Immune regulation at the foetal-maternal interface; implications for healthy and complicated pregnancies

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**Immune regulation at the foetal-maternal interface; implications for healthy
and complicated pregnancies**

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“And so, does the destination matter? Or is it the path we take? I declare that no accomplishment has substance nearly as great as the road used to achieve it. We are not creatures of destinations. It is the journey that shapes us. Our callused feet, our backs strong from carrying the weight of our travels, our eyes open with the fresh delight of experiences lived.”
— **Brandon Sanderson, *The Way of Kings***

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Abstract

For a successful pregnancy, the maternal immune system must acquire tolerance towards the paternal antigens present in the semi-allogeneic foetus. This tolerance is mainly established locally at the foetal-maternal interface, where foetally-derived trophoblasts invade the maternal endometrium (called decidua during pregnancy) and come in close proximity to maternal immune cells. The decidua is populated by maternal immune cells of a unique composition, characterised by their suppressive phenotypes that are essential for maintaining tissue homeostasis. Accordingly, failure of immune tolerance can lead to pregnancy complications. Macrophages and regulatory T-cells are enriched in the decidua and are believed to play important roles in the establishment of tolerance. However, there is limited information regarding the factors that regulate their functions and if their function is compromised in pregnancy complications.

The aim of this thesis was to further elucidate which factors are responsible for induction of the regulatory phenotypes of macrophages and T-cells found in the decidua, how tissue resident cells in the decidua contribute to this and if this system is compromised during pregnancy complications, such as preeclampsia and recurrent pregnancy loss.

Decidual stromal cells (DSCs) constitute the largest population of tissue resident cells in the decidua. In an *in vitro* system of macrophage differentiation, we found that Isolated peripheral blood monocytes cultured in conditioned medium (CM) from DSCs acquired a high expression of the regulatory M2 markers CD163, CD209 and CD14, and a low expression of CD86, characteristics of decidual macrophages. This induction was in part mediated by macrophage-colony stimulating factor (M-CSF), as neutralising its effects reduced the expression of CD163. However, since only a partial reduction was reached, other factors are involved. Another likely candidate for this polarisation is interleukin (IL)-34, a second ligand for the M-CSF receptor. We showed that IL-34 is expressed by both DSCs and the foetal placenta. Further, *in vitro*, IL-34 was able to induce macrophages with similar properties as that of M-CSF-induced macrophages, with high expression of CD163, CD209 and CD14. This was also coupled to a cytokine secretion profile similar to M-CSF-induced macrophages, with high production of IL-10, low production of tumour necrosis factor (TNF) and no production of IL-12. We found no evidence of IL-34 being aberrantly expressed in placentas from preeclamptic women.

In addition to promoting induction of macrophages with a regulatory phenotype, CM from DSCs promoted expansion of Foxp3+CD25^{bright} regulatory T (Treg) cells in an *in vitro* polarisation system, in a SMAD3 dependent manner. Protein profiling of DSCs revealed limited production of the Th2 related IL-13, IL-4, IL-33 and thymic stromal lymphopoietin (TSLP), as well as no production of the Th17 related IL-17A and chemokine (C-C motif) ligand (CCL) 20. Instead we found that DSCs were more prone to production of regulatory factors, such as M-CSF, leukaemia inhibitory factor (LIF) and transforming growth factor (TGF)- β , albeit with addition of the more pro-inflammatory IL-6, chemokine (C-X-C motif) ligand (CXCL) 8 and the Th1-related CXCL10.

We also investigated if the placenta's ability to induce Treg cells and regulatory M2 macrophages is compromised in women with a history of unexplained recurrent pregnancy loss (uRPL) and if the placental secreted protein profile is skewed to a pro-inflammatory response in uRPL. Using surplus materials from chorionic villous sampling (CVS), we generated CM from placental tissue taken from healthy and uRPL pregnancies and used this to polarise macrophages and T-cells *in vitro*. We found no difference in the ability to induce Treg cells and regulatory M2 macrophages between the healthy group and the uRPL group. Likewise, no differences in the protein profile was observed between the two groups.

Taken together, our findings imply that DSCs produce a variety of factors promoting foetal tolerance by induction of Treg cells and regulatory M2 macrophages. Furthermore, we also showed that the placenta retained its ability to induce Treg cells and regulatory M2 macrophages in women with a history of uRPL.

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Sammanfattning

Graviditet utgör en stor utmaning för mammans immunförsvar eftersom fostret till hälften består av gener från pappan, vilket innebär att fostret bär på gener som är främmande för mammans immunförsvar. Detta betyder att mamman måste utveckla en tolerans för att förhindra en avstöttningsreaktion, men samtidigt också bevara skyddet mot potentiellt farliga infektioner. Tolerans skapas främst lokalt i kontakten mellan livmoderslemhinnan (kallad decidua under graviditet) och placentan (moderkakan), där placentalceller från fostret kommer i nära kontakt med mammans immunceller. Således har dessa immunceller en central roll i att skapa tolerans. Två celltyper som är anrikade i deciduan under en graviditet är specialiserade makrofager och regulatoriska T-celler. Dock är det inte fastställt hur dessa celler får sina immundämpande egenskaper. I denna avhandling studeras hur mammans immunceller utvecklar tolerans genom kommunikation med andra celler och genom att interagera med varandra. Fokus i avhandlingen är stödjeceller, stroma-celler, i decidua, som inte bara bygger upp vävnaden utan också deltar genom produktion av tillväxtfaktorer och hormoner som bidrar till skapande av tolerans.

De övergripande målen i avhandlingen var att kartlägga faktorer som styr utvecklingen av decidua-makrofager och regulatoriska T-celler, var dessa faktorer produceras och om kvinnor som utvecklat graviditetskomplikationer har en försämrad förmåga att utveckla immundämpande immunceller lokalt.

I en delstudie visar vi att de stödjeceller som utgör majoriteten av alla celler i deciduan, deciduala stromaceller, har förmågan att stimulera utvecklingen av makrofager som liknar de som finns i deciduan. För utveckling av immundämpande makrofager visade det sig att tillväxtfaktorn M-CSF var en drivande faktor. Dock dämpades uttrycket endast delvis, vilket tyder på att även andra faktorer är inblandade. En av de mest troliga är IL-34, som binder till och aktiverar samma receptor som M-CSF. I en annan delstudie visade vi att IL-34 bildas både i placentan och av stromaceller i deciduan. IL-34 var, i likhet med M-CSF, också drivande i utvecklingen av makrofager som är mycket lika makrofager i deciduan. Utöver effekten på makrofagerna drev de deciduala stromacellerna även utvecklingen av regulatoriska T-celler, troligen via produktion av en annan tillväxtfaktor, TGF- β .

Då IL-34 och M-CSF var involverade i utveckling av tolerans hos makrofager, undersökte vi även om havandeskapsförgiftning (preeklampsi) var associerad med en försämrad förmåga att producera dessa faktorer i placentan. Dock hittade vi inga skillnader i förekomst av IL-34 och M-CSF hos placenta-celler från kvinnor med havandeskapsförgiftning.

Sammantaget visar våra studier att deciduala stromaceller är viktiga för att producera en repertoar av faktorer som skapar och bibehåller immundämpande egenskaper hos makrofager och regulatoriska T-celler under en graviditet. Vi visar att M-CSF, IL-34 och TGF- β är centrala för denna toleransutveckling och att de bildas av deciduala stromaceller. Dock verkar dessa faktorer inte vara avvikande vid havandeskapsförgiftning.

Original publications

1. Interleukin-34 is present at the fetal–maternal interface and induces immunoregulatory macrophages of a decidual phenotype *in vitro*

R Lindau, R B Mehta, G E Lash, G Papapavlou, R Boij, G Berg, M C Jenmalm, J Ernerudh, J Svensson-Arvelund

Hum Reprod. 2018, 1;33(4):588-599.

