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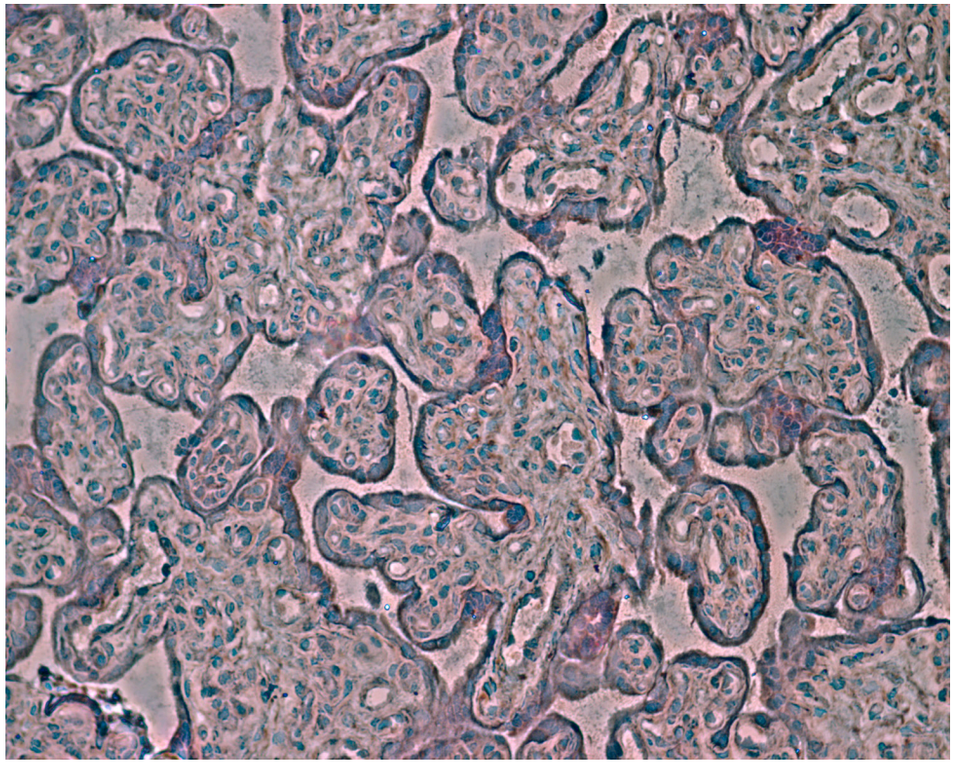

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MAIT CELLS IN PLACENTAL TISSUES AND THEIR RECONSTITUTION FOLLOWING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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The cover image is a photograph of a cross-section of a term placenta showing fetal villi and the surrounding empty intervillous space.

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The placenta is a temporary organ of human reproduction. Both the fetus and placenta are covered in a membrane of maternal origin, the decidua. After the 1st trimester, the fetus is supplied with oxygen and nutrients by maternal arterial blood that penetrates the decidua and fills the intervillous space of the placenta. Fetal blood vessels covered in a thin cell layer, the villi, protrude down into this intervillous blood (IVB), and gases and molecules are transported in and out of the fetal circulation. The maternal immune system recognizes fetal antigen as foreign, yet in the case of a successful pregnancy, it tolerates the fetus while still maintaining immunity against pathogens. Maternal immune cells come into contact with fetal antigen in the decidua and the intervillous space, and these immunological sites are referred to as the feto-maternal interface.

Mucosal associated invariant T (MAIT) cells is a large subset of antigen-specific T cells. MAIT cells are activated by metabolites from the synthesis of vitamin B_2 by certain species of bacteria and fungi, which are presented on the MHC class I related molecule (MR1). As humans cannot synthesize vitamin B_2, MAIT cells can thus discern between self and non-self.

In this thesis, we have studied the immune cell composition and function at the feto-maternal interface with special focus on MAIT cells. We also studied the reconstitution of MAIT cells in the conceptually interesting context of allogeneic hematopoietic cell transplantation (HCT).

We found that IVB has a fundamentally different composition of immune cells compared to peripheral blood (PB) from the same donors. The IVB was enriched in MAIT cells, effector memory T cells and B cells. The MAIT cells in IVB had a more potent response when stimulating mixed mononuclear cell cultures with bacteria compared to PB. MR1 was readily expressed by fetal macrophages inside the villi.

We compared immune cells isolated from the two different parts of the decidual membrane, the decidua basalis (DB) that covers the placenta from the maternal side, and the decidua parietalis (DP) that is attached to the edge of the placenta, and covers the fetus and amniotic fluid. DP contained more T cells and $CD56^{high}$ NK cells, whereas DB was enriched in MAIT cells, B cells, monocytes and $CD56^{dim}$ NK cells. Immune cells in DP had a higher expression of co-inhibitory markers such as PD-1, TIM-3 and LAG-3. In spite of this expression, MAIT cells, T cells and NK cells from both DP and DB were functional when stimulated with bacteria or PMA/Ionomycin.

Non-pregnant women had a higher frequency of MAIT cells in PB compared to pregnant women at term. Using supernatants from placenta tissue explant cultures, we observed that MAIT cells and $CD8^+$ effector T cells were selectively recruited in migration assays. When analyzing the chemokine and cytokine patterns in these supernatants and plasma samples from IVB, many similarities were seen. In contrast, the levels of chemokines in PB plasma was strikingly different compared to that of IVB plasma. Among these, macrophage migration inhibitory factor (MIF) was 182-fold higher in IVB compared to PB plasma. The frequency of MAIT cells in both IVB and DP correlated with levels of MIF in IVB. When blocking MIF together with CCL20 and CCL25, the migration of MAIT cells towards the placenta tissue explant supernatant was reduced. Using recombinant MIF protein, we could show that MIF attracted MAIT cells, probably by binding the chemokine receptor CXCR4.

IVB contained a higher proportion of B cells compared to PB, and the IVB B cells were primarily of the mature/naïve phenotype. This subset of B cells was correlated with levels of CCL20 in IVB plasma. Mature/naïve B cells all expressed the receptor for CCL20, CCR6, and they had a higher median fluorescent intensity of CCR6 compared to immature B cells. Using the same migration assay as previously, we could see that placenta tissue explant supernatant attracted B cells.

Lastly, we investigated the reconstitution of MAIT cells following HCT. MAIT cells did not start to increase in total number until two years after the transplantation, and as the non-MAIT T cells proliferated during this time, the relative proportion of MAIT cells remained at the same low levels during the observation period. When stimulating mixed mononuclear cells with bacteria, the MAIT cells from 2-6 months after HCT had an impaired interferon-γ response, whereas the response at 24 months was similar to that seen in healthy controls. Lastly, we showed that proliferating MAIT cells were more sensitive to common immunosuppressive drugs used in patients after HCT.

In conclusion, the immunological constitution and function of IVB seems to be shaped by soluble factors secreted by placental tissue. IVB is a specialized immunological environment enriched in MAIT cells and other effector T cells, as well as mature B cells, and challenging IVB mononuclear cells with bacteria led to a more potent MAIT cell response. This points to that the placenta attracts certain subsets of immune cells in order to uphold immunity at the feto-maternal interface.

MAIT cell reconstitution following HCT was impaired, both in terms of cell number, frequency and function. This could partly be explained by an increased sensitivity of dividing MAIT cells to common immunosuppressive drugs used after transplantation. The impaired MAIT cell reconstitution could partly be an explanation of the increased risk of infectious complications following HCT.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Den grundläggande frågan för immunförsvaret är ”vad är jag, och vad är främmande?”. Det första skall skyddas från det senare, och stora resurser läggs av individen på att öva immunförsvaret i denna distinktion.

Immunsystemet delas klassiskt upp i två delar, det medfödda och det anpassningsbara, eller adaptiva försvaret. Det medfödda immunförsvaret har under evolutionens gång anpassats att känna igen olika mönster som är ofta återkommande på olika patogener. Det finns troligen mycket stora likheter mellan hur det medfödda immunförsvaret fungerar för oss idag, och hur det fungerade för människor för flera tusen år sedan. Det adaptiva immunförsvaret byggs istället upp inom varje enskild individ under dennes liv. Genom ett trial-and-error-maskineri skapas slumpmässigt ett enormt antal olika receptorer för mönsterigenkänning hos så kallade T-celler och B-celler. De celler som sedan binder patogener med sin fram-slumpade receptor väljs ut och tillåts att skapa tusentals kloner genom att dela på sig. De som inte binder till något går in i programmerad celldöd, och återvinns inom kroppen. På så sätt har vårt immunförsvar möjlighet att ”känna igen” patogener som kanske till och med inte finns ännu. En mindre andel av de kloner som har visat sig fungera sparas till efter att infektionen är utläkt, så att samma patogen inte skall skapa samma problem igen. På så sätt byggs ett sorts immunologiskt bibliotek upp inom varje individ under dess liv där den immunologiska kompetensen ökar för varje år, jämför exempelvis skillnaden i sjukdomsbörda mellan ett förskolebarn och dess föräldrar.

Graviditet är ur en immunologisk synvinkel ett intressant koncept. Fostret är endast till hälften likt mamman, och om man stimulerar mammans immunceller med fostrets celler i provrör så tolkar de fostret som främmande, och fostrets celler dödas. Mammans immunceller är i kontakt med fostret i moderkakan och fosterhinnorna, men trots det så klarar sig fostret undan mammans immunförsvar under graviditetens 9 månader. Man skulle kunna tänka sig att immunförsvaret kring fostret är dämpat eller blockerat, men det förekommer förvånansvärt få infektioner i och omkring fostret och moderkakan. Bakgrunden till detta är inte klarlagd.

MAIT-celler är en sorts T-celler (adaptiva delen av immunförsvaret) som aktiveras av bi-produkter av bakteriers tillverkning av vitamin B_2. Eftersom människan inte kan tillverka eget vitamin B_2 gör detta att förekomsten av dessa bi-produkter betyder att det finns patogener där. MAIT-celler har alltså inte T-cellernas framslumpade specificitet, utan är mer principiellt lika det medfödda immunförsvaret i sitt sätt att avgöra vad som är kroppseget och vad som är främmande. Till sin funktion är de dock klart en del av det adaptiva immunförsvaret.

Moderkakan är ett tillfälligt reproduktionsorgan. Fostret försörjs med syre och näring genom att mammans blod pumpas in i moderkakan. I detta blod sticker så

kallade villi ned, likt grenarna från ett uppochnedvänt träd. Varje gren innehåller fostrets blodkärl som är täckta av ett tunt cellager, och syre och näring går över cellerna och in i blodkärlen. Dessa samlas sedan ”uppåt” i trädet, och blir tillslut till navelsträngen som går in i fostret. Blodet som pumpas in i moderkakan har setts mest som ett transportsystem för syre och näring, och dess sammansättning av immunceller och deras funktion är relativt okänd. Detta blod i moderkakan, det ”intervillösa blodet” eller IVB, är denna avhandlings huvudsakliga fokus. Vi har framför allt fokuserat på MAIT-celler i IVB, men även andra immunceller.

Utöver detta har vi också studerat ett annat tillstånd där två olika immunsystem samexisterar. Allogen hematopoetisk stamcellstransplantation är en medicinsk behandlingsmetod för olika sorters blodcancer, immunbristsjukdomar och enzymbristsjukdomar. Man behandlar patienter med kombinationer av cellgifter, strålning och antikroppar för att trycka ned det befintliga immunsystemet och stamcellerna i benmärgen. Efter det utförs själva transplantationen genom att nya stamceller och immunceller från en donator introduceras i kroppen där de får fäste och börjar dela på sig. Dessa celler bygger så upp ett nytt immunsystem som håller blodcancern borta, eller ersätter den tidigare sjukdomsskapande bristen.

Vi har studerat immunceller från moderkakor som donerats av friska kvinnor efter okomplicerade graviditeter som förlösts med kejsarsnitt (**delstudie I**). Vi såg att immunsystemet hade en annan sammansättning i IVB jämfört med mammans blod. Andelen MAIT-celler var ökad, och vi såg också en ökning av erfarna mogna T-celler, och B-celler som var redo att producera antikroppar. MAIT-cellerna i IVB var inte mer aktiverade från början, men när vi stimulerade immunceller med bakterier hade MAIT-cellerna ett starkare immunsvar i IVB jämfört med mammans blod. Genom att färga tunna snitt av moderkaksvävnad kunde vi se att den aktiverande receptorn för MAIT-celler uttrycktes av celler från det medfödda immunförsvaret inuti villi.

Moderkakan, fostret och fostervattnet omges av fosterhinnorna. Vi gjorde en jämförande studie mellan immunceller i två olika delar av fosterhinnan som bildats av mammans kropp, decidua basalis (täcker själva moderkakan) och decidua parietalis (täcker fostret och fostervattnet) (**delstudie II**). Vi såg att immuncellspopulationerna var mycket olika i dessa två delar, basalis innehöll en större andel MAIT-celler och B-celler, emedan parietalis innehöll en större andel T-celler. Flera immuncellspopulationer i parietalis var kraftigt immunhämmade jämfört med basalis. Båda miljöerna innehöll en hög grad av erfarna och mogna T-celler. Vi stimulerade också immunceller med bakterier och kunde se att MAIT-celler från båda delarna av decidua kunde aktiveras mot bakterierna. Detsamma gällde för T-celler som trots sitt kraftiga uttryck av immunhämmande molekyler hade sin förmåga att aktiveras intakt.

I **delstudie III** fortsatte vi att undersöka MAIT-celler i IVB. Vi såg att kvinnor som inte var gravida hade en högre andel MAIT-celler i blodet jämfört med gravida kvinnor. Vi tog små bitar av vävnad från moderkakor och lät de släppa ifrån sig olika signalmolekyler i provrör. Denna vätska, så kallad supernatant, visade sig dra till sig MAIT-celler i migrationsexperiment. Vi mätte 40 olika lösliga faktorer i supernatanter, i IVB och i mammans blod. Vi såg att IVB och mammans blod hade en helt olik profil vad det gällde lösliga signalmolekyler. Dessutom fanns det stora likheter mellan mängderna molekyler i supernatanterna och i IVB. Vi kunde också visa att andelen MAIT-celler i både IVB och decidua parietalis var associerade med den lösliga faktorn macrophage migration inhibitory factor (MIF) som också fanns i riklig mängd i både IVB och i supernatanterna. Genom att blockera signalerna från MIF och två andra signalmolekyler kunde vi stoppa effekten supernatanten hade på MAIT-celler i migrationsexperiment. Vi visade sedan att MAIT-celler dras till MIF i migrationsexperiment.

I **delstudie IV** studerade vi B-celler i IVB jämfört med mammans blod. Vi såg att IVB och mammans blod innehöll olika sorters B-celler, och att dessa var associerade med olika sorters lösliga signalmolekyler i IVB. Genom att studera uttrycket av receptorer för signalmolekyler kunde vi se att det troligen var CCR6 på B-celler som band till CCL20 i IVB. Vi visade också att supernatanter från moderkaksvävnad drog till sig B-celler.

I **delstudie V** studerade vi hur MAIT-celler återbildades efter stamcellstransplantation, vilket var okänt innan dess. Vi såg att andelen MAIT-celler är kraftigt sänkt efter transplantationen, och att en mycket liten ökning ses först två år senare. Genom att stimulera immunceller med bakterier kunde vi se att MAIT-celler från det första halvåret efter transplantationen inte svarade som förväntat, emedan reaktionen var normal efter två år. Vi visade också att MAIT-celler som genomgår celldelning är mer känsliga än andra T-celler för vanligt förekommande immundämpande läkemedel som ges till i stort sett alla patienter efter stamcellstransplantation.

Sammanfattningsvis har vi visat att IVB skiljer sig från mammans blod vad det gäller den immunologiska miljön och sammansättningen av immunceller. Andelen MAIT-celler var ökad, och svarade starkare på stimulering, vilket skulle kunna vara ett sätt att försvara sig mot bakterieinfektioner under graviditeten. Moderkakan släpper ifrån sig lösliga signalmolekyler som drar till sig just de immunceller som vi också såg var mer vanligt förekommande i IVB. Moderkakan verkar alltså själv forma det lokala immunförsvaret under graviditeten. Immuncellerna i decidua var kraftigt immunhämmade, troligen för att skydda fostret från mammans immunförsvar. Dock så hade de fortfarande möjligheten att aktiveras mot patogener och alltså samtidigt skydda fostret mot infektioner. Efter stamcellstransplantation sågs ett försenat återskapande av MAIT-celler, och deras funktion var normal först två år senare. Detta skulle delvis kunna förklara att patienter är mycket infektionskänsliga efter HCT.

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CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
CMV	Cytomegalovirus
CsA	Cyclosporine A
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CTB	Cytotrophoblast
DB	Decidua basalis
DC	Dendritic cell
DSC	Decidual stromal cell
DP	Decidua parietalis
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
ER	Endoplasmic reticulum
FMO	Fluorescence-minus-one control
GrzB	Granzyme B
GvHD	Graft-versus-host disease
GvL	Graft-versus-leukemia
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HCT	Allogeneic hematopoietic cell transplantation
ICOS	Inducible T cell co-stimulator
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
IVB	Intervillous blood
MAC	Myeloablative conditioning
MAIT	Mucosal Associated Invariant T
MFI	Mean/median fluorescence intensity
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MR1	MHC class I related molecule
MSC	Mesenchymal stromal cell

NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PMA	Phorbol myristate acetate
PB	Peripheral blood
PBS	Phosphate buffered saline
PD-1	Programmed cell death-1
RIC	Reduced-intensity conditioning
S1P	Sphingosine-1-phosphate
SIR	Sirolimus
STB	Syncytiotrophoblast
TCR	T cell receptor
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
UCB	Umbilical cord blood

1 INTRODUCTION

1.1 The immune system

The human immune system is classically divided into two parts, the innate and the adaptive. The innate immune system is the immunological memory of the species. It has been shaped by evolution to recognize common surface molecules of major groups of pathogens to find and eliminate them. The adaptive immune system on the other hand forms the immunological memory of the individual. In most cases, the inflammatory processes of the innate system provide samples of infectious pathogens (antigens) to antigen presenting cells (APCs) which in turn aid the critical education of the cells of the adaptive immune system.

It is through the tightly controlled mechanisms of the adaptive immune system that each individual in most cases has to suffer only once from a manifest infection from a certain strain of bacteria, virus or fungi. Adaptive immune cells are dependent on their ability to sense whether target cells belong to the individual (self) or not (non-self). Cells in the body constantly expose a sample of themselves on their major histocompatibility complex (MHC) molecules, similar to information in a theoretical "immunological passport". If the MHC molecule contains non-self material, cells of the adaptive immune system will recognize this as foreign, and can either kill these cells directly, or use antibodies and cytokines to direct the innate immune system towards designated targets (1, 2).

If infected cells try to evade MHC-based recognition by altering, or simply hiding their MHC molecules, surveying natural killer (NK) cells will sense the lacking "immunological passport", and based on this information eliminate this cell. NK cells is the most common example of a cell type existing in between the classical division of the immune system into innate and adaptive (1).

During my PhD, I have studied mucosal associated invariant T (MAIT) cells, another subset of unconventional immune cells. The specificity of pathogen recognition by MAIT cells has been honed throughout the evolution of the species rather than within the individual, similar to innate immune cells. However, as MAIT cells share both developmental pathways and effector functions with T cells from the adaptive immune system, MAIT cells can hardly be sorted into either the innate or adaptive side (3).

This thesis will primarily focus on studies of MAIT cells and conventional T cells in two immunologically challenging settings. In pregnancy, non-self cells and tissues from one individual exists inside another for nine months, and in stem cell transplantation, one individual's whole immune system is transferred into the non-self environment of another individual, and resides there for the rest of the individual's life.

1.1.1 Human leukocyte antigen system

MHC molecules are called human leukocyte antigens (HLA) in humans. The classical HLA molecules are polymorphic, resulting in many different versions of HLA between individuals (4). Different HLA molecules have different functionality. HLA-A, -B and C forms the class I molecules and present antigen to $CD8^+$ T cells, whereas HLA-DP, -DQ and -DR are class II molecules, and present antigen to $CD4^+$ T cells. HLA is inherited in blocks called haplotypes containing a full version of all six polymorphic HLA-molecules (5). The fetus receives one haplotype from each parent, and as HLA is co-dominantly expressed, each cell expresses twelve different HLA class I and II molecules. Apart from these classical HLA molecules, other molecules are also involved in antigen presentation and immune activation. The oligomorphic class I molecules HLA-E, -F, -G and the non-polymorphic CD1 family members and the MHC class I related (MR1) molecule are more evolutionarily conserved between individuals compared to their classical counterparts, and all exert important immunological functions (6, 7).

The HLA molecules provide a unique immunological fingerprint of the individual, and during the thymic maturation process, heavy emphasis is put on the ability of T cells to recognize this signature (8). The ability to discriminate between self and non-self is then utilized in the host's defense against bacteria, viruses and mutations.

However, in a transplantation setting, the HLA profile becomes a barrier between individuals. A transplanted organ will contain cells with a foreign HLA profile, and dendritic cells (DCs) of both donor and recipient will present antigen from the graft to the T- cells of the recipient. The immune system is thus educated and primed against the graft, and immune cells of the recipient will destroy the donated organ (9). Today, the only way to perform organ transplantations with a meaningful outcome is to heavily immunosuppress the recipient, often by targeting the interleukin (IL)-2 signaling pathways (10). This comes at a price of increased susceptibility to infections and risk of secondary malignancies (11). To find alternative paths to tolerance has been the focus of transplantation immunologists for decades.

1.1.2 Chemotaxis

A crucial function of the immune system is to be able to move its cells throughout the organism between sites of interest. A central part of cell movement is achieved by the use of chemokines and chemotactic cytokines secreted by both immune cells and cells in various tissues and organs (12). The secretion of chemokines creates a concentration gradient, used by immune cells to guide themselves towards the target site (13). All immune cells express chemokine receptors, and subpopulations of leukocytes can be identified by their expression of certain combinations of receptors (13). Chemokine receptors also often have the ability to bind several

different chemokines, and in a physiological setting, cells are rarely, if ever, exposed to a single signal, but rather a mix of many chemokines in various concentrations. This results in a combination of signals that will either provide a strong attraction force, no attraction at all, or an attraction force in between these two hypothetical strengths on the immune cell subsets. Together this makes it possible for organs and tissues to be more specific in their attraction of immune cells, thus shaping the local immune cell composition. Chemokine signals are critical in the development of T cells, both in bone marrow, thymus, secondary and tertiary lymphoid organs, as well as in the homeostatic maintenance of cells in organs and tissues (13, 14). On top of such homeostatic functions, chemokines are also crucial in the immune system's response to inflammation. Local immune cells and tissue resident cells will then change their pattern of secreted chemokines in order to attract the desired immune cell subsets to the site of inflammation.

An account of the full present knowledge of chemokines and chemokine receptors is out of the scope of this thesis, and well-written introductions to this field have been written by others (reviewed in (12-14)). However, some of the well-established axes for T cell trafficking can function as an illustration of T cell chemotaxis. CCL19 and CCL21 binds to the receptor CCR7 on T cells and CCL21 attracts T cells to lymph nodes. CCL19 and CCL21, together with signals transferred between Sphingosine-1-phosphate (S1P) and the Sphingosine-1-phosphate receptor 1 then steer T cell movement between different lymph node zones by sensitizing and de-sensitizing mechanisms, and in the end the T cells are navigated into the efferent lymphatic (15). Signals transferred through CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 are well-known in orchestrating a Th1-response, attracting, among others, $CD8^+$ central memory and effector memory T cells (13). The expression of CCR4 and CCR10 is associated to migration by T cells and other immune cells to skin by the binding of many different ligands. Homing of T cells to the intestinal tract is instead steered by the ligand CCL25 and the receptor CCR9, and the up-regulation of CCR9 is influenced by metabolism of dietary vitamin A (15). Interestingly, vitamin D metabolites (typically found in skin) induce expression of the skin homing chemokine receptor CCR10, while suppressing expression of the gut homing CCR9 (16).

1.1.3 T cells

T cells originate from the bone marrow, where a hematopoietic stem cell (HSC) have divided along specified differentiation pathways, resulting in all the major cellular components of the immune system. The last differentiation step of T cells in the bone marrow results in a vast quantity of premature T cells, or thymocytes. The thymocytes then migrate from the bone marrow to the primary lymphoid organ, the thymus (17). In the thymus, T cells undergo further differentiation, mostly focused

on their T cell receptor (TCR) and its associated molecules. In distinct maturation steps, the T cells acquire the characteristic surface proteins cluster of differentiation (CD) 3 and CD4 or CD8. Initially, the genes of each T cells TCR are randomly rearranged. The β chain is rearranged first, with the T cells having no expression of CD4 or CD8. After this stage, T cells have a simultaneous expression of both CD4 and CD8, and rearranges the α chain of their TCR. After the unique TCR has been randomly arranged, the ability of the TCR to bind to the MHC with a self-peptide is tested. If they bind MHC I, they commit to CD8 expression, and if they bind MHC II, CD4 expression is chosen. The expression of the non-binding molecule (CD4 or CD8) is suppressed, and T cells failing this positive selection undergoes apoptosis. In the final step, the cell is once again subject to testing the quality of the binding to self-MHC, self-peptide. In this step of negative selection, T cells that bind too strongly are selected for apoptosis, and the remaining fraction leave the thymus as naïve T cells (17).

Driven by chemokine gradients, the naïve T cells enter the secondary lymphoid organs and encounter DCs with samples (antigen) from the environment of the infected site. The antigens are presented on MHC molecules on the surface of the DCs. In the lymph nodes, the naïve T cells scout the DCs for the pattern of a matching MHC molecule, with a matching antigen (18). At this point, each naïve T cell clone has a unique TCR profile following the thymic maturation. So, together with the MHC-binding, each randomly produced unique TCR is now tested against the set of antigen sampled from the site of infection (18). The migration of naïve T cells between blood, secondary lymphoid organs and lymph is elegantly orchestrated by changes in sensitivity to the chemokines and signaling molecules S1P, CCL19 and CCL21. If the antigen-MHC complex does not bind specifically to the TCR, the T cell will move on to the next DC, and eventually the next lymph node (15). This enables a DC to encounter up to 500 T cells each hour.

