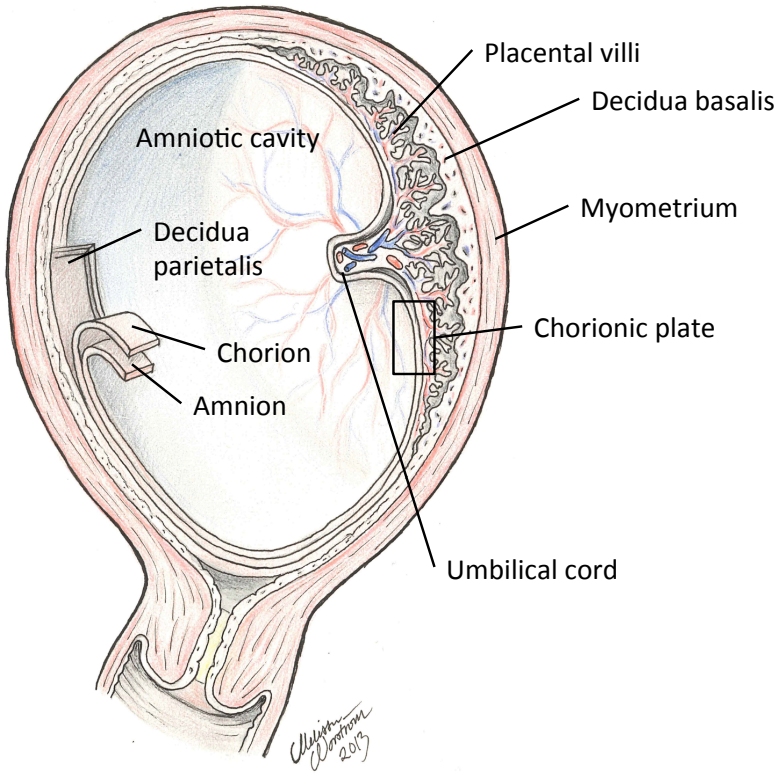


Figure 5. The structure of the placenta and its adjacent tissues.

An interesting thought would be to regard the placenta as a haploidentical transplant. However, transplantation of a solid organ or hematopoietic stem cells will lead to rejection by/of the host, while pregnancy is tolerated. Thus, there must be fundamental differences in these two entities in the priming and effector responses of the immune system to non-self. As mentioned, acute rejection is driven by direct and indirect allo-recognition. Donor or recipient tissue-resident APCs will collect graft antigens and migrate to proximal lymphoid organs. Presentation of a foreign peptide to a T cell by a foreign APC (direct allorecognition) will elicit a stronger response in a larger quantity of T cell clones than if a foreign peptide is presented by self APCs (indirect allorecognition). Interestingly, studies have indicated that indirect allorecognition—and not direct allorecognition—is the major pathway for the maternal immune system to recognize fetal antigens[242,243]. The reason for this may depend on a few already identified factors. First, trophoblasts have no expression of MHC class II molecules, limiting priming of CD4+ T cells in the placenta[241]. The trophoblasts also have an unusual expression of HLA-C, HLA-G, and HLA-E, while expression of the highly polymorphic HLA-A and HLA-B is limited. In addition, the dendritic cells that are resident in the decidua are restricted in their ability to leave the tissue and migrate to the lymph nodes[244], with a reduced transcription level for the RNA encoding chemokine CCL21, one of the ligands for CCR7. Another explanation could be the absence of lymphatics in the decidua[245], limiting the possibility of primed DCs to migrate to lymphoid organs. This does not rule out the possibility of fetal-derived peptides reaching the lymph nodes and being taken up by APCs resident in the lymph node, but the priming of the T cells will be less effective without

the help of APCs that have migrated from the tissues. As if limitation of DC migration for efficient T cell priming was not enough, studies have suggested that gene silencing in the decidua prevent migration of effector T cells to the feto-maternal interface[246]. Specifically, upon activation by TNF-α, myometrium upregulates the levels of transcription for RNAs encoding Ccl5, Cxcl9, and Cxcl11 whereas stromal cells from decidua do not. The chemokines produced by these transcripts are ligands for CXCR3, which is present on Th1 cells. Altogether, the results of these studies indicate that one way of maintaining feto-maternal tolerance is to limit the allorecognition and reduce infiltration of effector T cells.

Despite the factors mentioned above, immune cells are present at the feto-maternal interface, but the composition differs from that in the non-pregnant state. A pregnancy-specific subtype of decidual NK (dNK) cells is abundant in the decidua and plays a pivotal role in the intravascular trophoblast invasion of the uterus. This cell population represents a major part of the total lymphocyte population in the uterine compartment. The number of cells is reduced at term relative to the early stages of pregnancy, and they are phenotypically different from NK cells in peripheral blood[247]. Also, the cytotoxic ability of dNK cells is reduced by recognition of HLA-E expressed on trophoblasts[248,249]. Instead, dNK cells have secretory functions, and produce for example IFN-γ, IL-8, and TNFα[247]. Together with macrophages, dNK cells can contribute to the induction of Tregs[250].

Macrophages in the decidua are a fairly large proportion of the leukocytes located there. Macrophages are tissue-resident phagocytes that are potent in clearing the extracellular space from apoptotic cells, bacteria, and debris. They originate from monocytes that differentiate when stimulated and migrate to the tissues. Macrophages are potent APCs. Like T cells, macrophages can be divided into specific lineages. They were previously designated M1 or M2(a,b,c) macrophages, depending on whether they have a Th1 or a Th2 immune function and differentiation profile. The nomenclature regarding these lineages can be based on the conditions in which isolated monocytes differentiate[251]. Human monocytes cultivated in colony stimulating factor-1 (CSF-1), also named M-CSF, differentiate into macrophages. Additions to the culture may give macrophages with a specific profile. The factors commonly used are LPS, IFN-γ, glucocorticoids (GCs), ICs, TGF-β, IL-10, and IL-4. The subtypes produced can consequently be referred to as M(IL-4), M(ICs), M(IL-10), M(GC+TGF-β), M(GC), M(LPS), M(LPS+IFN-γ), and M(IFN-γ). The latter three fall into the commonly referred to M1 spectrum of macrophages, while the others can be placed in the M2 spectrum. Phenotypically, decidual macrophages fall close to M(IL-10), as decidual macrophages that spontaneously produce IL-10 have been found to be enriched compared to monocytes/macrophages in peripheral blood[252]. In the same study, there was no difference in the secretory patterns of IL-4 and IFN-γ between decidual macrophages and monocytes/macrophages in peripheral blood. Others have shown that M-CSF and IL-10 are the two most important factors for driving the differentiation of monocytes towards macrophages with a gene expression profile similar to *ex vivo* separated decidual macrophages[253]. Interestingly, decidual macrophages are highly functional phagocytes and are therefore potentially potent APCs[254]. In contrast, decidual macrophages have low

expression of the co-stimulatory molecule CD86[255], which is important for a sufficient T cell activation. Indeed, decidual macrophages are more potent than macrophages from peripheral blood in suppressing alloreactive PBMCs[256]. In addition, studies have suggested that decidual macrophages can suppress Th1 effector functions and induce Tregs through their expression of PD-L1, IL-10, IDO[257], and TGF-β[258]. IDO is an enzyme that depletes tryptophan in the microenvironment, causing T cells to undergo cell-cycle arrest. The metabolites from tryptophan degradation have also been seen to render Th1 rather than Th2 cells sensitive to apoptosis[259]. Altogether, these data suggest that decidual macrophages have the ability to maintain homeostasis through phagocytosis and to contribute to a tolerogenic niche at the feto-maternal interface.

T cells are also important in feto-maternal tolerance. Compared to the NK and macrophage compartment, the number of T cells in the feto-maternal interface is low[260]. Compared to peripheral blood, where the T cell compartment contains more CD4+ cells than CD8+ cells, the feto-maternal interface mainly contains CD8+ T cells. A recent study has shown that there is an accumulation of virus-specific T_{EM} CD8+ T cells in the decidua during uncomplicated pregnancy, which may suggest that the skewing of the CD8+ T cell compartment may be due to management of infections rather than allogeneic responses against fetal tissue[261]. Tregs are also enriched at the feto-maternal interface[90,262], but not in peripheral blood during pregnancy[263]. Th1 cells are more abundant in the decidua, while Th2 and Th17 ratios are lower compared to peripheral blood[90]. This is in line with the high number of CD8+ T cells, where Th1 cells may be important for enhancement of the probability of activation of the CD8+ cells, while Tregs support tolerance of the fetus. Induction of Tregs at the feto-maternal interface has been linked to activity through the IDO and PD-L1 pathways[242,264]. One experimental study has shown that IL-10 is not necessarily needed for successful pregnancy[265]. This may suggest that other immunosuppressive functions of Tregs (see above) may have a greater influence on feto-maternal tolerance. The presence of Tregs is, however, still important for a successful pregnancy. It has been shown that miscarriage and pre-eclampsia are associated with reduced levels of Tregs[266].

Another cell type that has been shown to be of importance in maintaining tolerance is the decidual stromal cell. I will continually discuss DSCs and their immunosuppressive functions—and consequently, their role in feto-maternal tolerance—throughout the remainder of this thesis, based on the theory that some of the properties of DSC-mediated immune suppression can be translated in adoptive DSC therapy in order to restore homeostasis in patients with GVHD.

1.3 STROMAL CELLS

Stromal cells are crucial for maintenance of the extracellular space and preservation of the structure of connective tissue. Depending on their localization, they have different specialized assignments. In this thesis, I will focus on decidual stromal cells (DSCs) and their

immunoregulatory properties, as well as the properties that are important for isolation and *in vitro* expansion. Indeed, the role of stromal cells in immunity is diverse, ranging from creation and maintenance of the bone marrow niche[267], development of adaptive immune responses in lymphoid tissue[268], maintenance of mucosal homeostasis[269], feto-maternal tolerance[270], and induction of immune privilege close to tumors[271]. A large part of the literature available is focused on mesenchymal stromal cells (MSCs), mostly isolated from the bone marrow (unless otherwise stated, the term MSCs always refers to bone marrow-derived MSCs). Some of the differences between DSCs and MSCs have been elucidated in the Results and Discussion sections of **Papers I–V**, and these two subsets will therefore be introduced in most depth. When mentioned and discussed, the term "stromal cells" alone is used to refer to all stromal cells, including DSCs and MSCs, and it also applies to studies where there is confusion regarding the origin of the stromal cells.

1.3.1 Characterization

Stromal cells can be isolated from many different compartments of the placenta and its adjacent tissues, including amniotic fluid[272], decidua basalis, decidua parietalis[273,274], chorionic villi[275], umbilical cord[276], amnion and chorion[277]. MSCs can also be isolated from a wide variety of other connective tissues, but isolation from bone marrow is most common[267]. In the initial study comparing the different types of MSCs isolated from placenta, despite the fact that stromal cells are a heterogeneous cell type, these stromal cells appeared to have some similarities[274]. These features are also the ones that are used to identify MSCs in general. First, stromal cells usually have differentiation capability *in vitro* towards mesodermal cell types such as bone, fat, and cartilage[278]. Stromal cells from the placenta have been shown to be able to differentiate to other lineages as well, for instance neuroglia[279], hepatocytes[280], and skeletal muscle cells[281]. This is consistent with the broad function and *in vivo* distribution of stromal cells. However, there have also been studies showing that some stromal cells have limited differentiation ability[273]. Especially when isolating stromal cells from placental tissue, it may be important to test the origin of the cells, since they may be of maternal or fetal origin[282]. Moreover, MSCs show positive expression of CD73, CD90, and CD105[283]. The cells also lack expression of markers indicating endothelial, myeloid, and hematopoietic lineage. Normally, MSCs do not have any expression of HLA-DR, but this can be induced by IFN-γ[284]. *In vitro*, the cells adhere to plastic under normal culture conditions. After initial seeding, the cells proliferate and form colony-forming units[283]. The characteristics of the DSCs that we isolated and described in **Papers I–V** will be discussed further later on.

1.3.2 Stromal cell-mediated immune modulation

Perhaps one of the features of MSCs that initiated an extensive exploration of their immunomodulatory properties was when these cells were reported to inhibit activated T cells

in experimental models and in the human setting *in vitro*[285-288]. This is a feature that has been reported in many different types of stromal cells. This inhibition does not appear to be dependent on HLA matching. It was subsequently shown that MSCs could inhibit the generation and function of DCs (reduced expression of MHC class II, CD11c, and CD83), which can result in a reduced ability of DCs to activate an adaptive immune response[289-291]. Although they are considered to have immunosuppressive properties and to be immunoprivileged, MSCs can promote proliferation of unstimulated peripheral blood mononuclear cells (PBMCs) to some extent[288], as well as activate the complement system[292].