2. Decidual stromal cells support tolerance at the human fetal-maternal interface by inducing regulatory M2 macrophages and regulatory T-cells

R Lindau, *S Vondra, *§J Spreckels, M Solders, J Svensson-Arvelund, G Berg, J Pollheimer, H Kaipe, MC Jenmalm, J Ernerudh

Manuscript

3. First-trimester trophoblasts obtained by chorionic villus sampling maintain tolerogenic and proteomic features in successful pregnancies despite a history of unexplained recurrent pregnancy loss

V Bruno, R Lindau, MC Jenmalm, C Ticconi, E Piccione, A Pietropolli, J Ernerudh

Am J Reprod Immunol. 2020; 00:e13314

Additional publications

The Human Fetal Placenta Promotes Tolerance against the Semiallogeneic Fetus by Inducing Regulatory T Cells and Homeostatic M2 Macrophages

J Svensson-Arvelund, RB. Mehta, **R Lindau**, E Mirrasekhian, H Rodriguez-Martinez, G Berg, GE. Lash, MC. Jenmalm and J Ernerudh

J Immunol. 2015, 194 (4) 1534-1544

Genome amplification and cellular senescence are hallmarks of human placenta development

P Velicky, G Meinhardt, K Plessl, S Vondra, T Weiss, P Haslinger, T Lendl, K Aumayr, M Mairhofer, X Zhu, B Schütz, RL. Hannibal, **R Lindau**, B Weil, J Ernerudh, J Neesen, G Egger, M Mikula, C Röhl, AE. Urban, J Baker, M Knöfler and J Pollheimer

PLOS Genetics 2018 14(10): e1007698

Abbreviations

APC	Antigen Presenting Cell
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of Differentiation
CM	Conditioned medium
CSF	Colony Stimulating Factor
CTB	Cytotrophoblast
CVS	Chorionic Villous Sampling
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DSC	Decidual stromal cell
ELISA	Enzyme-linked Immunosorbent Assay
EVT	Extravillous Trophoblast
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GW	Gestational Week
HLA	Human Leukocyte Antigen
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
KIR	Killer Immunoglobulin Like Receptor
LIF	Leukaemia Inhibitory Factor
LPS	Lipopolysaccharide
M-CSF	Macrophage-Colony Stimulating Factor
MHC	Major Histocompatibility Complex

MMP	Matrix Metalloproteinases
NK	Natural Killer
PBMC	Peripheral Blood Mononuclear Cell
PD-L	Programmed Death-Ligand
PEA	Proximity Extension Assay
RPL	Recurrent Pregnancy Loss
STB	Syncytiotrophoblast
TGF	Transforming Growth Factor
Th	T helper
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T Regulatory
uRPL	Unexplained Recurrent Pregnancy Loss
VEC	Vascular Epithelial Cell
VEGF	Vascular Endothelial Growth Factor

Introduction

The immunological paradox of pregnancy

The most important feature of the immune system is the ability to distinguish between self and non-self, and in recognition of non-self, mount protective immune responses towards potentially dangerous and harmful pathogens. Because of this, the maternal immune system has to acquire tolerance towards the semi-allogeneic foetus, since it contains genes from both the mother and the father, thus being partly self and partly non-self to the mother's immune system. At the same time, the maternal immune system must maintain an adequate defence against harmful pathogens. Already in the 1953, Sir Peter Medawar postulated three hypotheses on how the mother cope with the presence of the semi-allogeneic foetus; 1) anatomical separation between the mother and the foetus by the placenta, 2) immaturity of foetal antigens, and 3) an acquired inertness of the maternal immune system during pregnancy (1). Although these hypotheses have been proven mostly inaccurate, the paradox of pregnancy still remains to be fully elucidated (2). In this thesis, I will discuss the immunological adaptations that occur during pregnancy, with focus on the interplay of cells at the foetal-maternal interface and how this contributes to a tolerant microenvironment and also, the implication of the immune system in pregnancy complications.

Establishment of pregnancy

For a successful implantation of the blastocyst into the endometrium to occur, the endometrium must first undergo a remodelling process termed decidualisation. Decidualisation of the endometrium begins in the mid-secretory phase of the menstrual cycle, as a response to the post-ovulatory rise of progesterone and oestrogen (3). Under the influence of these steroid hormones, endometrial stromal cells undergo differentiation into specialised decidual stromal cells (DSC), accompanied by increased vascularisation and an influx of maternal immune cells into the endometrium, preparing it for the potential receiving of a blastocyst. This period, in which the decidualisation process begins, is known as the window of implantation, which lasts approximately 5 days (4).

Implantation starts with attachment (apposition) of the blastocyst to the decidualised endometrium (decidua), followed by the development of a stronger, integrin-mediated adhesion. Thereafter, 6-7 days post fertilization, the blastocyst will have invaded and embedded itself into the decidua (5). After the implantation is complete, cells from the trophoblast will differentiate into the more specialised trophoblast cell lineages and form mononuclear cytotrophoblast (CTB cells) and a multinuclear primary syncytium, containing fluid-filled lacunae, that will further penetrate and expand into the decidua (6). Initially, the underlying CTB cells are not in contact with the maternal side, but rapid proliferation will create protrusions penetrating through the multinucleated primary syncytium, forming the primary villi structure of the early placenta, consisting of a CTB core that is surrounded by a

syncytiotrophoblast (STB) cell layer. Further proliferation and branching of the primary villi structure will create the villous tree and transform the lacunae into the intervillous space. Eventually, the CTB cells will breach the primary syncytium, merge, and form the CTB shell, creating what is known as the foetal-maternal interface (discussed later). This is followed by emergence of extraembryonic mesenchymal stem cell from the CTB core that branch off, creating secondary villi. During the third week post-conception, the secondary villi develop into tertiary villi containing foetal capillaries. As the placenta grows, the CTB shell will become discontinuous and form CTB cell columns that anchor the placenta to the decidua (7).

CTB progenitor cells found in the villi structure can differentiate along two different pathways. Some CTB cells fuse and form the multinucleated STB that covers the villi of the placenta and act as a barrier towards maternal blood, while at the same time regulating nutrition and gas exchange between mother and foetus. The other differentiation pathway for CTB cells is to differentiate into the invasive extravillous trophoblast (EVT) cells. EVTs migrate from the tips of the cell columns that anchor the foetal placenta to the maternal decidua, in which they take an active part in remodelling of decidual spiral arteries and extracellular matrix. Remodelling of the spiral arteries will remove vascular smooth muscle cells, resulting in dilated, non-vasoactive vessels that can provide adequate blood supply to the growing foetus (4).

The foetal-maternal interface

The foetal-maternal interface is composed of the foetal part of the placenta, the maternal decidua and the underlying myometrium. The most profound immunological changes during pregnancy take place at the foetal-maternal interface since it is here that foetally-derived EVT cells invade the maternal decidua and come in direct contact with maternal leukocytes (8). During pregnancy, around 40% of all cells in the decidua are immune cells and they are composed of a unique distribution of leukocytes, of which ~70% are natural killer (NK) cells, ~20% are macrophages, ~10% are T-cells and ~1% are dendritic cells (DC) (9) (Figure 1). An extensive mapping of cells and cellular interactions was recently reported using single-cell transcriptomic methodology (10), providing further insights in the cellular composition at the foetal-maternal interface.

Figure 1: Schematic representation of the foetal-maternal interface. Extravillous trophoblasts (EVT) break off from the cytotrophoblast (CTB) cell column and invade the maternal decidua to remodel the spiral arteries. In the decidua, the invading EVTs will come in contact with the unique composition of maternal leukocytes present in the decidua, the majority being uterine natural killer (uNK) cells (~70%), macrophages (~20%) and T-cells (~10%). CTB, cytotrophoblast cell, DSC, decidual stromal cell, EVT, extra villous trophoblast, uNK, uterine NK cell, VEC, vascular epithelial cells.

Trophoblasts in the decidua

As discussed earlier, trophoblasts originate from the trophectoderm of the blastocyst and differentiate into several lineages of trophoblast cells, each with different functions.

An important feature of trophoblast cells is their limited expression of major histocompatibility complex (MHC) proteins (also called human leukocyte antigen (HLA) if it refers to humans). The limited expression of HLA allows them to invade the decidua and interact with maternal immune cells without eliciting a rejection response. The HLA proteins are highly polymorphic and their function is to bind peptides and present them to T-cells. (11). HLA proteins are divided into two groups, class I and class II, which present antigens to CD8+ and CD4+ T-cells, respectively. EVT's invading the maternal decidua are devoid of HLA class II proteins, rendering them incapable of presenting antigens directly to CD4+ T-cells (12). Also, EVT's lack expression of the highly polymorphic class I proteins HLA-A and HLA-B (11). They do, however, express HLA-C molecules as well as the non-classical HLA-E and HLA-G molecules (13). In contrast, the STB cells lining the floating villi in the intervillous space, acting as a barrier towards maternal blood, are devoid of any HLA molecules (12).

Trophoblast cells actively take part in modulation of immune responses at the foetal-maternal interface. Previous data from our group has shown that tissue (in the form of small pieces, explants) from 1st trimester placenta, as well as isolated CTB and EVT cells, can induce polarisation of macrophages into decidual-like regulatory M2 macrophages and selectively expand the proportion of regulatory T-cells (Treg) *in vitro*, while not affecting other T helper (Th) cell populations (14).

Induction of M2 macrophages was dependent on trophoblast derived macrophage-colony stimulating factor (M-CSF) and interleukin (IL)-10, whereas induction of Treg cells could be attributed to IL-10, transforming growth factor (TGF)- β and tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (14). Besides these cytokines, trophoblasts also produce some pro-inflammatory related cytokines, such as IL-1 β , IL-6 and TNF (14). Furthermore, as pregnancy is established, trophoblasts become the main producers of pregnancy-related hormones such as human chorionic gonadotropin, progesterone and oestrogen (15).

The location of EVT's around and inside maternal blood vessels and their ability to produce a variety of chemokines suggests that they are involved in recruitment of leukocytes into the decidua. For example, they have been shown to produce the chemokine, chemokine (C-X-C motif) ligand (CXCL) 12, which *in vitro* recruited NK-cells in a transwell system (16), as well as chemokine (C-C motif) ligand (CCL) 2 (14), a chemokine that predominantly acts on monocytes, but also has the ability to recruit T-cells (17).