Upon binding of an MHC-antigen complex with a molecular match of a certain TCR on a T cell, said T cell receives additional activating signals from the DC, and begins to divide. The most important co-stimulatory signal is sparked when CD28 on the T cell binds CD80 and CD86 on the DC (19). Other signals potentiating T cell activation are the inducible T cell co-stimulator (ICOS), CD40L and CD27 (20). The last major step of T cell activation is cytokine stimulation. IL-2 is essential to the differentiation and expansion of all T cells (21, 22), and the balance between IL-12 and IL-4 determines the direction of maturation of CD4 T cells into different T helper cell compartments (23, 24).

The activated T cell clones all have the exact same TCR, and thus all are reactive against the same antigen-MHC complex presented by the DC. After the T cells are de-sensitized against CCL19 and CC21 and conversely sensitized to S1P throughout

their time in the lymph node, they will migrate back into blood. Sensing the pro-inflammatory milieu, the T cells leave the blood circulation and enter the tissue at the site of infection (15). The activated specific T cells will now eradicate the infection. This will in turn lead to less pro-inflammatory signals being released by phagocytes in the area. In the increasing absence of the signals necessary for a continued immune response, anti-inflammatory feed-back mechanisms will overcome the pro-inflammatory drive, and both the innate and adaptive immune system will revert back to a resting state. Most of the specific T cells become apoptotic in the absence of a sufficient cytokine drive, among which IL-2 is the most influential (21, 22). However, some of the T cells will survive and form a long-lasting memory population with specificity against the previous infection. In theory, if the same pathogen infects the individual again at a later time point, the innate immune system will perform in the exact same manner as it did the last time. The difference is that the memory T cells remaining from the previous infection will be activated days earlier this time, and the infection will be cleared within hours-days instead of days-weeks. The individual's adaptive immune system has thus developed an immunological memory against the pathogen (25, 26). This experience will however not be transferred to the offspring, and the immunological memory of the species is left untouched.

1.1.4 B cells

B cells reside in the B cell zone of the secondary lymphoid organs, and survey soluble antigen using their membrane-bound immunoglobulin (Ig) as receptors. After activation, the B cell internalize the antigen, transits to the T cell zone, and presents the antigen on MHC II. $CD4^+$ T cells with specificity for the exact combination of MHC and antigen binds the B cell, providing additional stimulation with CD40 ligand and cytokines. B cells and a certain subpopulation of $CD4^+$ T cells then again transit to a germinal center. In the germinal center, the B cells start a rapid proliferation. The original type of Ig that they produce is changed in a process called class-switching, providing a more suitable type of antibody against the target antigen. The binding strength of the Ig is then improved by a process call somatic hypermutation. Each dividing B cell has a high mutation rate in the Ig genes, as well as a high tolerance for these mutations. The B cells with these various Ig specificities are then tested against antigen bound by previously produced Ig, either in soluble form or presented by specialized follicular DCs. The B cells with Ig specificities that bind the antigen get survival signals, the ones that do not bind go into apoptosis. The antigen is then again internalized, presented on MHC II, and if a specialized follicular $CD4^+$ T cell, binds, the B cell receives the final signals of survival. Thus, the Ig produced by the B cells will have an increasing affinity over the course of an immune reaction. Most of the mature B cells enter the circulation, producing antibodies. Some will enter the bone marrow or mucosal

tissues and reside as long-lived plasma cells. Others will transition to memory B cells, which both circulate in blood, and lie dormant in tissues until a potential re-infection (1, 2, 27). B cells can also be activated independently of T cells by binding antigen with toll-like receptors (TLRs), complement receptors and the B cell receptor. If enough B cell receptors are bound by the same antigen, called cross-linking, the B cell can also be sufficiently activated by this alone. The antibodies produced by a T cell independent B cell activation are short-lived, and do not undergo the process of affinity maturation described above (1, 2).

1.1.5 MAIT cells

MAIT cells is a subset of innate like T cells with a unique capacity to respond to bacterial-derived stimuli. MAIT cells can be functionally activated by either TCR or cytokine stimulation, and respond with up-regulation of both cytotoxic molecules and pro-inflammatory cytokines.

The proportion of MAIT cells in peripheral blood is reported to be around 0.2%-20% in different publications, with a median of 1-2% of total $CD3^+$ cells (28-30) (**paper III**). The frequency of MAIT cells is very low at birth and increases during childhood and over early adolescence (31). In a very small cohort, no difference was seen when comparing the frequency of MAIT cells between heterozygous and homozygous twins, pointing to environmental factors being more influential than genetics in determining the MAIT cell frequency shortly after birth (31). The highest relative amount of MAIT cells is found in the third and fourth decenniums of life, after which a steady decline generally is seen until the end of life (29, 30, 32). When comparing men and women between 15 and 50 years of age, women had significantly higher frequencies of MAIT cells, but no differences were discernable between other age cohorts (29). The proportions of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ MAIT cells also changes with age. The relative amount of $CD8^+$ MAIT cells decreased with age, and were replaced with either $CD4^+$ (30), or $CD4^-CD8^-$ MAIT cells (29). In spite of these age-related changes, no differences were seen when comparing the MAIT cell production of IFN-γ or IL-17 upon phorbol myristate acetate (PMA) and ionomycin simulation between older and younger individuals (30).

1.1.5.1 The TCR of MAIT cells

Conventional T cells as a group consists of many different clones of T cells, the potential diversity of the TCR specificity within one individual ranging up to 10^{18} unique antigen specificities (1, 2). MAIT cells on the other hand utilizes a limited diversity in their TCR specificities. Most use the Vα7.2 which is in turn most often coupled with the Jα33 segment, but sometimes Jα12 or Jα20 instead (33). The preferred expression of Vα7.2 by MAIT cells is utilized by most researchers in

the field to identify these cells with monoclonal antibodies. Coupled with any of these three α chains, the β chains within the MAIT cell population have a limited diversity, the number of sequences being 10-100 times fewer than that of conventional T cells (33). The Vβ2 and Vβ13.2 are the most commonly used β chains of the MAIT cell TCR (33, 34), and the MAIT cells with these β chains generally seem to respond with more IFN-γ and TNF-α production upon *E. coli* stimulation than other β chains found in smaller subsets of MAIT cells (34).

1.1.5.2 The MR1 molecule and its role in TCR-mediated MAIT cell activation

The difference in the TCR diversity between conventional T cells and MAIT cells is most likely due to the conceived antigen of the respective TCR. Conventional T cells fill the role of being capable of defending the host against any possible pathogen, even evolutionarily honed species yet unknown to us. The TCRs of MAIT cells are not aimed at antigens specific for a certain pathogen or part of a pathogen. Instead, MAIT cells are defined by their ability to be activated by metabolites from the synthesis of riboflavin (vitamin B_2) (35) bound by the non-classical MR1 molecule (36). The MR1 gene is non-polymorphic (37), and the gene for MR1 is located on chromosome 1 (1q25.3) (37). The stability of the MR1 gene is not only apparent between humans, but also between species. The ligand binding domains of MR1 is highly conserved among the great apes (*Hominidae*), but also to a large extent among all mammals. The most well studied example is the mouse (*Mus musculus*), with whom we share 90% of the gene encoding the antigen binding domain (37, 38). The MR1 protein is almost ubiquitously expressed, albeit at varying intensity in all human tissues, as well as in various common cancer cell lines (37, 38). Looking at the MR1 localization on cells in general, and APCs in particular, many studies have found extracellular MR1 expression to be virtually absent on resting cells (39-41). Instead, MR1 predominantly resides in an incompletely folded conformational state intracellularly in the endoplasmic reticulum (ER). At this stage, the MR1 molecule is missing one of its subunits (β_2m) (39). In a yet unknown fashion, riboflavin synthesis metabolites are taken up by the cell, and transported to the ER. The antigen is then bound by MR1, which associates with the β_2m subunit, and folds into its functional shape. The functional MR1 with the bound riboflavin antigen then relocates to the cell surface through the Golgi apparatus by yet unknown mechanisms (39). The whole complex is then reinternalized into the cell, and most MR1 molecules are degraded (39). Recycling of the MR1 molecule has been shown during *Mycobacterium tuberculosis* infection, where the recycled MR1 molecules are loaded with riboflavin synthesis metabolites in endosomes (42). It has also been shown that loaded MR1 molecules could be completely stripped from the cell surface by blocking NF-κB signaling (40). Thus, in the majority of the cells, MR1 molecules probably reside in the ER until bound with riboflavin synthesis metabolites, upon which they relocate to the cell surface in a NF-κB dependent manner. Some of these

MR1 molecules are recycled and reloaded with new metabolites in endosomes and transferred back to the surface, although most are degraded in lysosomes. For a continuous riboflavin synthesis metabolite presentation, the cell then needs to synthesize new MR1 proteins, providing a potentially limiting step in the ability to activate MAIT cells during conditions with high levels of available activating ligands. Another interesting logical conclusion is that due to riboflavin synthesis metabolites being secreted by pathogens, as well as their diffusible nature, they could provide a sensing opportunity for MAIT cells, similar to the well-known function of chemokine gradients in general immune cell homing (35).

The MR1 expression has been shown to be functional in a wide range of cells. DCs (43, 44), monocytes (28, 44-46), macrophages (44, 47), B cells from both liver (47) and blood (41), as well as epithelial cells from bile ducts (47) and lungs (43) have all been shown to be able to activate MAIT cells in an MR1 dependent manner *in vitro*. However, which APC that is most important for MR1 dependent MAIT cell activation *in vivo* remains to be studied.

Due to the limitations and functions of the MAIT cell TCR and the MR1 molecule, it is logical that only microorganisms with a functional riboflavin synthesis pathway have been shown to be able to directly activate MAIT cells (43, 45). Mammals on the other hand cannot synthesize riboflavin. This provides a wholly separate conceptual method of discrimination between self and non-self by the immune system, where instead of meticulous T cell education in the thymus, the separation is based on metabolic capabilities. As the species made discoverable to the immune system by this method do include many of the old and most common disease-causing pathogens we know, it further strengthens the concept of an evolutionary conserved rapid activation pathway of this subset of invariant T cells (48). MAIT cells have a rather broad antibacterial spectrum in both mice and humans, including *Escherichia*, *Pseudomonas*, *Staphylococcus*, *Klebsiella*, *Salmonella*, *Mycobacteria* and *Lactobacillus* species (35, 45). On the other hand, the bacterial species *Streptococcus* group A, *Enterococcus faecalis* and *Listeria monocytogenes*, which all lack the riboflavin synthesis pathway, also lack the ability to activate MAIT cells (43). The fungi species *Candida* and *Saccharomyces* can also produce riboflavin, and have consequently been described to activate MAIT cells *in vitro* (45). Another mode of activation MAIT cells are superantigens produced by bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes*. These molecules cross-link the TCR of the MAIT cell with MHC class II on APCs, and MAIT cells have been shown to be hyperreactive to this mode of stimulation compared to conventional T cells (46).

The technical development of MR1 tetramers loaded with certain metabolites of riboflavin synthesis has enabled and simplified the investigation of MAIT cells with standard immunological methods such as flow cytometry. Furthermore, it

has been shown that by staining the C-type lectin CD161 together with the TCR Vα7.2, a population of cells with a high correspondence to the population identified by tetramer staining is isolated (34, 49) (**paper III**).

1.1.5.3 TCR independent activation of MAIT cells

MAIT cells are predominantly $CD8^+$ T cells, although a small proportion is double negative or $CD4^+$ (49). MAIT cells have a high expression of the full functional receptors for the cytokines IL-7, IL-12, IL-18, and IL-23 (28, 45). The expression of the α unit of the IL-15 receptor is not expressed on resting MAIT cells, but strongly up-regulated upon stimulus with IL-15 (50). The receptors for the cytokines IL-2, IL-7 and IL-15 share the common γ chain (CD132), and IL-2 and IL-15 also share use of the β subunit (CD122). These receptors are then distinguished by their different α subunits CD25 (IL2Rα), CD127 (IL7Rα) and CD215 (IL15Rα). Interestingly, stimulating PBMCs solely with IL-2 or IL-15 strongly stimulate MAIT cells to up-regulate expression of IFN-γ and the cytotoxic molecules granzyme B (GrzB) and perforin (the latter only shown for IL-15) (50). On the other hand, IL-7 stimulation only showed an effect on cytotoxic molecules, and not IFN-γ (51). This would suggest a CD122 dependent pathway of MAIT cell IFN-γ up-regulation, but the effect by adding IL-15 to PBMCs was shown to be mediated via secondary increase of IL-18 by monocytes, and the IL-18 was responsible for the actual MR1-independent activation of MAIT cells. The pathway of IL-2 mediated IFN-γ secretion by MAIT cells was not investigated further (50). Both IL-7 and IL-15 increase the response of MAIT cells to MR1-dependent stimuli. In this setting, IL-15 still exerted effects through IL-18 signaling, but also by itself (50). IL-12 alone has been shown to have a weak activating effect on MAIT cells in PBMC cultures, but a strong additive effect when added together with either IL-15 or IL-18 (34, 44, 52). Both IL-12 and IL-18 are also significant parts of the sum of totally activating signals when using bacteria to stimulate MAIT cells in PBMC cultures (40, 50) (**paper I**). Finally, IL-23 stimulation has been shown to independently increase production of IL-17 in MAIT cells (53), while others did not observe this effect for IL-23, but only for IL-7 (54). In conclusion, certain cytokines can themselves activate MAIT cells in PBMC cultures and in cultures of purified MAIT cells, as well as augment the MAIT cell-mediated response to bacteria, but the exact pathways of cytokine-dependent MAIT cell activation remains unknown.

1.1.5.4 Thymic development of MAIT cells

MAIT cell development requires a functional thymus, although the exact maturation process remains unknown. In mice, MAIT cell development in the thymus is dependent on selection by $MR1^+CD4^+CD8^+$ thymocytes (55). Koay *et al*. used both mice and human subjects to demonstrate a probable 3 stage maturation process of MAIT

cells. Stage 1 are $CD161^{-}CD27^{-}$ and $CD4^{+}$ or $CD4^{+}CD8^{+}$, stage 2 are $CD161^{-}CD27^{+}$ and $CD4^{+}$, $CD8^{+}$ or $CD4^{+}CD8^{+}$, and stage 3 consists of $CD161^{+}CD27^{pos\text{-}lo}$ MAIT cells which are either $CD4^{-}CD8^{-}$ or $CD8^{+}$. A minor fraction of stage 3 MAIT cells can also be $CD4^{+}$. Stage 1 MAIT cells were only present in the thymus, whereas stage 2 MAIT cells were found in the thymus, cord blood and peripheral blood from individuals younger than 14 years, but not in peripheral blood from adults. About 10% of the MAIT cells in the thymus were stage 3, whereas the corresponding numbers were 80% in cord blood, 90% in peripheral blood from young individuals, and close to a 100% in adults. Interestingly, cytokine production was limited to the stage 3 MAIT cells upon stimulation with PMA and Ionomycin (56). Diverseness in the differentiation status between the 3 MAIT cell stages of development has not been investigated. However, the general MAIT cell population in the thymus of mice (57), as well as human cord blood MAIT cells (58) (**paper I**) partly express a naïve phenotype. Similar findings were made when investigating MAIT cells from various tissues from human fetuses from 2nd trimester. MAIT cells from both primary and secondary lymphoid organs expressed a more naïve phenotype, and did not respond with IFN-γ upon bacterial stimulation. Interestingly, tissue resident MAIT cells from the same donors expressed a more effector memory phenotype, and had a stronger cytokine response upon stimulation (59). Altogether, the present knowledge suggests a probable development course of MAIT cells similar to that of conventional T cells. Following cell division from stem cells (yet unknown) in the bone marrow, MAIT cell precursors are selected and educated both initially in the thymus, as well as later in secondary lymphoid organs. MAIT cells then travel through the circulation, and enter tissues where they become resident. Maturation is probably continuous following exit of lymphoid organs, and as germ free mice lack MAIT cells altogether, it is logical to assume that MR1 ligand stimulation is a limiting step in the final maturation (36). This also illustrates the importance of the microbiome of the developing fetus in shaping the immune system during pregnancy. Indeed, meconium of neonates display a diverse microbiome, including many of the bacterial species with the functional riboflavin synthesis pathway necessary to activate MAIT cells (60).

The β chain repertoire is more diverse in cord blood MAIT cells compared to cells from adults, and they express a naïve phenotype, whereas MAIT cells from adults are differentiated effector memory or terminally differentiated cells (31) (**paper I**). Compared to cells from adults, cord blood MAIT cells have a stronger proliferative capacity upon stimulation with phytohemagglutinin (PHA), but the IFN-γ response to bacterially infected cells is virtually absent (31). Altogether, this points to a gradual, partly TCR dependent maturation of MAIT cells after birth, resulting in a gradual increase in the proportion of MAIT cells with β chains with a higher affinity for binding MR1 bound riboflavin synthesis metabolites.

1.1.5.5 Effector functions of MAIT cells

In the absence of stimulation, MAIT cells express the molecules granzyme A, granulysin and perforin. Upon TCR-mediated activation, MAIT cells up-regulate the extracellular expression of the α-chain of the IL-2R (CD25), CD69 and the marker of de-granulation CD107a (45, 61, 62). Intracellularly, MAIT cells increase their expression of perforin, and start expressing granzyme B (28, 51, 63) (**paper I**). Perforin acts by creating a hole in the target cell, so that granzymes and granulysin can be delivered to the cytoplasm of the target cell. The granzymes induce apoptosis whereas the granulysin kills intracellular bacteria by disruption of their membranes (64). Many studies have shown that co-cultures of infected target cells are killed in an MR1-dependent manner when MAIT cells are added to the cultures (51, 61, 63), and one group have shown by time-lapse microscopy that an MR1-transformed cell line pulsed with *E. coli* turned apoptotic in the presence of sorted MAIT cells (61).

Apart from cytotoxic functions, MAIT cells respond to stimulation with production and secretion of the cytokines IFN-γ and TNF-α. MAIT cells in the liver has been shown to be potent producers of IL-17 upon either PMA/Ionomycin stimulation or TCR stimulation together with IL-7 (65). MAIT cells from the female genital tract did produce more IL-17 and IL-22, but lower amounts of IFN-γ and TNF-α compared to MAIT cells from peripheral blood when stimulated with *E.coli* (66). Hence, the secretory profile of MAIT cells seems to be linked to the environment of the cell. The factors behind this is not known, and it would be of great interest to determine such factors behind MAIT cell functionality specialization based on tissue localization.

As MAIT cells can be functionally activated by cytokines alone in an MR1 independent manner, it is not surprising that MAIT cells becomes activated when stimulating cell cultures with viruses. Stimulating cell cultures with influenza virus, dengue virus or hepatitis C virus activates MAIT cells in a predominantly IL-18 dependent manner, and the IFN-γ produced by MAIT cells aids in reducing the viral replication (44, 67).

1.1.5.6 MAIT cells in health and disease

In vitro, the anti-bacterial activity of MAIT cells have been proven as MAIT cells are activated within PBMC populations when adding bacteria to the cultures, as well as directly by co-culture with bacterially loaded APCs. Mice where the MR1 gene had been knocked out were found to have a higher bacterial load measured in the spleen (45). These mice also died to a higher extent when challenged with *Klebsiella pneumonia* (68), or even a live vaccine strain of *Francisella tularensis* (69).

In humans, decreased number of MAIT cells in peripheral blood has been correlated to infections with the bacteria *Mycobacterium tuberculosis* (43, 45, 70), *Pseudomonas aeruginosa* (71), *Helicobacter pylori* (72), *Vibrio cholera* (only in children) (73), *Shigella dysenteriae* (61) and typhoid fever caused by *Salmonella enterica* (74). In a study where healthy participants were prospectively infected with Plasmodium falciparum sporozoites, the MAIT cell frequency initially decreased, and then rebounded to higher levels than before the infection (75). Depletion of MAIT cells and impaired functionality has also been shown in human immunodeficiency virus type 1 (HIV-1) infection, and successful anti-retroviral therapy had no effect on MAIT cell numbers (76, 77). Similar findings were seen when studying MAIT cells in patients with co-infection of HIV-1 and *Mycobacterium tuberculosis* (78). In patients infected by hepatitis C, as well as co-infected with hepatitis C and HIV-1, MAIT cells were more activated, but fewer in number both in the liver and peripheral blood (44, 79-82). However, this effect was not seen when investigating samples from patients infected with hepatitis B (83). The impact of viral infections on MAIT cells can also be measured in the blood from patients with Dengue fever and influenza. Both cohorts had markedly lower frequencies of MAIT cells following infection compared to healthy controls (44, 67), and during the acute phase of dengue fever, the patients' MAIT cells were more activated, and expressed more granzyme B compared to controls (44). In mice, MAIT cells have been shown to contribute to the protection against lethal influenza, independent of MR1 activation (84). Together, this points to a significant effect of bystander activation of MAIT cells during viral infections, and a subsequent decrease in relative frequency following clearance of the infection, perhaps due to MAIT cell apoptosis following the activation. Clinical consequences of this decrease in MAIT cell frequency are not known. In the case of influenza, the risk of secondary bacterial infections is well established, and a common cause of morbidity and mortality, especially in the elderly population (85). It would be interesting to investigate whether the induced relative MAIT cell deficiency following influenza could be part of this susceptibility to secondary infections.

The co-inhibitory marker programmed cell death-1 (PD-1) has been correlated with an impaired IFN-γ production in MAIT cells (70). Increased PD-1 expression on MAIT cells has further been associated with *Mycobacterium tuberculosis* infection (70, 86), HIV-1 (87) and hepatitis C (82).

Furthermore, MAIT cells have been investigated in a wide range of autoimmune disorders, as well as in patients suffering from various types of cancer. MAIT cell frequencies are decreased in rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, ulcerative colitis, Crohn's disease, chronic obstructive pulmonary disease, coeliac disease, obesity and asthma (88).

To conclude, MAIT cells are T cells, although their mode of activation more closely resembles that of the innate, rather than adaptive, immune system. MAIT cells are potentiated by joint innate activation, but their primary mode of activation is similar to the conventional T cells. MAIT cells also both originate from the same stem cell differentiation line, and share effector functions with the conventional T cells of the adaptive immune system. However, MAIT cells are typically activated by local APCs, whereas conventional T cell activation takes place in secondary lymphoid tissues. In terms of timing and effector functions, the responsiveness of MAIT cells resembles that of conventional memory T cells with a fast and potent local response upon activation. Looking at the wider picture though, MAIT cells are rather part of the evolutionary memory of the species than of the memory of the individual.

1.2 Pregnancy

1.2.1 Placental development

A full overview of the human placental development is out of the scope of this thesis, although a short summary based on the excellent review by Turco and Moffett (89) is provided below for introducing important background concepts.

A few days following fertilization, the embryo implants itself in the uterine mucosa. The inner cell mass that is to develop into the fetus is surrounded by the outer cell layer, the trophectoderm. About a week after fertilization, the trophectoderm attaches and then invades the uterine mucosa. In the absence of pregnancy this mucosal layer is shed as menstruation. Following fertilization, the mucosa is instead converted under the influence of progesterone to become the specialized decidual tissue (90). Two weeks after fertilization, the embryo has been fully embedded in the decidua. The trophectoderm cells, now termed the syncytium, that has been invading into the decidua, now differentiate and starts forming a trabecular system of fluid-filled spaces called lacunae, a system later to develop into the intervillous space. Fetal trophoblast cells now branch out into the lacunae, away from the embryo, and form the primary villi. These are made up of a cytotrophoblast (CTB) core, covered by syncytiotrophoblasts (STB). CTBs also eventually form a continuous barrier between the decidua and the intervillous space. During the third week after fertilization, mesenchymal stromal cells originating from the chorionic plate (the boundary between the fetal cavity and developing placenta) migrate through the primary villi, creating fetal capillaries and differentiating into supporting cells such as the fetal macrophages, Hofbauer cells. These now fully developed tertiary villi continue to branch out from the base at the chorionic plate, and thus eventually forms a mature system of large villous trees protruding down into the intervillous space. A macroscopically dissected part of the villous

tree from a term placenta can be seen in Figure 1. Some of the CTBs in contact with the decidua will then migrate into the decidua, becoming the extravillous trophoblasts. By the end of the 1st trimester, the base structure of the placenta is formed, and will then grow in parallel with the developing fetus to match its need for oxygen and nutrients (89).

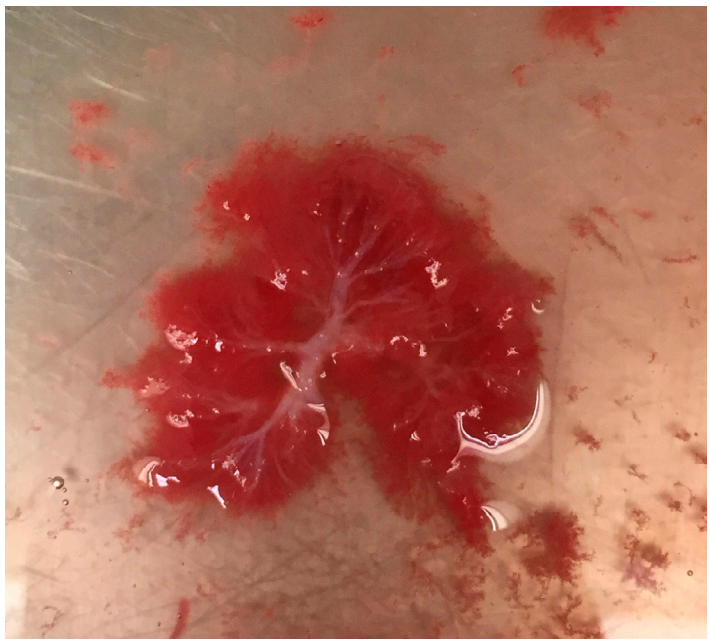

Figure 1. *A macroscopically dissected part of the villous tree from a term placenta, including a larger portion of the fetal blood vessels seen in white.*

The CTB is the highest hierarchy of trophoblast differentiation, and divide and differentiate into the STB, as well as into the extravillous trophoblasts. Early in pregnancy, the CTB form a continuous layer between the STBs and the villous blood vessels. As the pregnancy proceeds and the villous tree grows, the CTB layer is thinned out, and at term, most of the villi is covered only by the STB layer (91). The STBs are the cells in direct contact with the maternal intervillous blood and as such, they contain transporter proteins for glucose and amino acids, as well as neonatal Fc receptors that facilitates the transport of maternal IgG to the fetus (92). They are totally devoid of HLA expression, and thus provides an immunological barrier between mother and fetus. A cross-section of a fetal villi from a term placenta stained with hematoxylin and eosin can be seen in Figure 2.