Furthermore, MSCs have been associated with a variety of immunomodulatory factors, which is consistent with the idea of being a supportive cell type with the role of maintaining homeostasis in the tissue of residence. The immune modulation by stromal cells may be broadly divided into three categories: direct or indirect immune suppression, APC ability, and anti-apoptotic/other supportive functions.

No definitive pathway for stromal cell-mediated immunosuppression has yet been identified. However, one interesting feature is that many of the suggested pathways of immune suppression by stromal cells is initiated when the cells are primed by cytokines (e.g. IFN-γ, IL-1α/β, and/or TNF-α)[291] or engagement of toll-like receptors (TLR-3). IDO is upregulated by IFN-γ/TNF-α in stromal cells and has been reported to induce a switch towards macrophages with phenotype within the M2 spectrum (possibly M(IL-10))[293] and to suppress PBMCs by depletion of tryptophan[294]. Moreover, studies have also suggested that IDO production in stromal cells in part promote Tregs and inhibit Th17 differentiation[295]. This has also been seen in other cell types, such as macrophages[258] and dendritic cells[296] that produce IDO. Production of IDO is not exclusive for stromal cells, but appears to be more linked to the production of IFN-γ. Indeed, IDO production initiated by IFN-γ is suggested to be one explanation for why IFN-γ can be regarded as both a proinflammatory and an anti-inflammatory cytokine. Another soluble factor that has been shown to be of importance for stromal cell-mediated suppression of adaptive immune cells is PGE_2. Just like IDO, PGE_2 production is highly elevated in MSC cultures with added IFN-γ or TNFα[297]. By the addition of a competitive inhibitor to PGE_2 (indomethacin), the antiproliferative effect of stimulated PBMCs *in vitro* was found to be abrogated[297]. Moreover, nitric oxide (NO) has also been shown to be a factor of importance for prevention of GVHD in mice by primed MSCs[298]. Many of these factors are soluble and do not require direct contact between the stromal cell and the target cell. NO is, however, quickly degraded.

Aside from immunomodulatory effects that can be directly correlated to primed stromal cells, these cells have been shown to have constitutive expression of PD-L1, which can interact with PD-1 on lymphocytes and inhibit their activation[36,299]. A soluble factor, HLA-G5, has also been shown to be secreted by stromal cells[300]. HLA-G5 has a low polymorphism compared to other HLA class I molecules. Its known ligands are a specific NK-cell receptor (CD158d) and two leukocyte immunoglobulin-like receptors (CD85j and CD85d), which are expressed on myeloid cells and monocytes, DCs, and lymphocytes, respectively.

Interestingly, HLA-G is also expressed on cytotrophoblasts and may be of importance in feto-maternal tolerance[301]. Blocking of soluble HLA-G5 in allo-stimulated cultures reduced the frequency of Tregs in one report[300]. Additional factors that have been implicated in stromal cell-mediated suppression of immune cells is secretion of galectins[302] and exosomes containing suppressive factors (e.g. miRNA)[303], expression of the adhesion markers ICAM-1 and VCAM-1 to facilitate suppression[304], and increased adenosine production through expression of CD39 and CD73[305]. As mentioned previously, prevention of migration of T cells by DSCs may also be one way of reducing immune responses at the feto-maternal interface[246].

As already described, many factors have been identified that mediate suppression of immune responses by stromal cells. However, stromal cells may also trigger immune responses. IFN-γ can increase expression of HLA class II molecules on MSCs[306], which enables priming of adaptive immune responses[284]. Apart from from priming CD4+ T cells by HLA class II, stromal cells may also cross-present exogenous antigens to CD8+ T cells[307]. This can happen despite the fact that stromal cells do not normally express CD80/CD86 (also presented in **Paper I**).

Lastly, stromal cells may have anti-apoptotic features. For instance, MSCs have been suggested to prolong the survival of T cells under co-culture. The expression of Fas ligand and CD95 is reduced when T cells are cultured with MSCs[308].

1.3.3 Stromal cell therapy

The field regarding stromal cell-based therapy has literally exploded during the past ten years. As of October 2015, more than 550 clinical trials involving stromal cell-based therapy (with MSCs mainly) were registered at NIH (ClinicalTrials.gov). The range of application is wide, and includes GVHD, boosting of engraftment after HSCT, Crohn's disease, type-1 diabetes, acute respiratory distress syndrome, and ischemic cardiomyopathy among others. All of these have shown effects of MSCs in preclinical models of the diseases[267].

MSCs were first introduced as a treatment for GVHD in 2004[231]. In that case study, severe GVHD was successfully reversed in a boy twice by intravenous infusion of 2×10^6 cells/kg and 1×10^6 cells/kg, respectively. Subsequent follow-up studies showed that MSCs may reduce GVHD[230,232]. Long-term survival in this patient group was not altered with stromal cell therapy[309,310]. Other studies in which MSCs have been used to treat GVHD are summarized in reviews by Kaipe[311] and Luk[312], with some exceptions of recently published studies[222,310,313-315]. To conclude, from the data that are currently available, it is difficult to determine whether treatment is efficient or not. This is based on the fact that there have been few randomized trials, that there have been mixed results in published studies, and that there was limited surveillance in the studies apart from clinical response. There is currently one ongoing phase-III academic study where the efficacy of MSCs is being evaluated in the

context of GVHD[316]. The cells used in these studies were mostly from a third party, meaning that the cells were not derived from the recipient or from the donor. The origin of the MSCs in the clinical setting is bone marrow, umbilical cord, or adipose tissue.

Since MSCs have a role in creation of the bone marrow niche, one theory is that co-transplantation of MSCs and HSCs might enhance engraftment and reconstitution. This was first performed in 2005[317], where HLA-identical MSCs were co-infused with the graft. The outcome of this therapy varies. Co-transplantation was found to be associated with faster lymphocyte and platelet recovery[318-320]. A subsequent study by the same group showed that MSC co-infusion did not reduce the risk of graft failure but might reduce the risk of severe GVHD[321]. In umbilical cord transplants, data from our center have indicated that co-infusion of MSCs can be associated with an impaired adaptive immune reconstitution[320,322]. Other studies have suggested an increased rate of leukemic relapse[323] and development of post-transplant lymphoproliferative disease (PTLD)[324].

The fate of MSCs following infusion has not been fully elucidated. An experimental GVHD model has shown distribution of MSCs to the sites of inflammation[325]. Migration patterns of intravenously infused stromal cells in acute or chronic GVHD in man indicate limited homing or engraftment at sites of inflammation[118,326]. Interestingly, one study showed that the ability to home to bone marrow is reduced when the cells are cultured *in vitro*, compared to primary cells[327]. The way of administration may also affect *in vivo* distribution. For instance, intra-arterial administration in rabbits has a different distribution pattern compared to observations of intravenous infusion in man[328]. Factors such as survival of the cells may have a large influence on the *in vivo* distribution. Studies have indicated that MSCs are quickly attacked by the complement system upon administration, as seen in experimental models and *in vitro*[292,329].

2 AIMS

The general aims of the work for this thesis were to:

- Investigate the immunoregulatory effects of decidual stromal cells isolated from term placentas in allogeneic settings *in vitro*, and to determine how these cells affect adaptive immunity.
- Increase our understanding of how the immune system is affected by cellular therapy.

The specific aims of **Papers I–V** were to:

Paper I

- Isolate stromal cells from the fetal membranes, placental villi, umbilical cord, and bone marrow and to characterize the cells with regard to cell-surface expresssion of known stromal cell markers and integrins that have been shown to be of importance in MSC-mediated immune modulation.
- Investigate the basic immunomodulatory properties of the different stromal cell populations in allogeneic settings *in vitro*.

Paper II

- Introduce the use of DSCs as cellular therapy for GVHD.
- Further characterize DSCs used clinically regarding origin, cell-surface expression, and expansion potential.

Paper III

- Further investigate immunomodulatory effects of isolated DSCs, concentrating on parameters that are known to be of importance for feto-maternal tolerance and MSC-mediated immunomodulation.
- Provide a basis for further studies of isolated and expanded DSCs.

Paper IV

- Based on the findings in **Paper III**, further explore the ability of DSCs to modulate IL-2 production, IL-2R expression, and IL-2R signaling in alloantigen-stimulated T cells *in vitro* and *in vivo*.

Paper V

- Monitor immune parameters that have been shown to be of importance in GVHD and other inflammatory settings in patients following treatment with DSCs for GVHD.
- Identify factors of importance that can be used to identify or predict response to treatment with DSCs, and gain a better understanding of how DSCs affect immunological parameters *in vivo*.

3 MATERIALS AND METHODS

For the exact manufacturers of reagents, consumables, and hardware, please see each individual paper, as this may have varied slightly between papers. In the following section, the basic methodology of the techniques that were most used in **Papers I–V** will be briefly described and discussed. The isolation of DSCs will be presented in more detail.

3.1 ETHICAL CONSIDERATIONS

Ethical approval for all the studies presented in this thesis was obtained from the regional ethics committee at Karolinska Institutet. This includes: isolation of mesenchymal stromal cells (DNR 446/00) and stromal cells from placenta (DNR 2009/418/31/4, 2010/2061-32). Clinical use of DSCs and follow-up of patients treated with DSCs was also approved (DNR 2010-452-31/4, 2014/2132-32) by the same committee. All patients signed informed consent and were treated in accordance with the Declaration of Helsinki.

3.2 ISOLATION AND EXPANSION OF STROMAL CELLS FROM PLACENTA

One advantage of stromal cells isolated from placental tissue is that the cells are obtained from tissue that would otherwise be discarded directly after delivery. Prior to planned, uncomplicated caesarean section, the mothers sign informed consent to donate their placentas. After caesarean section, the placenta is placed in a sterile metal container and transferred to the laboratory where the isolation takes place. The metal container is directly placed in a class II laminar airflow cabinet. The isolation protocol was inspired by the isolation protocol of amniotic epithelia and mesenchymal cells by Ellis and Strom[330,331]. The DSCs were used in all the papers presented in this thesis.

First, excess blood was washed away with phosphate-buffered saline (PBS). Then, the fetal membranes (containing amnion, chorion, and decidua parietalis) were dissected from the rest of the placental tissue. The cut in the membranes was made approximately 1 cm from the chorionic plate. This can be viewed in detail in **Figure 6**. The membrane was then cut into 3–4 pieces, placed in 50-ml falcon tubes, and washed another few times. This was followed by trypsination performed in four steps. First, the membranes were swirled in 10 ml trypsin/EDTA solution for 30 seconds. The trypsin digests were then discarded. Twenty-five ml fresh trypsin/EDTA was added and the tubes were incubated for 10 min at 37°C. The trypsin digest was discarded in this step also. Trypsin/EDTA was added a third time and was then incubated for 40 min. This step was repeated once. This yielded two products: the trypsin digests and the fetal membranes, which both contained DSCs. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin (hereon referred to as DMEMcomp), was added in the same volume as the trypsin/EDTA to inhibit the trypsin. The products were centrifuged and

washed twice in DMEMcomp. The cells in the trypsin digests were pooled, counted, and seeded in 185 cm^2 flasks at a concentration of 10^5 cells/cm^2. Four or five pieces of membrane were seeded in separate flasks. The total volume DMEMcomp in each flask was 20 ml. The medium was changed every 3–4 days. The stromal cells migrate from the tissue explants and adhere to the bottom of the culture flask. When colony forming units (CFUs) had developed (after ≥ 10 days), the membrane pieces were removed and the cells were expanded until 90% confluency had been reached (in 25 ml DMEMcomp). The cells were subsequently harvested by depletion of DMEMcomp and addition of 4 ml trypsin/EDTA followed by washing in DMEMcomp. The DSCs were frozen in aliquots (in DMEMcomp supplemented with 10% dimethylsulfoxide (DMSO)) until use. The cells were then at passage 0. For expansion to higher passages, 0.5×10^6 DSCs were added to a 185 cm^2 culture flask with 25 ml DMEMcomp (final volume). This was cultured as described above until 90% confluency was reached. This protocol was the same in all papers. Quality control and characterization of the DSCs will be presented in the Results section. The methods used were mixed lymphocyte reactions (MLRs) to investigate the immunosuppressive capacity of DSCs in the allogeneic setting, PCR to determine the origin of the cells, and flow cytometry (FC) for identification of cell-surface markers. Karyotyping and investigation of differentiation capabilities were also carried out, but not on DSCs from all donors.