Decidual stromal cells

Decidual stromal cells (DSCs) are the largest population of cells in the decidua. They are of fibroblast origin (3) and develop from endometrial stromal cells under the influence of post-ovulatory increase in oestrogen and progesterone (18). The decidualisation reaction incur both morphological and biochemical changes in the stromal cells, including acquisition of polyploid nuclei, increased amounts of rough endoplasmic reticulum and ribosomes (19). DSCs produce a variety of different factors like prolactin, leukaemia inhibitory factor (LIF), IL-11, IL-15 and insulin growth factor binding protein 1 (3,18,20). Furthermore, they also serve as an energy reservoir for the implanting embryo, by accumulating lipids and glycogen, until the placenta is formed (21). Although DSCs are involved in many important processes at the foetal-maternal interface including hormone and growth factor production, angiogenesis/vascularisation and tissue remodelling (20), these processes are beyond the scope of this thesis and I will focus on discussing the role of DSCs in modulating the local immunological milieu.

DSCs produce a variety of chemokines, including CCL2, CXCL8, chemokine (C-X3-C motif) ligand (CX3CL) 1, CXCL12 (22), CXCL10 and CXCL11 (23), which suggests involvement in recruitment of peripheral leukocytes into the decidua. For example, the ligand for CXCL12 is expressed on NK-cells and it has been suggested that the CXCL12/ C-X-C chemokine receptor type (CXCR)4 axis is involved in recruitment of NK-cells to the decidua (24). Furthermore, Croxatto *et al.* showed that DSCs inhibit upregulation of activating receptors in response to IL-15 in peripheral blood-derived NK-cells. This was also accompanied by a reduction in cytolytic activity, proliferation, and interferon (IFN)- γ production (25). Similarly, Yang *et al.* showed that in a co-culture system of DSCs and decidual (d)NK-cells, neutralisation of the cytokine IL-24 led to a reversal of dNK-cell characteristics, with increased expression of CD16, granzyme B and perforin, while the levels of inhibitory receptors as well as the production of anti-inflammatory cytokines (TGF- β and IL-10) were decreased (26). Collectively, these data show that DSCs play a major role in dNK-cell homeostasis. In addition, DSCs have shown effect on other cell types, like inhibition of blood monocytes differentiation into DC and reduced capacity of DCs to activate T-cells (25), effects that, at least in part, were mediated by MIC-1 (27). Furthermore, DSCs reduced the proliferation of alloreactive T-cells in mixed lymphocyte reactions, a feature attributed to DSC-derived Indoleamine-pyrrole 2,3-dioxygenase (IDO), and they also expanded the proportion of Treg cells, in a proximity dependent manner (28). In addition, DSCs were found to express the checkpoint molecule programmed death (PD)-ligand (L)1 and the anti-inflammatory TGF- β (28). Although DSCs produce a multitude of factors and perform many different tasks, including immune modulation and hormone production, little is known about their role in macrophage homeostasis and Treg induction.

NK-cells

NK-cells are part of the body's "frontline" defence, the innate immune system. NK-cells are involved in the defence against intracellular infections, especially viral infections, as well as recognising and destroying malignant tumour cells. NK-cell function and activity are coupled to their expression of a wide array of inhibitory or activating cell surface receptors, divided into the following families; the killer immunoglobulin-like receptors (KIR), the C-type lectin heterodimer family, the natural killer cytotoxicity receptors and the immunoglobulin-like receptors (29). In addition, NK-cells can also respond to and become activated when exposed to cytokines like IFN- α , IL-12, IL-15 and IL-18 (30).

Although dNK-cells are the largest population of leukocytes in the decidua (~70%), their origin is not established. It may involve a combination of different sources, including differentiation of CD34⁺ NK cell precursor in the endometrium (31) or recruitment of NK-cells from the peripheral blood via CXCR4/CXCL12 axis (24), where they acquire a decidual phenotype under the influence of for example TGF- β (32) and IL-24 (26).

dNK-cells have a distinctive phenotype compared to their blood counterparts. dNK-cells are CD56^{bright}CD16⁻ (9), whereas in blood 90% are CD56^{dim}CD16⁺ and only 10% are CD56^{bright}CD16⁻ (29). The decidual CD56^{bright}CD16⁻ NK-cells are less cytotoxic and more prone to production of cytokines and growth factor (33) as well as angiogenic factors (34), supporting a role in spiral artery remodelling and trophoblast invasion.

Interestingly, single cell reconstitution of cells present at the foetal-maternal interface suggests that dNK-cells are not one singular entity, but rather three different subsets (dNK1, dNK2, and dNK3), all with different properties (10). Notably, dNK-cells, in general, express a different repertoire of receptors compared to their blood counterpart (10). These receptors are of both the activating and inhibitory type (35). A balance between activating and inhibitory signals are necessary for proper placentation. For example, binding of HLA-C to the activating KIR2DS1 leads to enhancement of cytokine production of dNK-cells, promoting EVT invasion (36). Conversely, HLA-G binding to leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) inhibits the cytolytic function of NK-cells (37). This suggests that a balance between activating and inhibitory signals is required for proper cytokine and chemokine production, and for controlling the cytotoxicity that is important for proper trophoblast invasion and spiral artery remodelling (4). Even though dNK-cells have a reduced cytotoxic capacity, treatment with IL-15 restored their cytotoxic capacity and they were able to kill virus-infected cells *in vitro* (38), indicating that dNK-cells still retain their ability to control viral infections at the foetal-maternal interface.

Macrophages

Macrophages constitute a heterogeneous cell type of the innate immune system. They are specialised in phagocytosis and neutralisation of harmful pathogens while at the same time

maintaining tissue homeostasis by removal of cellular debris. Macrophages are present in almost every tissue in the body, and they have specialised functions coupled to their location. Examples of tissue-resident macrophages include Kupffer cells in the liver, microglia in the brain or osteoclasts in the bone. Their most generic function is guarding our body against insults. To be able to perform their task as sentinels, macrophages are equipped with a range of different sensor molecules, such as the toll like receptors (TLR), scavenger receptors, C-type lectin receptors, RIG (retinoic acid-inducible gene)-like receptors, and NOD (nucleotide oligomerization domain)-like receptors. These receptors provide the macrophages with the means to recognise and orchestrate an inflammatory response towards pathogens like bacteria, viruses, fungi, and parasites, but also to recognise danger signals from tissue damage (39–41).

Figure 2: Schematic illustration over macrophage polarisation and factors contributing to polarisation of each subset, examples of effector cytokines and function. Adapted from Martinez et al *F1000Prime Rep*;2014, 6:13

Another aspect of macrophage function that needs to be considered is their plasticity, *i.e.* their ability to switch between different polarisation phenotypes in response to different stimuli (Figure 2). In the Th1/Th2 era of immunology, it was originally postulated that macrophage polarization followed the same paradigm as CD4+ Th-cells, where the classically activated, pro-inflammatory M1 macrophage represented the Th1 response and the “alternatively activated” (alternatively because not classically activated) M2 macrophages represented the Th2 pathway (42). However, this M1/M2 paradigm has been challenged as being too simplistic to classify macrophages, since their plasticity and ability to adapt to different stimuli, allows them to act across these boundaries (43). In addition, M2

macrophages constitute a very heterogeneous set of cells (see below). Nevertheless, for simplicity the M1/M2 paradigm will be used when discussing macrophage function and polarization.

Classically activated M1 macrophages constitute our first line of defence against intracellular pathogens and acquire this phenotype in response to IFN- γ , lipopolysaccharides (LPS) and TNF, but also to granulocyte macrophage-colony stimulating factor (GM-CSF) (43). Activated M1 macrophages produce high quantities of reactive oxygen and nitrogen species (44) as well as pro-inflammatory cytokines like IL-12, IL-6, TNF, IL-1 β and IL-23, cytokines that drive induction of Th1 and Th17 adaptive immune responses (45). In addition, M1 macrophages produce the Th1-associated chemokines CXCL9, CXCL10 and CXCL11 (46), and they show a high expression of the co-stimulatory molecules CD80, CD86 and CD40, which drive activation of the adaptive immune system (46).

Originally, the term alternatively activated macrophage arose from the observation that macrophages stimulated with IL-4 acquired a distinct phenotype that did not fit within the classically activated macrophage phenotype (47). Subsequently, the concept of alternatively activated macrophages has been further expanded to include three main subtypes, M2a, M2b and M2c, to better classify M2 macrophages (48). M2a macrophages represent the original alternatively activated macrophage. They are induced by IL-4/IL-13, express CD86, CD200R, CD206 (Mannose receptor) and CD209 (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN)), produce IL-10, IL-1Ra, CCL13, CCL17 and CCL22 and are involved in Th2 responses (46,48,49). M2b macrophages are induced by immune complexes, IL-1 receptor ligand and TLR agonists, they produce both the anti-inflammatory IL-10, and pro-inflammatory IL-6, IL-1 β and TNF and are involved in Th2 activation and immune regulation (46,48). Finally, the M2c macrophages are induced by glucocorticoids, M-CSF and IL-10, and they produce large amounts of the anti-inflammatory IL-10, as well as the chemokines CCL18 and CCL16 (46), and show high expression of the scavenger receptor CD163, CD14 and CD209 (50), while low expression of CD86 and HLA-DR (46). Their main function is immune regulation and maintenance of tissue homeostasis.