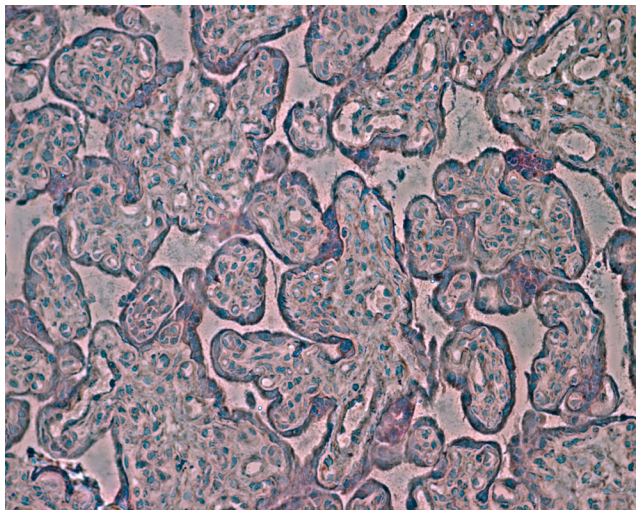

Figure 2. *Cross-section of a term placenta stained with hematoxylin and eosin showing fetal villi and the surrounding empty intervillous space.*

The extravillous trophoblasts invading the decidua ends up in three different locations, in the decidua, in the vessel wall of the spiral arteries, or in the lumen of said blood vessels. The trophoblasts in the decidua penetrate as deep as into the deep muscular layer of the uterus. Here they reside throughout pregnancy, and the exact function of these cells is not known (89). The extravillous trophoblasts also invade the spiral arteries penetrating through the decidua and emptying into the intervillous space. During the 1st trimester, the extravillous trophoblasts inside the spiral arteries have formed a temporary plug, preventing high pressure arterial blood flow to enter the intervillous space, at this time also not yet prepared for a high-oxygen environment (89). The extravillous trophoblasts invading into the wall of the maternal spiral arteries works together with the non-cytotoxic uterine NK cells in performing the vital task of a thorough and aggressive reforming of the spiral arteries. The smooth muscle is broken down, and replaced by a stiffer, non-vasoactive tissue. This results in a permanent situation where the arterial blood is delivered to the intervillous space at a very low pressure, which has been shown to protect the villous tree and fetal blood vessels from mechanical damage. This also counteracts the ability of the maternal circulatory system to down-prioritize the delivery of oxygenated blood to the placenta and the fetus, as the effect of arterial constriction is limited (91). After delivering oxygen and nutrients, the blood leaves the placenta through veins penetrating the decidua. Dysregulation of the extravillous trophoblast invasion of the decidua and spiral arteries and their interaction with uterine NK cells has been shown to be strongly linked to many of the major pregnancy complications such as fetal growth restriction, pre-eclampsia, preterm labor and unexplained stillbirth (89).

During the 1st trimester, during the formation of the mature villous tree, the trophoblast plugs prevents the influx of maternal arterial blood. Thus, the placenta is perfused by maternal plasma, and the fetus develops in a low-oxygen environment. At the end of the 1st trimester, the trophoblast plugs disappear, and during the 2nd trimester, the fetus becomes dependent on maternal arterial blood for the supply of oxygen and nutrition (93).

1.2.2 Anatomy and physiology of the term placenta

At the end of an uncomplicated pregnancy, maternal blood flows through spiral arteries penetrating the decidua, and is drained into the placenta in the intervillous space at a low pressure. The fetal villi protrude down into the intervillous space filled with maternal blood (intervillous blood) that fill the spaces between the villi. The space in the villi containing the blood vessels is surrounded by a discontinuous layer of CTBs, which is in turn covered by a continuous layer of STBs. All these structures are made up of fetal DNA. The gas exchange then takes place over the STB and CTB membrane (Figure 3) (94).

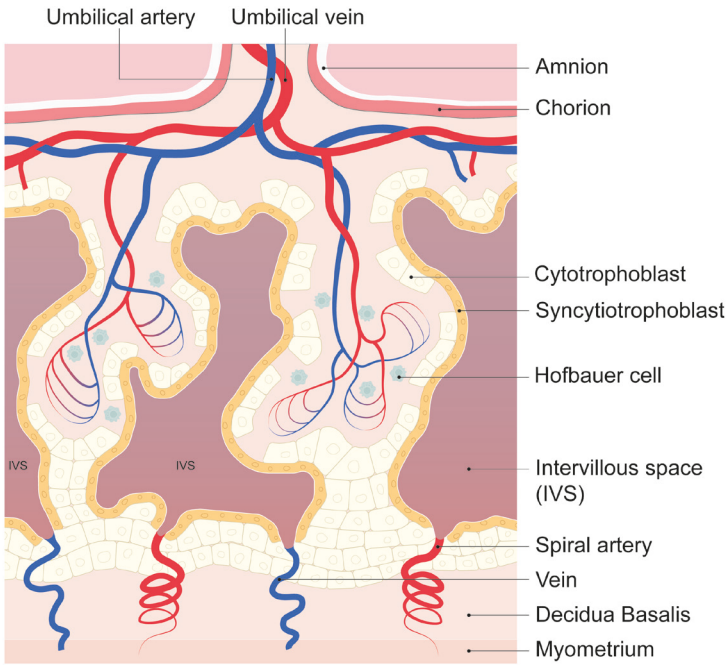

Figure 3. *A schematic cross-section of the placental circulation. Villi are formed around fetal vessels which are covered by first a layer of cytotrophoblasts, and then an outer layer of syncytiotrophoblasts. Maternal blood enters the intervillous space in between the villi through the spiral arteries, and gas and nutrients are exchanged over to the fetal cord blood circulation.*

The maternal side of the placenta is covered by the decidua, the maternal membrane that differentiated from endometrial cells under the influence of progesterone during the 1st trimester (90). The fetal side of the placenta is covered by the two fetal membranes amnion and chorion. At the edge of the placenta the three membranes meet and fuse together. These three joint membranes are often referred to as the fetal membranes, and they surround and protect the fetus and amniotic fluid (Figure 4) (95). The decidua covering the placenta is called the decidua basalis (DB), and the part fused with the fetal membranes is called decidua parietalis (DP).

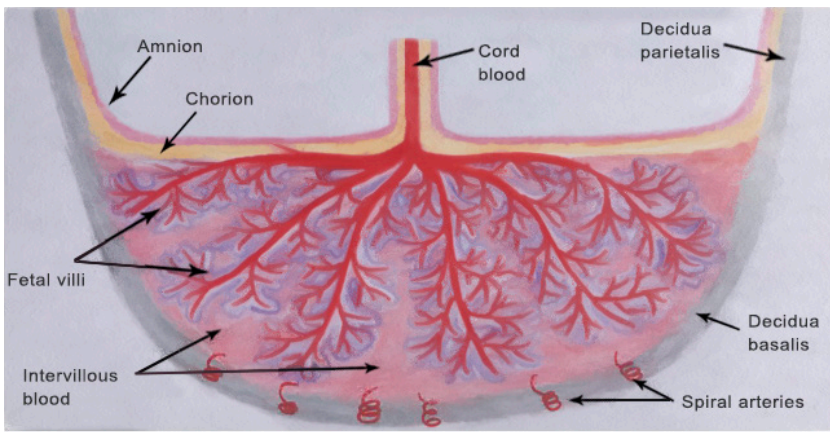

Figure 4. *A schematic cross-section overview of the term placenta and fetal membranes, without the fetus. The fetal membranes chorion and amnion cover the fetal side of the placenta, whereas the maternal decidua encompasses the maternal side. At the edge of the placenta, the three membranes fuse together to form a singular membrane, commonly referred to as the "fetal membranes" in laymen terms. The chorion and amnion encloses the fetus and amniotic fluid, whereas the decidua covers the placenta as well. The decidua covering the placenta is called the decidua basalis, and the part fused with the fetal membranes is called decidua parietalis. From the 2nd trimester, maternal blood flows through the spiral arteries, filling the intervillous space between the fetal villi with intervillous blood.*

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1.2.3 Immunological changes

The contact between the fetal tissue and the maternal immune system is commonly referred to as the feto-maternal interface. This contact is established in the intervillous space between maternal blood and fetal STBs. Maternal and fetal tissues are also in direct contact in the fetal membranes where the DP is fused with the chorion and amnion. DB is also in contact with fetal tissue as extravillous trophoblast cells invade the decidua during the formation of the placenta. Maternal immune cells are resident in both DP and DB, and thus come into direct contact with allogeneic fetal antigen (96).

1.2.3.1 Tolerance

The human pregnancy has been regarded as a transient 9-month transplantation, without the need for the systemic immunosuppression mandatory for successful organ transplantation (97). The maternal immune system can recognize the semi-allogeneic antigens of the fetus (98), and maternal peripheral lymphocytes have been shown to react vividly against fetal antigens *in vitro* (99). Still, it remains tolerant in the majority of pregnancies. Several mechanisms of tolerance have been demonstrated at the feto-maternal interface. There is an increased local production of immune inhibiting factors, such as IDO and TGF-β (100), which provides an immunosuppressive microenvironment. Another important factor is the low immunogenicity of the fetal trophoblasts. After the 1st trimester, STBs are completely devoid of HLA-expression (101), and the trophoblasts in contact with maternal cells in the decidua lack the expression of classic polymorphic HLA molecules (102). Extravillous trophoblasts only express low amounts of HLA-C, and instead of HLA-A or B, they express the non-classical HLA-E, -G and –F molecules. HLA class II is thought to be absent on trophoblasts. During the 1st trimester, the majority of decidual immune cells are $CD56^{high}CD16^{-}$ NK cells, a specialized subset with limited cytotoxic potential against the fetus (103). Apart from the low base cytotoxicity, NK cells interacting with these non-classical HLA molecules appear to lose their cytotoxic functions (104, 105). T cells only constitute about 10% of the $CD45^{+}$ population in early pregnancy decidua (106). As pregnancy proceeds, the proportion of T cells increases to almost 50% at term, with a higher proportion in DP than in DB (107) (**paper II**).

The immunological environment in the decidua is enriched in T regulatory cells (108, 109) (**paper II**), suggesting an increased threshold of activation of the adaptive immune system (110). The tissue resident lymphocytes also express increased levels of co-inhibitory markers such as TIM-3 (111) and PD-1 (112) (**paper I**) compared to peripheral blood. Macrophages expressing a regulatory phenotype, that can be induced by M-CSF and IL-10, dominate the overall macrophage population (113). It has been speculated that this anti-inflammatory environment could be induced in order to protect the fetus against an alloreactive response from the mother's immune system, and/or to prevent an inflammatory process in the fetal membranes that might lead to rupture and premature delivery (114). This is supported by the findings by Vinnars *et al*. who found that when comparing pre-eclampsia to normal pregnancies, villous tissue had a dysregulated expression of cytokines and molecules important in NK cell regulation. This points to that the intervillous environment is in a pro-inflammatory state, and that local attempts to downregulate the activation is insufficient (115). Little is known about tolerance mechanisms in the intervillous space, where the quantitatively largest part of feto-maternal immune interaction takes place.

1.2.3.2 Immunity

While maintaining tolerance towards the semi-allogeneic fetus, the maternal immune system at the feto-maternal interface also has to provide immunity against bacteria and other infectious agents. Placental barriers such as the decidua and placental tissue can be overcome by bacteria and other microorganisms, which can the reach the fetus itself. With an infection that would trigger a full innate and adaptive inflammatory response, the increased levels of cytokines could cause premature contractions, or even rupture of the placental membranes (114). Trophoblasts have been shown to produce a wide variety of anti-viral (116), but also anti-bacterial substances (117). There is an accumulation of memory $CD8^+$ T cells specific for both CMV and Epstein-Barr virus (EBV) in decidual tissues, especially DP (118), and decidual NK cells have been shown to be able to control cytomegalovirus (CMV) infections (119). Decidual macrophages can phagocytose bacteria, and produce pro-inflammatory cytokines (120). Apart from this, little is known how immunity against bacteria is maintained in the generally immunosuppressed environment of the placenta.

1.2.3.3 Intervillous blood from an immunological perspective

The intervillous space is the compartment in between the villi, and the blood filling this space is called intervillous blood (IVB). In 2003, Moore *et al*. described the phenotype of immune cell populations in IVB compared with peripheral blood (PB), and found a slight IVB enrichment of monocytes and NK cells (121). Vega-Sanchez *et al.* observed a decreased frequency of T cells and a slight increase in monocytes in IVB following both vaginal as well as caesarian delivery (122). Others have studied the cytokine profile in IVB, both in health and disease. Bouyou-Akotet *et al.* showed an increased amount of IL-15 in IVB compared to PB, and a generally decreased level in women infected with *Plasmodium falciparum* (123). Singh *et al.* showed that the chemokine-like cytokine macrophage migration inhibitory factor (MIF) was strongly increased in IVB compared to both PB and cord blood, and found that *Plasmodium falciparum* positive women had higher levels of MIF (124). The MHC class I chain (MIC)-related proteins A and B (MICA/B) molecules are released in high levels by tumors, and down regulate NK and T cell cytotoxicity via the receptor NKG2D. MICA/B have been shown to be released by STBs into IVB, and increased levels can be measured in PB of pregnant women throughout the pregnancy (125). The same group also showed that other types of ligands for NKG2D, the UL-16 binding proteins, are secreted by STBs. Both of these groups of NKG2D ligands were secreted into the IVB bound to exosomes (126). Placental exosomes released by STBs also express TRAIL and FasL, and trigger apoptosis in activated PBMCs, including T cells. This could provide a potent anti-inflammatory reserve capacity, as the multivesicular bodies protect the TRAIL and FasL content, and can then be rapidly deployed when needed (127).

Little is known about the immune cell composition, phenotype as well as cytokine and chemokine levels in IVB in healthy individuals. The maternal blood volume in the placenta is exchanged 2-3 times every minute to provide nutrient and gas exchange (128). The cells and molecules of the immune system uses the blood circulation to travel throughout the body, using selectins, integrins and chemokines to stop at the target tissue. Whether the intervillous space of the placenta is merely a vessel for the gas and nutrient exchange, or if it should be regarded as an immunologically active organ remains to be determined.

1.3 Allogeneic hematopoietic cell transplantation

Allogeneic hematopoietic cell transplantation (HCT) is a well-established treatment for leukemia (129), other hematological malignancies, certain immunodeficiencies and some rare inborn errors of metabolism (130). The transplantation is preceded by a conditioning regimen given to the patient, consisting of different cytotoxic drugs, sometimes in combination with irradiation and/or antibodies. Conditioning is crucial in damaging the existing immune system so that the transplanted stem cells can re-populate the bone marrow, and divide and differentiate into a new functional immune system. Conditioning can be divided into two groups, myeloablative conditioning (MAC) (131, 132) and reduced-intensity conditioning (RIC) (133, 134). With a MAC regimen, the immune system will not recover, and the patient is dependent on a subsequent transplantation to survive. For older patients who would not tolerate a full conditioning, a RIC will be enough to shift the odds in favor of a favorable outcome of the coming transplantation, although the cancer is not treated as heavily as with a myeloablative conditioning.

The patient is then transplanted with a graft containing stem cells and other immune cells from either a relative or an unrelated donor. Careful measures are taken to match the graft for the MHC molecules HLA-A, -B, -C, -DP, -DQ and -DR (135). If an unrelated donor is to be used, the patient is more heavily treated with immunosuppressive drugs to dampen potential harmful reactions from the new immune system (136). After the transplantation, the patient is still neutropenic, and must be kept in isolation. After approximately two weeks, enough innate immune cells have differentiated from the stem cells of the donor to stave of the most immediate threat of bacterial infections, and the patient can be discharged from the hospital, although still requiring close monitoring.

1.3.1 Immunosuppression

As the transplanted immune system develops from the stem cells of the donor, the T cells will sense slight mismatches in the MHC molecules between the patient and donor. These cells, together with mature T cells transferred with the graft, will

attack the patient's cells and tissues with varying severity. This alloreactivity against the donor defines the outcome of the transplantation (137). A moderate reactivity provides the crucial graft-versus-leukemia effect (GvL) that keeps patients with malignant diseases in remission, whereas a higher reactivity paves way for graft-versus-host disease (GvHD) (138). In GvHD, the alloreactive cells from the donor attack not only the malignant cells, but also other cells in the body of the recipient.

To prevent GvHD, patients receive immunosuppressive drugs prior to, and following transplantation to dampen the proliferative potential and threshold of activation of the T cells. Some of the most commonly used immunosuppressive drugs are cyclosporine A (CsA) (139) and Sirolimus (SIR) (140). Both these drugs intervene in the downstream signal of IL-2 bound to the high affinity IL-2 receptor, and thus activated T cells are deprived of the crucial IL-2 signal that lets them survive, differentiate and proliferate (21, 22, 141). To date, there is no effective treatment of severe acute GvHD, and for patients with acute GVHD who does not respond to steroids, the outcome is poor (142). Therapies for severe acute GVHD include Ruxolitinib,Vedolizumab and Mesenchymal stromal cells (143-145).

1.3.2 Immune reconstitution

Following HCT, the immune system must be rebuilt. During the first months after HCT, the innate immune system is reconstituted to adequate levels. To reconstitute the adaptive immune system to competence levels similar to that of healthy individuals take years. The development is highly influenced by donor age, GvHD and conditioning regimen (146). The transplanted graft contains mature T cells, and initially, the majority of the patient's T cells are descendants of these transplanted T cells (147). After approximately three months following HCT, new thymocytes divide and differentiate from the transplanted stem cells in measurable amounts (148). As education of thymocytes into naïve T cells is tightly linked to thymic function, and as thymic output decreases with age, it is not surprising that children have a relatively fast and qualitative reconstitution of the T cells, whereas older patients have a much poorer development (149). The first year after HCT, the T cell repertoire is characterized by a high number of $CD8^+$ T cells in relation to the number of $CD4^+$ T cells (150) (**paper V**). With insufficient number of T cells, patients remain at a higher risk of infections for at least two years following transplantation. If the patients experience complications such as GvHD, relapse or certain infections, the immune reconstitution is hampered further (151).

2 AIMS

The general aims of this thesis were to investigate the cellular components of placental tissues. Based on the findings in Paper I, aims were broadened to include a special focus on MAIT cells, both in placental tissues and in the conceptually interesting setting of HCT.

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

Develop and test methods of isolating immune cells from the IVB, DP and DB of term placentas (**Paper I and II**). Characterize the general immune cell components of IVB, DP, DB, and compare IVB and DP to PB (**Paper I**) and DP to DB (**Paper II**). Perform a more focused investigation of conventional T cells and MAIT cells in regard to their phenotype and function (**Paper I and II**).

Based on the findings in **Paper I**, further explore differences between IVB and PB, in regard to T cells as well as soluble factors (**Paper III**) and B cells (**Paper IV**). Investigate potential factors and possible mechanisms for the attraction of MAIT cells (**Paper III**) and B cells (**Paper IV**) to placental tissues.

Investigate the reconstitution of MAIT cells and conventional T cells following HCT, both in regard to cell number, phenotype and function. Compare MAIT cell number and phenotype between patients with and without GvHD. Examine the effect of common immunosuppressive drugs used in HCT on the proliferation of MAIT cells and T cells (**Paper V**).

3 METHODS

Here a brief description of the various methods used in the included papers will be described. For a more detailed description of the methods used in papers I-V, including reagents, manufacturers, and hardware, please see the respective article. Following the paragraph addressing the ethical considerations, the different methods will be described roughly in the order they appear in **papers I-V**.

3.1 Ethical considerations

The vast majority of the experiments performed in biomedical science require access to biological material. If the results are supposed to have impact on the knowledge of human physiology and pathology, there is a need for experimentation on human tissues and cells.

The results included in this thesis are based purely on work on human material. We have obtained human tissues and cells from three main sources: blood from healthy blood donors, placentas and blood from healthy mothers giving birth and blood from patients who have undergone HCT. For all samples except the ones from blood donors, we have also accessed personal information from the individual donor to reassure that we have a balanced material in terms of sex, age, treatment (if applicable) etc., and to make our results understandable and reproducible by others.

The use of blood from blood donors as a source of immune cells does not involve any additional pain, suffering or breach of integrity as we use a by-product of the donated blood. When donating red blood cells, the blood is filtered, and the immune cells are removed from the product given to the patient. The immune cells are then normally discarded, unless scientists or companies buy them for scientific purposes. The cells that we get are anonymized, we only receive an assurance that the donor was healthy, the sex of the donor, as well as the blood group.

The majority of the experiments performed in this thesis uses cells from placental tissues. The placenta is normally discarded following delivery, and thus, donation does not include any invasive procedures for the donor. For **paper I**, **III** and **IV**, we also collected a blood sample from the mother giving birth. The mothers have undergone previous blood collection during the course of their pregnancy, as well as in preparation for the approaching caesarian section. The risks associated with a venous blood sample are small, but may include pain and hematoma at the site of skin penetration, and also a very small risk of local infection. During preparations of the manuscripts, we also accessed limited personal information about the donors to make our results easier to compare with others researchers'.

By performing research in the medical sciences and providing basis for new knowledge based on donated human material, there is a risk of making discoveries beneficial for the medical sciences, whereas the donor had rather remained unknowing of the findings, even if the data is presented on a group level. Thus, being a study subject always includes a risk of getting to know things about yourself that you in retrospect regrets getting to know. Personally, I do not think that the discoveries presented in this thesis have given the study participants insights about the health of them or their child that they would rather have been without, but this remains a speculation.

Being a study subject can in some cases (i.e. pharmaceutical or interventions trials) potentially result in a possible health benefit for the subject. The donors of placental tissues and peripheral blood have hardly benefitted directly from the results of the research in this thesis, but the knowledge might provide a basis for improved future maternal health care.

The last source of human material is the peripheral blood samples from stem cell transplantation patients. These patients have given repeated venous blood samples during years following transplantation. The same risks of venous puncture are applicable here as above. The samples included in **paper V** were most often taken at times when the patients were already subject to routine venous sampling, though some were taken solely for study purposes. The patients donating blood for this study will probably not receive any direct benefits due to their participation. It is rather the scientific field of stem cell transplantation that will be widened, and the increased knowledge in immune reconstitution can be applied into improved practices in the care of future patients.

Overall, the human individuals donating cells and tissues for use in the work presented in this thesis are at little risk of any adverse events related to their participation. Also, the breach of personal integrity has been limited if any, and has not been accessed by anyone not related to the different projects. These risks stand in contrast to the possible gains of the research project. Reproductive immunology is one of the more overlooked aspects of immunology, and we still do not know the basis for the major complications to pregnancy such as miscarriage, preeclampsia or premature birth. Without the access to human material, we will not come any closer to preventing or treating such conditions. I think that the possible gains with the projects in this thesis justify the relatively minor risks associated with participation. Regarding the stem cell transplantation patients, the same line of reasoning applies, the risks associated with donation of blood and the breach of integrity are relatively small compared to the possible increased knowledge in how the immune system is rebuilt following allogeneic HCT.

The research behind the papers included in this thesis was approved by the regional review board of ethics in Stockholm. This includes collection of placentas and

peripheral blood (2009/418-31/4, 2010/2061-32, and 2015/1848-31/2), and the collection of peripheral blood from HCT patients (2010/760-31/1) as well as from a cohort of HCT patients suffering from GvHD (2010/452-31/4 and 2014/2132-32).

3.2 Cell isolation

A detailed description of our method of immune cell isolation from placental tissues can be found in our publication from 2020 (152), and also in less detail in **papers I-IV**.

In short, the placentas that were used in **papers I-IV** were donated by healthy women giving birth by caesarian section at term (gestational week 38-42), following uncomplicated pregnancies. Prior to the surgery, venous blood samples were taken into heparin tubes. During the procedure, after the baby was delivered, the placenta was removed from the womb, and placed directly in a sterile metal container, which was then taken by us directly to the lab without delay (Figure 5). Working in a class II laminar airflow cabinet, the placenta was washed with phosphate buffered saline (PBS). After placing it with the umbilical cord facing up, the cord was clamped with a hemostatic clamp. The fused fetal membranes were then cut along the edge of the placenta, and placed on a sterile petri dish and covered with PBS. The placenta was then turned around so that the umbilical cord faced down.