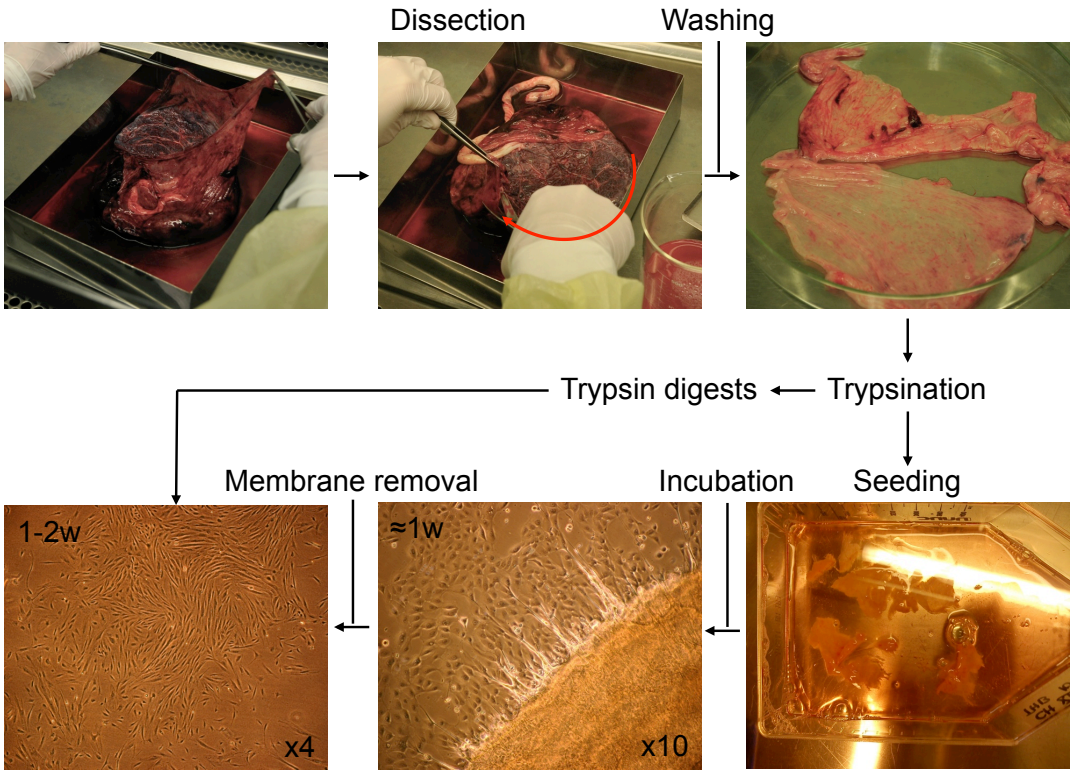


Figure 6. Schematic presentation of work flow for the isolation of decidual stromal cells from term placental tissue.

3.3 THE ALLOGENEIC SETTING *IN VITRO*

Most of the *in vitro* work in **Papers I–IV** regarding DSC-mediated immune modulation was based on the simple MLR. Briefly, PBMCs were obtained from buffy coats by Lymphoprep gradient centrifugation. The cells were washed twice in PBS and resuspended in RPMI 1640 medium supplemented with 5–10% human AB serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (hereon referred to as RPMIcomp) at a concentration of 3×10^6 cells/ml. These PBMCs were stimulated with the alloantigens from an irradiated pool of PBMCs from at least six donors. The ratio of responder cells to stimulator cells was 1:1. DSCs were added to these cultures, either directly in order to interact with the MLR through cell-cell contact, or in a transwell, which precluded cell contact-dependent interactions. The incubation period for the assay was normally 6 days. In addition to these standard conditions, a wide range of agents was added to these cultures. The DSCs may also have been pretreated with soluble factors before addition to the MLR. The readouts for this assay were proliferation by ^{3}H-thymidine incorporation (**Papers I–IV**), extracellular or intracellular phenotyping by FC (**Papers I**, **III**, and **IV**), determination of cytokine concentration by ELISA (**Papers I**, **III**, and **IV**) or by Luminex (**Paper IV**), and/or RNA expression by PCR (**Paper IV**).

Although the assay is methodically straightforward, it has limitations—especially regarding the specificity of the parameter of investigation. The MLR contains all cells of the lymphoid lineage, as well as monocytes. This allows a biologically direct and indirect allorecognition. The impurity of the system also limits the reliability of specificity of an interaction, which may very well be the result of an intricate cascade involving many cell types. This was of particular interest in **Papers I**, **III**, and **IV**, where specific pathways were investigated. In **Paper IV**, one of the explanations for why the MLR was favored over other stimulation assays—for instance, the use of anti-CD3/CD28 antibodies on purified T cells or stimulation with DCs—was that the interaction under investigation was not detectable in the anti-CD3/CD28 system due to the high level of T cell activation.

Proliferation was measured using ^{3}H-thymidine. Every time a cell divides, its genome is duplicated, which leads to incorporation of ^{3}H-thymidine. Based on the level of proliferation, this is detected as an amplified signal relative to the control. This method measures the proliferation over the last 16–24 h. An alternative technique for measurement of proliferation is CFSE staining, which is a dye that binds to the cell membrane and that is added to the cells before the incubation. This method has the advantage of showing the proliferation during the entire culture period. For every cell division, the dye will be diluted, theoretically halving the signal from the CFSE during FC analysis. However, in our hands the CFSE staining negatively affected both the proliferation characteristics and the phenotype of the activated T cells (**Paper III**, data not shown). We observed that the amount of activated T cells (CD25+) was systematically reduced when CFSE was used. Others have also found this[332], but toxicity may be avoided with optimized concentrations of CFSE and addition of FCS to the labeling medium[333]. Results on proliferation in MLRs only labeled with CFSE are given in **Paper I**.

3.4 FLOW CYTOMETRY

FC allows detection of cellular phenotype at the single-cell level. By using specific fluorochrome-conjugated antibodies, millions of cells with a very specific phenotype can be swiftly characterized. The instrument uses lasers, which hit droplets that have been preformed to contain a single cell stained with fluorchrome-conjugated antibodies. The emission spectrum for all the fluorochromes in each droplet is detected after the excitation. In **Papers I–V**, FC was the most commonly used instrument of analysis. The major limitation of the method lies in the overlap in the emission spectra of the different fluorochromes used. The panels used in **Papers I–V** had a maximum of nine colors. The number of parameters analyzed can be increased by using more recent technologies involving rare metals instead of fluorochromes[334], or by determining multiple RNA expression intensities at the single-cell level[335].

The staining procedure is described in each paper (**Papers I–V**). In the analysis, we used fluorescence minus-one controls (FMOs) to define the negative populations[336,337]. In high-dimensional FC, this is the preferred type of control. The FMO controls were especially important in **Papers IV** and **V**, where the intensity of expression of some parameters was very low. In those parameters, FMO controls were the only reliable way of differentiating positively stained cells from unstained cells. This also limits the reliability of the results to some extent, especially when taking the increased autofluorescence on activated T cells into account. In **Paper V**, other limiting factors such as sample size also determined the outline of the panels. Intracellular staining reduces the antigen epitopes on the cells, making it difficult to combine intracellular staining with other markers. This increases the number of specimens needed for each sample. We therefore chose to characterize the common T cell subsets with surface markers instead of using intracellular staining of their signature transcription factors (**Figure 2**). This allowed further phenotyping, without compromising the number of events collected in each specimen.

3.5 STATISTICAL ANALYSIS

For **Papers I–IV**, Wilcoxon matched-pair signed rank test was performed on related samples, whereas the Mann-Whitney U-test was used for continuous unrelated variables when comparing two groups.

For **Paper V**, Fisher's exact test was performed on non-parametric categorical data including two groups and two variables. Where additional parameters were included, Chi-square test was used. The D'Agostino and Pearson omnibus normality test was used to determine normal distribution. Since the patient data were related for each patient over time, Friedman's test was used to compare all time points in each group (responder/non-responder/all patients) for

each parameter. The pairwise comparison that followed required Bonferroni adjustment to reduce the p-value for what was regarded as a significant finding.

Orthogonal projection to latent structures by means of partial least-squares discriminant analysis (OPLS-DA) was used to find parameters that differed between the responders and the non-responders among the parameters assessed by FC and Luminex. This analysis scales all values for each parameter, calculates the difference between the groups, and presents the parameters of importance that discriminate the groups. The parameters of importance generated by the OPLS-DA were subsequently analyzed with the Mann-Whitney U-test.

4 RESULTS AND DISCUSSION

4.1 ISOLATION, CHARACTERIZATION, AND EXPANSION OF DECIDUAL STROMAL CELLS

MSCs have immunomodulatory properties and they have been used successfully as a cellular therapy against GVHD. However, various aspects of the protocol required improvement. For instance, isolation of the cells was invasive for the donor and the expansion potential of the cells was highly variable and depended on the donor. In order to establish a clinical therapy, it is of importance to be able to obtain the cells of interest easily, and to be able to expand them to numbers sufficient for efficient clinical therapy. The aim of **Paper I** was therefore to find stromal cells that were easily isolated and had potent immunosuppressive functions. Stromal cells from placental tissue have a crucial role in feto-maternal tolerance, and should therefore have immunomodulatory properties to sustain the fetal allograft during pregnancy[238]. Cells from placental tissue are easily obtained, since the placenta is normally discarded after delivery. Studies published before **Paper I** had indicated that stromal cells from the placenta could be isolated and that the cells had a comparatively great expansion potential[274]. These papers, however, lacked comparative functional and characterization data that would be of importance for possible implementation in cellular therapy. With the rationale of finding stromal cells of potential clinical interest, with comparable immunomodulatory properties to those of MSCs isolated from bone marrow, we isolated stromal cells from different parts of the term placenta. The sites from which stromal cells where isolated included the two feto-maternal interfaces, the blood-bathed placental villi (PVSC) and the fetal membranes (DSCs are described as FMSCs in **Paper I** and as FMCs in **Paper II**). Additionally, stromal cells were isolated from Wharton's jelly of the umbilical cord (UCSCs). The stromal cells from these three compartments were then characterized and compared to MSCs derived from the bone marrow. Morphologically, cells from all three sources are quite similar (**Paper I**), and have spindle-like fibroblast morphology (see **Figure 6**). Moreover, the expression of surface markers that are used to identify MSCs[283] is the same when comparing the four sources (**Paper I**), and includes expression of CD29, CD73, CD90, and CD105. Moreover, of the markers that we investigated, we found that DSCs and MSCs also expressed CD44 and PD-L1 (**Paper I**), and DSCs were positive for PD-L2 also (**Paper II, Figure 7**). Stromal cells from all sources were negative for CD14, CD31, CD34, CD45, CXCR4, SSEA-3, and negative or slightly positive for SSEA-4 (only on MSCs) (**Paper I**). Additional markers on DSCs were investigated in **Paper II**, including CD14, CD86, EpCAM, VCAM-1, and HLA-G. DSCs have no expression of these markers. Of the markers, VCAM-1 has been shown to be inducible on MSCs in mice by activated splenocytes, or IFN-γ with IL-1 or TNFα, but it is not constitutively expressed[304].

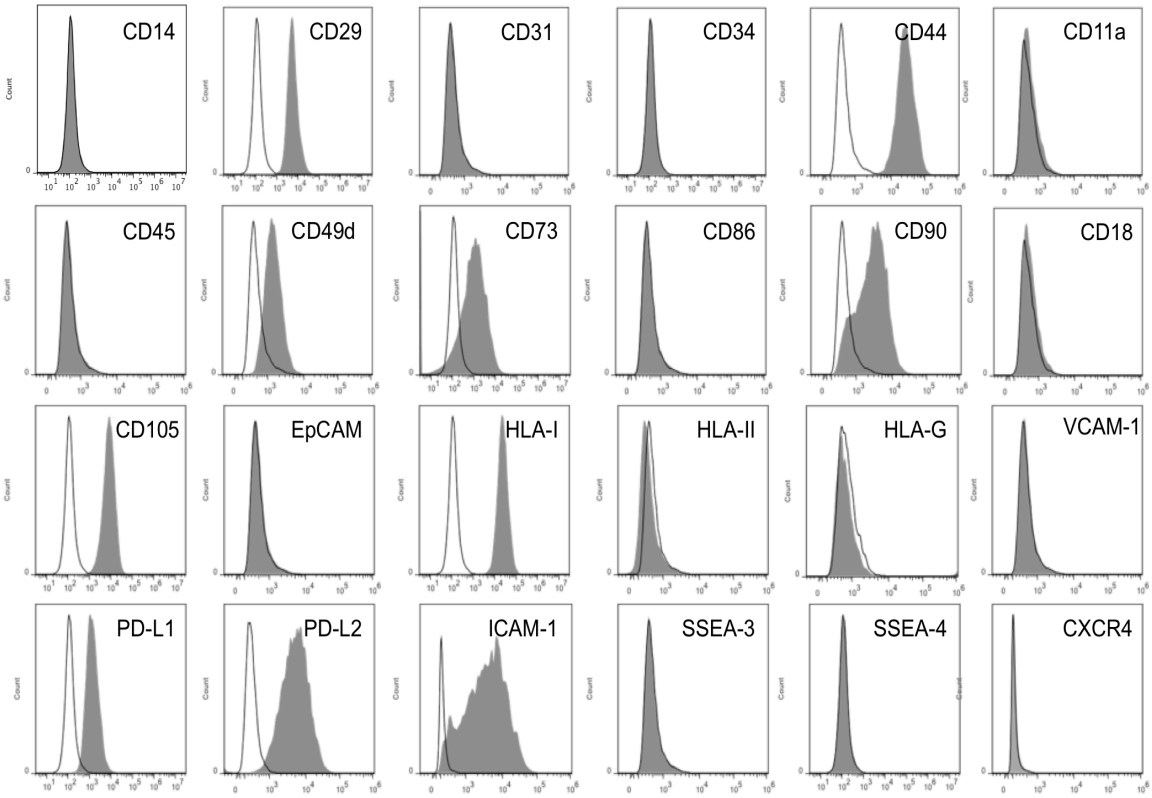


Figure 7. Representative plots of the surface phenotype of decidual stromal cells. Filled histograms represent stained samples and transparent histograms represent isotype controls.