The dogma of macrophage polarisation is further expanded when taking into account the prominent ability of colony stimulating factors (CSF) to influence polarisation. There are three CSFs, granulocyte-CSF (G-CSF), M-CSF and GM-CSF (51). Of these, the most relevant to consider for macrophage polarisation are M-CSF and GM-CSF. M-CSF (also called CSF-1) is constitutively expressed by a variety of cells and is detectable in plasma at a resting state, whereas GM-CSF (CSF-2) requires pro-inflammatory stimuli to be detectable (52). Macrophages cultured with M-CSF have been shown to produce high levels of IL-10 (53,54), and low amounts of IL-12p70 and IL-23 following LPS challenge (55). M-CSF-macrophages show low expression of CD80/86 (54), indicating a reduced capacity to activate T-cells. Previous studies from our group have also shown that M-CSF polarised macrophages acquire high expression of CD163, CD209 and CD14 (14,50). On the contrary, macrophages cultured with GM-CSF showed different characteristics, with low expression of CD163, CD209 and CD14 (14,50). Furthermore, GM-CSF induced macrophages, as opposed to M-CSF induced macrophages, produce low amounts of IL-10 and high amounts of pro-inflammatory TNF, IL-

12p70 and IL-23 (53–55). Interestingly, in 2008, it was discovered that M-CSF shares its receptor with another ligand, IL-34 (56). IL-34 has been shown to be important for generating and maintaining certain tissue macrophage subsets, such as, osteoclasts (57), Langerhans cells and microglia (58,59). In addition, macrophages cultured in the presence of IL-34 developed immunosuppressive abilities, including inhibition of T-cell proliferation, high production of IL-10 and expression of CD163 (60).

At the foetal-maternal interface, macrophages are the second largest leukocyte population, constituting around 20% of the total leukocyte population. The origin of decidual macrophages remains largely unknown, and as with the dNK-cells, different hypotheses exist (61). Several cell types, including trophoblasts, DSCs and dNK-cells produce the monocyte chemoattractant CCL2 (62), suggesting that monocytes can be recruited to the foetal-maternal interface from peripheral blood. Once in the decidua, monocytes differentiate into macrophages under the influence of the local microenvironment, as shown by the ability of the placenta and isolated trophoblast cells to polarize monocytes into a decidual type of macrophages, a polarization that was dependent on M-CSF and IL-10 (14). There is also evidence of tissue resident, proliferating macrophages in the endometrium before pregnancy occurs, which may be contributing to the local pool of macrophages during pregnancy (63).

Decidual macrophages are predominantly of the regulatory M2c phenotype, as revealed by upregulation of a large number of genes associated with immune regulation and M2 polarization in a global gene expression screening (64). Furthermore, decidual macrophages show a high expression of M2 associated surface markers such as CD163, CD14 and CD209 (50,65), and they are main producers of IL-10, compared to the other decidual leukocyte populations (66). However, in contrast to M2c macrophages, decidual macrophages also produce inflammatory cytokines such as TNF, IL-1 β , IL-6, IL-8 and IFN- γ (67), which may be important for implantation and invasion in early pregnancy (68).

Decidual macrophages are the largest population of antigen presenting cells (APC) in the decidua (9,69). Despite this, they are poor activators of T-cells, shown by a reduced capacity to promote IFN- γ production by T-cells (70) and a failure of isolated CD14+ cells from decidua to elicit proliferation of allogeneic T-cells, in contrast to monocyte derived DCs (63). Furthermore, decidual macrophages might also be important players in Treg cell induction at the foetal-maternal interface. We observed that conditioned medium (CM) from isolated 1st trimester decidual macrophages directly induced expansion of Treg cells *in vitro* (Svensson-Arvelund unpublished observation). It has also been shown that interactions between dNK-cells and decidual macrophages, resulted in a macrophage-dependent expansion of Treg cells, effects that were mediated by TGF- β and IDO (71).

Apart from being major contributors to foetal tolerance, decidual macrophages have been implicated to partake in several other pregnancy promoting processes. Macrophages are enriched around remodelling spiral arteries (72), inside the walls of remodelled vessels (67), and at locations where masses of trophoblasts invade the decidua (73). They produce angiogenic factors (Epidermal growth factor and vascular epithelial growth factor (VEGF)) (62), as well as a multitude of matrix metalloproteinases (MMP) (67), indicating an active

role of decidual macrophages in the remodelling of spiral arteries. Another function of the decidual macrophages is to remove the cellular debris and apoptotic cells that are generated through the rapid turnover of cells during spiral artery remodelling and invasion of trophoblasts (61,74), which could otherwise result in a detrimental inflammatory response. There is also evidence for an active contribution of decidual macrophages in promoting CTB proliferation and triggering invasion of EVT_s into the decidua from the CTB cell columns by secretion of IL-33 (75).

Decidual macrophages are the second largest maternal leukocyte population in the decidua during pregnancy. They perform a variety of important functions during pregnancy to maintain a homeostatic foetal environment. However, little is known about how they acquire their regulatory properties, although several factors, including M-CSF, IL-10, and IL-34, have been shown to induce regulatory properties *in vitro*. Although, scarce information exists of their expression pattern in the decidua.

T-cells

T-cells are part of the adaptive immune system and are involved in directing and orchestrating immune responses towards pathogens. They also kill infected and malignant cells, as well as create a long-lasting memory of infections. T-cells are generally divided into the main populations of CD4⁺ Th-cells and cytotoxic CD8⁺ T-cells. A common defining feature for these cell types is their T-cell receptor (TCR), the most common being the $\alpha\beta$ TCR. T-cells require binding of their TCR to antigenic peptides in complex with MHC molecules, coupled with engagement of CD28 to co-stimulatory (CD80/CD86) molecules on APCs to become fully activated. The Th-cells recognise MHC class II molecules and CD8⁺ cytotoxic T-cells recognise MHC class I molecules (76).

Activated Th-cells can differentiate down several different pathways, to become either Th1, Th2, Th17 or Tregs (see Figure 3), depending mainly on the cytokine environment at the activation site and what kind of infection is present. Other subsets have also been described, including Th9 and Th22, but their function is less understood (77).

Th1-cells express the defining transcription factor T-bet and are important for defence against intracellular pathogens. They differentiate down the Th1 path in response to TCR engagement and APC-derived IL-12p70. Once induced, Th1-cells produce large amounts of IFN- γ , which in turn will further increase macrophage activation and killing, enable CD8⁺ T-cell mediated killing of virus-infected cells and induce IgG isotype switching in B-cells (78).

Figure 3: Schematic illustration over T helper (Th) cell polarisation. Adapted from Walsh et al Trends Immunol. 2013;34:521–30.

Th2-cells express GATA-3 as their defining transcription factor, and this subset is important for defence against parasites and helminths, which are many times larger than bacteria and viruses, thus requiring a different route to fight (78). The main polarizing cytokine for Th2 differentiation is IL-4. However, it is not entirely established what the source of IL-4 is. Basophils have been suggested as an early source (79) and some studies show that TSLP (80) and IL-33 (81,82) prime APCs to produce Th2 cytokines. It is also possible that Th2 polarization can occur without any cytokine input, requiring only a low affinity TCR engagement (83). Th2-cells produce IL-4, IL-5, and IL-13 as effector cytokines, promoting IgE isotype switch, eosinophil activation and mucin production to name a few (78).

Th17-cells are defined by their expression of the ROR γ t transcription factor, encoded by the *RORC* gene (84), and they mount defences against extracellular bacteria and fungi (78). The differentiation of Th17 is a bit more complex than the other subtypes. In mice, Th17 differentiation is dependent on IL-6 and TGF- β , and to a lesser extent IL-21. A cytokine also required for expansion of the lineage is IL-23, which acts as a stabiliser (85). In humans, TGF- β together with IL-21 is sufficient to promote Th17 expansion, however, combinations of TGF- β with IL-6 and IL-23 have also been shown to induce ROR γ t expression, with IL-6 and IL-1 β working as amplifiers, and IL-23 as a stabiliser (85). The main effector function of Th17-cells is to recruit neutrophils to the site of infection (78).