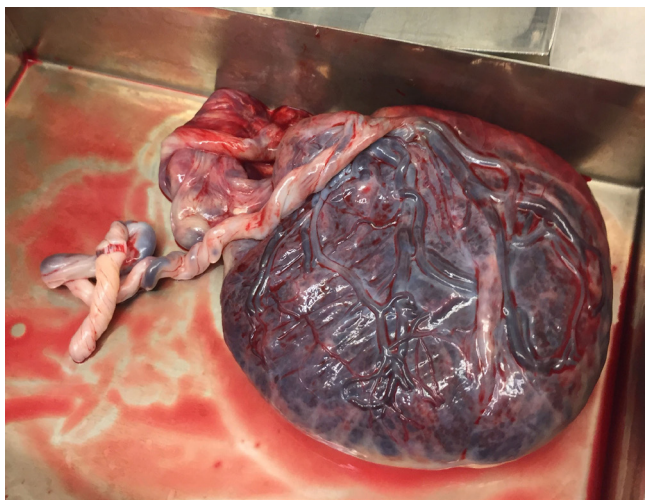

Figure 5. *A term placenta a few minutes after delivery by caesarian section. The placenta is seen from the fetus' perspective, with the umbilical cord attached at the side of the placenta, at the top of the picture. The fetal side of the placenta with the clearly bulging fetal blood vessels are covered by the membranes amnion and chorion. The now collapsed joined membranes amnion, chorion and decidua parietalis are seen in the top left. The placenta is lying with the decidua basalis, the part previously attached to the womb, facing down.*

The surface was inspected for rifts or cuts, and if any were found, the placenta was discarded as the integrity between mother and fetus was assumed to be broken. The surface was then washed with PBS, and visible blood clots were removed. The placenta was then lifted so that intervillous blood dripped or flowed from the placenta and could be collected in heparin tubes. The placenta was then turned around, washed again, the hemostatic clamp removed from the umbilical cord, and a fresh cut was made with scissors. Umbilical cord blood was then collected from the cord into heparin tubes. The tubes from IVB, PB and UCB were centrifuged, the plasma supernatant was collected and frozen in -80 °C. The blood was then diluted with PBS, and mononuclear cells were isolated by density gradient separation.

The placenta was then placed down with the umbilical cord facing down. After washing with PBS, the thin gray membrane (decidua basalis) was scraped off the surface by using a scalpel angled at 45°. The tissue was placed in a 50ml tube, which was filled with PBS. The fetal membranes were placed with the DP facing up. After washing with PBS and removing visible blood clots, the DP was scraped of the chorion and amnion using a cell scraper placed at a 45° angle. The tissue was then disintegrated into as small pieces as possible with a scalpel, and placed into a 50ml tube, which was filled with PBS. Both of the tubes with decidual tissue was then washed by repeated short centrifugations and removal and subsequent addition of clean PBS, until the PBS was no longer visibly colored by blood. Tissue resident cells were then released by using the gentleMACS Dissociator. The resulting mix of dissociated tissue and cells was filtered successively through a 100µm metal net, a 70µm and lastly a 40µm cell strainer. After washing, the cells were either frozen, stained for analysis by flow cytometry after red cell lysis, or mononuclear cells were isolated by density gradient separation for the later use in cell cultures.

Cells from healthy blood donors were delivered in a bag of concentrated cells. This concentrate was diluted with PBS, and mononuclear cells were isolated by density gradient separation. Cells were then frozen for the later use in experiments.

3.3 Flow cytometry

Flow cytometry is a method which allows analysis of the cell phenotype down to the single cell level. Cells are labeled with specific antibodies directed to the targets of interest. The antibodies are in turn attached to a fluorochrome, a molecule that when hit with light of a specific wavelength, emits light at another wavelength. Labeled cells are then loaded into the flow cytometer, which creates a very thin stream of the cell suspension. Inside the machine, lasers are aimed at detectors, and when the stream of labeled cells interrupt the laser signals, the resulting changes in how the light scatters can be measured and used to calculate the size and granularity of cells. If the antibodies have bound the target epitopes, the lasers will hit the attached fluorochromes, and the resulting emitted light will also be measured

by the detectors. Thus, if light of a certain wavelength is detected, it means that a certain antibody-fluorochrome was bound to the cell, and that the cell expressed the molecule that the antibody was targeted against. However, when you increase the number of antibody-fluorochromes measured simultaneously, things do get more complicated. Because light is not only emitted at a specific wavelength, but rather as an emission spectrum, an antibody-fluorochrome complex is unfortunately often detected by multiple detectors. The false positive results due to this spectral overlap can be adjusted for by the use of control beads and the careful decisions on which antibodies are conjugated to which fluorochromes, and also which combinations of antibody-fluorochromes you use together. The procedure of staining for flow cytometry is described in **papers I-V**, and in more detail in our recent publication (152). For the analysis, we utilized fluorescence-minus-one controls (FMOs) where a control sample is stained with all markers except one. This provides a negative control that takes any possible remaining false positives from that certain combination of antibody-fluorochromes into account. Data can then be measured as whether a cell is possible or negative for a certain marker, information which is then often translated to that cell having certain properties. Another way to interpret flow cytometry data is to measure the relative fluorescence, called mean/median fluorescence intensity (MFI), of a cell. The more target molecules a cell expresses, the more antibody-fluorochromes will bind to the cell, and the higher the intensity of the emitted light will become. As an effect in biology very rarely results from a binary signal, but rather a competition/combination between positive/negative signals, giving a resulting vector of the combination of the added signals, it can often make sense to interpret your data in such a way. If a cell type from two different organs both express the same receptor molecule, but the cells from one organ have 3 times the number of molecules on the cell surface, these cells will have a lower threshold of ligand activation, which might result in a relevant biological difference.

Thus, flow cytometry can answer the questions whether a cell expresses a marker, and also if one positive cell type expresses more markers than another. This is then scaled up the various combinations you can get from the co-expression of 10 markers (the upper limit of the flow cytometer used in this thesis), measured on millions of cells, with ability to look at each single cell separately. Other flow cytometers have the ability of analyzing as many as 30 parameters on the same cell. Because of the vast amount of data that can be obtained from each sample, flow cytometry is the dominating method of the ones used in this thesis.

3.3.1 Tetramers

Instead of an antibody that targets a molecule, so called tetramers can be used. Tetramers most often refers to four linked MHC molecules containing a certain peptide. By mixing such tetramers with T cells, the T cells with a TCR specific for

the peptide presented by the tetramers will be bound. If the tetramers were then bound to a fluorochrome, the sample could then be analyzed by flow cytometry, and the frequency of T cells specific for the peptide of interest can be measured. In **paper III**, we got access to MR1 tetramers loaded with either 5-OP-RU or 6-FP. The MR1 tetramers were produced by the NIH Tetramer Core Facility as permitted to be distributed by the University of Melbourne, and the MR1 tetramer technology was developed jointly by Dr. James McCluskey, Dr. Jamie Rossjohn, and Dr. David Fairlie. As the MAIT cell TCR binds MR1 with bound 5-OP-RU, but not 6-FP (negative control), these tetramers provided a way to select MAIT cells from a mix of mononuclear cells. As the tetramers were bound to a fluorochrome, we could then measure the frequency of MAIT cells in a sample with a flow cytometer based on the TCR specificity of the MAIT cells. In **paper III**, we used the MR1-tetramer to validate our extracellular staining used in **paper I**, **II**, **III** and **V**.

3.4 Bacterial stimulation cell cultures

To study MAIT cell activation, an assay of bacterial stimulation was set up. It was known that *E. coli* was a bacterial species able to activate MAIT cells in cultures of mononuclear cells. We used a resident *E.coli* strain isolated from a fecal sample of a child (153) **paper II**. Others have used laboratory strains such as DH5α (61, 63) or D21 (34) or other bacteria with a functional riboflavin synthesis pathway (43, 45). Prior to the experiments, the bacteria were fixed using paraformaldehyde. The use of a clinical *E. coli* strain meant that we could argue that our results were more applicable to a physiological context compared to the laboratory strains. Before we started our experiments, we titrated how many bacteria we needed to add to our culture to get a measurable activation of MAIT cells. In order to get a measurable reaction form MAIT cells, we had to add a notably higher number of bacteria compared to what most other groups were using for their laboratory strains. After isolation of mononuclear cell suspensions by density gradient centrifugation, cells were incubated with bacteria and anti-CD28 antibodies for 16 hours. During the last 4 hours, Brefeldin A, a chemical that prevents protein transport from the ER to the Golgi complex, thus trapping newly synthesized proteins inside the cell, was added to the cultures. After this, cells were harvested, and intracellular proteins could be stained and measured by flow cytometry. An example of the resulting staining can be seen in Figure 6.

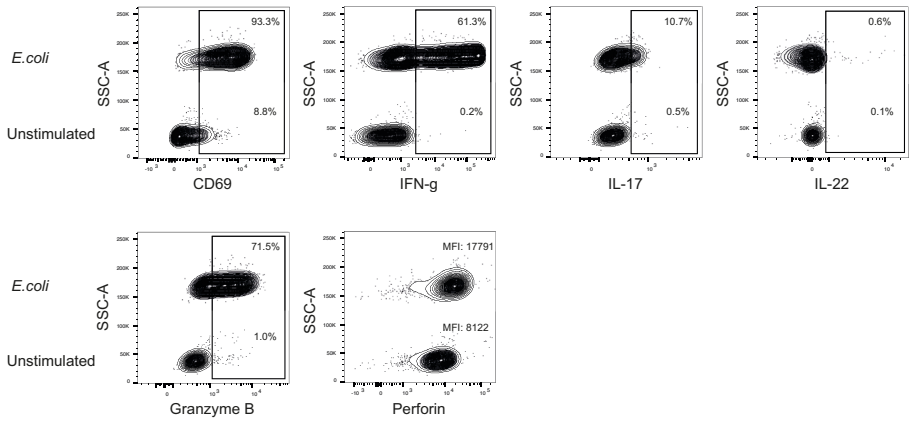

Figure 6. *Example of increased expression of extra- and intracellular MAIT cell markers in response to bacterial stimulation.*

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3.5 Mitogenic stimulation

A common and standardized, yet unspecified method activating T cells and NK cells is the use of PMA and Ionomycin. PMA activates intracellular protein kinase C, mimicking a TCR activation for T cells, and other activating receptors for NK cells, and Ionomycin raises the levels of intracellular calcium. Together, the signals result in an activation of NF-κB and NFAT, and the resulting gene transcription imitates a potent T and NK cell activation. PMA/Ionomycin was used in **paper II** to demonstrate the activation potential of T cells and NK cells in decidual tissues.

3.6 ELISA

The enzyme-linked immunosorbent assay (ELISA) is a widely used technique for the detection of soluble particles within a sample (Figure 7). A plate is coated with antibodies against the target antigen. The sample is then added to the plate, the antigen binds to the antibody, and the rest is washed away. A biotin-labeled detection antibody is added which also binds to the antigen. An enzyme (labeled HRP in figure 7) bound to streptavidin is then added, and as streptavidin has a strong affinity to biotin, this bind to the antibody-antigen-antibody-biotin complex. A substrate is then added which, when converted by the enzyme, leads to a color reaction that can then be measured. Protein levels are then determined by the use of comparison to controls of known protein concentrations. ELISA was used in **paper I** and **V** to measure levels of soluble IFN-γ, grzB, IL-10, IL-12 and IL-18.

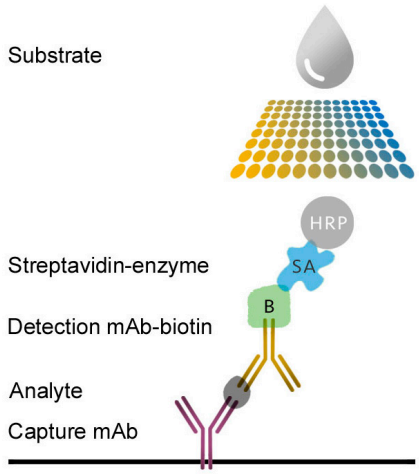

Figure 7. *Schematic representation of the ELISA setup used in this thesis.*

3.7 Multiplex assay

The multiplex assay is a development of the principles of an ELISA, enabling more than one analyte to be measured from the same sample. A well is pre-coated with capture antibodies, but in a multiplex, they are connected to a color-coded bead. After this, the sample is added, the analyte bound, then bound by a biotin-coated detection antibody, which is in turn bound by a streptavidin bound fluorochrome. The sample is then read on an instrument similar to a flow cytometer which uses two lasers. One sorts the beads to identify the analyte, and the other the strength of the fluorochrome signal, measuring how much of the analyte that was in the sample. With this technique, up to 40 analytes can be measured in the same sample. A multiplex assay, Luminex, was used to measure cytokines and chemokines in **paper III** and **IV**.

3.8 Immunofluorescence microscopy

Immunofluorescence is in many ways similar to flow cytometry. An antibody is added to a sample, which can be cells or very thin pieces of tissue. The antibody can be either directly bound to a fluorochrome, or the fluorochrome is bound to a molecule that in turn binds the primary antibody. When the fluorochrome is hit with light of a certain wavelength, light of another wavelength is emitted, and can be measured and photographed by a camera attached to the microscope. The difference is that whereas flow cytometry analyzes single cells in suspension, with immunofluorescence microscopy, you can investigate the expression of proteins on cells in their physiological surroundings. However, where you can

combine 10-30 different fluorochromes in flow cytometry, immunofluorescence microscopy is often limited to 3-6 different of antibody-fluorochromes to stain the same sample. Immunofluorescence microscopy was used in **paper I** to investigate expression of MR1 and CD68. We used pieces of placental and decidual tissues that were snap-frozen in liquid nitrogen, cut into 6-10µm sections and mounted on glass slides. The tissue was then fixed, permeabilized, stained and analyzed by immunofluorescence microscopy (Figure 8).

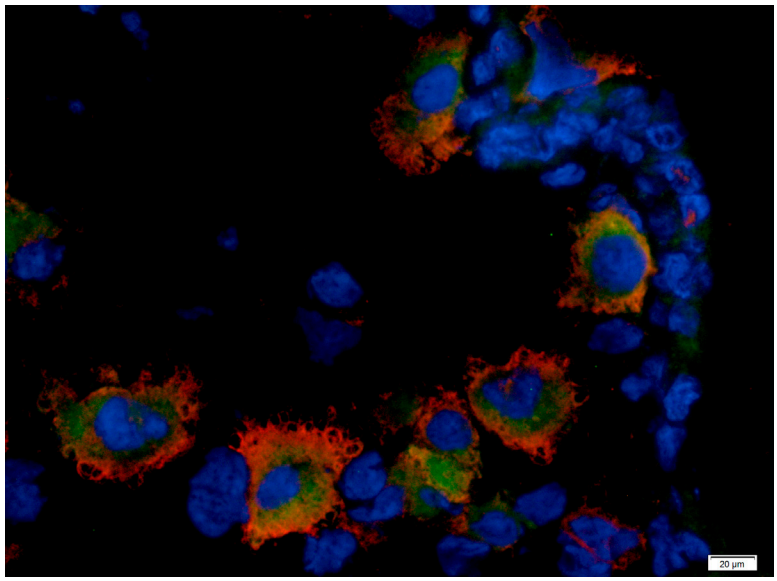

Figure 8. *Immunofluorescence staining of cross-section of a 2nd trimester placenta in 40x magnification. Cells are stained for DAPI (cell nuclei, blue), MR1 (red) and CD68 (green). Pictures are then overlaid over each other to demonstrate co-expression.*

3.9 Placental explant culture supernatants

Following the cell isolation from placental tissues, the placenta was placed with the umbilical cord facing down. The DB layer was removed with scissors. Between 5 and 10 cm^3 sized biopsies were then cut at different places from the underlying tissue of fetal villi. The tissue was dissected into as small pieces as possible, macroscopically identifiable blood vessels were removed, and the tissue pooled together. By a series of short centrifugations with exchange of the PBS in between, the blood was removed from the tissue pieces. The tissue was then weighed, and put into culture with a specified volume of cell culture medium per gram of tissue. After 48 or 72 hours of incubation, the supernatant was harvested, and frozen in aliquots. This placental explant culture supernatant was used in **papers III** and **IV**.

3.10 Migration assay

In order to investigate the migratory attraction of the placental explant culture supernatants, a migration assay was set up. The supernatant was diluted 50% with medium, and put into wells in a culture plate. Transwell inserts with a membrane with 5µm pores were inserted into the wells, and cells from healthy donors, either PBMCs, enriched T cells, or enriched $CD8^+$ T cells were added into the transwell inserts (Figure 9). After incubation, the transwells were removed, and the cells that had migrated through the membrane were collected and analyzed by flow cytometry. To measure migratory effects in more detail, blocking agents can be added to the bottom well to neutralize certain signals, or recombinant proteins could be used instead of the placental explant culture supernatants. The migration assay was used in **paper III** and **IV**.

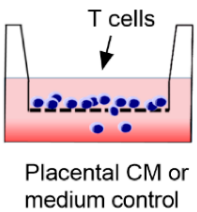

Figure 9. *Schematic representation of the migration assay setup. Medium, supernatant, blocking agents or recombinant protein was added to the bottom chamber. The transwell insert was then put into the well, and cells, in this example T cells, were added on top of the membrane. Cells then migrated through the membrane, and could be harvested from the bottom of the well after removal of the transwell insert.*

Solders M et al. Front Immunol. 2019 Jun 6;10:1300

3.11 CFSE proliferation assay

In order to measure the antiproliferative effect of two different immunosuppressants on MAIT cells in **paper V**, cells were stained with the dye carboxyfluorescein succinimidyl ester (CFSE). CFSE is a fluorescent dye that binds to intracellular molecules, and with each cell division, the fluorescence intensity is halved. We stained PBMCs with CFSE, stimulated them with an anti-CD3 antibody to start the proliferation, and then added various clinically relevant concentrations of either CsA or SIR. After five days, cells were harvested, stained and analyzed by flow cytometry, with the CFSE being detected in one of the common fluorescence wavelengths (Figure 10).

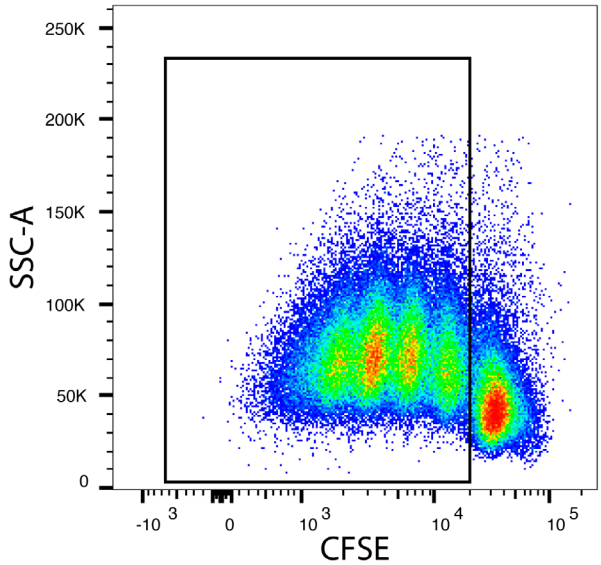

Figure 10. *Representative flow cytometry plot showing a CD3$^+$ cell population labeled with CFSE and stimulated with anti-CD3 antibodies for five days. Five distinct populations can be seen. The right one are cells that have not proliferated, and then each population to the left represents a cell division step from the original cells, as the intensity of CFSE is roughly halved. The gate thus comprise of proliferated cells.*

3.12 Chimerism

Chimerism analysis is used in HCT to determine if a patient's immune cells are of donor origin, or if some of the patient's original immune system remains. Samples are taken before the transplantation, and after the transplantation, samples are compared to this original sample. So called microsatellites, certain positions in the genome known to have a high variation between individuals, are used to determine the genetic origin within the sample. Routine data on patient chimerism after HCT was compiled and presented in **paper V**. The microsatellite polymorphism used in chimerism testing is also used in paternity testing. We further utilized this technique in **paper I** in order to measure the amount of fetal DNA in our IVB samples. As we collected blood from the maternal side of the placenta after birth, part of the feto-maternal barrier could have been broken, resulting in more or less contamination of fetal/umbilical cord blood in our samples. We used both cord blood and amniotic tissue as samples for determining fetal DNA, or more precisely to localize fetal microsatellites of paternal origin. For the maternal origin, maternal peripheral blood was used. After localizing one or more microsatellite positions were one allele had been inherited from respective parent, the DNA of the IVB was compared, and the small percentage of paternally-derived DNA represented fetal contamination in our isolation procedure.

3.13 Statistics

The work included in this thesis was almost exclusively based on smaller sample sizes of $n \leq 25$. The absolute majority of these had a non-normal distribution, and hence, all data was regarded as such, and analyzed by non-parametric methods. Two-tailed tests were used where applicable, and an alpha value of 0.05 was used to define significance throughout the work of this thesis. The majority of the analyzes in **paper I** was done comparing three groups of paired data. We first used the Friedman test to answer whether there was any difference between the three groups. If a significant difference was detected, post-testing was performed using the Wilcoxon signed rank test rather than Dunn's test, as the latter assumes the included groups to be interdependent, which they were not. We used a Bonferroni corrected *p* value to correct for multiple testing. When only two groups were compared, the Wilcoxon signed rank test was used directly. **Paper II** was completely based on comparisons between two groups of paired data, though compared to paper I, the number of analyzes had increased significantly. In order to minimize the risk of type 1 errors, to present the data in a general view, and most importantly, to utilize the strength of the large quantity of data available, we used the orthogonal projection to latent structures by means of partial least-squares discriminant analysis (OPLS-DA). OPLS-DA is a multivariate tool which scales the values for the included parameters and uses them to create a model of separation between the two groups. The ability of the model to separate the two groups based on the data included can then be illustrated in an observation plot which then illustrates how well the two groups can be separated from each other, utilizing the included data. The different parameters can then be ranked in order of how much they contribute to the model's ability to differentiate between the two groups. We used this ranked OPLS-DA loading plot as a basis to identify parameters of interest, which were then subsequently analyzed by the Wilcoxon signed rank test. In **Paper III**, we still worked mostly with two groups of paired data. Similar to **paper II**, we used the loading plot from an OPLS-DA analysis as a foundation for univariate post-testing using the Wilcoxon signed rank test. In the one instance of un-paired data, the Mann-Whitney test was used. In order to investigate which soluble factors (X variables) were associated with a certain cell type (Y variable), a linear OPLS analysis was used. The X variables are then ranked between a negative and positive association with the Y variable. Along the same line of reasoning as above, we used this a basis for further testing of potential correlations using the Spearman correlation test. For analysis of *in vitro* experiments, the Wilcoxon signed rank test was used to compare experimental conditions with the negative or positive control, where applicable. In **paper IV**, we used the same statistical methodology as in **paper III**, and also the Friedman test followed by Dunn's multiple comparison test to compare three groups of paired data. In **paper V**, the Friedman test followed by Dunn's multiple comparison test was used to compare

repeated measures data over four timepoints. The Kruskal–Wallis test followed by Dunn's multiple comparison test was used to compare the four interdependent timepoints to a set of unrelated controls. Comparisons between two groups of unpaired data was done using the Mann-Whitney test. For analysis of *in vitro* experiments, the Wilcoxon signed rank test was used to compare experimental conditions with the negative or positive control, where applicable. Although the data was non-parametric, a two-way ANOVA was used to compare the differences between two groups over multiple time points, as no such suitable non-parametric test exists. The Spearman correlation test was used to investigate potential correlations between certain patient characteristics and cell frequencies. The Friedman test followed by the Wilcoxon signed rank test with Bonferroni correction was used to compare groups of paired but not interdependent data. Statistical testing was performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) and SIMCA (SIMCA software, Sartorius Stedim Biotech, Umeå, Sweden).

4 RESULTS AND DISCUSSION

4.1 Cell isolation

In **paper I**, we used immunofluorescence microscopy to analyze cells in their physiological surroundings. For the other methods used throughout **paper I-IV**, the cells needed to be isolated from the placenta.

4.1.1 IVB

We utilized a simple and straightforward technique of IVB collection. During previous projects in our group where the focus was on isolating DSCs (154-156), it was noted that blood leaked out of the maternal side of the placenta at a slow and steady pace, even after more than one hour of lying in room temperature while the DSC isolation was finished up. Intrigued, we tried to collect this blood from another specimen, and found that by simply lifting the placenta, blood slowly oozed from the placenta, forming drops that could be collected either on a petri dish and transferred to heparin tubes, or be collected in heparin tubes directly. We also collected UCB from the umbilical cord, and when comparing these two specimens with flow cytometry, we found large differences in the composition of common immune cell subsets such as T cells, B cells, NK cells and monocytes (Figure 11), as well as in T cell differentiation with an increased frequency of effector memory T cells. For the following specimens, we compared IVB with maternal PB (Figure 11), and saw that from an immunological perspective, our isolated IVB did not seem to be PB that simply circulated in and out of the intervillous space. Especially interesting was the findings that MAIT cells were consistently higher in IVB compared to PB, whereas MAIT cells in UCB were very few (**paper I**) (Figure 12).