Distinct differences were observed when the expression of adhesion molecules was determined. DSCs had significantly higher expression of CD49d, CD29, PD-L1, and ICAM-1 compared to MSCs (**Paper I**). Interestingly, ICAM-1 has previously been reported to be involved in MSC-mediated contact-dependent suppression of immune responses[304]. The expression of CD49d and CD29 may also indicate an ability to home to inflamed tissue, although no evidence for this was given in a subsequent paper where DSC distribution *in vivo* was tracked in patients with cGVHD[118].

In contrast, while MSCs from bone marrow are able to differentiate into bone and fat, all stromal cells isolated from the different parts of the placenta did not differentiate into bone and fat. Whether or not stromal cells from term placentas have differentiation capabilities is debated. Work by In'T Anker *et al*. and many others have shown that stromal cells from placenta and decidua have the ability to differentiate[274,338,339]. Conflicting reports by Kanematsu[273] *et al*. and Pilz[340] *et al*. among others showed results in line with ours. It is important to note that the isolation techniques used in these papers and in **Papers I–V** were similar, but they differed in some respects. Kanematsu and In´T Anker both only used the trypsin digests for isolation of the stromal cells, and they did not culture membrane pieces in addition to the trypsin digests. Pilz used collagenase and cultured pieces of membrane. A recent review by Kmiecik *et al*. discussed the various results regarding differentiation potential to (above all) osteoblasts and how the osteogenic potential can be enhanced depending on the site of isolation and on selection of stromal cells based on cell-surface

marker expression[339]. Although most MSCs are cultured with passaging, the gradual loss of differentiation potential following *in vitro* expansion of MSCs has not been discussed[341,342]. Many factors may influence the diverse results, including cell origin, isolation and expansion of the stromal cells, and the methods used for determination of the characteristics.

Following the initial isolation of stromal cells from the various placental tissues, a pure population of stromal cells is not obtained. For instance, the isolates contain epithelial cells. When isolating cells from the fetal membranes, epithelial cells are present both in the trypsin digests and on the fetal membranes. When the cells are seeded for the first time, epithelial cells adhere to the plastic. However, following the incubation period, the epithelial cells are unable to proliferate, yielding a pure stromal cell population after the first harvest (passage 0 to passage 1). Amniotic epithelium (AE) is one of the placenta-derived cell populations that have also been identified as a source of cells that may be used as cellular therapy in regenerative medicine[343]. These cells also have differentiation capability[344], and immunomodulatory capability. However, expansion of epithelial cells requires a different cultivation medium[331,345]. The DMEMcomp medium that we use is therefore selective for stromal cells and not epithelial cells. Due to the fact that AE has differentiation capability; there is the possibility that AE could differentiate into stromal cells during the expansion[346,347]. In **Paper I,** we cultured AE in DMEMcomp. We did not observe differentiation into stromal cells, and these cells were not able to expand in the culture medium. AE is of fetal origin and is easily isolated from the amnion that is attached to the chorionic plate. A pure AE population can therefore be obtained. One thing that is certain about AE is that it is of fetal origin. Additional proof for no AE being present in the cultures was given in **Paper II,** where PCR with primers using microsatellite polymorphism in the mother and child was used to determine the origin of the cells. We could clearly see that the cultured DSCs were of maternal origin. The maternal blood and cord blood (or AE) were used to identify the mother and the child, respectively. The conclusion from this analysis was that the stromal cells that we cultured did not originate from chorion or amnion, since these tissues are of fetal origin. One issue in **Paper I** when we investigated stromal cells isolated from different parts of the placenta was that the origin of the cells was not determined. In our hands, DSCs appear to be favored by our isolation and cultivation protocol. This may very well have led to contamination of DSCs in the stromal cell isolates from chorion. The anatomical difference in the amnion (mechanically separated from the chorionic plate, **Figure 5**) increase the probability of these cells originating from the fetus. The stromal cells isolated from the amnion also had reduced proliferative ability compared to the other cell isolates. Chorionic stromal cells were isolated from the fetal membranes. We therefore concluded that these cells are most likely DSCs, like the cells isolated from whole fetal membrane (FMSCs). The anatomical separation from maternal tissue in the isolation of PVSCs and UCSCs limits the possibility of these cells originating from the mother. PVSCs were isolated from the fetal side of the chorionic plate (**Figure 5**)[348]. In retrospect, it can be debated whether PVSCs should have been referred to as stromal cells from the chorionic plate rather than from the placental villi.

Before publication of **Paper II**, the origin of DSCs was unknown. Despite the fact that the cells are isolated from fetal tissues, they are of maternal origin. A recent review has highlighted the confusion regarding the origin of stromal cells isolated from placental tissue[282]. Among the studies in which cells have been isolated and cultured from placental tissue, the characterization of the cells has been poor. Many of the studies published have actually shown that the incidence of maternal origin of the stromal cells isolated is high (approximately half of the studies investigated), especially in papers where cells were isolated from the fetal membranes.

One of the main findings in **Paper II** was the *in vitro* expansion potential of DSCs. In **Paper II**, DSCs from four donors were isolated and expanded. To date, DSCs from a total of seven placental donors have been expanded and used (**Papers II** and **V**). The total number of cells expanded from each donor is presented in **Table 1** (last updated June 2015). Others have published data where the expansion of stromal cells from different sources has been compared[273,274]. With addition of the results in **Paper II** and in **Table 1**, it can be concluded that DSCs have a great expansion potential, and a large number of cells can be obtained at low passage number. An exponential expansion of DSCs *in vitro* raises the concern of altered properties of the cells. As presented in **Paper II**, DSCs have a normal karyotype following expansion, suggesting that no severe chromosomal alterations have occurred.

Table 1. Presentation of the total number of decidual stromal cells (DSCs) expanded from seven donors. Passage refers to the passage number to which the DSCs have been expanded. Expansion completed shows the present expansion status for each donor. For the donors where DSCs are still available, an estimate for the completion of expansion to passage 4 was made. This was based on the growth coefficient of each donor, and the number of DSCs available in passages lower than 4.

Placenta ID	Cells expanded ($\times 10^9$)	Passage	Expansion completed (P4) (%)
FM1	1.8	2-3	100
FM3	3.6	2-4	100
FM7	2.7	2-4	100
FM8	5.3	2-4	40
FM11	1.5	2-4	20
FM13	1.8	2-4	18
FM14	1.9	2-4	13

4.2 IMMUNE MODULATION *IN VITRO*

Evaluation of the immunomodulatory functions of stromal cells by determining their ability to inhibit alloantigen-stimulated proliferation in MLRs was the first step in **Paper I**. This was an important characteristic for one of the specific aims of **Paper I,** which was to compare the features of tissue-specific stromal cells in the allogeneic setting. Stromal cells from all sources except placental villi were able to suppress proliferation in the MLRs (**Paper I**). The lowest proliferation was observed in the MLRs with added MSCs or DSCs. As reported earlier in the literature[288], we also determined that all stromal cells alone are capable of

inducing a small proliferative response when cultivated with PBMCs of allogeneic origin (**Papers I** and **III**). These data suggest that stromal cells are immunogenic, despite having an immune inhibitory function in a highly inflammatory setting such as the MLR. The hypothesis regarding MSCs being immune privileged has also been questioned recently[349]. The data presented in **Papers I** and **III—**as well as other reports and the suggested role of MSCs as functional APCs**—**indicate that MSCs can induce innate[292] and adaptive immune responses[307]. In the clinical setting, DSCs have been shown to induce anti-HLA antibodies in immunocompetent individuals, whereas immune suppressed patients do not develop antibodies[350]. Also, there are only limited data on whether stromal cells avoid cytotoxic T cell activity. However, one study has shown that allogeneic T cells primed with PBMCs from the same donor as the MSCs, lyse MSCs to a lesser extent than PBMCs[351]. This is based on the assumption that PBMCs from the same donor as the MSCs will prime the same T cell population. This may exclude activation of T cells by tissue-specific minor histocompatibility antigens presented by the MSCs. The conclusion is that MSCs are not immune privileged. Still, third-party stromal cells from various tissues are immunosuppressive in the allogeneic setting. To explore this further, and the possible differences between stromal cells from various compartments of the placenta and MSCs, cytokine concentrations of IFN-γ, IL-10, IL-17, and IL-6 in supernatants from the MLR cultures was determined (**Paper I**). Addition of stromal cells from either source induced a small production of IL-17, IL-10, and IL-6 when added to PBMCs. In the MLR setting, the presence of DSCs or UCSCs reduced the concentration of IFN-γ in the MLRs. In the same setting, IL-10 was increased. In contrast, while UCSCs increased the concentration of IL-17 in the allogeneic setting, DSCs reduced the IL-17 concentration. This is also in line with the IL-6 results, where DSCs had the lowest background production of IL-6 and a low median production of IL-6 in the MLR setting compared to other stromal cells. As mentioned, IL-6 is a key cytokine for induction of Th17 cells[71]. IL-6 was increased under all conditions where stromal cells were added. MSCs did not significantly alter any cytokine concentrations other than that of IL-6, when added to the MLRs. Other reports have suggested that MSCs can either induce[352] or suppress IL-17 and Th17 production and differentiation[353,354]. In a setting with purified T cells and anti-CD3/CD28 stimulation, DSCs did not increase the concentration of IL-10 after 3 days (**Paper I**). This may be interpreted in two ways: either the induction of Tregs and their subsequent IL-10 production takes longer than 3 days (Tregs are induced by DSCs in MLRs, **Paper III**), or one of the cell types removed in this particular assay (e.g monocytes[355]) is the main producer of IL-10 in the MLR setting. Either way, IL-10 does not appear to be crucial for DSC-mediated suppression. Blocking of IL-10 with an anti-IL10 antibody does not impair the antiproliferative ability of DSCs (**Paper III**). IL-10 is not necessary for a successful pregnancy[265], but the levels of IL-10 are increased during pregnancy and reduced IL-10 concentrations are also associated with spontaneous abortion[356]. Provocatively, this might suggest that IL-10 is just a factor that results from the generation of Tregs and M(IL-10), which are of greater importance for the maintenance of feto-maternal tolerance. A more rational explanation would be that IL-10 is important in pregnancy, but that cells other than DSCs maintain IL-10 production.

One of the prerequisites for finding a new cell source for the clinical setting was the ability to suppress the inflammatory profile in the MLR. PVSCs did not suppress the proliferation in MLR, and although the UCSC setting showed a high IL-10 concentration, IL-17 was significantly increased and the concentration of IL-6 was high compared to DSCs. DSCs had a consistent antiproliferative ability, reduced IFN-γ and IL-17, increased IL-10, and a high expression of integrins that might be of importance in the clinical setting. Based on this, further studies in **Paper I** and **Paper III** were therefore focused on DSCs and MSCs. In **Paper IV**, we concentrated entirely on DSCs.