Treg cells are defined by high expression of CD25 and the transcription factor Foxp3 (86). Tregs are indispensable immune suppressing cells that control immune responses,

preventing over-activation of the immune system, preventing autoimmunity, and maintaining homeostasis (87). There are two modes of induction of Tregs, they can either be induced in the thymus (tTregs) or in the periphery (pTregs). tTregs are believed to be a result of intermediate TCR engagement during T-cell maturation in the thymus (87). pTregs are induced by TGF- β and retinoic acid from naïve CD4⁺ cells (88). Tregs exert their suppressive functions in several different ways, for example by production of anti-inflammatory cytokines such as IL-10 and TGF- β , induction of IDO (89), endocytosis (90) and anergy upon CTLA-4 binding to and stripping off co-stimulatory molecules CD80/86 on APCs, scavenging IL-2 through CD25 expression, removal of ATP through CD39, and secretion of granzymes, lysing the target cell (91,92).

T-cells comprise only ~10% of the decidual leukocyte compartment, *i.e.* much lower than in blood. Of the $\alpha\beta$ T-cells, 30-45% are CD4⁺ T-cells and 45-75% are CD8⁺ T-cells, *i.e.* a relative higher abundance of CD8 than in blood. Invariant T-cells ($\gamma\delta$, CD4-CD8-TCR $\alpha\beta$ and NKT cells) are also present at high frequencies compared with blood (93). In addition, unpublished observations from our group also show the presence of mucosal associated invariant T-cells (MAIT) in the decidua. These MAIT cells have higher expression of anti-inflammatory molecules compared to their blood counterparts. They are, however, more abundant in blood than in the decidua (Raffetseder et al. unpublished).

It was long ago suggested that pregnancy is biased towards Th2 responses, with a weakening of Th1 responses (94,95). However, more recent data show that there is no increase in Th2 cells in the decidua compared to blood and that Th17 cells are less abundant in the decidua (96). Many mechanisms for limiting T-cell activation at the foetal-maternal interface have been described, including receptor mediated suppression of proliferation by PD-1, apoptosis induction by FasL interaction on activated T-cells and depletion of tryptophan by IDO, halting T-cell proliferation (97). Furthermore, in mice, it has been shown that decidualized endometrium loses its ability to produce Th1-associated chemokines due to epigenetic modifications, effectively restricting access of effector Th1 cells to the decidua (98).

Out of the CD4⁺ pool in the decidua, ~5% are Treg cells (93). The Treg cells have been shown to be crucial for successful murine pregnancy (99,100). In humans, Treg cells are enriched in the decidua compared to blood and show a phenotype associated with immune suppression (96,101,102), although the origin of these cells remains elusive (103). It has been suggested that expansion of Treg cells is induced by exposure to seminal fluid, which contains TGF- β (104). Factors in the microenvironment in the decidua could be another source of Treg cell induction. Indeed, we have shown that trophoblast cells, both CTBs and EVT, induce CD25^{bright} Foxp3⁺ Treg cells, expressing CTLA-4 and CD39 and suppressed proliferation of allogeneic T-cells *in vitro* (14). It is also possible that Treg cells are recruited from the peripheral blood by local production of CCL4 and CCL17, binding to CCR4 and CCR5 on Treg cells (96,105). It has also been suggested that recognition of foetal HLA-C, presented by APCs to CD4⁺ T-cells could promote induction of Treg cells (106).

Treg cells are enriched in the decidua during a normal pregnancy and are crucial for a successful pregnancy. Accordingly, aberrations in Treg responses have been implicated in pregnancy complications. However, the contribution of local tissue resident cells in the decidua to induction of Treg cells, both in normal and complicated pregnancies, is poorly understood.

Systemic immune adaptation

Although the most pronounced immunological changes occur at the foetal-maternal interface, systemic adaptations to pregnancy are also present. This is clearly evident by the observations that auto immune diseases, like rheumatoid arthritis (RA) and multiple sclerosis (MS) improve during pregnancy (107). The systemic alteration during pregnancy may be due to placenta-derived factors released into the maternal circulation, factors such as steroid hormones, cytokines and microvesicles, or it may be due to direct contact of maternal leukocytes to foetal trophoblasts inside the intervillous space (8).

During pregnancy it has been observed that there is an increased number of circulating leukocytes, due to an increased output of innate immune cells, in particular, granulocytes and monocytes (108). These cells also exhibit increased levels of activation (109,110), with higher levels of baseline inducible nitric oxide synthase (iNOS) and oxidative burst capacity (110). This is accompanied by a reduced capacity of CD3+ T-cells and CD56^{dim} NK-cells to produce the Th1 cytokine IFN- γ compared to their counterparts in non-pregnant women (111). Furthermore, peripheral blood mononuclear cells (PMBC) from pregnant women challenged with different viruses showed a lower IFN- γ production and a higher IL-10 production compared to non-pregnant controls (112). Due to the reduced capacity to mount a Th1 response, pregnant women during the third trimester have more severe infections regarding in particular, hepatitis E, herpes simplex (generalised type), malaria, and influenza (113). The level of Treg cells in the circulation during pregnancy has been debated, and has reported to be increased, decreased or unchanged. The discrepancy might be due to differences in gating strategies and also in which trimester the samples were obtained in (103,114–118).

Pregnancy complications

Preeclampsia

Preeclampsia is a pregnancy complication that affects 2-8% of pregnancies worldwide and is one of the leading causes of maternal and perinatal morbidity and mortality (119). Preeclampsia occurs in the second half (>20 gestational weeks (GW)) of pregnancy and can be divided into the early onset (<34 GW), often more severe form, or the late onset (>34

GW), which is usually milder and has less impact on maternal and foetal health. The clinical manifestation of preeclampsia includes high blood pressure and proteinuria as well as severe systemic inflammation and endothelial dysfunction. The clinical symptoms are a result of inadequate trophoblast invasion and remodelling of spiral arteries, resulting in high resistance vessels that compromise placental blood flow (120). The only cure for preeclampsia is delivery and removal of the placenta (121). However, early screening and acetylic acid supplementation may prevent development of preeclampsia in risk pregnancies (122).

The risk of developing preeclampsia is higher in the first pregnancy and declines in subsequent pregnancies. However, if there is a partner change for the subsequent pregnancies, the reduction in risk for developing preeclampsia is abolished. Furthermore, prolonged exposure to paternal antigen (semen and seminal fluids) also reduces the risk of developing preeclampsia, implicating protective priming of the maternal immune system towards paternal antigens (123).

Development of preeclampsia is believed to be caused by maladaptation of the maternal immune system towards paternal antigens (123), leading to severe local and systemic inflammation. It has been shown that serum taken from preeclamptic women and inject it into mice creates symptoms of preeclampsia, while this phenomenon is not seen with serum from healthy pregnant women (124). Furthermore, preeclamptic women show an increase in pro-inflammatory cytokines and chemokines, such as TNF, IL-6 (125,126), IFN- γ (127) CXCL10 and CXCL11 (128), and a decrease in the anti-inflammatory IL-10 (125) in the circulation. This change in cytokine balance was suggested to be caused by a shift in NK cell phenotype rather than a change in Th1/Th2-cells (129). Circulating NK-cells also showed higher cytotoxic potential in preeclamptic patients (130). Along with this, no difference in frequencies of circulating Treg cells was found in preeclamptic compared to normal pregnant women (116). Furthermore, aberrant NK-cell numbers in the decidua have been reported, although these findings have been inconsistent including both increased (131,132) and lower amounts (133,134) of NK cell in the decidua.

Also for macrophages, the reports on their cell numbers in preeclampsia diverge, some studies report a decrease in decidual macrophage numbers (134,135) whereas some report increased numbers (136,137). Disregarding the discrepancies in macrophage numbers, evidence exists of altered macrophage function. Increased presence of activated macrophages is found in spiral arteries in preeclamptic pregnancies, and at the same time, presence of trophoblasts is near absent (138). This absence of trophoblasts might be explained by macrophage-derived TNF-induced apoptosis of trophoblasts around the spiral arteries (139). Furthermore, the balance of macrophage polarization factors is altered in the decidua in preeclampsia. It has been reported that the M1-promoting factor GM-CSF is increased in the preeclamptic decidua (140), and along the same line, lower abundance of the M2-promoting IL-10 (136) has been reported. It was shown *in vitro* that IL-10 abrogated TNF production in macrophages, restoring trophoblast invasion (141). In line with the

skewed cytokine profile, lower frequencies of M2 macrophages have been reported in decidua from preeclamptic women (136,142).

Recurrent pregnancy loss

Recurrent pregnancy loss (RPL) is a pregnancy complication that manifests early in pregnancy and affects 1-2% of pregnancies (143). The European Society for Human Reproduction and Embryology define RPL as 2 or more consecutive pregnancy losses before GW 24 (143). Although there are several factors known to cause RPL, including chromosomal abnormalities, hormonal factors, uterine malformations, autoimmunity, thrombophilia, and antiphospholipid antibody syndrome, around 50% of the cases remain unexplained. In these unexplained cases, a dysregulated immune response is believed to be a potential underlying cause (144). In support of this, PBMCs from women with a history of RPL had a higher Th1-to Th2-ratio in response to challenge by placental antigens (145). Similarly, the Th1/Th2 cytokine ratio was skewed in favour of a Th1 response in women with a history of unexplained RPL (uRPL), who had a subsequent abortion compared to women with a history of RPL who had a subsequent normal pregnancy, when PBMCs were challenged with placental antigens (146). Furthermore, lower frequencies and impaired suppressive function of peripheral Tregs (147) as well as increased proportions of peripheral Th17 cells (148) have been reported in RPL patients. In line with this, the decidual CD4+ T-cell compartment is also skewed in favour of a Th17 response (148,149). The importance of Treg cells in preventing rejection is evident in mice models where depletion of Treg cells increased the amounts of foetal rejections (150). Also, transfer of Treg cells from normal mice to abortion-prone mice reduced the rejection rate (151).