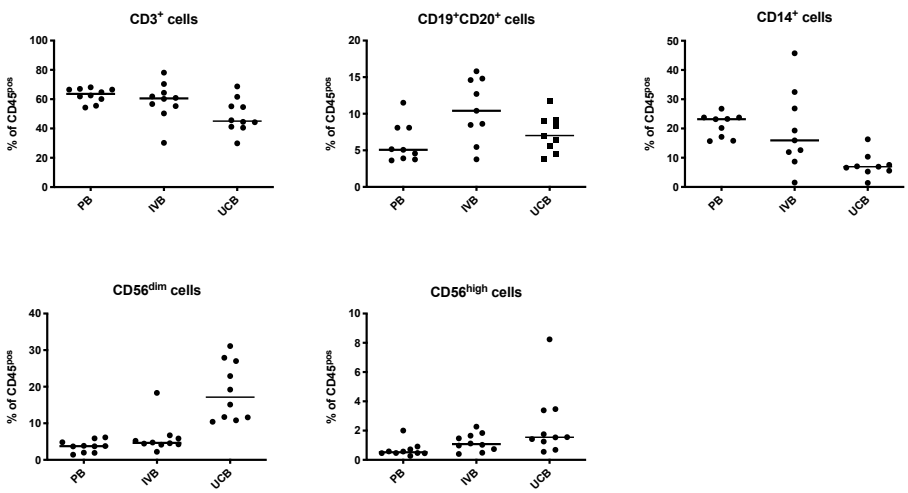

Figure 11. *Composition of common immune cell subsets in paired samples of PB, IVB and UCB. Cells are expressed as percentage out of the CD45+ gate. n = 10*

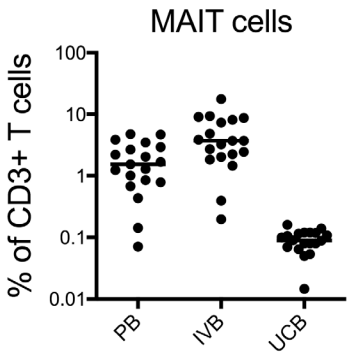

Figure 12. *MAIT cells in paired samples of PB, IVB and UCB, expressed as % of total CD3+ cells. n = 19.*

This led us to believe that our method of collecting IVB did not entail a large UCB contamination. To confirm this, we used chimerism analysis to determine the fetal DNA content in our IVB samples. In the six donors that were analyzed, the median content of fetal DNA in matched IVB samples was 12.1% (range 5.2-19.4) (**paper I**), results from a representative donor can be seen in Figure 13. It should be noted that as we measured total fetal DNA, the contamination could originate from any local source. It could be due to broken integrity of the feto-maternal barrier, and hence be a straight up mix of UCB in the IVB. However, it could also be loose cells and debris from the STB layer, as well as the known secretion of exosomes by STB into the IVB (125, 126). As many of the cell populations that were higher in IVB compared to PB were lower in UCB, it led us to the logical conclusion that the differences between IVB and PB could be assumed to be true. In fact, if the fetal DNA contamination were indeed originating from immune cells, it would mean that most of the observed differences between PB and IVB were indeed even larger than what we measured, that the differences were measurable in spite of the UCB contamination (Figure 12). With the focus on MAIT cells in **paper I**, we also did a two-step chimerism analysis. Using IVB from three donors, a portion was set aside and frozen. The remaining cell suspensions were depleted of non-T cells, and then positively selected for CD161 using magnetic bead separation. This resulted in a cell population enriched in T cells, and within this, a 3-5 fold increase in the frequency of MAIT cells. When comparing the chimerism results pre- and post-enrichment for these three donors, the amount of fetal DNA was almost halved. Thus, there was a constant presence of a small amount of fetal DNA, but the MAIT cells were almost exclusively of maternal origin (Figure 14).

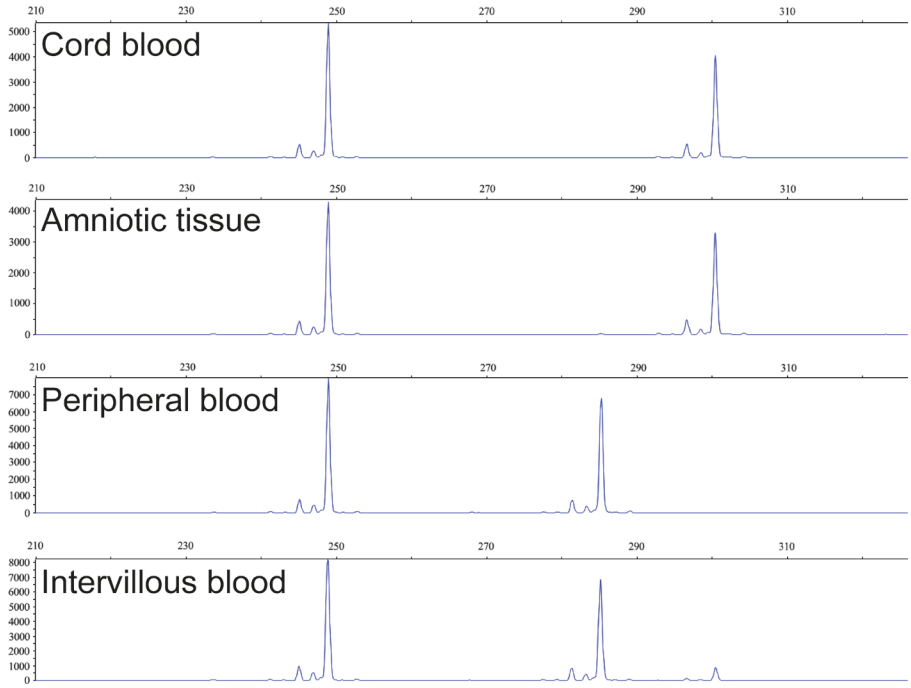

Figure 13. *Chimerism analysis of one placental donor. The amount of fetal DNA contamination into intervillous blood is represented by the small peak in the bottom right corner. Solders M et al. Sci Rep. 2017 Jul 21;7(1):6123*

For isolation of IVB, multiple techniques have been proposed over a period of time 20-30 years ago. Common for these techniques were the intentional damage to the placenta, and thus the inability to prevent breach of the feto-maternal barrier (157). Moore *et al*. described a perfusion-based technique in 1997 where multiple small non-piercing catheters were inserted a few mm into the placental tissue. The catheters were then connected to a pump which pumped PBS with added EDTA into the placenta, and the diluted blood that oozed from the placenta was collected and used for analysis. These methods yielded a product with low contamination of fetal red blood cells, and no large visible bands when using a PCR-based method of comparing fetal DNA content of IVB to UCB and maternal PB (157). Another method published by Camelo *et al*. in 1995 as a modification of one previously published in 1972 was performed by simply pricking the placenta with a sharp stylet, and collecting the blood that dripped out of the placenta (158). These, and other methods of IVB collection was then compared by Othoro *et al*. They found that either pricking or perfusion were better than the other methods which were more destructive to the placenta (159). If their isolated IVB product did not contain any fetal red blood cells, the method was regarded as free of fetal contamination.

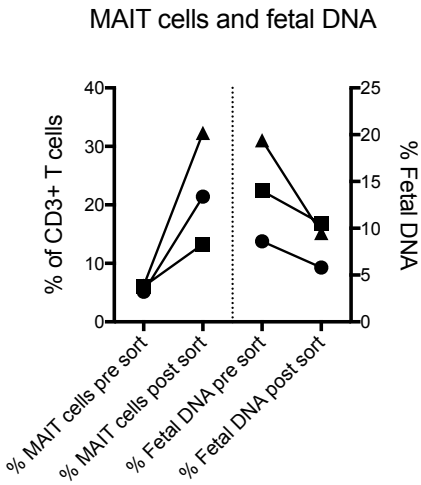

Figure 14. *MAIT cells were enriched by positively selecting CD161^{+} cells following a negative T cell enrichment. The amount of fetal DNA was then compared between the immune cells pre- and post-enrichment. n = 3.*

Solders M et al. Sci Rep. 2017 Jul 21;7(1):6123

We did not perform a separate study of our method of IVB isolation compared to that the other published methods. We do however think that our method has certain advantages. The method proposed by Moore *et al*. is based on creating an overpressure in the intervillous space by a continuous injection of PBS, which leads to the IVB being flushed out, probably through the existing venous system of the placenta (157). With our method, the force of gravity is sufficient. Personally, I do not think that these variation results in any major differences in the end product. The major disadvantages of the method used by Moore *et al.* is in my mind the risk of damage when inserting the catheters, and most importantly the inevitable dilution of the IVB with PBS. This leads to difficulties in interpreting results when analyzing levels of soluble factors, as the dilution factor cannot be specified. The method proposed by Camelo *et al*. where the placenta is punctured, and the IVB drips out through the hole does yield undiluted blood (158). However, the yield of IVB is not stated, though the variant of said method published by Othoro *et al*. is noted to yield about 1 ml of IVB (159). This is a severe limitation, as 1ml of IVB is nowhere near enough to perform the extensive phenotypic and functional assays that we had planned. With our way of IVB collection, we tend to get between 10 and 100ml of IVB from a term placenta. On top of this, as with the method described by Moore *et al*., puncturing the placenta in order to access the intervillous space is associated with a risk of damaging the villi, and thus creating a source of fetal contamination. To summarize, by just using the gravitational force and letting the IVB drip out through the vein of the placenta, we get a large volume of undiluted IVB, without using any object to pierce the placenta. It is hard to compare the level of fetal contamination between the methods, as different assays were used in the publications (152, 157-159) (**paper I**).

4.1.2 Decidua

Cell isolation from tissues requires the tissue to be broken down. This can be achieved by mechanical disruption, or by the use of different enzymes that breaks down the tissue integrity. The former yields a smaller amount of cells of a higher viability and intact protein expression, whereas the later yields a larger number of cells, but the viability tends to be lower, and the surface expression of proteins can be altered in the process (160, 161). We were inspired by colleagues in our lab, and used a variant of the method published by Norström *et al*. (162), our adaptation of this method is described in detail in our recent publication (152), and in brief in the methods section above, as well as in **paper I-II**. We used a combined mechanical approach where we first scraped the decidua off from the underlying tissue, followed by further mechanical disaggregation by the gentleMACS Dissociator. The resulting mix of PBS, cells and pieces of tissue was then filtered and washed. If cells were to be used in functional assays, a mononuclear cell suspension was achieved by density gradient centrifugation using Ficoll. If only phenotypic analysis by flow cytometry was to be used, erythrocytes were lysed using a lysis buffer, the remaining cells washed in PBS, and then stained and analyzed by flow cytometry. If cells were to be frozen and analyzed at a later time, no lysis was used as the freezing process itself lyses the erythrocytes. As we wanted to analyze extracellular markers, and use the cells for functional assays, the viability of the cells and integrity of protein expression were more prioritized over total cell numbers, thus leading us to the use of mechanical disaggregation rather than enzymatical. Inspired by the method published by Nagaeva *et al*., we did try to use a discontinuous Percoll gradient for the isolation of mononuclear cells (160). However, in contrast to their results, our yield of mononuclear cells was larger using Ficoll, and thus, we continued using Ficoll (data not shown).

4.2 Immune cell subsets in placental tissues

A successful human pregnancy relies on the placenta to shelter the fetus from the mother's immune system. In case of discovery, the fetus would be categorized as non-self, and subsequently killed by effector cells from the mother (98, 99). The exact mechanisms are unknown, but the placenta uses physical barriers, local immunosuppressive signals (100), absence of antigen presentation on classical HLA-molecules (102), absence of the actual HLA molecules themselves (101) and induction of the differentiation of a specialized sub group of NK cells (103) in order to prevent discovery and rejection by the maternal immune system. Many of these mechanisms are strikingly similar to the ones used by many tumors for their own protection, which highlights the dominating importance of the concept self and non-self for the function of our immune system.

4.2.1 IVB

Initially, we used a flow cytometric panel where we stained for known major immune cell populations. This was later expanded, and more data was acquired on T cell frequencies and phenotypes. As IVB originates from the maternal blood circulation, we compared IVB to paired PB. In **paper I**, we also compared DP with cells from IVB as well as PB, as the DP resident cells at some point originated from PB, and they could also have been part of the IVB for a short while.

We did not observe any differences in the frequency of total T cells out of total lymphocytes (**paper I**) (Figure 15). Regarding the CD4/CD8 phenotype of T cells, no differences were detected between PB, IVB and DP in paper I, although a trend towards decreased $CD4^+$, and increased $CD8^+$ and $CD4^-CD8^-$ t cell frequencies in DP compared to PB could be seen (**paper I**).

With the increased statistical power in **paper III**, it was clear that IVB contained fewer $CD4^+$ T cells, with a corresponding increase in $CD8^+$, and $CD4^-CD8^-$ T cells. B cell frequencies were increased in IVB compared to PB and DP, and lower in DP, and monocytes were decreased in DP compared to PB (**paper I**) (Figure 15). NK cells ($CD56^{dim}$) were increased in IVB compared to both PB and DP. NK cells with a $CD56^{high}$ expression were also increased in IVB compared to PB, and high levels were seen in DP (**paper I**) (Figure 15). Moore *et al*. found higher frequencies of $CD14^+$ and $CD56^+$ cells in IVB compared to PB (121). In our material, both $CD56^{dim}$ and $CD56^{high}$ NK cells were more numerous in IVB compared to PB. No statistical difference was seen regarding $CD14^+$ cells, but the trend was rather that there were fewer $CD14^+$ cells in IVB compared to PB (Figure 15). It should also be noted that the median frequency of $CD14^+$ cells in PB was 23% out of $CD45^+$ cells, whereas the corresponding mean number was 5.5% in the publication by Moore *et al*. (121). An apparent difference is that they collected the PB by capillary sampling up to 24 hours after delivery, whereas we took venous samples a few hours before delivery. As physiological vaginal birth is characterized by potent pro-inflammatory signals, both locally and systemically, the analyzed samples could be influenced by this fact (116). Altogether, this calls for caution when comparing results. Vega-Sanchez *et al*. used a cell isolation method similar to ours, though the way they present data as percentage of the lymphocyte gate/total events/total $CD3^+$ cells makes comparison with our data difficult (122). All in all, very few scientific articles are published on immune cell composition in IVB, and the ones that exist are hard to interpret in the context of our scientific work due to differences in sample collection, cell isolation and technical advancements during the time between publications.

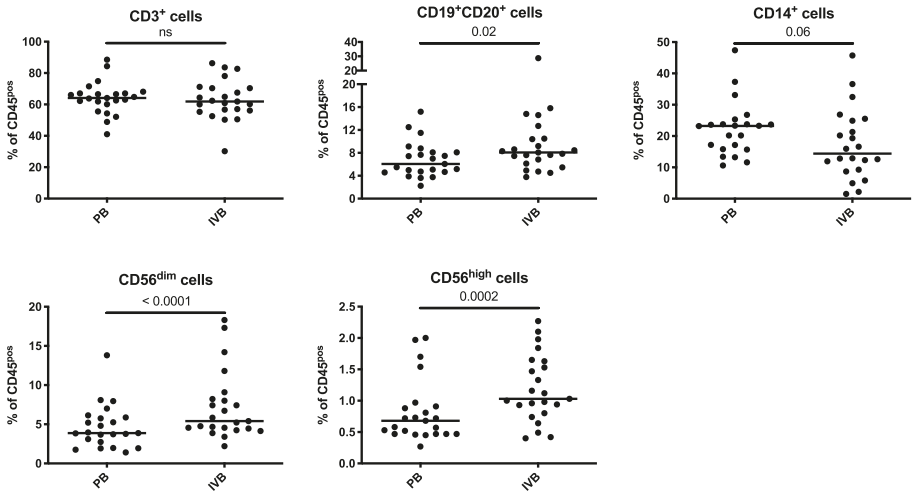

Figure 15. *Composition of common immune cell subsets in paired samples of PB and IVB. Cells are expressed as percentage out of the CD45$^+$ gate. Comparisons between samples were performed by Wilcoxon test, numbers indicate p value. n = 23 for all except for CD14+ cells where n = 22.*

In **paper I** and **III**, the main focus was T cells, and especially MAIT cells, which will be discussed further below. CD45RA is one isoform of the CD45 molecule, and upon activation and conversion to memory cells, the CD45RA of T cells is converted to CD45RO. Thus, a T cell more positive for CD45RA will be more negative for CD45RO, and vice versa. CCR7 is one of the chemokine receptors for T cell homing to secondary lymph nodes, and as such it is expressed on naïve and central memory T cells. Dividing the non-MAIT conventional T cells dependent on their expression of CD45RA and CCR7 thus makes it possible to estimate the proportions of naïve (CD45RA$^+$CCR7$^+$), central memory (CD45RA$^-$CCR7$^+$), effector memory (CD45RA$^-$CCR7$^-$) and terminally differentiated (CD45RA$^+$CCR7$^-$) T cells. We found that IVB was enriched in effector memory T cells at the expanse of naïve cells (**paper I** and **III**) (Figure 16).

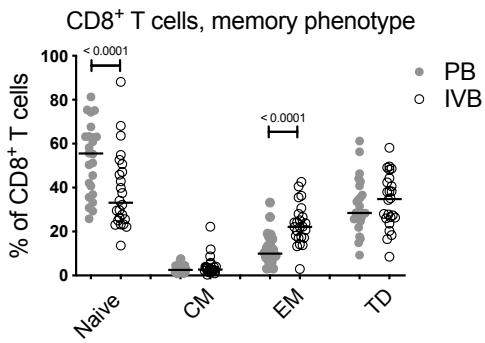

Figure 16. *Memory phenotype of CD8$^+$ T cells in paired samples of PB and IVB. n = 24. Comparisons between samples were performed by Wilcoxon test, numbers indicate p value. n = 24.*

Solders M et al. Front Immunol. 2019 Jun 6;10:1300

This was more strongly pronounced in DP where the majority of T cells were of the effector memory phenotype. This was however not unexpected, as tissue-resident T cells are well-known to be mostly of this phenotype (15). With the focus of this thesis on MAIT cells, we have not explored this notable finding of an increased frequency of effector memory T cells further. If we assume that the accumulation of these cells has happened due to evolutionary pressure to adjust to one or many factors that limits survival of the species, we can speculate in the effect of these cells in this setting. Effector memory T cells are antigen experienced T cells, that has a decreased need for co-stimulation upon binding the right antigen-MHC complex with its TCR. Thus, they can rapidly up-regulate their ability to locally support innate cells and B cells as well as direct cytotoxic killing. The alternative to a fast memory-based adaptive response is a more classical response of innate reaction, increased inflammation, migration of APCs, selection of naïve T cells in secondary lymph nodes, clonal expansion, and migration to the inflamed tissue. To reach the same level of local immune "competence", a de novo response takes a couple of days longer than a memory-based one. As the target tissue is the placenta, with the membranes surrounding the fetus in close proximity, a prolonged inflammatory phase could well result in premature rupture of the membranes, increasing the risk of local infection and pre-term birth (163). The accumulation of effector memory T cells in both IVB and decidua could consequently be a way of the immune system to increase the chance of a memory response instead of an innate one, although that remains to be studied.

We did not observe any differences between PB and IVB in the expression of the activation markers CD69, CD25 or HLA-DR on non-MAIT T cells (**paper I**). The IVB non-MAIT T cells did have a significantly higher median expression of the co-inhibitory marker PD-1 compared to PB. The non-MAIT T cells from DP generally expressed high levels of both activation markers as well as PD-1 (**paper I**). This probably in part reflects their status as tissue-resident cells, and hence the comparison with T cell in blood can be a bit misleading. However, there are accumulating data supporting that the decidual microenvironment also induces a steady but reversible state of immunosuppression via co-inhibitory markers (164). With the increased statistical power in **paper III**, we could discern a pattern of a significant but discreet increase in the expression of CD69, HLA-DR, TIM-3 and PD-1 of non-MAIT T cells in IVB compared to PB (**paper III**). This points to that IVB is a slightly more inflammatory environment than PB, also supported by our charting of the chemokine/cytokine profile in IVB and PB where the levels of a number of both pro-and anti-inflammatory soluble factors were higher in IVB compared to PB (**paper III**).

4.2.2 Decidua

The decidua has been studied more extensively than IVB with regard to local immune cell phenotype and function, though the majority of published studies have been performed on 1st trimester samples. In **paper II**, we performed an extensive measurement of immune cell phenotypes in DP and DB from term placentas, including a limited functional evaluation. Part of the data on DP was taken from **paper I**, but were complemented with new data, and the DB data had not been published previously. Using OPLS-DA and data on 81 different immune cell variables, we could see that DP and DB immunologically were two distinctly different sites. In the OPLS plot, we could observe that both DP and DB had their own characterizing features. Overall, when comparing general immune cell subsets, DP contained more T cells, $CD56^{high}$ NK cells, and $CD3^{+}CD56^{+}$ cells. DB were instead dominated by monocytes, B cells and $CD56^{dim}$ NK cells (**paper II**) (Figure 17).

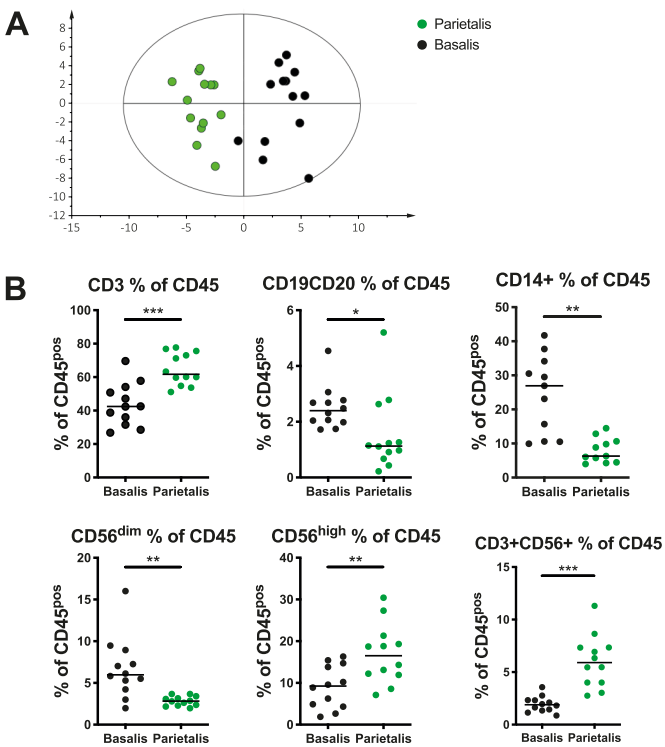

Figure 17. *(A) OPLS-DA observation plot showing the separation between the groups decidua parietalis and basalis based on immune cell phenotypes. (**B**) Distribution of the indicated immune cell populations between decidua basalis and parietalis. Comparisons between samples were performed by Wilcoxon test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. n = 8-13.*

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Both DP and DB were dominated by non-MAIT T cells with an effector memory phenotype, both within $CD4^+$ and $CD8^+$ T cells, and the $CD8^+$ T cells also had a significant proportion of terminally differentiated cells (**paper II**). Tilburgs *et al*. have showed similar findings in term decidual tissues, and also that the mRNA transcription of perforin and granzyme B seemed to be blocked (165). DP T cells generally had a higher expression of CD69, CD25, and a tendency of higher HLA-DR expression (**paper II**). The expression of the alpha chain of the IL-7 receptor, CD127, was also significantly lower in DP for both $CD4^+$ and $CD8^+$ T cells, also indicating a more activated and antigen experienced phenotype compared to DB (166) (**paper II**). The expression of the chemokine receptor CXCR3 was expressed on approximately half of the T cells in DP. The CXCR3 expression was lower in DB, though still higher than what is normally seen in blood (**paper II**). CCR6 expression was also higher in DP T cells, but the levels were generally low, between 5 and 20% on $CD4^+$ and $CD8^+$ T cells in DP and DB (**paper II**). CXCR3 and CCR6 based migration to placental tissues is further discussed below.

When investigating the expression of co-inhibitory markers, DP was associated with a generally higher expression than DB (**paper II**). About 80% of the $CD4^+$ and $CD8^+$ T cells in DP expressed PD-1, compared to 60-70% in DB. Similar patterns were seen for LAG-3 and TIM-3 which were generally expressed by a larger proportion of T cells in DP (**paper II**). Interestingly, a higher expression of co-inhibitory markers was also seen on B cells and NK cells, especially in DP (Figure 18). With the difference in activation and effector function between these cell types, one can speculate if there are unknown factors in decidual tissues that has a broad and unspecific effect on upregulating co-inhibitory markers on immune cells in general (**paper II**). The high expression of PD-1 on decidual T cells has been shown by others in 1st trimester samples (112, 167). The PD-1 ligands have been shown to be expressed by local macrophages and stromal cells, and the interaction do suppress proliferation and cytokine secretion (155, 167, 168). Wang *et al*. elegantly showed that by blocking the co-inhibitory effect of TIM-3 and/or PD-1 in mice, miscarriage rates increased, pointing to an important role of PD-1 in immunological tolerance during pregnancy. In 1st trimester decidual samples from miscarriages, the proportion of T cells expressing co-inhibitory markers was decreased, and the T cells produced more pro-inflammatory cytokines upon PMA/Ionomycin stimulation compared to samples from abortions due to non-medical reasons (112). We did not find any apparent major difference in IFN-γ production between DP, DB and healthy control PB upon stimulation with PMA/Ionomycin (**paper II**). This is supported by a paper by van der Zwan *et al*. who showed a high protein expression of co-inhibitory markers both in 1st and 3rd trimester DP, but $CD8^+$ T cells still proliferated, de-granulated and produced cytokines upon in vitro stimulation (164).

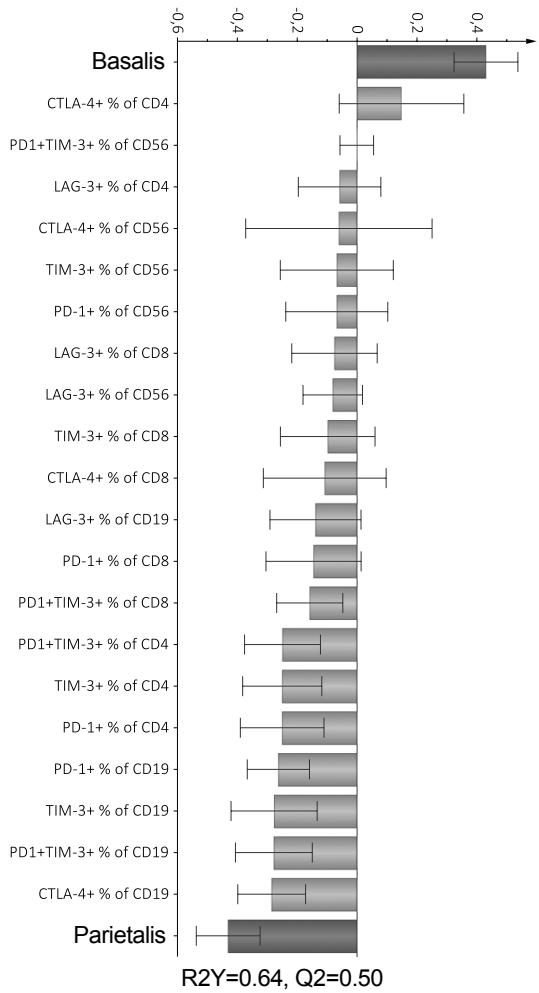

Figure 18. *OPLS plot showing associations between either decidua basalis or parietalis, and coinhibitory markers on T cells, B cells and NK cells.*

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T regulatory cells have been shown to be enumerated in decidual tissues compared to PB by many others (169-171). We could see a significantly higher proportion of T regulatory cells in DP compared to DB, a finding supported by others (98, 169) (**paper II**). Tilburgs *et al*. demonstrated a correlation between DP T regulatory cells and HLA-C mismatch between mother and fetus, indicating that allo-recognition does indeed play a role in immune cell activation and local immunosuppression (98). Salvany-Celades *et al*. continued to investigate this link and revealed that decidual macrophages and extravillous trophoblasts did induce T regulatory cells from $CD4^+$ T cells in a partly HLA-C dependent manner (172).