Many of the immune-modulatory effects of MSCs are dependent on initial priming of these cells—with, for example, IFN-γ, IL-1α/β, and/or TNFα [291]. Following pretreatment with IFN-γ, MSCs upregulate the expression of HLA class II[306], which may enable them to prime CD4+ T cells[284]. In **Paper I**, we also found that the expression of PD-L1 and ICAM-1 was increased by IFN-γ (**Figure 8**). Interestingly, DSCs did not express HLA class II upon IFN-γ stimulation. In addition, the intensity of expression of PD-L1, CD49d (α4 integrin), and ICAM-1 was higher on DSCs than on MSCs. Although not confirmed in **Paper I**, the low expression of HLA class II and CD86 may reduce the possibility of DSC-mediated priming of CD4+ T cells. This finding contradicts the study by Olivares *et al*., which found that DSCs treated with progesterone *in vitro* and isolated from the first trimester could express both HLA class II and CD86[357]. Another study by Nagamatsu *et al*. suggested that term DSCs (although isolated differently from those in **Papers I–V**) may upgregulate expression of HLA-DR when treated with IFN-γ, but not when treated with TNF-α[358]. Another finding that characteristically distinguishes DSCs from MSCs[294] is constitutive expression of IDO, as presented in **Paper III**. According to immunofluorescence staining, as well as FC, DSCs have a constant expression of IDO and this expression is not significantly increased by IFN-γ. However, subsequent analysis of RNA expression showed low transcriptional levels of IDO RNA in unstimulated DSCs, while levels were increased when the DSCs were stimulated with IFN-γ (Solders *et al*., unpublished). The same analysis for HLA class II molecules has not been performed with DSCs yet. These data may indicate that IFN-γ may have a slightly different role in immune suppression in DSCs than in MSCs. Priming of MSCs with IFN-γ increases their antiproliferative effect, while the same pretreatment of DSCs actually gives reversed results (**Paper III**). Despite this, blocking of much of the IFN-γ present in the MLRs leads to a reduced antiproliferative effect being exerted by the DSCs (**Figure 9**). This may lead to the conclusion that DSCs need a small amount of IFN-γ to retain certain functions—such as production of IDO and expression of PD-L1, HLA class I molecules, and integrins. On the other hand, high concentrations of IFN-γ may increase functions on the DSCs that support the alloproliferation, and this was not investigated in **Papers I–V**. We did not observe an increased expression of HLA class II molecules, which should to some extent prevent DSCs from acting as APCs for CD4+ cells. But this does not exclude the possibility that DSCs cannot provide co-stimulatory signals to CD4+ T cells. The increased expression of ICAM-1 should enhance the interaction with T cells and allow interaction through, for example, PD-L1 or HLA class I, the expression of which is also elevated in the same setting.

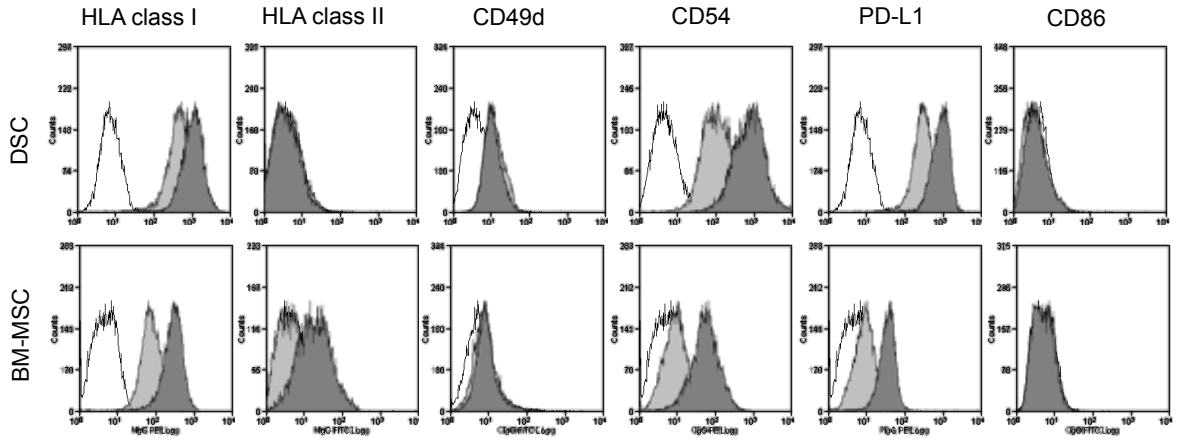


Figure 8. Representative plots of phenotype of decidual stromal cells (DSCs) and bone marrow-derived mesenchymal stromal cells (MSCs). The transparent histograms are isotype controls, light gray histograms are untreated cells, and dark gray histograms are plots where the cells were stimulated with 100 U of interferon-γ for 48 h prior to analysis.

Further studies regarding these findings may explain how IFN-γ affects DSCs, which would be of great interest. For instance, the expression levels and responsiveness of the IFN-γ receptor (CD119) in DSCs are unknown, as is the level of activation of the major signaling pathways following JAK1/2 phosphorylation on the IFN-γ receptor. The findings in **Papers I** and **III** should be confirmed with alternative methods, but the conflicting results in the same setting with different cell types still indicate that the responsiveness to IFN-γ may differ between DSCs and MSCs. Other suggested mechanisms that may influence the differences seen with IFN-γ priming could be gene silencing of, for example, HLA class II, which has been reported to inhibit production of chemokines in the decidua[246]. Additionally, there are other factors (apart from IFN-γ) that can increase the expression of IDO, such as PGE_2[359,360]. PGE_2 is another factor that may be of importance for DSC-mediated suppression (**Paper III, Figure 9**). As PGE_2 may induce expression of IDO, the results in **Paper III** do not explain whether the immunosuppressive effect seen when blocking PGE_2 is a direct consequence of the immune-modulatory effect of PGE_2 or an indirect mechanism that is the result of impaired IDO production. The functions of PGE_2 are diverse, and they can be regarded as being both proinflammatory and anti-inflammatory, as PGE_2 is a main activator of early inflammation (e.g. attraction of innate immune cells to sites of inflammation, vasodilation), while affecting some adaptive immune cells to promote an anti-inflammatory shift. In adaptive immunity, PGE_2 has been shown to inhibit IL-2 production[361] and reduce the amount of JAK3 in T cells[362], indicating a reduced responsiveness to IL-2 in T cells. In contrast, studies have shown that PGE_2 may also induce the IL-2-dependent Treg subset[363,364], Th17 cells[365], and promote a shift towards Th2 cells by inhibiting the cytokine that promotes Th1 cells—IL-12[366]. Based on the results in **Papers III** and **IV,** PGE_2 may be involved in the production of IL-10 and generation of Tregs, but the results in **Paper IV** contradict the role of PGE_2 and its inhibitory effect on IL-2 production in this setting. However, whether or not PGE_2 might affect STAT5 phosphorylation (pSTAT5) through reduced JAK3 levels was not determined in **Paper IV**. Interestingly, PGE_2 is a factor that plays a pivotal role during implantation and promotes tolerance during the first trimester.

In response to IL-1α, stromal cells in the uterus have been reported to produce PGE_2 in experimental models[367]. IL-1 was present at high levels in our *in vitro* experiments (data not shown), which supports the idea of PGE_2 being a suppressive mediator induced by DSCs in the allogeneic setting *in vitro*.

One of the factors that appears to be of major importance for DSC-mediated suppression in our setting is IDO. Inhibition of IDO activity reduces the ability of DSCs to suppress the MLR (**Paper III**). In addition, inhibition of IDO reduces the frequency of Tregs in the cultures (**Paper III**). These results are in line with the work of many others[295,368-370]. Interestingly, many of the findings in **Paper III** may be derived from the presence of IDO. We are not aware of any link between IDO and PD-L1. However, even though IDO appears to be important for the immunosuppression *in vitro*, the data in **Paper III** also show that IDO cannot be the only mediator of suppression. The proliferation of the MLRs is reduced when stromal cells are added to the culture. However, if the stromal cells are added in a transwell, the antiproliferative effect in the MLR is reduced, as is the frequency of Tregs. This supports the idea that the DSC-mediated suppression *in vitro* is also at least partly dependent on contact with the cells in the MLR. The frequency of Tregs is increased compared to control MLRs when the DSCs are also placed in the transwell, which could in part be a result of the soluble products originating from IDO activity—or it could be due to the DSC's constitutive production of TGF-β. MSCs are able to suppress the MLR in a transwell, which is also in line with their ability to upregulate secretory immunomodulatory functions due to the presence of IFN-γ, IL-1α/β, and/or TNFα in the cultures. The results in **Papers I** and **III** indicate that DSCs are affected differently by IFN-γ, which could hypothetically reduce the ability of DSCs to suppress the MLRs from a distance. As presented in **Papers I** and **II**, DSCs express PD-L1 and PD-L2. When we blocked PD-L1 in the cultures, the proliferation was increased (**Figure 9**). The interaction between PD-L1/PD-L2 and PD-1 requires cell-to-cell contact. One of the drawbacks of this assay is that PD-L1 is blocked on the DSCs and on any other cells that may express this in the culture (e.g. macrophages). Even so, the addition of an anti-PD-L1 antibody increased the proliferation in the MLRs, indicating that PD-L1/PD-1 interactions are involved in immune modulation by DSCs. Engagement of PD-1 by PD-L1 inhibits signaling through the TCR[371,372]. The inhibitory signaling by PD-1 can, however, be overcome by a strong co-stimulation of CD28 and/or IL-2. In **Paper IV**, we found that the MLR and DSC co-cultures resulted in a high concentration of IL-2 in the supernatant. One could therefore speculate whether the high concentrations of IL-2 in the cultures would mask a higher significance of the PD-L1/PD-1 axis in this setting. Apart from interaction between PD-L1 and PD-1, PD-L1 has been shown to bind to CD80, making it an additional competitive binding molecule besides CD28 and CTLA-4[373]. In **Paper III**, blocking of PD-L2 was also done. This, however, had no effect on the proliferation of the MLR, and there was no additive effect of combined blocking of PD-L2 and PD-L1.

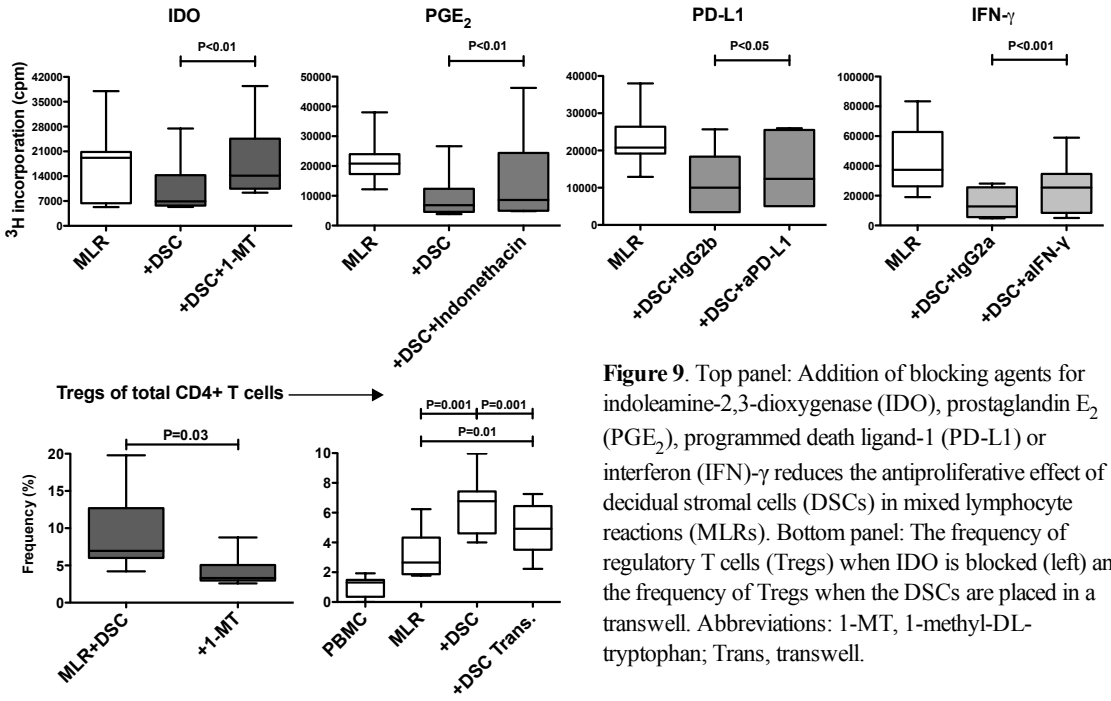


Figure 9. Top panel: Addition of blocking agents for indoleamine-2,3-dioxygenase (IDO), prostaglandin E_2 (PGE_2), programmed death ligand-1 (PD-L1) or interferon (IFN)-γ reduces the antiproliferative effect of decidual stromal cells (DSCs) in mixed lymphocyte reactions (MLRs). Bottom panel: The frequency of regulatory T cells (Tregs) when IDO is blocked (left) and the frequency of Tregs when the DSCs are placed in a transwell. Abbreviations: 1-MT, 1-methyl-DL-tryptophan; Trans, transwell.

Moreover, we did not observe that some of the other factors that we blocked had an influence on the proliferation of the MLRs. Blocking of the activity of IL-17, TGF-β, or HLA-G did not affect the antiproliferative ability of the DSCs.