Aberrations in the innate immune system have also been associated with RPL. For instance, lower proportions of the dNK CD56^{bright}CD16⁻ phenotype have been found in the endometrium of RPL patients, and with this, an increase in the more cytotoxic CD16+ NK cell population (152), showing abnormal receptor expression and cytokine profile compared to pregnancies in control women (153). Similar observations have been made for peripheral blood NK-cells in women with a history of RPL (154). Macrophage dysregulation has also been reported in RPL, where macrophages in the decidua of RPL patients had higher expression of the co-stimulatory CD80 and CD86 and a lower production of IL-10 compared to normal pregnancy (155). Another, more recent study showed that deciduas from RPL patients contained more iNOS+ M1 macrophages compared to healthy controls, and lower amounts of IL-10+ macrophages (156), suggesting an inability to properly polarize macrophages in RPL.

Onset of labour and parturition is a physiological event characterised by a coordinated switch from the anti-inflammatory state during pregnancy to a cascade of intrauterine pro-inflammation, characterised by pro-inflammatory cytokines like IL-6, CXCL8, IL-1 β and TNF, as well as prostaglandin E2 (157,158). Preterm birth is, by WHO's definition, delivery before 37 weeks of gestation, and can further be subdivided into extremely preterm (<28 GW), very preterm (28-32 GW) and moderate to late preterm (32-37 GW) (159). In 2014, preterm births accounted for approximately 11% of all live births globally (160), and it is the leading cause of perinatal morbidity and mortality. (159). Preterm birth can manifest in three different ways, spontaneous labour with intact membranes, premature rupture of membranes or medically induced labour/caesarean section (161). Two-thirds of premature births occur spontaneously, the remaining ones are due to maternal or foetal complications (162). Spontaneous preterm birth has been proposed to be a multifactorial disease involving several mechanisms, including stress, vascular disorders, infection and breach of foetal-maternal tolerance (162).

One of the main causes for preterm birth is intrauterine infections. Most of these infections are subclinical. However, among patients who had a preterm labour and delivered preterm with intact membranes, 22% were positive for infection and from patients with premature rupture of membranes the rate was 32% (163). Furthermore, it has been shown in many animal models that administration of infectious or inflammatory agents, both systemically and intrauterine, will lead to preterm delivery (164). Given the prominent role of the innate immune system and its receptors in recognising pathogens, their involvement in preterm birth could be expected. Indeed, mice deficient in IL-10 experienced excessive inflammation and increased resorption and preterm deliveries when challenged by a TLR9 agonist (165). Similarly, LPS administration to IL-10-deficient mice increased rates of preterm deliveries (166). Administration of recombinant mouse IL-10, TNF neutralisation and removal of dNK-cells prevented preterm labour and preterm births in these mice (166). Also, mice carrying a TLR4 mutation were shown to be less prone to deliver preterm (167). Furthermore, increased amounts of inflammasome components and mature IL-1 β and IL-18 were found in membranes of preterm labour patients with acute histological chorioamnionitis (168). Also, reduced number of M2 macrophages were found in the decidua of preterm patients, and these macrophages produced more TNF and IL-12 compared to term controls (169). A recent study from Gomez-Lopez *et al.* demonstrated that preterm deciduas contained more CD8+ IL-17-producing cells compared to healthy controls, and this was also accompanied by a lower proportion of Treg cells. Furthermore, depletion of systemic Treg cells increased the incidence of preterm birth in mice, which was rescued by adoptive Treg cell transfer. Systemic depletion of Treg cells also increased susceptibility to LPS-induced preterm birth (170).

Taken together, there are several lines of evidence to suggest the involvement of immune aberrations in the major pregnancy complications uRPL, preeclampsia and preterm labour.

Aims and hypotheses

The general aim of this thesis was to increase the understanding of how the interplay between cells in the decidua, normally and in pregnancy complications, can promote adaptation and tolerance towards the semi-allogeneic foetus. In particular, the role of placental and decidual tissues in the induction of regulatory M2-like macrophages and Treg cells was elucidated.

In **Paper I** the aim was to investigate if IL-34 is present at the foetal-maternal interface and if it has a regulatory role similar to that of M-CSF.

We hypothesised that IL-34 is produced by foetal and maternal tissues, that IL-34 would be able to induce macrophages similar to the regulatory M2-like macrophages found in the decidua, and that a dysregulated IL-34 response could be involved in preeclampsia.

In **Paper II** the aim was to assess the potential role of decidual stromal cells in shaping immune regulatory phenotypes of macrophages and T-cells in a manner similar to that in the decidua.

We hypothesised that decidual stromal cells produce factors that could induce polarisation of macrophages into decidual-type regulatory M2-like macrophages as well as promote the expansion of Treg cells.

In **Paper III** the aim was to establish an *ex vivo* model to assess functional effects of trophoblasts as obtained by chorionic villous sampling (CVS), and to evaluate if the immune function is compromised in pregnancies of women with a history of RPL.

We hypothesised that placental tissue from women with a history of RPL would have a reduced capacity to induce regulatory M2-like macrophages and Treg cells and have a protein profile associated with an inflammatory response, as compared with women without RPL.

Study designs and methodology

Subjects

In paper I, to study the *in vitro* effects of IL-34 on macrophage polarization, we isolated peripheral monocytes from 14 healthy, non-pregnant women. To investigate the presence of IL-34 at the foetal-maternal interface, placental and decidual tissues from 49 women undergoing elective termination of pregnancy were collected at the Department of Obstetrics and Gynecology (Kvinnokliniken) at Linköping University Hospital, Sweden or at the Royal Victoria Infirmary, Newcastle upon Tyne, UK. Prior to surgery, Misoprostol was administered to all women. In addition to 1st trimester samples, plasma samples were collected from 83 healthy pregnant women and from 86 women with preeclampsia. Lastly, placenta biopsies from 15 normal pregnancies and 9 early-onset and 12 late-onset preeclamptic placentas were used for immunohistochemistry staining (IHC).

In paper II, CM was obtained after culturing of decidual stromal cells from 8 term placentas and from 4 1st trimester placentas obtained from elective pregnancy terminations. For evaluation of CM's functional effects on polarisation of macrophages and T-cells, we isolated monocytes and CD4+ T-cells from either buffy coats (n = 26) or peripheral blood (n = 7) obtained from healthy non-pregnant women.

In paper III, 36 pregnant women were included. Of these, 9 had a history of RPL, defined as 3 or more consecutive pregnancy losses before GW 20. For functional studies, 9 healthy and 9 women with RPL were included. Monocytes and CD4+ T-cells were isolated from 9 healthy non-pregnant women. For proteomic analysis, all 36 women were included. In the RPL group, 2 women had minor thrombophilia and 3 had hypothyroidism. In the remaining 4, no explanation for the RPL was found. The groups were matched for age and smoking.

Experimental Design

In this section I will give a general description of the experimental designs used in papers I-III. Detailed information is available in each paper.

In vitro macrophage polarisation

In papers I-III, we utilized our established macrophage polarisation assay (14,50) to generate macrophages *in vitro*. In brief, monocytes were isolated from peripheral blood from healthy non-pregnant women and cultured for 6 days in presence of growth factors and with addition of the CM to be studied. The presence of M-CSF induces an M2-like macrophage phenotype, similar to those found in the decidua. These are distinctly different compared to monocytes cultured with GM-CSF, which instead induce a pro-inflammatory M1-macrophage

phenotype (14,50,54,171). Or from IL4/IL-13, which induce a Th2-associated M2 macrophage phenotype (14,47,48,50). To identify the polarisation status of the macrophages we evaluated the surface expression of macrophage markers including CD14, CD86, CD163, CD206, CD209 and HLA-DR. Of these, CD163 and CD209 are considered to be M2 macrophage-specific in humans (172), and they are expressed by decidual macrophages (50). CD206 is also expressed by decidual macrophages and is commonly used as an M2 marker (50,64,173). However, we (14,50) and unpublished observations) and others (174) have noticed that CD206 is not specific for M2 macrophages since GM-CSF-polarised macrophages also express this marker and thus CD206 in itself cannot be used to differentiate between M1 and M2 macrophages (50).