4.3 MAIT cells in placental tissues

Like other T cells, MAIT cells are also dependent on differentiating between self and non-self as a safety threshold for activation, though their mechanism differs from both the innate and adaptive immune system. Humans are dependent on dietary resources for vitamin B_2. On the other hand, certain pathological species of bacteria and fungus do synthesize their own vitamin B_2, releasing metabolites from this synthesis into their surroundings (35, 48). These metabolites are then sampled by APCs, and presented on the MR1 molecule, similar to the MHC for conventional T cells. MAIT cells are then activated by binding the MR1 with the bound vitamin B_2 synthesis metabolite, and exert immediate, potent and local killing of pathogens and infected cells, as well as driving local inflammation (48).

4.3.1 Presence and phenotype

4.3.1.1 IVB

Prior to the work included in this thesis, MAIT cells had not been investigated in placental tissues. MAIT cells were consistently increased in IVB compared to PB, both measured as percentage of $CD3^+$ T cells as well as out of total $CD45^+$ cells (**paper I** and **III**). MAIT cells made up a median of 4% of the total T cells in IVB, slightly more than double the median frequency in PB (**paper I** and **III**) (Figure 19).

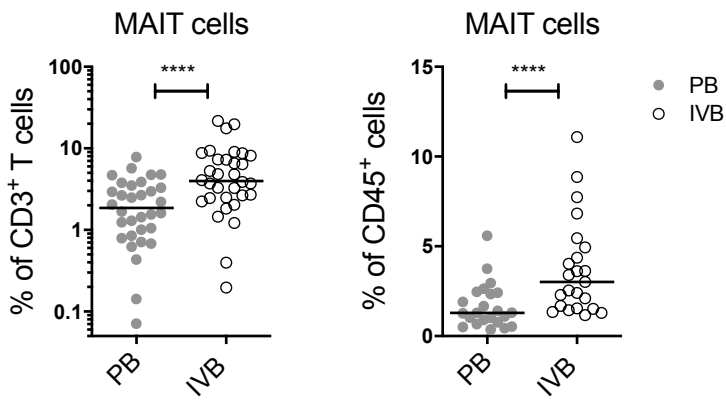

Figure 19. *Frequency of MAIT cells in paired samples of PB and IVB measured as % of $CD3^+$ cells (left, n = 24) and $CD45^+$ cells (right, n = 23). Comparisons were performed by Wilcoxon test. **** $p < 0.0001$.*

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Throughout **paper I** and **III** we used the extracellular co-staining of CD161 and Vα7.2 to identify MAIT cells with flow cytometry. In **paper III** we verified that we were indeed staining MAIT cells by the use of fluorochrome-conjugated 5-OP-RU (positive) and 6-FP (negative) loaded MR1 tetramers. The overlap in staining when comparing the population identified by co-staining of CD161 and Vα7.2 with those stained with the 5-OP-RU tetramer was about 90-95% in both PB and IVB, similar to what has been published by others (173, 174) (Figure 20). No apparent differences were seen regarding CD4/CD8 phenotype of MAIT cells when comparing the two staining strategies (**paper III**).

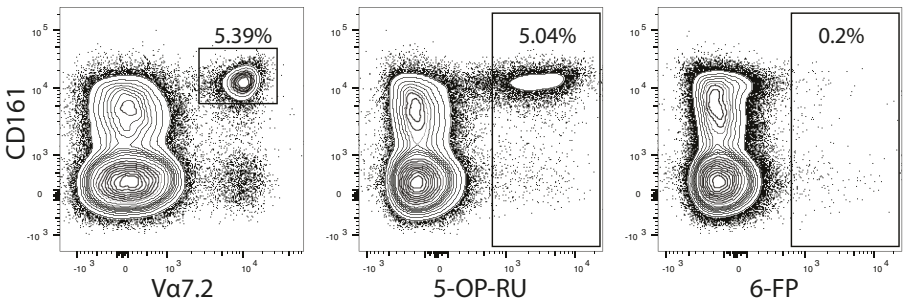

Figure 20. *Representative plots of staining of MAIT cells with Vα7.2 (left), 5-OP-RU loaded tetramer (positive, middle) and 6-FP loaded tetramer (negative, right).*

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Similar to the non-MAIT T cells, more MAIT cells in IVB were of an effector memory phenotype compared to PB (**paper I** and **III**). MAIT cells are known to have an effector memory phenotype and functions (34), and it is not clear what effects a decreased relative expression of CD45RA has on MAIT cells (**paper I**). What can be said is that the MAIT cells in IVB have made a more thorough shift in CD45 isoform expression compared to PB MAIT cells. In mice, it has been shown that $CD45RO^+$ T cells have increased production of pro-inflammatory cytokines (175). The mechanisms behind altered CD45 expression are unknown for MAIT cells, but it would be interesting to investigate any associated maturation processes with a potential effect on MAIT cell effector functions.

Activation status in freshly isolated MAIT cells was compared between PB, IVB and DP in **paper I**. No differences were seen between PB and IVB MAIT cells when comparing the expression of CD69, CD25, HLA-DR or CD127 in **paper I**. With the increased statistical power in **paper III**, IVB MAIT cells were shown to have a slightly lower expression of CD25. DP MAIT cells were generally more activated, much like the non-MAIT T cells (**paper I**). An interesting finding was that the expression of PD-1 was significantly lower on IVB MAIT cells, but higher on the non-MAIT T cells (**paper I** and **III**). This was not seen for the expression of TIM-3, which was slightly higher on all T cells in IVB (**paper III**). Whether

pathways of PD-1 regulation differs between MAIT cells and non-MAIT T cells is unknown. As MAIT cells are even more prone to a rapid activation than non-MAIT T cells, the latter might need a higher activation threshold at the feto-maternal interface to prevent unnecessary activation, though this remains a speculation.

4.3.1.2 Decidua

When comparing MAIT cells in decidual tissues using OPLS-DA, it was clear that the local microenvironment in DP and DB differed in its polarizing effect on MAIT cell presence and phenotype (**paper II**). MAIT cells were more frequent in DB compared to DP measured as percentage out of $CD3^+$ cells. There was a slight shift from $CD8^+$ to $CD4^+$ MAIT cells in DP compared to DB. MAIT cells in DP were generally more activated and exhausted than in DB, based on the expression of the parameters CD69, CD25, HLA-DR, CD127 and PD-1 (**paper II**). Making a general overall comparison between IVB, DB and DP MAIT cells based on extracellular phenotype, the DB MAIT cells seemed to have an activation status in between that of IVB and DP. As the maternal blood enters the intervillous space via the spiral arteries piercing the DB, it is maybe not too surprising that local factors influence MAIT cells in both of these compartments close to each other. MAIT cells in DP on the other hand are physically far from both IVB and DB, and it can be speculated that they have stayed in that tissue for a long time during pregnancy, even since the parietalis part of the membranes grew away from the placenta with the maternal arterial blood supply. Following this line of thought, it is also interesting to speculate on whether it is factors in IVB that influences DB MAIT cells, vice versa, or a cross-talk between the two compartments.

4.3.2 Function

4.3.2.1 IVB

In order to investigate MAIT cell functionality, we stimulated mixed mononuclear cell suspensions with fixed *E. coli* and CD28 for 16 hours. Brefeldin A was added during the last 4 hours of culture, and intracellular cytokine production was measured by flow cytometry. Amounts of released cytokines were measured in culture supernatants by ELISA. We found that a higher proportion of IVB MAIT cells had an intracellular production of IFN-γ compared to both PB and DP (**paper I**). The same difference between IVB and PB was seen when measuring GrzB expression (**paper I**). The MFI of perforin was higher in IVB compared to both PB and DP in unstimulated conditions (**paper I**). The increased response in IVB over PB MAIT cells regarding IFN-γ and GrzB was also seen when comparing amounts of soluble protein in the supernatants from the same experiments (**paper I**) (Figure 21). A small but still significant increase in expression of GrzB and MFI of perforin, but not IFN-γ expression, was also seen for $CD4^+$ and $CD8^+$ non-MAIT T cells from the same cultures, pointing to generally increased levels of pro-inflammatory cytokines in the cultures, leading to a secondary activation of non-MAIT T cells.

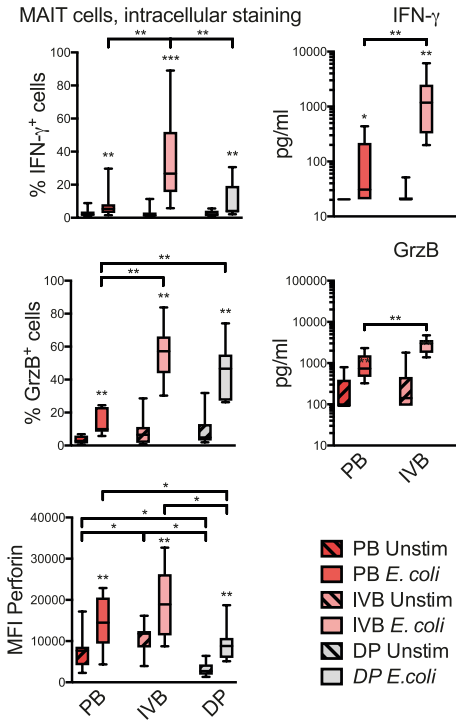

Figure 21. *Following bacterial stimulation for 16 hours, cells were analyzed for intracellular expression of IFN-γ (top left, n = 12), GrzB (center left, n = 10) and perforin (bottom left, n = 9) by flow cytometry. Culture supernatants were analyzed for soluble IFN-γ (top right, n = 10) and GrzB (center right, n = 10) by ELISA. Symbols on top of bars indicate statistical significance compared to unstimulated conditions. Two paired samples were compared with the Wilcoxon test. Three paired samples were compared by the Friedman test followed by a Bonferroni-corrected Wilcoxon test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.*

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In line with this reasoning, we measured IL-12p70 and IL-18 in both bacterial culture supernatants, as well as in IVB and PB plasma samples. No obvious differences were discerned regarding these cytokines (**paper I**). IVB plasma had a somewhat lower level of IL-18, and similar levels of IL-12p70 as PB. IL-10 was higher in *E.coli* activated PB cultures compared to IVB, which could provide some explanation as to why PB MAIT cells had a generally lower response to the bacterial stimulus than IVB MAIT cells, either by affecting monocytes, or the T cells directly (176). It would need further investigation to verify if IVB mononuclear cell cultures have a lower IL-10 feed-back response to bacterial stimulus compared to PB, and if so, mechanisms behind such an effect. To further try to elucidate reasons behind the stronger response of IVB MAIT cells, we blocked MR1, IL-12p40 and IL-18 alone and in combinations. It seemed that all three factors played a substantial role in the activation of MAIT cells, measured both as intracellular expression of IFN-γ and soluble IFN-γ in supernatants, for both PB and IVB (**paper I**). Based on our data, it can also be speculated that the activation pathways overlap, as blocking MR1 alone gave approximately half of the IFN-γ production, but addition of MR1 blocking to blocking of IL-12p40 and IL-18 resulted in only a modest addition of the blocking effect (Figure 22).

Sattler *et al*. showed that IL-12 and IL-18 does not activate MAIT cells alone, but in combination. They also showed that IL-18 was the limiting factor in activating MAIT cells, as the combination of IL-15 and IL-18 gave a MAIT cell activation similar to IL-12 and IL-18, whereas IL-12 and IL-15 in combination failed to stimulate purified MAIT cells (50). They also showed that monocytes were the source of IL-18 in their cultures, and it would thus be of interest to investigate any functional differences between monocytes in PB and IVB. In conclusion, MAIT cells in mononuclear cell cultures from IVB were more activated by bacterial stimuli than PB. Whether this was an effect due to differences in MAIT cells, or in some other cell type in the cultures would be interesting for future studies. At the same time, by using freshly isolated samples without purification or manipulation, our *in vitro* findings lie closer to what is actually found *in vivo*.

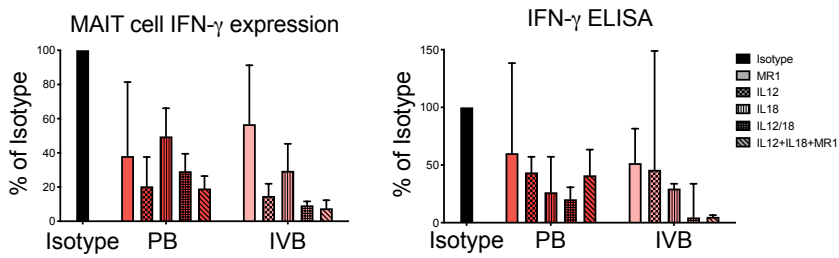

Figure 22. *Blocking factors important for MAIT cell activation in PB and IVB in bacterial stimulation cultures. The factors blocked are indicated in the figure, and after incubation, cells were analyzed for intracellular expression of IFN-γ by flow cytometry, and soluble IFN-γ in supernatants by ELISA.*

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4.3.2.2 Decidua

MAIT cell functions were investigated for DP in **paper I**, and again for both DP and DB in **paper II**, though then for only two donors. DP MAIT cells had an IFN-γ response similar to that of MAIT cells in PB, and their GrzB response was higher (**paper I**) (Figure 21). The same trends were seen when analyzing soluble factors with ELISA (**paper I**) (Figure 21). Both the background expression and increased levels in response to bacterial stimuli of perforin was lower in DP MAIT cells compared to PB (**paper I**) (Figure 21). The low expression of perforin in resting DP MAIT cells could be a reflection of their immunosuppressive microenvironment, as MAIT cells require licensing by either TCR binding, cytokine stimulation, or both to upregulate cytotoxic markers (63). It is also interesting that the two key molecules of MAIT cell cytotoxicity GrzB and perforin have separate modes of upregulation. GrzB requires TCR stimulation together with stimulation of either CD28 or cytokines, whereas perforin upregulation is TCR-independent (50, 63).

Thus, our findings in **paper I** indicate that there is sufficient antigen presentation by MR1, but that there is a relative impairment of cytokine production in DP cultures, maybe reflecting the immunosuppressive microenvironment from where the cells were isolated. Levels of IL-10 in the culture supernatants were lower in DP compared to both PB and IVB (**paper I**). This is a bit surprising, as the level of MAIT cell activation was similar in PB and DP, and DP contains more T regulatory cells than PD (Figure 23). It could be that monocytes are the primary source of IL-10 in bacterial cultures such as ours, and as monocytes were very few in DP, IL-10 levels were low (**paper I**). This could in turn be due to that our method of mechanical cell isolation did not release all macrophages and DCs. This though remains a speculation.

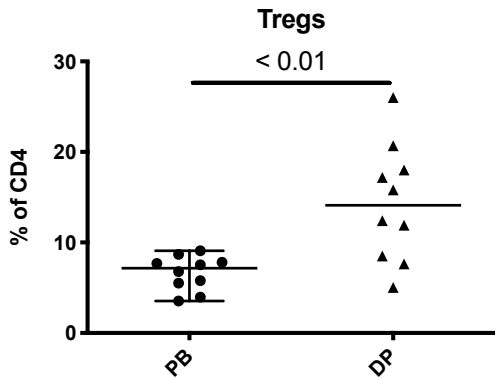

Figure 23. *T regulatory cells ($CD25^{high}CD127^{low}$) measured as percentage of $CD4^{+}$ T cells in paired samples of PB and DP. Comparisons between samples were performed by Wilcoxon test, number indicate p value. n = 10.*

4.3.3 MR1 expression

4.3.3.1 IVB

In **paper I**, we stained snap-frozen samples of fetal villi from the intervillous space from both 3rd and 2nd trimester samples, and analyzed them with fluorescence microscopy. MR1 expression clearly localized to inside of the villi. In line with the absence of HLA-molecules, expression of MR1 on STBs was totally absent, both in 3rd and 2nd trimester samples (**paper I**). Not all cells inside the villi stained positive for MR1. We suspected the MR1 positive cells to be villous macrophages, so called Hofbauer cells. Co-staining of CD68 and MR1 revealed complete co-expression of these proteins, thus indicating that the Hofbauer cells were indeed responsible for the intra-villous MR1 expression (**paper I**) (Figure 24).

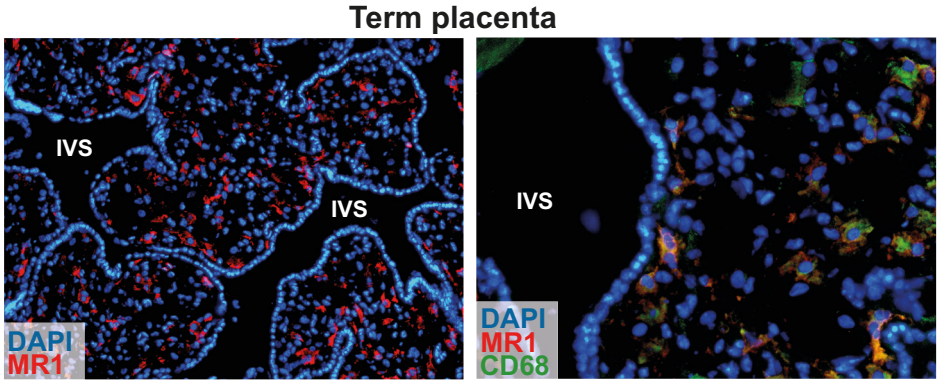

Figure 24. *Immunofluorescence staining of cross-section of a 3rd trimester placenta in 10x (left) and 20x (right) magnification. Cells are stained for DAPI (cell nuclei, blue) and MR1 (red) (left) and also for CD68 (green) (right). Pictures are then overlaid over each other to demonstrate co-expression. IVS, intervillous space.*

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The use of nitrogen to snap-freeze tissue leads to destruction of all IVB cells, leading to the empty black spaces seen in the fluorescence microscopy figures in **paper I**. Thus we were not able to investigate MR1 expression on cells in the intervillous space, or try to stain MAIT cells at the site of interest for that matter. Trying to interpret the findings of $MR1^+$ cells inside the fetal villi, we speculate that the Hofbauer cells lie ready with a high MR1 expression in the case of an inflammatory process that would disrupt the STB border. In the case of a concomitant bacterial infection, riboflavin synthesis metabolites would then be released around this site, internalized by the Hofbauer cells, and presented to the IVB MAIT cells in close proximity. This is supported by reports of maternal T cells being found inside the fetal villi in the condition villitis of unknown etiology, a chronic inflammatory disorder of villus inflammation that affects about 10% of pregnancies (177). It would be highly interesting to study MAIT cell infiltration of villi in cases of local bacterial infections.

4.3.3.2 Decidua

Performing the same staining on frozen sections of the fused fetal membranes revealed MR1 expression throughout DP, but not chorion and amnion. Co-staining of MR1 and CD68 revealed some cells expressing both markers, and some MR1 positive cells that did not express CD68 (**paper I**). Gibbs *et al*. examined endometrial $MR1^+$ cells in non-pregnant women, and found them to co-express HLA-DR and CD11c, indicating either DC or macrophage origin (66). The $MR1^+CD68^-$ cells in DP are likely to be some kind of APC, though whether they are DCs or B cells, or a different cell type completely remains unknown. As the decidua forms the outer barrier function of the placenta, it should in theory be the first site of discovery of a pathogen. Thus, it can be speculated that the high MR1 expression together with the presence of MAIT cells in DP could be a part in the immunological defense of the placenta and fetus against bacterial infections.

4.4 Migration

4.4.1 Placental secretome

After the publication of **paper I**, we were more interested in the fact that MAIT cells and effector memory T cells were enriched in IVB compared to PB. As both MAIT cells and non-MAIT T cells in both PB and IVB had a low expression of Ki67, we deemed a local proliferation unlikely to be the factor behind the increased cell frequencies in IVB (**paper I**). By comparing the frequency of MAIT cells in PB samples from pregnant women with non-pregnant blood donors, we could see that women at the end of their pregnancy had lower proportions of MAIT cells in PB compared to non-pregnant women (**paper III**) (Figure 25). We then hypothesized that MAIT cells, and perhaps also other cell subsets enriched in IVB, were recruited/retained in the intervillous space, and decided to try and investigate any such factors.

Meggyes *et al*. investigated PB MAIT cell frequencies in a small cohort of healthy pregnancies and cases of pre-eclampsia. In the patients with pre-eclampsia, MAIT cell frequencies was decreased out of the $CD8^+$ T cells, and the MAIT cells had a lower PD-1 expression. However, as they gated MAIT cells out of the $CD3^+CD8^+$ cells, their results on healthy pregnancies are hard to compare to ours (178). Ravi *et al*. investigated PB MAIT cell proportions in HIV+ and HIV- women. In the HIV- cohort, they observed a median MAIT cell frequency of 1.5% out of total $CD3^+$ cells (179), comparable to our findings of 1.8% (Figure 25).

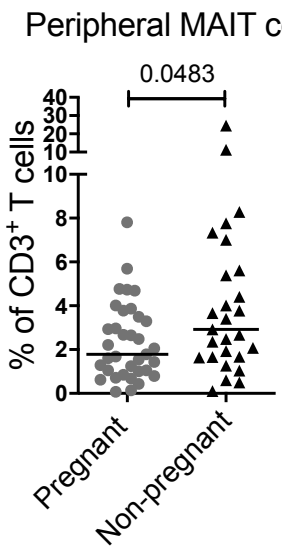

Figure 25. *Comparison of frequency of MAIT cells in PB between women at term pregnancy (n = 37), and non-pregnant female blood donors (n = 27). Comparison made with the Mann-Whitney test, number indicate p value.*

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If the levels of cytokines and chemokines were to be altered in IVB compared to PB, we thought it was likely that this was due to local production and release of said proteins. In order to investigate this, we took large biopsies from the villous portion of term placentas, cut the tissue into small pieces, pooled the tissue to get a representative sample from the whole placenta, washed away adherent blood, and put tissue pieces in culture for 2 days. These placental explant culture supernatants were then analyzed by Luminex. We found that the supernatants contained high levels of many cytokines and chemokines, among these MIF, IL-6, CXCL8, and CCL2 (**paper III**) (Figure 26).

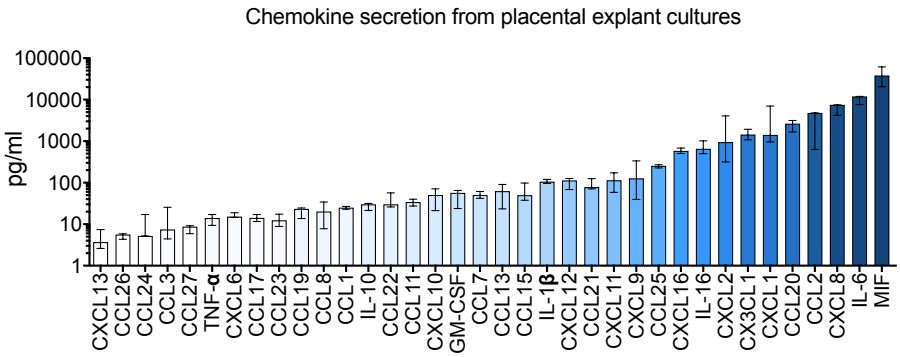

Figure 26. *Chemokine secretion from placental explant culture supernatant. n = 5. Data is displayed as median with range.*

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We were inspired by Svensson-Arvelund *et al*. in the experimental setup for our placental explant culture supernatants (180). They used 1st trimester placentas instead of our 3rd trimester samples. We used the same proportions of tissue and medium, but Svensson-Arvelund *et al*. used a 24 hour incubation whereas we used 48-72 hours. We also analyzed the supernatants with different multiplex kits. However, for many of the analytes that overlapped, we could see similar results, for example for the chemokines IL-6, CXCL8, CCL20, CCL2. They also isolated CTBs and extra-villous trophoblasts, and saw that they both secreted high levels of CCL2, CXCL8 and CXCL10. Thus, we can see some similarities between our results and theirs, though we have differences in both experimental setup and analysis. Svensson-Arvelund *et al*. showed that the supernatants from placenta tissue explant cultures did induce M2 macrophages and T regulatory cells in cell culture, thus promoting a tolerogenic phenotype of the local immune cells (181).

4.4.2 Migration towards placental explant culture supernatants

Next, we set up an assay of immune cell migration to investigate the attraction properties of placental explant culture supernatant. We could see that the supernatant attracted both T cells in general, $CD8^+$ T cells, $CD8^+$ effector memory T cells and MAIT cells. A more relevant readout is the relative change in T cell frequencies, as this could explain the relative differences observed between PB and IVB. Interestingly, the supernatant attracted both MAIT cells and $CD8^+$ effector memory T cells to such an extent that their relative proportions increased compared to medium control (**paper III**) (Figure 27). Thus, villous tissue secretes factors that selectively attracts MAIT cells and $CD8^+$ effector memory T cells, two T cell subsets that were found to be increased in IVB compared to PB (**paper I** and **III**).