The increase in Tregs in the MLRs with DSCs is interesting (**Figure 9**). It has also been shown that Tregs are enriched in decidual tissue[90]. It can unfortunately only be speculated what subtype of Tregs is present in the cultures, and whether these cells have a central or an effector phenotype. The increase seen at 6 days may depend of a variety of factors: an expansion of inducible or naturally occurring Tregs (nTregs), a differentiation of inducible Tregs (iTregs), or increased survival ability of the Tregs in the culture compared to other cell types in this setting. The work in **Paper III** shows that the frequency of Tregs is to some extent increased when the DSCs are placed in a transwell. As DSCs are a source of TGF-β, this may induce FOXP3 expression in CD4+CD25+FOXP3− cells[98]. One interesting hypothesis that might be investigated is also whether the contact-dependent and non-contact-dependent DSC interactions promote different types of Tregs. Functional studies and further in-depth analysis of the Tregs may explain the importance of these cells in this setting. Such analysis might include expression of CCR7 and CD62L[92,93], to determine whether the cells are of an effector or central phenotype. Cytokine secretion and suppressive capacity can be used to explore the functionality. Helios has previously been suggested as a marker that can be used to identify Tregs that originate from the thymus (naturally occurring Tregs)[374]. This has, however, been questioned in a study where this Treg subset was shown to have an inconsistent expression of Helios, while still having functional similarities[375]. A more recent study found that CD15s were expressed on Tregs with a high suppressive capacity[376]. Combined analysis of these factors may explain the importance of Tregs in this setting. A guess in this case would be that DSCs promote differentiation of inducible Tregs, as the population that contributes to the increased frequency has lower intensity of expression of

FOXP3 than the control MLR setting. This may indicate unstable expression of FOXP3 and the inducible state of FOXP3 gene expression seen in T cells that are dependent on TGF-β to maintain a FOXP3+ phenotype[377]. The expression of FOXP3 in nTregs is regarded as being fairly stable[378,379]. However, stability of the FOXP3 expression in iTregs and nTregs is still debated. Interestingly, differences in the frequencies of Tregs could not be seen in **Paper V** between patients who had a lower grade of GVHD and patients who deteriorated after systemic administration of DSCs.

Furthermore, the plots in **Paper III** that show the frequency of Tregs in the MLRs was also one of the underlying reasons for the investigation of IL-2 in this setting, which was part of **Paper IV**. Here, we observed for the first time that DSCs in contact with the MLR had drastically increased expression of CD25 (IL-2Rα). This is an indication of IL-2 production. Determination of the concentration of IL-2 in the supernatant of these cultures showed a high concentration of IL-2 in the MLR + DSC condition. If the DSCs were put in a transwell, the IL-2 concentration in the supernatant was low, indicating that the DSCs may contribute to this. The DSCs themselves are unable to produce IL-2. The concentration of IL-2 peaked at day 3–4 of incubation. Interestingly, others have presented data of high IL-2 levels under conditions with stromal cells[380-383], but the phenomenon has not been addressed in detail. However, the induction of IL-2 in the allogeneic setting is not peculiar, and it fits into the context of the MLR as a highly T cell proliferative milieu and supports the findings of MSCs inducing Tregs[95,384,385]. This leads to the question: why are the levels of IL-2 high in these cultures? IL-2 is regarded as an autocrine/paracrine cytokine that is produced and rapidly consumed, leading to T cell expansion and proliferation[119,120].

In **Paper III**, the expression of CD25 was investigated, but little was known regarding the expression of the other IL-2R subunits, the common γ-chain (γ_c, CD132) and the β-chain (CD122) on T cells in this setting. In **Paper IV**, the expression of CD25 was mapped in a large number of experiments. In our hands, the frequency of CD25 was not changed when DSCs were added to the MLR. The intensity of expression of CD25 was, however, consistently elevated to a high degree. *In vivo* data on cells isolated from the decidua parietalis and peripheral blood have shown that CD25 expression is elevated during pregnancy[386], indicating that our findings may to some degree be associated with the *in vivo* situation during pregnancy. CD132 was constitutively highly expressed on T cells, but its frequency and intensity of expression was reduced in the MLR + DSC setting. CD122 has low constitutive expression; it was upregulated in the MLR, and the expression was comparable to the unstimulated situation when DSCs were added to the MLR. The intensity of expression in the MLR+DSC setting was reduced compared to the MLR. Finally, when investigating the combined frequency of expression of the high-affinity IL-2R, we observed that the expression was low in the MLR + DSC setting on day 6 of incubation (**Figure 10**). These results were associated with reduced pSTAT5 expression in both CD4+ (trend) and CD8+ cells (**Figure 10**), but not in Tregs, although the intensity of pSTAT5 was also reduced in the Tregs (**Paper IV**). Tregs are regarded to be more dependent on IL-2 for expansion[94,95]. While not all CD4+ cells or CD8+ cells were able to phosphorylate STAT5 upon IL-2

stimulation, all Tregs were pSTAT5+ following IL-2 stimulation at the end of the incubation period.

There are some factors that may influence this setting. Soluble IL-2Rα expression is increased in response to IL-2. Increased levels of this factor are also associated with GVHD[387,388].We did not detect any difference in soluble IL-2R concentration when stromal cells were added to alloantigen-stimulated PBMCs *in vitro*. Another factor that has been identified and must be taken into account in this setting is the possibility of alternative splicing of IL-2 RNA, where IL-2 derived from spliced versions may block the IL-2R[389,390]. We could not, however, identify any differences between our *in vitro* conditions regarding alternative splicing. Taken together, these results suggest that soluble IL-2 and alternative splicing of IL-2 have little impact in our setting. Additionally, conditioned medium from MLR + DSC cultures was also capable of stimulating phosphorylation of STAT5 in T cells to the same extent as 10 ng/ml rIL-2.

Previous studies in mice by another group have shown that addition of IL-2 to a culture with allo-stimulated T cells and MSCs reverse the T cell anergy induced by the MSCs, and restore proliferation in this setting[391]. In **Paper IV**, IL-2 was added at the beginning of the experiment and the response was measured on day 3. On that day, expression of IL-2R was still high (Erkers *et al*., unpublished observations), and addition of IL-2 would stimulate further expansion of the PBMCs. Addition of IL-2 at this stage may overcome the negative signaling of, for instance, PD-L1—as discussed previously. However, the data in **Paper IV** suggest that if the experiment is given further incubation, the T cells will become less responsive to IL-2. This will ultimately leave the cells unable to be saved by addition of exogenous IL-2.

Due to the kinetics of IL-2R expression, experiments in which DSCs were exchanged for recombinant IL-2 showed that the reduction in CD122 expression could be seen with addition of an equal concentration of IL-2 to that detected in the supernatants on day 3. The high IL-2 concentration may in part be responsible for the depletion of CD122 expression. In **Paper IV**, we suggested that the reduction in pSTAT expression might be due to the depletion of IL-2R. Combining this with the results of **Paper III**, PGE_2 could also be a factor that influences the expression of pSTAT5[362]. Other cytokine receptors that express CD132 include IL-4[392], IL-7[393], IL-9[394], IL-15[395], and IL-21[396]; they may also influence the output in these *in vitro* assays. In comparison, the levels of these cytokines are very low in our setting compared to that of IL-2 (Erkers *et al*., unpublished observation). We did not do any further investigation of IL-15 in this setting, despite the fact that it shares CD122 and CD132 with IL-2, and the signaling pathway is very similar[397]. One way of finding out whether reduced pSTAT5 expression is due to depletion of the IL-2R is to add ^{125}indium-labeled rIL-2 to the cultures (**Figure 10**). Our hypothesis is that [^{125}I]rIL-2 is taken up by the cells with IL-2R, and that this may vary depending on the expression of IL-2R.

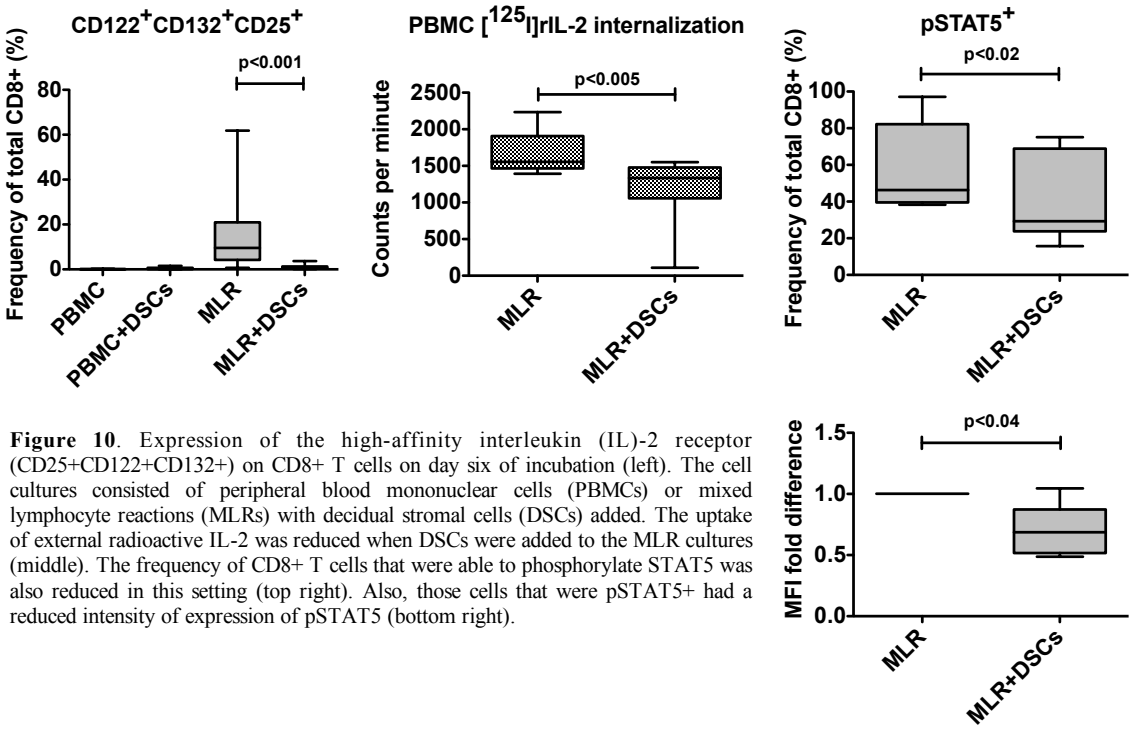


Figure 10. Expression of the high-affinity interleukin (IL)-2 receptor (CD25+CD122+CD132+) on CD8+ T cells on day six of incubation (left). The cell cultures consisted of peripheral blood mononuclear cells (PBMCs) or mixed lymphocyte reactions (MLRs) with decidual stromal cells (DSCs) added. The uptake of external radioactive IL-2 was reduced when DSCs were added to the MLR cultures (middle). The frequency of CD8+ T cells that were able to phosphorylate STAT5 was also reduced in this setting (top right). Also, those cells that were pSTAT5+ had a reduced intensity of expression of pSTAT5 (bottom right).

Although there was a high amount of IL-2 (low IL-2 levels being an indication of exhaustion[398]), the data presented in **Paper IV** regarding IL-2R expression may indicate that the PBMCs in the cultures had a higher rate of exhaustion. Previous studies have also indicated that high concentrations of IL-2 have a pro-apoptotic effect[124]. We therefore determined the expression of PD-1 and CD95, which may be associated with exhaustion in chronic infection[399,400]. Although IL-2 expression is increased and IL-2R expression is reduced in the MLR + DSC setting, no differences in PD-1 or CD95 expression could be seen compared to the control MLR setting. Interestingly, addition of a large dose of IL-2 to the MLRs increased the frequency of CD95+ cells, while the PBMCs incubated with DSCs in the MLR did not upregulate expression of CD95. This is in line with other data indicating that DSCs prevent apoptosis in lymphocytes[401], and that IL-2 in high concentrations induces CD95[402] and apoptosis [124].

The underlying reason for increased IL-2 concentration in the presence of DSCs was not determined in **Paper IV**. Experiments where intracellular staining was performed showed that the production of IL-2 occurs in T cells (**Paper IV**). The number of experiments was, however, low, and other cell subsets were not investigated. Further experiments to reveal factors of importance for the induction of IL-2 in this setting are important. Despite the low expression of HLA class II molecules on DSCs, these cells are a source of allo-recognition that, while they still work regularly in a suppressive fashion, are able to provide stimulation (TCR) that may trigger IL-2 production. While the data in **Papers I–III** may indicate that DSCs have a reduced ability to provide co-stimulation (no expression of CD80 and CD86, **Figure 7**), the allogeneic setting probably play a large role in providing co-stimulatory signals, as PBMCs cultivated with allogeneic DSCs do not appear to alter IL-2 production significantly. This is an important point, as MLRs in particular are an *in vitro* model for the

HSCT setting rather than for gestation. The data support the idea that DSCs promote IL-2 production, which subsequently leads to depletion of the full IL-2R complex—which may in turn explain the reduced pSTAT5 and IL-2 uptake in alloantigen-activated T cells *in vitro*.