Similarly, CD14, CD86 and HLA-DR are expressed on both M1 and M2 macrophages, but there is a discrepancy in their expression pattern on the different subsets. In particular, CD14 is highly expressed on decidual macrophages (50) and has a higher expression in M2 compared with M1 macrophages, which show lower expression (50,53,174). Decidual macrophages also express high levels of HLA-DR, but at the same time have low expression of the co-stimulatory molecule CD86 (65).

In our model system (Figure 4), GM-CSF and M-CSF are used as reference factors for induction of M1-like and M2-like macrophages, respectively, where we know that M-CSF is able to induce macrophages that are very similar to decidual macrophages (50). In addition, in order to investigate the M2 polarising ability of secreted products from cells and tissues, we also add different kinds of CM in combination with GM-CSF and use the ability to override the M1 promoting effects of GM-CSF as a readout. In this setting, addition of blocking agents was done to evaluate which factor(s) could reverse the M2-inducing properties. This principle was used in paper II to evaluate effects of factors secreted by decidual stromal cells.

In paper I, we polarised macrophages with IL-34 in addition to M-CSF and GM-CSF, which was used as representation of M2 and M1 states of polarisation. IL-34 was also used in combination with these factors. In paper II-III, macrophages were polarized using GM-CSF to which we also added DSC-CM or CVS-CM in different concentrations and analysed the ability of DSCs and the CVS to over-ride the pro-inflammatory properties of GM-CSF. In addition, in paper II, we also polarised macrophages using only DSC-CM, without any additional basic growth factor (Figure 4).

In vitro Th cell polarisation

In Paper II, we investigated if DSCs-derived factors could promote expansion of Treg cells. CD4+ T-cells were isolated from peripheral blood and incubated for 5 days in 100% DSC-CM (Figure 5). In paper III we investigated if placental tissue from women with a history of RPL had a compromised ability to generate Treg cells by culturing, isolated CD4+ T-cells with either 12.5% or 25% CVS-CM from women with a history of RPL or healthy controls for 5 days (Figure 5). In our model of Treg induction, CD4+ T-cells in a non-activated state are

exposed to CM from cells or tissues. It has previously been suggested that induction of Treg cells require TCR engagement for Treg induction (175). However, in previous work (14), we observed that CM from placental explants on its own induced expansion of Treg cells. This allowed us to imitate the normal resting state of the decidua, without any pro-inflammatory insults.

Figure 4. CD14+ monocytes isolated from peripheral blood were polarised into macrophages over 6 days in the presence of M-CSF, GM-CSF, IL-34, DSC-CM, CVS-CM or in combinations of these factors and CMs. At day 0 all stimuli were added with or without neutralizing antibodies, at day 3 the medium was changed. At day 6 the cells were harvested and analysed with flow cytometry. CVS, chorionic villous sampling, CM, conditioned medium, DSC, decidual stromal cell, GM-CSF, granulocyte macrophage-colony stimulating factor, M-CSF, macrophage-colony stimulating factor.

Figure 5. CD4⁺ T-cells isolated from peripheral blood were incubated either in culture medium or in decidual stromal cell-conditioned medium (DSC-CM) or chorionic villous sampling-conditioned medium (CVS-CM) for 5 days, with or without the specific inhibitor of SMAD3 (SIS3). On day 5 the cells were harvested and analysed by flow cytometry. CVS, chorionic villous sampling, CM, conditioned medium, DSC, decidual stromal cell, SIS3, specific inhibitor of SMAD3

Placenta and decidual explants

In paper I, we aimed to determine if IL-34 was produced by foetal and maternal tissues. For this purpose, 1st trimester placental villi and decidua were dissected into smaller pieces (explants) and incubated in culture medium for 20-24 h to allow for production of soluble factors (Figure 6). This CM was later used to run an IL-34 ELISA. Furthermore, some of the dissected tissues were also saved for IHC staining.

Term and 1st trim DSC-CM

DSCs from term placentas were isolated from women undergoing elective caesarean section. Briefly, decidual tissue was placed in flasks allowing DSCs to migrate out, colonising the

plate, and at 90% confluency they were harvested. These cells were subsequently re-plated, passaged and re-seeded into 24-well plates for 20-24 h to generate CM (Figure 6).

First trimester DSCs were isolated from elective pregnancy termination. Briefly, decidual tissues were dissected into smaller pieces and degraded enzymatically, creating a single cell suspension which was plated and allowed to grow until confluent. As for the term DSCs, these cells were subsequently re-plated, passaged and re-seeded into 24-well plates for 20-24 h to generate CM (Figure 6).

CVS-CM

CVS is an invasive, prenatal technique, in which a needle is inserted into the placenta, guided by ultrasound, and chorionic villi are collected through aspiration. The CVS technique is used to obtain foetal villous placental biopsies for diagnostic purposes, mainly to identify foetal chromosomal abnormalities. Surplus material from the procedure was incubated in culture medium for 20-24 h to generate CM (Figure 6).

Figure 6. DSCs isolated from 1st trimester and term placentas were expanded *in vitro*, cultured for 20-24 h to generate CM. Placenta from 1st trimester abortions or CVS sampling and 1st trimester decidua were cultured for 20-24 h to generate CM.

Throughout papers I-III we have been interested in determining the cytokine/chemokine profile of the tissues we used for polarisation, and also in paper I, we investigated secreted proteins from the IL-34 polarised macrophages. Measurements were done using different methods including IHC for tissue expression, and ELISA, multiplex bead assay (Luminex) and proximity extension assay (PEA) for secreted proteins.

In paper I, one aim was to determine if IL-34 is produced by foetal and placental tissues, and we therefore utilized IHC staining. IHC is a commonly used technique to visualise intra-cellular proteins and their location. Briefly, formalin-fixed tissue explants are embedded in paraffin and subsequently sectioned into 4 µm thin slices and mounted onto an objective glass. To stain the tissue sections, the paraffin must be removed and the tissue re-hydrated and nonspecific binding neutralised. Depending on the target molecule, antigen retrieval is often needed as well. Staining of the tissue section is done by incubating the slides with primary antibodies directed towards the protein of interest, followed by incubation with a secondary antibody targeting the primary one (indirect IHC). The secondary antibodies are conjugated with an enzyme that will react with an added substrate, creating colour. However, one cannot differentiate intra-cellular from extra-cellular proteins and it is hard to quantify the amount of proteins using this method. So, in addition to IHC, we also set up an IL-34 ELISA to determine secretion and amount of IL-34 in various CMs, and to quantify intracellular levels of IL-34 in lysed placental and decidual tissues.

ELISA is a common and robust method for quantifying proteins in a solution. In a sandwich ELISA, the wells of a 96-well plate are coated with primary capture antibodies, directed towards the protein of interest, after which unspecific binding is blocked. Samples are added, allowing binding of the protein to the capture antibodies. This is followed by addition of enzyme-conjugated detection antibodies that bind to the protein. Finally, a substrate is added that will react with the enzyme producing a colour change that is measured spectrophotometrically. In our study, we were not able to detect any secreted IL-34, which may be due to complex formation between IL-34 and M-CSF (176), resulting in inaccessibility to the epitopes which the antibodies bind to.

Multiplex bead assay was used to determine the secreted cytokine profile of IL-34 polarised macrophages (paper I) and of DSCs (paper II). The advantages of using this method compared to ELISA include the ability to measure more analytes in the same run, which reduces sample volumes and work efforts. Multiplex bead assay is also an antibody-based method, in which antibodies are coupled to beads filled with different mixtures of usually 2 dyes, diluted to a certain concentration. This dilution is what distinguished each individual bead. These antibody-conjugated beads are incubated with the samples, followed by addition of biotin-conjugated detection antibodies directed against other epitopes. In the

next step, streptavidin-phycoerythrin (PE) is added, which binds to biotin, and the PE fluorochrome is excited by a laser, giving a fluorescence signal proportionate to the amount of proteins bound.

Lastly, in paper II and III we performed protein profiling of DSC-CM and CVS-CM using the PEA technology (Figure 7), from Olink Bioscience Uppsala, Sweden. The inflammatory panel was used, which includes 92 pro-and anti-inflammatory proteins. In this method, oligonucleotide-conjugated antibodies, directed towards two different epitopes of the same protein of interest, are incubated with 1 μ l sample. Next, when pair-wise binding of the antibodies occurs, the proximity of the oligonucleotides initiates proximity-dependent DNA polymerization, creating new PCR targets. Real-time PCR (Fluidigm® BioMark™HD System) is then used to detect, amplify, and quantify the PCR targets (Figure 7). The acquired data is expressed as normalised protein eXpression (NPX) values, which are arbitrary units in a Log2 scale. Therefore, these values are not directly comparable between analytes and between different methods. Because the PEA uses antibody pairs directed towards two different epitopes on the same protein and requires both antibodies to bind, it has a very high specificity, and the PCR-based amplification and detection gives a high sensitivity.

Figure 7. Schematic representation of the PEA technique. Sample incubation with antibody pairs allows binding of the antibodies to their epitopes, enabling proximity-dependent DNA polymerisation, amplification, and detection of the PCR products.