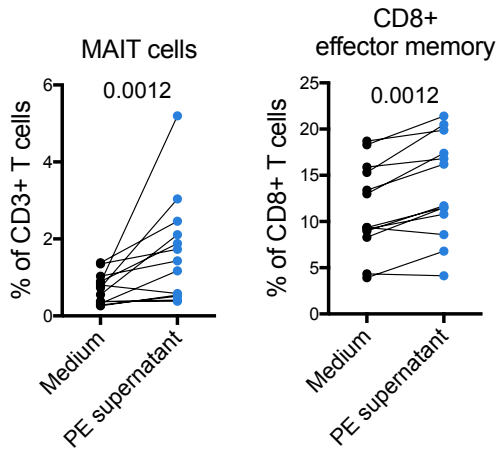

Figure 27. *Migration of MAIT cells and $CD8^+$ effector memory T cells towards placental explant culture supernatant (PE supernatant in figure). Comparisons between samples were performed by Wilcoxon test, numbers indicate p value. n = 13.*

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4.4.3 Soluble factors in IVB and PB

When isolating immune cells from PB and IVB, plasma was collected and frozen. These plasma samples were analyzed by the same Luminex as above, and revealed vast differences in cytokine and chemokine levels between PB and IVB (**paper III**). Most prominent was the difference in the levels of MIF, but also CXCL9, CXCL10 and CCL25, which were all higher in IVB compared to PB. On the other hand, certain soluble proteins were higher in PB, for example CCL27, CXCL12 and CCL21 (**paper III**) (Figure 28). As CCL27 is a chemoattractant for skin-resident memory T cell, and CCL21 attracts naïve CCR7-expressing T cells, the data on soluble chemoattractants seem to reasonably match the composition of immune cell subset

at the respective localization. The placental explant culture supernatants were only investigated by Luminex in five donors, and matched IVB plasma for only three of these donors. Thus, a comprehensive comparison between what cytokines and chemokines were secreted from the villous explants with the levels found in the IVB from the same donor was not possible. However, by visibly comparing the aggregated data, a pattern appeared where soluble factors higher in IVB compared to PB were generally also secreted by placental tissue explants (**paper III**). This is a mere indication, and it would require further studies to establish such a relationship, although it seems probable that the placental tissue does secrete factors that define the local immunological environment.

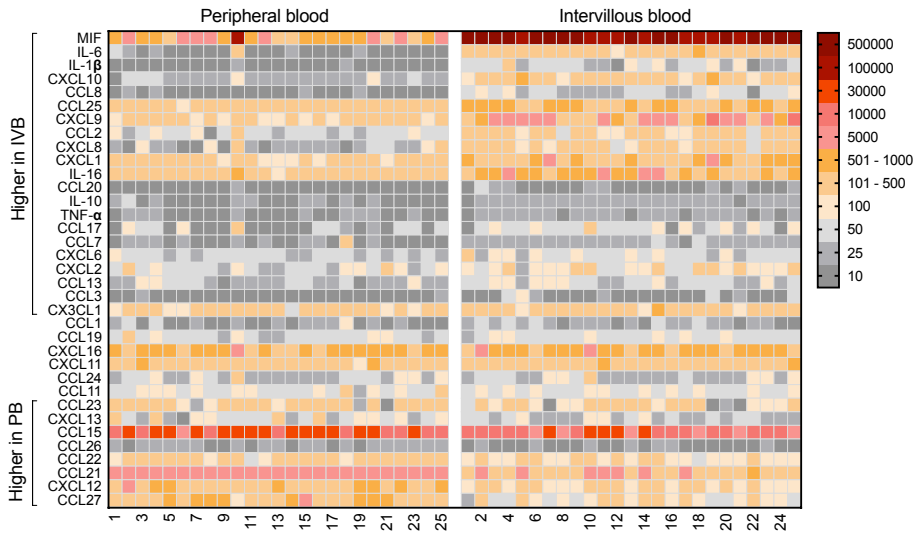

Figure 28. *Heatmap displaying each individuals (x axis) results of the measurements of the indicated parameters (y axis) in paired samples of PB and IVB plasma. Sorting is done according to previous OPLS-DA analysis, and the analytes significantly different between PB and IVB are indicated to the left of the y axis. n = 25.*

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The levels of soluble factors in IVB has only been investigated in a few previous publications. In line with our findings, Bouyou-Akotet *et al*. found increased levels of CCL2 in IVB compared to PB (123). Chaisavaneeyakorn *et al*. found similar levels of MIF in IVB compared to PB as we did, and interestingly, the levels of IVB MIF was significantly reduced in 3rd or later pregnancies compared to 1st or 2nd pregnancies (182). Singh *et al.* also found IVB MIF levels to be similar to our observations, and they saw an association between higher levels of MIF in IVB and stillbirth as well as low birth weight deliveries (124). Interestingly, in both of these publications, IVB from donors with placental malaria had significantly higher

levels of MIF (124, 182). These results have been interpreted as MIF being part of a pro-inflammatory drive produced by local cells, aimed to retain macrophages at the site of infection.

4.4.4 Cell and chemokine correlations

We utilized OPLS analysis to investigate associations between MAIT cell proportions and levels of cytokines and chemokines in paired plasma samples. MAIT cell frequencies were found to be most closely associated with levels of MIF and CCL25, and negative associations were found with CXCL12, CCL21 and CCL27, the same molecules found to be associated with PB rather than IVB when comparing levels in plasma (**paper III**). Performing correlation tests, we found a weak correlation between MAIT cell proportions and MIF levels, and a trend for levels of CCL25 (**paper III**) (Figure 29). Utilizing data on MAIT cell frequencies in DP from **paper I** and **II**, we could show significant correlations with levels of both MIF and CCL25 in IVB (**paper III**) (Figure 29). No correlations were seen between these chemokines and MAIT cell proportions in PB (**paper III**) (Figure 29). The levels of CCL21 and CCL27 were found to be negatively correlated with MAIT cell proportions in IVB, further demonstrating the differences between the immunological environment in PB and IVB (**paper III**). When performing the same tests for the proportions of $CD8^+$ effector memory T cells, there was no correlation with MIF or CCL25. Instead, the proportion of $CD8^+$ effector memory T cells was strongly correlated to the three ligands for CXCR3, namely CXCL9, -10, and -11 (**paper III**) (Figure 29).

We continued to focus on the migration of MAIT cells. Using antibodies and chemicals, we blocked the chemokines CCL25, CCL20 and MIF, alone and together. We could see that by blocking all three factors together, but not alone, the proportion of MAIT cells attracted by the placental explant culture supernatant decreased. No effect was seen on the proportion of $CD8^+$ effector memory T cells. Recombinant MIF exerted a general attraction of T cells, but in a similar fashion as the placental explant culture supernatant, it selectively attracted MAIT cells, but not $CD8^+$ effector memory T cells (**paper III**). Lastly, we showed that one of the receptors for MIF, CXCR4 was expressed by almost all MAIT cells, and that $CD8^+$ effector memory T cells had a lower expression. However, when comparing the cells that had migrated towards recombinant MIF, the $CXCR4^+$ proportion of $CD8^+$ effector memory T cells was significantly increased, almost to what was seen for MAIT cells, and the CXCR4 MFI was also significantly increased (**paper III**), suggesting that MIF-mediated attraction is mediated by CXCR4.

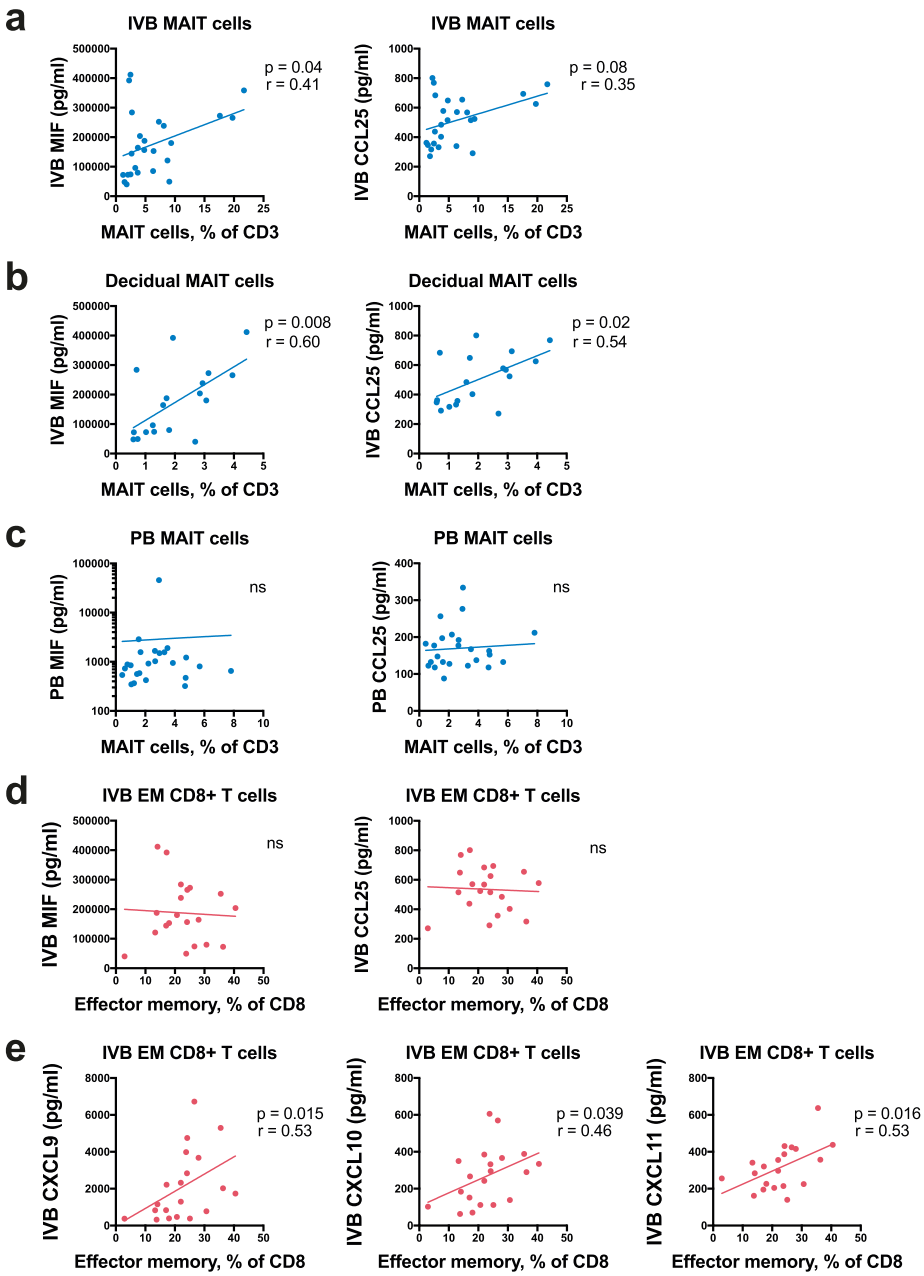

Figure 29. *Correlations between MIF (left) and CCL25 (right) and (a) MAIT cells in IVB, n = 25, (b) MAIT cells in DP, n = 18, (c) MAIT cells in PB, n = 24, and (d) CD8⁺ effector memory T cells in IVB, n = 20. (e) Correlations between CD8⁺ effector memory T cells in IVB and CXCL9 (left), CXCL10 (center) and CXCL11 (right), n = 20. Correlations between paired samples were made using the Spearman correlation test.*

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Most MAIT cells express the receptors for the chemokines CCL20 (CCR6), CXCL16 (CXCR6), and some MAIT cells also express the receptors for CCL25 (CCR9) and CXCL9, -10 and -11 (CXCR3) (183). In spontaneous bacterial peritonitis, MAIT cells were found to express CCR5, CXCR3 as well as the gut-homing α4β7 integrin. MAIT cells also migrated towards ascitic fluid from the patients, as well as towards CXCL10, CCL5 and CCL20 (184). MAIT cell migration towards CCL20 has also been shown by Won *et al*. though CXCL16 was seemed to have a stronger force of attraction on MAIT cells (183). We did not see any differences between PB and IVB regarding the levels of CXCL16 (Figure 28), and no correlation was seen between levels of CXCL16 and MAIT cell proportions in IVB (data not shown). CCL5 was unfortunately not part of the multiplex kit used to analyze our plasma samples.

Correlations between MIF or CCL25 and the proportions of MAIT cells have not been investigated by others. No associations were seen between MIF or CCL25 and PB MAIT cell proportions, while weak correlations were seen for IVB. This indicates that PB could be a sort of neutral ground from a chemotactic point of view, from which cells are recruited to organs with higher expression of chemokines as they passively pass through via the blood stream. It is intriguing that levels of chemotactic molecules in IVB could influence the immune cell composition in DP. It is not clear when and from where the maternal DP immune cells enter the tissue, and our findings indicate a connection between the maternal arterial blood supply of the placenta, and the cellular contents of the decidual membranes.

CXCR3-CXCL9, -10 and -11 interactions are a known pathway for orchestrating a Th1 response and for attracting $CD8^+$ effector memory T cells (13). Hence, the correlations are not surprising (Figure 29), but implicates that the cells in the placenta utilizes different factors of attraction to shape the local immunological environment.

In conclusion, we showed that IVB contains an altered chemokine and cytokine profile compared to PB. It seems as if the villous tissue is at least in part responsible for this difference. Soluble factors produced by the villous tissue selectively attracts MAIT cells and $CD8^+$ effector memory cells, two cell types that were previously found to be enriched in IVB. Proportions of $CD8^+$ effector memory cells correlated with the ligands for CXCR3 in IVB. MAIT cells were associated with MIF and CCL25. By blocking MIF, CCL20 and CCL25 in the placental explant culture supernatants, the attraction of MAIT cells was significantly decreased, while the $CD8^+$ effector memory cells were unaffected. Recombinant MIF attracted MAIT cells but not $CD8^+$ effector memory cells. Almost all MAIT cells expressed the MIF receptor CXCR4, and the $CD8^+$ effector memory cells that had migrated towards MIF had an increased expression of the receptor. Together, this points to the placenta shaping its own local immunological environment by chemokine

secretion. Immune cells that passively enter the intervillous space with the arterial blood are then kept in the IVB, thus gradually forming a local composition of T cells that can rapidly respond to threatening pathogens.

4.5 B cells in placental tissues

4.5.1 Presence and phenotype

4.5.1.1 IVB

In **paper IV**, we investigated B cells in paired samples of IVB and PB. Using OPLS discriminant analysis based on the frequency of B cells out of $CD45^{+}$ cells and 8 other B cell variables measured with flow cytometry, a clear separation between PB and IVB could be seen (**paper IV**). IVB contained more B cells, and the IVB B cells were to a larger degree of a mature/naïve phenotype ($CD24^{int}CD38^{int}$). The IVB B cells also had a higher expression of the transferrin receptor CD71. The PB predominately contained B cells with a memory ($CD24^{high}CD38^{lo/neg}$) or a transitional/immature ($CD24^{high}CD38^{high}$) phenotype, as well as a higher expression of the co-inhibitory marker LAG-3 (**paper IV**) (Figure 30).

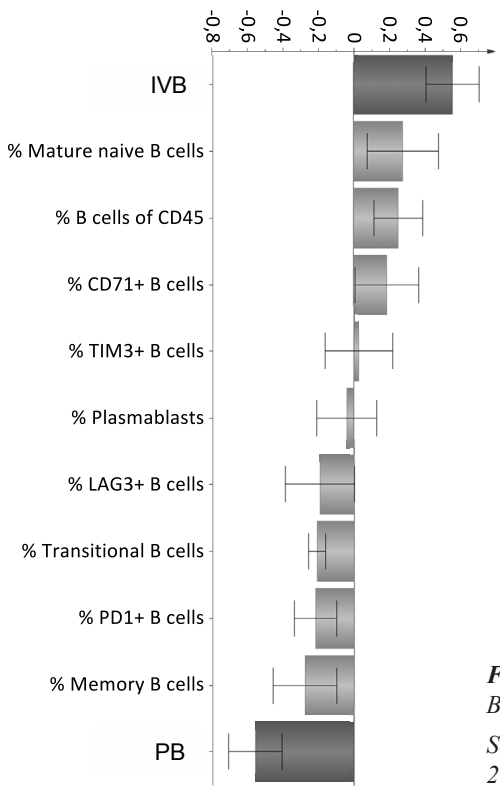

Figure 30. *OPLS-DA loading plot showing B cell variables associated with PB or IVB.*

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Pregnancy-associated B cell lymphopenia in PB is a known phenomenon in both human (185, 186) and mice (187, 188). The studies in mice have shown that B cell precursors in the bone marrow are diminished, and that this in part is due to a negative effect by the hormone estradiol (189). Whether this effect of a lower production of new B cells is sufficient to explain the peripheral B cell lymphopenia, or if the existing B cells also migrate away from PB is unknown. Othoro *et al*. also found the frequency of B cells to be increased in IVB when using the method of pricking the placenta (159), whereas Moore *et al*. and Vega-Sanchez *et al*. both saw a small but non-significant increase in B cells in IVB (121, 122). The function of mature/naïve B cells in IVB can only be speculated upon. Mature/naïve B cells are ready to be activated by antigen, either by the help of T cells in secondary lymphoid organs, or independently from T cells by TLRs or complement receptors together with the B cell receptor, or by cross-linking of the B cell receptor. This could be a function for the mature/naïve B cells in IVB, to be rapid responders to pathogens, independently of help from T cell. Proportions of IVB B cells in different maturation stages have not been investigated previously, but Muzzio *et al*. found that mature B cells were accumulated in uterus-draining lymph nodes in mice (188). This could be an indication supporting our findings of an increased frequency of mature/naïve B cells in IVB (**paper IV**). Maybe mature/naïve B cells accumulate in IVB in order to be rapidly deployed to nearby secondary lymphoid organs in response to a local infection.

4.5.1.2 Decidua

When comparing B cells in DP and DB, we found that DB contained more B cells (**paper II**). Similar to IVB, the DP B cells had more of a mature/naïve phenotype, and the DB contained more transitional/immature B cells. The DP B cells had a higher expression of the co-inhibitory markers PD-1, TIM-3 and CTLA-4 compared to DB (**paper II**).

In a small cohort, Lundell *et al*. showed results similar to ours regarding the number of B cells being higher in DB compared to DP (190). Rieger *et al*. examined B cell frequencies in DB in placentas from pregnancies with a median gestational age of 34 weeks. They found B cells to comprise about 2.5 % of the total $CD45^+$ cell population (191), almost the exact same number that we found in our DB samples (**paper II**). Xu *et al*. also demonstrated the presence of B cells in both DB and DP, but the way the data is presented makes it hard to compare their results with our (192). Leng *et al*. also found similar results to ours, with more B cells in DB compared to DP, and that the B cells in DB were more of a transitional/immature phenotype (193). They also found a large proportion of plasmablasts in DP, which we did not see. Apart from this, comparisons were hard to make due to differences in gating strategies. The expression of various co-inhibitory markers on decidual B cells in DP is intriguing. A direct effect of co-inhibitory expression by B cells

has not been established, although some strong indications exists that PD-1 plays a role in B cell impairment in the context of HIV infection (194). It could also be that the decidual environment has a strong effect of inducing co-inhibitory marker expression aimed at T cells, but that the signal overlap to the B cells. If the expression of co-inhibitory markers on decidual B cells has a function, or if it is merely a bi-product of their environment remains to be determined.

4.5.2 Chemokine correlations and migration

Using the same data on soluble factors in IVB and PB as in **paper III** and plotting this and the B cell maturation frequencies in a principal component analysis plot, it was clear that the three B cell maturation subtypes associated with different chemokines (**paper IV**) (Figure 31). Using OPLS analysis based on the previous principal component analysis, we saw that high levels of mature/naïve B cells in IVB were associated with certain chemokines in IVB (**paper IV**). With univariate statistics, we could then see that the levels of the chemokines CXCL8, CXCL11 and CCL20 in IVB had a positive correlation with the frequency of mature/naïve B cells in IVB. Comparing the levels of theses chemokines in PB and IVB, we found that IVB contained significantly higher levels of CXCL8 and CCL20 (**paper IV**).

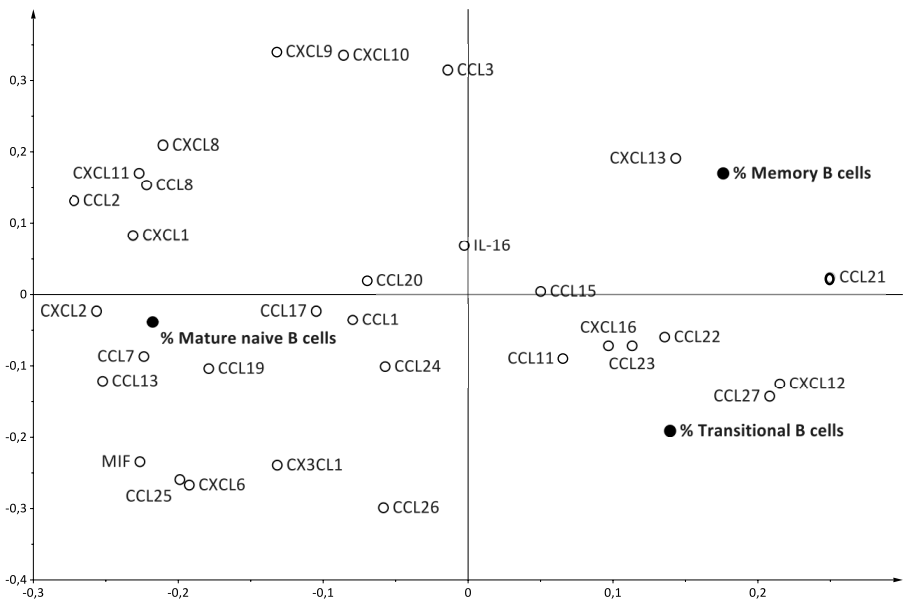

Figure 31. *Principal component analysis plot showing associations between the maturation status of B cells and soluble factors, both in IVB.*

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The expression of the chemokine receptors for CXCL8 (CXCR1), CXCL11 (CXCR3) and CCL20 (CCR6) was then measured on B cells from healthy donors. All B cells expressed CCR6, but the MFI was significantly higher on mature/naïve than on transitional/immature B cells (**paper IV**). No B cells expressed CXCR1, and expression of CXCR3 was most common on memory B cells, although among the $CXCR3^+$ B cells, the mature/naïve had a significantly higher CXCR3 MFI compared to transitional/immature B cells (**paper IV**).

Using the same assay for immune cell migration towards placental explant culture supernatants as in **paper III**, we could see that B cells were attracted by the placental tissue conditioned medium. The total number of B cells increased, as well as the relative proportion out of $CD45^+$ cells when comparing to the medium control (**paper IV**) (Figure 32).

Gomez-Lopez *et al*. has shown in two publications that explants from the joined fetal membranes amnion, chorion and decidua secreted soluble factors that attracted immune cells, including B cells (195, 196). They also showed that fetal membrane extracts from patients who had experienced labor were more potent in terms of immune cell attraction (195).

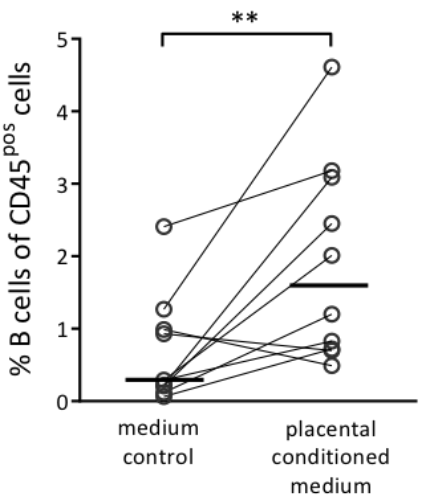

Figure 32. *Migration of B cells towards placental explant culture supernatants. ** $p < 0.01$. $n = 10$.*

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Concluding the results of **paper IV**, we showed that IVB contained a higher proportion of B cells than PB. The IVB B cells were skewed towards a mature/naïve phenotype, and this subtype correlated with levels of CCL20 in paired IVB samples. B cells had a ubiquitous expression of the CCL20 receptor CCR6, but mature/naïve B cells expressed a higher number of CCR6 molecules compared to transitional/immature B cells. Lastly, we saw that B cells were enriched after

migrating towards placental explant culture supernatants (Figure 32). Thus, it seems probable that the placenta attracts B cells, especially mature/naïve ones, by the local secretion of CCL20. Indeed, we did see in **paper III** that CCL20 was higher in IVB compared to PB, and that CCL20 was secreted in high levels from placental tissue. Thus, the pregnancy-associated B cell lymphopenia might not only be due to fewer cells released from the bone marrow, but could also be due to a selective migration from PB to IVB.

4.6 MAIT cell reconstitution following HCT

In the artificially created situation of HCT where an immune system is transplanted into a non-self host, balancing the immune system's reactivity to non-self signals from the host defines the patient outcome (137). With drugs that dampen the activation threshold of T cells, the aim is for the transplanted immune system to be triggered by the non-self malignant cells and pathogens, while left dormant to the signals from the healthy cells of the host. In these conditions the transplanted immune system needs to engraft and start to multiply to fill the patient with immune cells of such a quantity, diversity and quality that it meets the needs of the host. This reconstitution of the transplanted immune system is of utmost importance for the patient outcome. The reconstitution of MAIT cells following HCT, as well as their influence on immunity, GvHD, relapse and other aspects of HCT was completely unknown before the start of the project behind **paper V**.

Reconstitution of T cells in general and MAIT cells in particular was examined in 17 adult patients who had undergone HCT. We selected the patients based on them not having more than grade I GvHD, as GvHD is a known factor of impaired immune reconstitution following HCT. The study population was diverse in terms of diagnoses, age, donor type, GvHD prophylaxis and conditioning regimen. Patients were also selected based on sample availability, as we wanted to follow the reconstitution longitudinally in paired samples because the inter-individual spread of the data was expected to be large. Thus, we also indirectly selected patients that had survived for more than 2 years after the treatment. By this way of patient selection, we argued that our data would represent a broad type of best-case scenario of MAIT cell reconstitution following HCT (**paper V**).