In the HSCT setting, the use of immunosuppressive drugs is crucial to handling the alloreaction after transplant. One type of immunosuppressant—e.g. CyA and SRL—to some extent targets the downstream signaling of IL-2 (**Figure 3**). We therefore found it of interest to investigate the combined effect of DSCs and CyA or SRL in the allogeneic setting *in vitro*. From previous publications, it is known that IL-2 may reverse the immunosuppressive effects of CyA[403]. A high dose of CyA can then be used to overcome this. In contrast, several studies have reported various synergistic tolerogenic effects of MSCs and immunosuppressive drugs[404-407]. We therefore combined DSCs and CyA or SRL in MLRs, and measured proliferation between days 5 and 6 of incubation. Addition of DSCs or CyA/SRL independently suppressed proliferation of the MLRs. Combination of DSCs and CyA suppressed the culture, but the proliferation was not synergistically reduced further. In contrast, combination of SRL and DSCs did not result in reduced proliferation compared to control MLRs. The proliferation in the DSC + SRL situation was also significantly higher than in the situation with only SRL added to the MLR. This result is in line with the results of another study, by Buron *et al*., where a combination of MSCs with CyA or SRL increased proliferation in the allogeneic setting compared to adding MSCs alone[408]. The mixed results regarding this important interaction (in the context of cellular therapy) deserve further investigation. Determination of the mechanism of the interplay between DSCs and immunosuppressive drugs may be an important step to improving the therapy and safety. These results also indicate that the IL-2/IL-2R phenomena observed in **Paper IV** may be important for the reduction of proliferation by DSCs *in vitro.*

4.3 IMMUNOTHERAPY WITH DECIDUAL STROMAL CELLS

Based on the data presented in **Papers I** and **II** and the literature on MSC-based therapy, DSCs were introduced as a second-line therapy for severe GVHD (as decided by the principal investigators and the physician responsible). In **Paper II**, nine patients with severe GVHD were treated with DSCs. The dose of DSCs ranged between 0.9 and 2.8 × 10^6 cells/kg. All patients but one received RIC. All of them had gastrointestinal GVHD. The DSCs were given between 23 and 219 days after transplant. Five of the patients received more than one DSC infusion, and a total of 15 infusions were given. The three patients who were alive at 6 months after transplantation were still alive three years after HSCT (Solders *et al*., unpublished data). The patient material in **Paper II** was small, and no certain conclusions could be drawn regarding the safety or the efficacy of the DSC treatments. Several severe adverse events (SAE) occurred after the treatment to the end of the follow-up. Since many of these could be transplant-related complications, it is difficult to link the SAEs observed to the DSC treatment in **Paper II,** as there were too few patients and no consistent SAE occurred in most of them. Stromal cell treatment has not been associated with infusion toxicity, but later-occurring events have been reported, including reduced immune reconstitution[323] and an

increased risk of development of post-transplant lymphoproliferative disease (PTLD)[324] or death due to pneumonia[409]. A long-term follow-up including more patients should reveal the risk factors associated with DSC therapy.

The survival rate until 3 years after transplantation was comparable to those in other recent GVHD-related reports where MSCs were used[309,310]. In these studies, there was no difference in survival between the groups that received the treatment and matched historical controls. Other recent studies where conventional non-cell therapy approaches have been used to treat GVHD have shown limited results, and includes the addition of mycophenolate mofetil to standard steroid treatment[410] or the use of an IL-1R antagonist[411]. The conclusion is that at the moment, there are very few other reliable treatments besides corticosteroids for patients with severe GVHD.

Since **Paper II** was a pilot study, few parameters regarding the clinical protocol for DSC treatment had been subject to optimization. There are several factors that might be altered to improve the protocol. The dose was based on previously published data on MSCs. Interestingly, there have been no studies in man—except for one where the dosages of MSCs were investigated[412]. The literature has reports of MSC doses ranging between 0.4 and 9 $\times 10^6$ cells/kg[311]. Another parameter that may play a role is the number of interventions. Should treatment be one time only, or should it be given repeatedly? One study suggested that several infusions may be beneficial[413]. In **Paper II**, multiple infusions in five patients resulted in a reduction in GVHD in two out of the five after the treatment(s) following the initial treatment. The time point for the intervention may also influence the outcome of the treatment. For instance, should treatment be given at an earlier stage of GVHD progression in an attempt to prevent further progression? Is there a threshold in GVHD pathophysiology where intervention is meaningless? Moreover, handling of DSCs before treatment may also have importance for the efficacy of treatment. Replacement of the washing solution after thawing—from AB-plasma to serum albumin—considerably increased the viability of the DSCs administered to the patients (Solders *et al*., unpublished) (these patients are included in **Paper IV** and **V**). Further studies to establish a more optimized protocol for DSC dose, time of intervention, and cell handling are important for further clinical use of DSCs and other cell-based interventions for GVHD and other diseases.

Ex vivo analysis in **Papers IV** and **V** was based on the data obtained from patient samples that were obtained before and several time points after infusion with DSCs. Parts of these samples were subsequently analyzed by FC and Luminex.

To some extent, it was possible to analyze (*ex vivo*) the altered IL-2/IL-2R effects shown *in vitro* in **Paper IV**. The lack of analysis of CD132, CD122, and pSTAT5 was due to the fact that the work described in **Papers IV** and **V** was performed simultaneously. It was possible to analyze the peripheral blood concentration of IL-2, the expression of CD25, and the proportion of Tregs. Based on the different results when combining DSCs with SRL or CyA *in vitro*, the patients were divided based on which one of the two drugs they received as GVHD prophylaxis and as treatment for GVHD. This treatment was continued during the GVHD therapy. Also, all patients were combined to find changes in these parameters in the

whole patient material. *In vitro,* addition of DSCs to an MLR was associated with a consistent increase in the intensity of expression of CD25 in CD25+CD4+ T cells following 6 days of incubation. This could not be detected at any time point following infusion in either of the groups, nor in the combined patient material (**Paper IV**). Moreover, no significant change in the frequency of CD25+ cells, the proportion of Tregs, or the concentration of IL-2 was detected when analyzed in the same manner. These data are difficult to analyze and to put in context of the *in vitro* findings unless experiments in the DSC+SRL/CyA setting are explored in more detail *in vitro*. For instance, the expression of IL-2R subunits and IL-2 concentration should be determined *in vitro*, correlated with the proliferation response in the MLRs, and compared to the clinical data. Additionally, it is important to note that the expression of CD25 and production of IL-2 in the clinical setting may be heavily influenced by the immunosuppressive drugs and immune reconstitution following HSCT. The patients also received tacrolimus (which is comparable to CyA). This might conceal the separate effects of CyA or SRL alone. One randomized study involved surveillance of the levels of IL-2 and Tregs following treatment with MSCs for GVHD. In that material, the concentration of IL-2 and the frequency of Tregs were higher in the treated cohort than in untreated controls at one month[381]. Another study evaluated IL-2 concentrations in MSC-treated GVHD patients but used the data to correlate the concentrations to clinical outcome[222]. No findings regarding how IL-2 concentrations following treatment are correlated to outcome were reported.

The clinical data in **Paper IV** show no significant alteration in the SRL condition compared to the patients who did not receive SRL, indicating that no interference between the SRL and DSC therapy related to CD25 expression and IL-2 concentration occurs. For the clinical evaluation of the material presented in **Paper IV**, analysis where the patient material is divided based on type of first-line GVHD prophylaxis/treatment is nonetheless encouraged in future studies with a larger patient material.

The parameters investigated *ex vivo* in **Paper IV** were also included along with others in **Paper V**. In that paper, we followed immunological parameters following treatment with DSCs for severe GVHD. Three patients received a new DSC treatment later than one month after their first DSC treatment, whereas the new DSC treatment was regarded as a new intervention, making the maximum number of interventions investigated to be 25. A total of 27 soluble factors and over 50 cell subset parameters were investigated in these 25 interventions. The time points included in the analysis were as follows: before the start of DSC intervention, 3 hours after it, and one week, two weeks, and four weeks after the start of intervention. All the samples available from each patient were included. A clinical evaluation was done and the patients were divided in two groups (responders (n = 17) and non-responders (n = 8)), depending on GVHD status subsequent to the start date of DSC intervention. The data were analyzed to first investigate if there was a difference between the responders and the non-responders at the start of DSC infusion, or at any time point after that. We also wanted to investigate if any differences could be detected over time in each group or in the whole patient material. OPLS-DA with subsequent univariate analysis (Mann-Whitney) was used to identify factors of importance in the two groups, and Friedman's test

was used to discriminate differences between all time points of measurement in each group and in the whole material. In **Paper V**, the Luminex data and the FC data were analyzed separately.

Interestingly, there were no indications that the *in vitro* and the *ex vivo* findings were correlated in **Papers I**, **III–V** regarding changes in proportions of specific T cell subsets or the concentration of cytokines (e.g. Tregs, CD25 MFI^{high} CD4+ T cells, IL-10, IL-17, and IFN-γ) over time following introduction of DSCs in the allogeneic setting. Several other factors were, however, identified in **Paper V** that might give an indication of the changes in immunological status of the patients.

Three soluble factors were found to differentiate between the two groups before the start of the intervention: IL-6, IL-8, and IP-10 (**Figure 11**). The non-responders had significantly higher concentrations of all these factors before treatment. This was despite the fact that there was no difference in clinical parameters between the groups. Other factors associated with GVHD, such as IFN-γ, IL-1, and TNF-α, showed no significant differences between the groups. Interestingly, several studies have found that IL-6, IL-8, and IP-10 (although not analyzed together) can be elevated in GVHD. IL-6 concentrations are increased in the early stages of GVHD[414] and have been shown to be of importance for GVHD in experimental models[208]. In comparison, the concentration of IL-6 was increased in the presence of DSCs *in vitro,* although the DSCs did not appear to produce IL-6 constitutively to the same extent as MSCs (**Paper I**). This can be compared to stromal cells from placental villi, umbilical cord, and bone marrow that do produce IL-6 to some extent (**Paper I**). However, a significant increase in IL-6 could not be detected in either group over time, but the non-responders had a higher concentration of IL-6 than the responders four weeks after the start of DSC intervention. This is also in line with data indicating that IL-6 mediates GVHD. If the patients do not improve regarding their GVHD, they are more likely to have a higher concentration of soluble factors associated with GVHD. Of the factors mentioned above, IL-6 was the only cytokine that differed significantly between the groups at four weeks. Although IL-6 concentration was elevated in the non-responders, this group did not have an elevated proportion of Th17 cells, which may be induced by IL-6 and TGF-β. Clinical studies have suggested that an antibody to IL-6 may be used successfully to reduce the incidence of severe GVHD[415].

Moreover, IP-10 was highly elevated in the non-responders compared to the responders. This difference decreased over time, to finally become statistically insignificant between the groups at 4 weeks. Work of others has shown that IP-10 expression is increased in patients with skin GVHD[210]. In that study, IP-10 was produced by basal keratinocytes at the sites of skin GVHD. The levels of IP-10 were higher in our non-responder group than in the patients with skin GVHD in that report. The levels of IP-10 in GVHD patients in the paper by Piper *et al*. are comparable to the concentrations detected in the responders in **Paper V**. This would facilitate migration of cells that express CXCR3. As stated earlier, this chemokine receptor is expressed on Th1 cells. In **Paper V**, the median frequency of Th1 cells was lower in

peripheral blood of the non-responders than in that of the responders (but not significantly so). One of the weaknesses in **Paper V** was the lack of characterization of cell populations isolated from tissue sites of GVHD. Effector cells will migrate to tissues, and the data on these populations in peripheral blood should be interpreted with caution. Although not mentioned in patient characteristics in **Paper V**, subsequent analysis regarding the occurrence of skin GVHD, 6 of the cases in the responder group had skin GVHD, and 6 of the non-responder cases had skin GVHD ($p = 0.1$, non-significant). These data suggest that the trend of a higher occurrence of skin GVHD in the non-responders may have influenced the disparity of IP-10 concentrations between the groups. Additional analysis of other molecules associated with skin GVHD[220] may provide further information regarding disparity of skin GVHD between the groups. A separate analysis of the data in **Paper V** with the patients with skin GVHD excluded has not been performed, but is encouraged in future work.