4.6.1 T cell reconstitution

The non-MAIT T cells increased in total number during the first year after HCT, and then seemed to stabilize at that level (Figure 33). We did not observe any differences in non-MAIT T cell reconstitution when comparing patients depending on age, use of anti-thymocyte globulin, MAC or RIC conditioning or sex (**paper V**). The CD3 donor chimerism gradually increased, and at 12 months post HCT, all patients were full donor chimerism (Figure 33). As shown by many others, we

also observed an inverted ratio of CD4/CD8 expression on non-MAIT T cells. There was a trend towards an increase in $CD4^+$ non-MAIT T cells at the expense of $CD4^-CD8^-$ cells in the 24 months samples, but the inverted ratio remained (**paper V**). The non-MAIT T cells had a slight increase in CD69 expression during the first months post HCT. A median of 60% of the non-MAIT T cells expressed PD-1 during the first 2 months post HCT, this expression then gradually declined to normal levels after the first 12 months (**paper V**) (Figure 33).

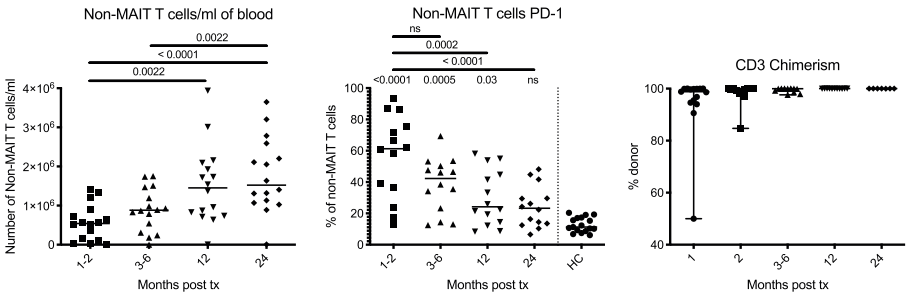

Figure 33. *Reconstitution of non-MAIT T cells/ml of blood (left, n = 16). Expression of PD-1 on non-MAIT T cells during the first 24 months following HCT, and compared to healthy controls (HC) (center, n = 14). CD3 chimerism in PB following HCT, expressed as % of donor DNA (right, n = 15, 12, 14, 11 and 7 for the 5 time points indicated). The Friedman test followed by Dunn's post-test was used to compare the four time points after HCT. For comparisons with the healthy controls, the Kruskal–Wallis test followed by Dunn's post-test was used (indicated by numbers above the datapoints).*

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Our results regarding non-MAIT T cells mostly confirm previous findings regarding timing of reconstitution and phenotypical changes. However, to our knowledge, the expression of PD-1 had not been investigated previously in this setting. Interestingly, there was a high expression of PD-1 during the first months after transplantation, followed by a gradual decrease towards levels seen in healthy blood donors. As the use of check-point inhibitors have been increasing for patients with hematological malignancies, many studies have shown an increased risk of acute GvHD for HCT patients with prior check-point inhibitor treatment (197, 198), and a clear correlation has been demonstrated in mice (199). The reconstitution of T cells was also affected, as PD-1 expression was significantly lower in patients with check-point inhibitor treatment prior to transplantation (197). As our patients had grade 0-1 GvHD, it could be speculated that low PD-1 expression on T cells after HCT could serve as a predictive biomarker for more severe GvHD. The reasons for the increased PD-1 expression shortly after HCT is unknown. It could be that these T cells are mostly transferred along with the graft, and then subject to increased levels of IL-7 and IL-15 after HCT (147), cytokines known

to increase T cells expression of PD-1 (200). When analyzing our experiments with immunosuppressive drugs, we could see that by addition of CsA, T cells had a lower PD-1 expression than the positive control (data not shown). This is also supported by Grant *et al*. who got similar results for a variety of immunosuppressive drugs (201). Thus, the increase in PD-1 expression on T cells seen in spite of treatment with immunosuppression, can probably be explained by proliferation due to cytokine stimulus, and could potentially serve as a biomarker for absence of severe GvHD.

4.6.2 MAIT cell reconstitution

MAIT cells measured as percentage of $CD3^+$ T cells were substantially lower in HCT patients compared to healthy blood donors. No increase was seen during the 24 months observation period. When estimating the number of MAIT cells per ml of blood, a significant increase was seen at 24 months post HCT compared to the first six months (**paper V**) (Figure 34).

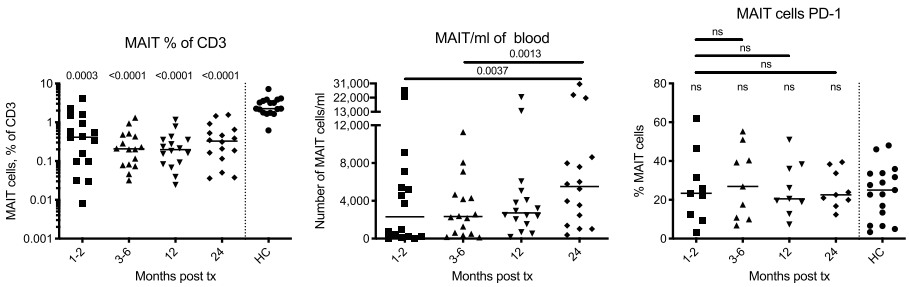

Figure 34. *Reconstitution of MAIT cells (n = 16) as % of CD3+ T cells, and compared to healthy controls (HC, n = 17) (left). Reconstitution of MAIT cells measured as cells/ml of blood (center, n = 16). Expression of PD-1 on MAIT cells (n = 9) during the first 24 months following HCT, and compared to healthy controls (HC, n = 17) (right). The Friedman test followed by Dunn's post-test was used to compare the four time points after HCT. For comparisons with the healthy controls, the Kruskal–Wallis test followed by Dunn's post-test was used (indicated by numbers above the data points).*

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Bhattacharyya *et al*. evaluated MAIT cell reconstitution at multiple time points after HCT. They saw an early rise in MAIT cell numbers during the first month after HCT, after which a plateau phase was reached that lasted throughout the 12 months observation period. Interestingly, they also measured a transient increase in KI-67 expression in MAIT cell during this time, indicative of MAIT cell proliferation (202). Kawaguchi *et al*. only measured MAIT cell reconstitution for 2 months post transplantation, but did not see any increase in MAIT cell proportions out of $CD3^+$ T cells during this period (203). Konuma *et al*. investigated MAIT

cells in a cross-sectional cohort of patients, some who were up to 30 years after transplantation. With time, the total number of MAIT cells in PB increased, but the proportion out of total T cells was lower for all patients, regardless of whether they were transplanted with grafts from siblings, matched unrelated donors, or cord blood (204). Thus, although there is not yet another publication following the same patients longitudinally for more than one year, the published literature supports our findings of a very slow MAIT cell reconstitution following HCT.

4.6.2.1 Factors influencing reconstitution of MAIT cells

We saw that patients who had not received anti-thymocyte globulin, and the ones who had received a MAC conditioning had significantly higher MAIT cells 24 months after the transplantation, both measured as percentage of T cells, and as number of cells per ml of blood.

Bhattacharyya *et al*. did not see any difference between MAC and RIC conditioning, and they did not include anti-thymocyte globulin in the protocol for any of the patients (202). That the use of anti-thymocyte globulin impaired MAIT cell reconstitution was not surprising, as this has a broad depleting effect on T cells. Bhattacharyya *et al*. found that patients who received post-transplant Cyclophosphamide, which is also cytotoxic for T cells, had a further impairment of their MAIT cell reconstitution (202). It thus seems as if MAIT cells are more sensitive to certain cytotoxic drugs than other T cells.

4.6.2.2 Phenotypic changes during reconstitution

MAIT cells are normally mostly $CD8^+$, with some being $CD4^-CD8^-$ and a small subset being $CD4^+$ (30). In our material, most MAIT cells had a $CD4^-CD8^-$ phenotype, which seemed to be gradually replaced by $CD8^+$ cells (**paper V**). MAIT cells had an increased expression of CD69 early after HCT, which then decreased at 24 months towards levels similar to what was seen in healthy blood donors. PD-1 expression on MAIT cells was similar to that of healthy blood donors all throughout the observation period (**paper V**) (Figure 34).

Bhattacharyya *et al*. also observed an initial increased level of CD69 expression on MAIT cells followed by a decrease towards normal levels. We both speculated that this is due to increased cytokine stimulation during the early months post HCT (202). MAIT cell CD4/CD8 phenotype in the context of immune reconstitution post HCT has not been studied by others. Dias *et al*. showed that $CD4^-CD8^-$ MAIT cells had lower expression of cytokine receptors and a weaker response to bacterial stimuli. They also showed that by exhausting $CD8^+$ MAIT cells, they converted to the $CD4^-CD8^-$ phenotype (205). In light of this, our findings could be interpreted as the MAIT cell reconstitution is characterized by early activation and exhaustion, resulting in MAIT cells with the less functional $CD4^-CD8^-$ phenotype.

4.6.2.3 MAIT cell functions following HCT

Using the same bacterial stimulation assay for MAIT cell activation as in **paper I**, we compared the response of MAIT cells in samples 2-6 months after HCT with samples taken 24 months after HCT. These samples were also compared to the reactivity of MAIT cells from healthy blood donors. Looking at the early samples from the HCT patients, the MAIT cells did not respond with an increase in IFN-γ. The samples taken 24 months after transplantation had an IFN-γ response similar to that seen in healthy controls. These results were shown both with intracellular flow cytometry, and by measuring soluble IFN-γ in the supernatants with ELISA (**paper V**) (Figure 35).

The MAIT cells from the HCT patients had an increased background expression of GrzB compared to healthy controls, and cells from both time points did significantly increase their expression. The background expression of perforin was normal in the 24-month samples, but significantly lower in the samples from the earlier time points. The increase in MFI was also higher in the 24 months samples compare to the 2-6 months samples (**paper V**).

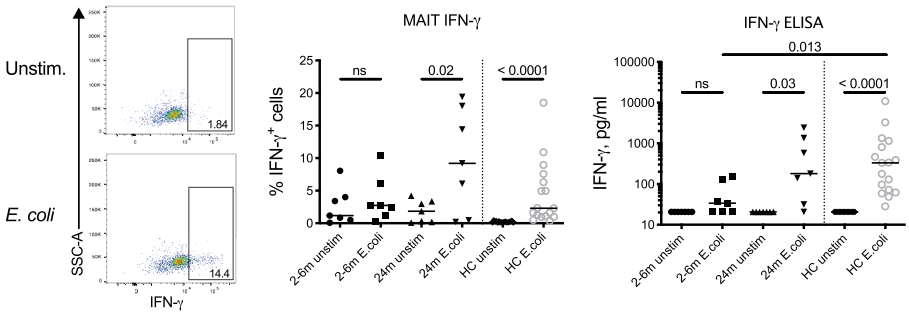

Figure 35. *Representative plot showing MAIT cell IFN-γ response to bacterial stimulation and in unstimulated conditions (left). Intracellular staining of MAIT IFN-γ response to bacterial stimulation early and late after HCT (n = 7), and compared to healthy controls (HC, n = 17) (center). Soluble IFN-γ in supernatants from the same experiments (right). Wilcoxon's test was used to compare paired unstimulated and stimulated samples, as well as between different timepoints. Comparisons with healthy controls were done using the Kruskal–Wallis test followed by Dunn's post-test, numbers indicate p value.*

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No other publication has investigated MAIT cell functionality at different time points of reconstitution after HCT. One of the reasons behind the impaired functional response of MAIT cells from the first 6 months following HCT could be their increased $CD4^{-}CD8^{-}$ phenotype, as $CD8^{+}$ MAIT cells have been shown to have a more potent response to stimuli (72, 205). Unfortunately, the MAIT cells were too few to analyze different response rates between $CD8^{+}$ and $CD4^{-}CD8^{-}$ MAIT cells

(**paper V**). The increased background expression of GrzB is intriguing, and has not yet been investigated by others. One explanation could be that high levels of circulating IL-7 early after HCT (147), as IL-7 alone has been shown to increase GrzB expression in MAIT cells (51). However, the supra-normal levels of IL-7 are not thought to last for years after transplantation, and we thus cannot explain the increased GrzB expression throughout the whole observation period. It is interesting to note that MAIT cells did seem to be reconstituted from a functional standpoint at 2 years after transplantation. This demonstrates that that in spite of the low reconstitution rate, the MAIT cells in HCT patients seem to go through an, albeit very slow, normalization process during the reconstitution following HCT.

4.6.3 Associations with GvHD

We also compared our data with data from another cohort of patients who suffered from GvHD grade 2-3. Samples from these patients were taken early after diagnosis, at a median of 3.3 months after HCT. We thus compared these data with the 3-month samples from our cohort of patients with non-mild GvHD. No difference was seen when comparing the proportion of MAIT cells out of total $CD3^+$ cells (**paper V**) (Figure 36). The patients with GvHD grade 2-3 had fewer T cells in general, both MAIT cells and non-MAIT T cells (Figure 36). The one difference that we observed was that the MAIT cells from the GvHD grade 2-3 were dominated by the $CD8^+$ phenotype, whereas the GvHD grade 0-1 patients mostly had the beforementioned $CD4^-CD8^-$ phenotype (**paper V**) (Figure 36).

Lower number, and lower frequencies of MAIT cells have been correlated to a large number of both bacterial and viral infections, as well as in a range of autoimmune disorders (see introduction 1.1.5.6). In HCT at the time when patients are at the highest risk of acute GvHD, classically said to be during the first 100 days after transplantation, their T cells are just beginning to reconstitute. As acute GvHD is known to negatively impact the reconstitution of T cells in general (206, 207), it is important to know whether a measured decrease in total MAIT cells is a MAIT cell specific event, or if it merely reflects a decreased thymic output due to the GvHD, affecting all T cells. This is a limitation in the two other studies I have found investigating MAIT cells in the context of GvHD. Bhattacharyya *et al*. found a non-significant drop in total number of MAIT cells at 1-month post transplantation, but did not give any data on how the non-MAIT T cells were affected (202). Kawaguchi *et al*. did find that patients with GvHD had lower frequencies of MAIT cells at day 90 after transplantation, which is interesting from a mechanistic point of view (203). However, they included all grades of acute GvHD when comparing to non-GvHD patients, and as grade I is desired from a relapse standpoint, the clinical relevance is questionable. When dividing the patients according to MAIT cell reconstitution, the half with the lowest numbers had significantly higher incidence of both total, and severe acute GvHD. Again

though, this was only shown for total number of MAIT cells, not proportion, and indeed, they do show a generally poorer reconstitution for total T cells as well in patients with acute GvHD (203). Judging by the available data, severe acute GvHD is associated with lower amounts of T cells, including the MAIT cell subset. If MAIT cells are more susceptible to the negative impact of the GvHD process, or if they are even a part of the pathological immune response remains to be shown.

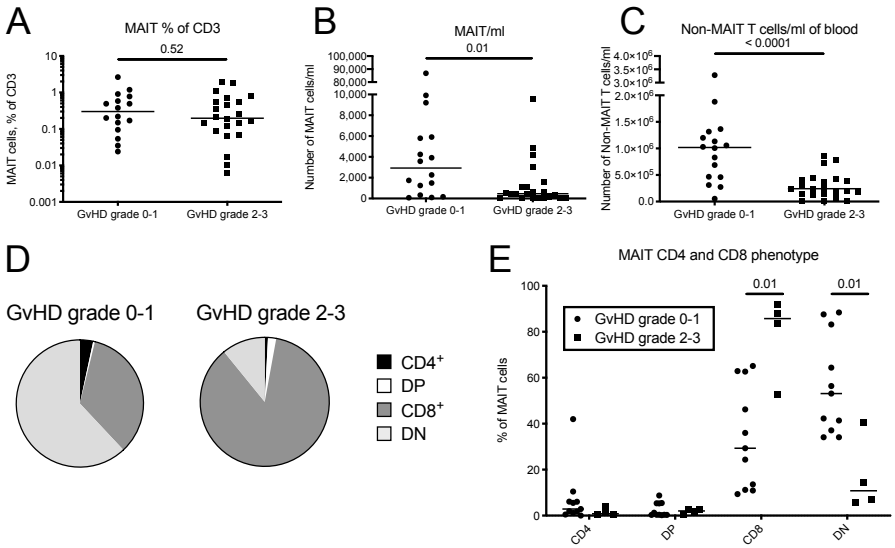

Figure 36. *(**A**) MAIT cells expressed as percentage of CD3$^+$ T cells did not differ with grade of GvHD. (**B**) The number of MAIT cells/ml of blood did differ with grade of GvHD, but so did the number of non-MAIT T cells (**C**). Grade 0-1 GvHD n = 16, and grade 2–3 GvHD n = 22 in **A-C**. **(D-E)** MAIT cells from patients with grade 0-1 GvHD (n = 11) were of a more CD4$^-$CD8$^-$ phenotype compared to grade 2-3 GvHD (n = 4), where the MAIT cells were more dominated by a CD8$^+$ phenotype (D-E). Horizontal lines in dot plots indicate the median value. Comparisons were made using the Mann–Whitney test, numbers indicate p value.*

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4.6.4 The effect of immunosuppressive drugs on MAIT and non-MAIT T cell proliferation

Lastly, we investigated the effect of two common immunosuppressive drugs on MAIT cell proliferation capabilities. Cells from healthy donors were labeled with CFSE and stimulated with anti-CD3 antibodies, and different concentrations of CsA and SIR were added to the cultures. As expected, both CsA and SIR decreased the proliferation of all T cells (**paper V**). However, MAIT cells were more sensitive to the immunosuppressive drugs, as their proliferation was decreased more compared to the non-MAIT CD8$^+$ T cells, both by CsA and SIR (Figure 37).

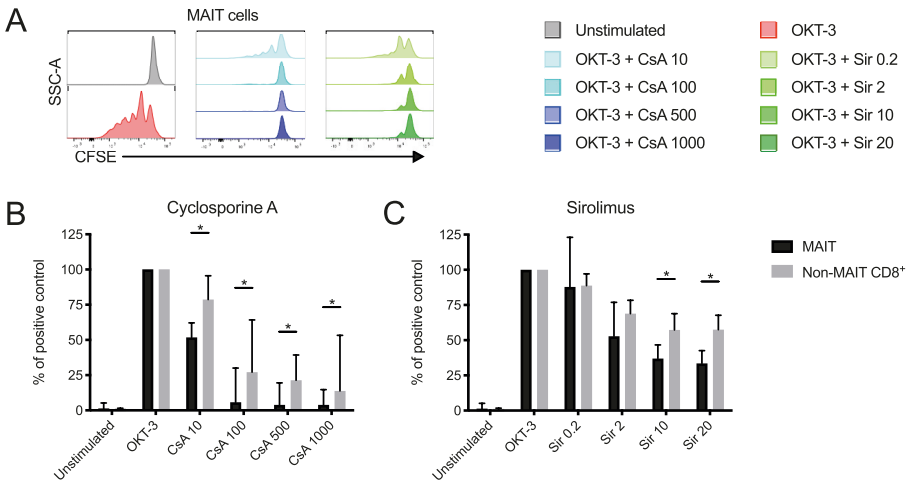

Figure 37. *(**A**) Representative histograms of MAIT cells unstimulated, stimulated with anti-CD3 antibodies or anti-CD3 antibodies in combinations with various concentrations of the immunosuppressive drugs CsA or SIR. After five days of culture, CFSE expression of cells was analyzed with flow cytometry. Data is shown for CsA (**B**) (n = 7) and SIR (**C**) (n = 8), each comparing MAIT cells with non-MAIT CD8$^+$ T cells. Bars end at the median, and error bars show the upper range of the data. Cell subsets were compared using the Wilcoxon test. * p < 0.05.*

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Van der Waart *et al*. showed that the ABCB1 multi-drug transporter protein was enriched in the CD8$^+$CD161^{high} population (208). It has since been confirmed that this population was likely MAIT cells, as virtually all MAIT cells do express ABCB1 (28, 209). The ABCB1 is able to efflux certain chemotherapeutic drugs, and CsA is both a substrate, and an inhibitor of this transporter protein (210). As the common immunosuppressants used in HCT are substrates for the ABCB1 transporter protein, the effect on MAIT cell reconstitution is of interest (210). Van der Waart *et al*. found that their CD8$^+$CD161^{high} population was less susceptible to CsA suppression compared to other T cells (208). Our findings contrast to theirs, as we saw that the proliferation of MAIT cells were more hampered by both CsA and SIR than non-MAIT CD8$^+$ T cells (**paper V**). We speculated that our different findings are due to differences in the assays. They used allogenic PBMCs, IL-2, IL-7, and IL-15 to stimulate their cells. Thus the blocking effect of CsA was exerted on the allogenic stimulus and IL-2, whereas the IL-7 and IL-15 pathways remained unblocked in their assay. We only used anti-CD3, and thus the stimulation was more directly targeted by the immunosuppressive drugs. The blocking effect on the ABCB1 transporter by CsA showed by others was only shown for levels of the drug about 10 times higher than what is used in patients (209), or nor stated at

all (28), and the biological relevance of this effect can thus be questioned. From our *in vitro* data, it seems as if the ABCB1 protein does not protect MAIT cells from the anti-proliferative effects of CsA, which is also supported by our observed overall hampered MAIT cell reconstitution following HCT.

In conclusion, we demonstrated an impaired reconstitution of MAIT cells following HCT. MAIT cells were not reconstituted to normal levels even two years after the transplantation. The functionality of MAIT cells was impaired during the first six months, but seemed to be restored at 24 months. We did not observe any correlation between MAIT cell numbers and GvHD, although MAIT cells in GvHD patients had a CD4/CD8 phenotype more reminiscent of that in healthy donors. MAIT cells were found to be more sensitive to immunosuppressive drugs commonly used in HCT, which could in part be an explanation to the observed slow reconstitution.

5 CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

The work included in this thesis includes the most thorough characterization of immune cells in general, and MAIT cells in particular, in the IVB of placentas following uncomplicated term pregnancies to date. We found that:

- IVB was enriched in MAIT cells, B cells, T cells with an effector memory phenotype and both $CD56^{dim}$ and $CD56^{high}$ NK cells.
- IVB contained increased levels of many cytokines and chemokines compared to PB, and lower levels of others. The cytokines and chemokines secreted by placental tissue closely resembled the IVB plasma secretome.
- IVB had MAIT cells that were in a resting state, and had a lower expression of PD-1.
- When stimulating IVB mononuclear cells with bacteria, MAIT cells from these cultures had a stronger response compared to PB cell cultures.
- MAIT cells were selectively recruited by soluble factors secreted by placental tissue, partly by interactions between secreted MIF and CXCR4 expressed on MAIT cells. $CD8^{+}$ effector memory T cells was not recruited by MIF, but probably rather by CXCR3 interacting with its ligands CXCL9, -10 and -11.
- B cells were selectively recruited by soluble factors secreted by placental tissue. B cells with a mature/naïve phenotype was enriched, probably by interactions between CCR6 on mature/naïve B cells and the ligand CCL20.
- Altogether, this indicates that the placenta creates a specialized immunological environment in IVB by secretion of signal molecules.

Apart from IVB, we also found that in decidual tissues:

- DP contained more T cells and $CD56^{high}$ NK cells, whereas DB was enriched in MAIT cells, B cells, monocytes and $CD56^{dim}$ NK cells.
- Immune cells in DP had a higher expression of various co-inhibitory markers.
- MAIT cells, T cells and NK cells from both DP and DB were functional when stimulated with bacteria or PMA/Ionomycin.

Finally, MAIT cells were analyzed in samples following HCT, and we observed that:

- MAIT cell reconstitution differed from that of non-MAIT T cells. MAIT cells did not start to increase in number until after the first year following HCT.

- MAIT cells had an impaired response to bacterial stimulation of mononuclear cell cultures during the first six months after HCT, this was normalized at 24 months.
- MAIT cells proliferating in response to anti-CD3 stimulation were more sensitive to common immunosuppressive drugs in clinically relevant concentrations.

5.2 Future work

This thesis lays a foundation of IVB as an interesting site of immunological processes during pregnancy. We have only studied placentas delivered by caesarian section following term uncomplicated pregnancies. Albeit interesting on its own, the goal has always been to move on and study IVB from pregnancies complicated by pathologies such as pre-eclampsia, intra-uterine growth restriction and intra-uterine infections. The logical next step is thus to collect IVB from such complicated pregnancies, and try to discern any differences when compared to the data on healthy pregnancies presented in this thesis. If a clear immunological dysfunction can be proven to be pathognomonic for a pregnancy complication such as pre-eclampsia, there is a good chance that with the wide range of immunomodulatory drugs available in modern medicine, one could find a suitable intervention with which to proceed to a clinical trial.

The development of the human placenta from an immunological perspective is still poorly understood. We have only studied IVB at term pregnancy. As we have showed that the placental tissue secretes factors that influence the local immune cell composition, it would be of great interest to study if and how these changes over the course of pregnancy. Is there a clear goal for the composition and function of immune cells in IVB from the beginning of pregnancy, or does it change along with the developing placenta and fetus? It would thus be of great interest to study IVB from placentas from earlier stages of pregnancy.

The enrichment of effector memory T cells and mature/naïve B cells in IVB and decidua is intriguing, and with the focus on MAIT cells in this thesis, these findings were a bit overlooked. It would be very interesting to examine the specificities of the effector memory T cells in IVB and decidua. Are these T cells specific for viral antigens? Or are they reactive against fetal antigens, but cannot be triggered by the STBs in contact with the IVB? Are the co-inhibitory signals in decidua strong enough to dampen any T cell response, or is it merely a higher threshold of activation? It would also be interesting to further investigate the mature/naïve B cells in IVB. Are they potential T cell-independent producers of antibodies? Or are they waiting in close proximity to local secondary lymphoid tissue?

The finding that non-pregnant women had a slightly higher proportion of MAIT cells in PB compared to pregnant women was an intriguing piece of the puzzle of potential MAIT cell migration to IVB. In order to further study this, another study would have to be performed. Women planning a pregnancy would have to be included before conception, and then followed longitudinally with blood samples throughout the pregnancy. The placenta would then be collected after birth, and the women followed continuously with blood samples for at least a year after birth. With such data, we could more closely investigate correlations between increasing or decreasing immune cell frequencies in PB over the course of the pregnancy, and the contents in IVB. If such a study was large enough, some participants would develop pregnancy complications, and it would be of great interest to study their PB immune cells prior to the disease development, as well as to study the placenta after delivery.

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