The levels of IL-8—together with those of sIL-2Rα, TNF receptor 1, and hepatocyte growth factor—have been suggested to be able predict survival in patients with GVHD[416]. The main function of IL-8 is to attract neutrophils to sites of early acute inflammation. The elevated levels of IL-8 may be explained by the tissue damage following transplant, and the levels of IL-8 are high in all patient groups after transplant, irrespective of complications after HSCT[417].

Decidual stromal cells have been suggested to exert gene silencing to reduce the production of chemokines, and by this reduce the migration of immune cells to the feto-maternal interface[246]. Addition of a blocking antibody to one of these chemokines (CCL5) appeared to reduce the incidence of severe GVHD in a phase-I study[418]. In **Paper V**, increased levels of CCL5 were detected in the responders four weeks after DSC intervention. Both innate and adaptive immune cells respond to CCL5 and may migrate towards sites of inflammation. With the lack of investigation of CCL5 and factors related to this in **Papers I–IV**, it is difficult to draw any conclusion regarding the impact of this finding other than that an elevated level of CCL5 may be linked to the state of inflammation at 4 weeks. This finding is therefore somewhat contradictory to the increased levels of IL-6, IL-8, and IP-10 in the non-responders, which suggest that lower levels of cytokines are linked to GVHD in the responders.

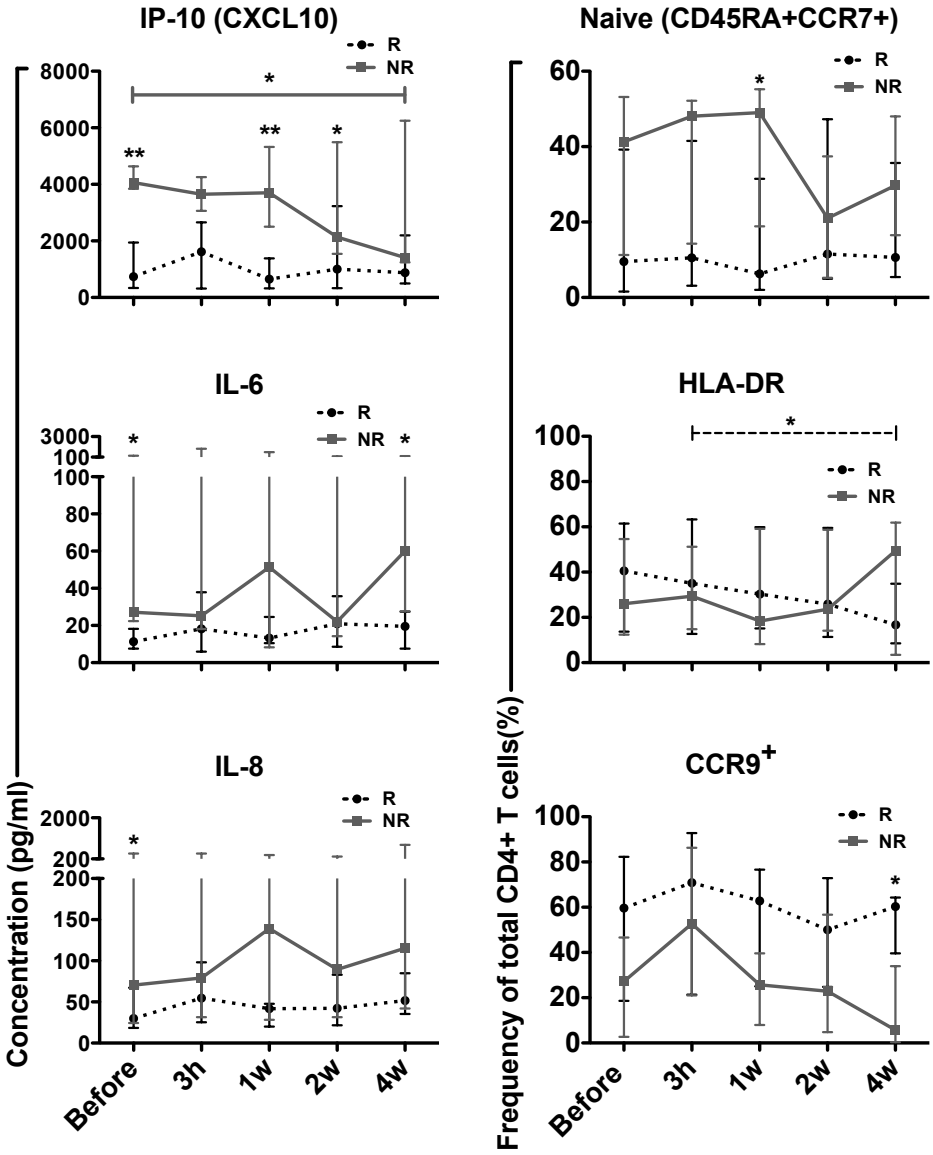


Figure 11. Graphs to the left show the concentration of interferon-γ-induced protein 10 (IP-10), interleukin (IL)-6, and IL-8 in peripheral blood of patients with severe GVHD who received decidual stromal cells (DSCs). The patients were divided into responders (R) and non-responders (NR) depending on improvement in GVHD status. Time points indicate the time from the start of DSC intervention. The graphs on the right present the expression of naïve, human leukocyte antigen DR (HLA-DR), and chemokine (C-C motif) receptor 9 (CCR9) on CD4+ T cells in peripheral blood in the same setting. Stars in the middle above a line represent a significant difference in a patient group over the two time points indicated. Stars directly above the 75th percentile show a significant difference between the two groups. *p = 0.01–0.05, **p = 0.001–0.01, ***p < 0.001.

In addition to systemic cytokine levels, we also determined the proportions of certain immune cell subsets and markers thought to be of importance in the GVHD setting or in inflammation. HLA-DR is upregulated upon T cell activation. Yet, elevated levels of HLA-DR on T cells have not been reported in GVHD[419,420]. In **Paper V**, a decline in HLA-DR-expressing CD4+ cells in the responder group was observed. In the CD8+ compartment, the median level of expression of HLA-DR was higher in the non-responders than in the responders, but the difference was not significant. In contrast, the proportion of naïve CD4+

cells in the non-responders was high. Interestingly, while most of the CD4+ T cells in the responders were of an effector phenotype, the frequency of cells with a distinct Th1, Th2, Th17, or Treg phenotype was not significantly different compared to the non-responders. Although not significantly different before DSC treatment, the increased disparity of naïve cells between the groups is intriguing. One recent report by Gauthier *et al*. suggested that low levels of IL-7 and DCs during GVHD limited the possibility of homeostatic expansion of naïve CD4+ cells in an experimental model[421]. No differences in IL-7 concentration between the groups were observed and monitoring of DCs was precluded in **Paper V** due the low occurrence of DCs in blood. There are several factors that influence the thymic output following HSCT, including, age, GVHD, source of graft, and T cell depletion[422]. No significant differences in these factors were detected between the non-responders and the responders in **Paper V**.

GVHD and other inflammatory conditions of the intestine are characterized by an influx of immune cells to the site of inflammation. The integrins α4β7 and the chemokine receptor CCR9 facilitate homing to the gut[423]. More specifically, an increased expression of α4β7 on T cells is associated with the development of GVHD in experimental models and in patients[214,424]. Therapy targeting α4β7 or CCR9 has been implemented in Crohn's disease[425,426] and ulcerative colitis[427]. The implementation of these strategies in GVHD has not yet been published, but it is an appealing concept. In **Paper V**, we investigated the expression of these molecules in most of the immune cell subsets evaluated. There was a difference between the responders and the non-responders in CD4+ T cells. At 4 weeks after the first DSC intervention, the responders had a higher frequency of CCR9 expression among the CD4+ T cells than the non-responders. Moreover, the responders had a higher frequency of B cells expressing CCR9. In the same group, among the monocytes that were CCR9+, the intensity of CCR9 expression was increased in both classical and non-classical monocytes at 2 weeks compared to before or 3h after DSC intervention. However, the frequencies of monocytes that were CCR9+ did not differ significantly in any monocyte population investigated—between groups or over time. A small but significant increase in the frequency of α4β7+ in activated CD4+CD25+ T cells distinguished the responders between 3h and 4 weeks following DSC treatment. The combined data suggest an increased homing ability of immune cells to the intestine following DSC treatment, and that this is associated with patients that have an improvement in their GVHD. The lack of findings in the subsets with a functional phenotype in the T and B cell compartment makes the determination of biological function in the CCR9+ and α4β7+ cells difficult.

Altogether, in **Paper V** we identified three soluble molecules (IL-6, IL8, and IP-10) that distinguished patients who had an improvement in GVHD status from non-responsive patients following DSC treatment. In addition, patients who had an improvement in GVHD appeared to have an effector phenotype in the T cell compartment, and increased expression of gut-homing markers over time.

5 CONCLUSIONS AND FUTURE WORK

5.1 CONCLUSIONS, PAPERS I–V

Stromal cells from the placenta can be isolated from various compartments. Depending of the compartment of the placenta, these cells have slightly different properties. In our pursuit of finding a cell subset suitable for cellular therapy for inflammatory conditions following HSCT, DSCs were found to:

- Be easily isolated and expanded from the fetal membranes of term placentas.
- Express surface markers associated with mesenchymal stromal cells.
    - Have elevated expression of CD49d, CD29, PD-L1,and ICAM-1.
- Have limited differention capacity.
- Be of maternal origin.

Moreover, our studies *in vitro* in the allogeneic setting suggested that DSCs:

- Have an immunosuppressive effect that may be partly contact-dependent.
- Can directly inhibit proliferation of stimulated T cells.
- Promote an anti-inflammatory cytokine profile.
- Can be affected by exogenous IFN-γ and
    - Upregulate expression of integrins and inhibitory markers, while HLA class II expression is unchanged.
    - Do not necessarily retain increased suppressive capacity.
- Are dependent on several molecules for their immunosuppressive function, including but not limited to: IDO, PD-L1, PGE_2, and IFN-γ.
- Promote Tregs in the allogeneic setting, partly dependent on IDO and cell contact.
- Reduce IL-2R expression, STAT5 phosphorylation and IL-2 internalization.
- May affect the antiproliferative effect of immunosuppressive drugs such as SRL.

In addition, patients treated with DSCs for GVHD:

- Can—based on GVHD response—be distinguished before treatment based on cytokine levels of IL-6, IL-8, and IP-10.
- Have increased expression of gut-homing markers and a reduced frequency of naïve CD4+ T cells correlated to clinical response.
- Showed a 75% overall response rate. Three out of eight evaluable patients were alive six months after transplantation.

5.2 FUTURE WORK

The work in this thesis shows some examples of how the DSCs that we isolate and expand may influence immune parameters, both *in vitro* and *in vivo*.

The focus was to investigate DSCs in the context of suitability for immunotherapy in comparison to existing sources of cells. Studies on DSCs and interaction with immune cells, such as macrophages, may further elucidate their function in tissues. Moreover, the putative role of DSCs in pregnancy success and their importance in feto-maternal tolerance need to be better established. Further investigations of immune cells at the feto-maternal interface could help identifying immune cell phenotypes that can be investigated in more detail.

A continuation based on **Papers III** and **IV** is to investigate in greater detail why DSCs promote IL-2 production. Also, the role of PGE_2 in this setting has not been determined. Are these results relevant *in vivo*, and do DSCs interact with immunosuppressants in a manner that is of clinical significance? The experiments in **Paper IV** begin to explore this interaction but further studies may unveil the full clinical impact.

In **Paper II**, DSCs were introduced as a treatment for severe GVHD. In order to determine the efficacy of these cells, there is a need for a larger (preferably randomized) trial to determine their clinical importance for reduction of GVHD. Apart from evaluation of the clinical effect following DCS intervention, the data in **Paper V** would benefit from a control group in determining markers for successful DSC immunotherapy. GVHD is a complex disease, and the potential influence of DSCs in restoring homeostasis might involve aspects other than immunoregulation. For instance, recent work regarding GVHD has highlighted the importance of regeneration of intestinal epithelium for the restoration of gut homeostasis. DSCs production of certain cytokines (e.g. IL-22) and other factors of importance have not been investigated.

The fate of DSCs and other stromal cells after administration in man has not been monitored in great detail, and to determine this in the clinical setting may be key in defining what contributes to a clinical effect. As mentioned, if DSCs are injured by complement upon administration, the cells would only exert a brief hit-and-run effect. In this sense, cellular therapy may be exchanged for exosomes or other membrane vesicles, which would be able to transport a wide variety of effector molecules derived from the cells of interest without the introduction of the entire cell to the patient.

In this thesis, the focus has been on GVHD treatment. However, there are many other inflammatory disorders where DSC therapy could be considered, such as acute respiratory distress syndrome, autoimmune neuropathies and inflammatory bowel disease.

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