Statistics

Detailed description of the statistics used is presented in the separate papers. Statistical analysis was performed using Graphpad Prism version 6, 7 or 8. In general, flow cytometry data was analysed using parametric tests, whereas cytokine data, apart from PEA, was analysed with non-parametric statistics. A p value <0.05 was considered statistically significant.

Ethical Statement

The studies have been approved by the local regional ethical review board in Linköping, and the samples collected elsewhere were approved by respective review boards (the regional ethical review board in Stockholm, the ethical committee of the Medical University of Vienna, the Institutional Review Board of Tor Vergata University Hospital, Rome and Newcastle and North Tyneside research ethics committee). Participants gave their written informed consent for participation in the studies. Samples collected are coded, data is presented at a group level, and participants do not receive information regarding individual tests. Participants were allowed to withdraw from the studies at any time.

Results and discussion

IL-34 is expressed by 1st trimester placenta and decidua

In paper I, we investigated if the cytokine IL-34 is present at the foetal-maternal interface by staining 1st trimester placenta and decidua tissues using IHC. In the placenta, the EVT cell columns (Figure 8A), as well as the chorionic villi, with their layers of cytotrophoblast and syncytiotrophoblast cells (Figure 8B), prominently stained positive for IL-34 (Figure 8C). We also identified cells positive for IL-34 in the decidua (Figure 8F and G), and to confirm that the IL-34+ cells were stromal cells, we stained sequential sections for either CD10 (stromal cells) (Figure 8J) or CD45 (leukocytes) (Figure 8I), showing a similar pattern of IL-34 and CD10 staining. Sequential staining of IL-34 and M-CSF revealed a similar expression pattern for the two CSF-1 receptor ligands in both the placenta (Figure 8D and E) and the decidua (Figure 8K and L). Next, we wanted to determine secretion of IL-34 in CMs from both placenta and decidua explants, but also in isolated EVTs, CTBs, STBs, CD10+ cells and placenta stimulated with LPS. Despite using three different ELISA kits, from different manufacturers, secreted IL-34 was not detected in any of the CMs. However, lysing the tissues and analysing the lysates revealed intracellular IL-34 (Figure 8 M). Similar findings were reported in a study investigating IL-34 production in epidermal tissue, where IL-34 could be detected in lysates of the tissue, but not secreted into the supernatant (177). Similarly, IL-34 mRNA was upregulated in a hepatocyte cell line challenged with hepatitis C virus, but no IL-34 could be detected in the supernatants (178). Since M-CSF and IL-34 showed a similar expression pattern in placenta and decidua, the inability to detect secreted IL-34 in cell supernatants might be because of the formation of a complex between M-CSF and IL-34 (176), which could impair antibody binding to IL-34.

IL-34 polarised macrophages have similar characteristics to that of M-CSF polarised macrophages and decidual macrophages

Since we found that IL-34 was expressed by both foetal and maternal tissues, and it has been shown to drive polarisation of macrophages to a more suppressive phenotype compared to that of GM-CSF and IFN- γ (60), we hypothesised that IL-34 is involved in generation of decidual macrophages. As hypothesised, macrophages polarised with IL-34 acquired a high expression of CD163, CD14 and CD209 and a low expression of CD86, similar to that of M-CSF induced macrophages and macrophages found in the decidua, while contrasting macrophages polarised with the more pro-inflammatory GM-CSF (Figure 9A). Although similar in several respects, we also observed differences between IL-34- and M-CSF-induced macrophages. These differences were most pronounced for CD14 and HLA-DR, with a higher median fluorescence intensity (MFI, *i.e.* expression per cell) in IL-34-induced macrophages (Figure 9A).

Figure 8. Interleukin-34 is expressed in first trimester human placenta and decidua.

Immunohistochemistry staining of formalin-fixed 1st trimester tissues shows staining of IL-34 in (A) cyto- and syncytiotrophoblast cells (CTB and STB), as well as in extravillous cell columns (EC, see arrows) in the placenta. (B) Higher magnification of IL-34 staining in CTBs and STBs in placenta, and (C) isotype-matched control. (D) IL-34 and (E) macrophage colony-stimulating factor (M-CSF) is expressed by CTB and STB in 1st trimester placenta (arrows). (F) Staining of IL-34 in decidual stromal cells (DSC), with arrows showing DSC, blood vessel (BV) and glandular epithelium (GE). (G) High magnification of IL-34 stained DSC. (H) Isotype-matched control for decidua. To confirm that IL-34 staining was confined to stromal cells, decidua was stained with (I) CD45 for leukocytes (non-stromal cells, see arrows) and with (J) CD10 for stromal cells. (K) IL-34 and (L) M-CSF is expressed by stromal cells in the decidua. Tissue lysates were analysed with IL-34 ELISA; medians are shown, and the dotted line denotes the detection limit. BV, blood vessel, CTB, cytotrophoblast, DSC, decidual stromal cell, EC, Extravillous trophoblast cell column, GE, glandular epithelium. STB, syncytiotrophoblast

To further elucidate the effect of IL-34 on macrophage polarisation, we analysed secretion of IL-10, IL-12p70, TNF, CCL2 and CXCL10 in collected supernatants. Again, a mostly similar pattern was noted for IL-34- and M-CSF-polarised macrophages, with lower amounts of the pro-inflammatory TNF and a tendency to higher IL-10 production compared to GM-CSF-polarised macrophages. A minor difference was that M-CSF macrophages showed higher secretion of CCL2 than IL-34 macrophages (Figure 9B). Since IL-34 and M-CSF can act in synergy with each other (176), and since they showed similar, but not identical, effects on the polarisation of macrophages, we also investigated their combined effects. A combination of M-CSF and IL-34 revealed a predominance of M-CSF features (for details see paper I).

The similar phenotype of IL-34-induced macrophages to that of M-CSF-induced macrophages and macrophages found in the decidua, indicates that IL-34 is involved in shaping the tolerant milieu at the foetal-maternal interface. The discrepancy we observed in the phenotype of IL-34- and M-CSF-induced macrophages, might be explained by a difference in intracellular signalling, following receptor activation (179), indicating non-redundant effects of IL-34. For example, IL-34-polarised macrophages express a different repertoire of genes compared to that of M-CSF-polarised macrophages (180), and IL-34, but not M-CSF, is needed for the development of microglia and Langerhans cells in mice (58,59). However, there is also evidence of a complementary role of IL-34, as it has been shown in M-CSF-deficient mice that IL-34 is able to compensate for the lack of M-CSF and rescue defects in osteoclasts, tissue macrophages and fertility (181). Interestingly, it was recently reported that IL-34 polarised macrophages produced high amounts of IL-10 when challenged with LPS and IFN- γ , which was in contrast to M-CSF-polarised macrophages (182). Thus, IL-34 might carry an important feature to ensure a tolerant milieu at the foetal-maternal interface during inflammatory insults.

Aberrant IL-34 expression was not found in preeclamptic women

Increased levels of IL-34 have been reported in patients with inflammatory disorders, such as RA and Sjögren's syndrome (183–185). Since preeclampsia is an inflammatory placental disorder, involving both local and systemic inflammation, we wanted to investigate if there was a deviation of IL-34 expression both locally and systemically in preeclamptic women compared to healthy pregnant women. We therefore used ELISA to measure IL-34 levels in plasma samples from healthy controls (n=83) and from preeclamptic women (n=85). However, no difference was found between the two groups, which was also true for M-CSF in the same material (128). Next, to investigate any local dysregulation of IL-34 expression, we stained placental biopsies from healthy controls (n=15), early onset preeclampsia (EOP) (n=9) and late onset preeclampsia (LOP) (n=12) for IL-34 using IHC. Again, no differences were found among the groups (Figure 10I). However, expression of M-CSF was higher in both EOP and LOP compared to healthy controls (Figure 10I), which might be interpreted as a countermeasure to combat the increase in inflammation and M1 scenario present during preeclampsia (140).

Figure 9. IL-34 induces macrophages with a phenotype similar to that of M-CSF and significantly different from GM-CSF-induced macrophages. (A) Blood monocytes from healthy non-pregnant women were differentiated to macrophages by culturing in the presence of GM-CSF ($n = 10$), M-CSF ($n = 10$), 50 or 100 ng/ml interleukin (IL)-34 ($n = 13$ and $n = 9$, respectively) for 6 days and the phenotype was assessed by flow cytometry. (B) Levels of secreted cytokines and chemokines were measured in cell-free supernatants using the same culture conditions ($n = 7$ for all conditions). Flow-cytometry data were analysed with one-way ANOVA and the Sidak multiple-comparison test, cytokine and chemokine data were analysed with Kruskal-Wallis test and Mann-Whitney. Graphs show the mean and SD (A) and the median (B). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. GM-CSF, granulocyte macrophage-colony stimulating factor, M-CSF, macrophage-colony stimulating factor, MFI, median fluorescence intensity.

Figure 10. Expression of M-CSF and IL-34 in placenta. Expression of M-CSF is increased in placentas from women with early onset (EOP) and late onset preeclampsia (LOP), compared with healthy controls, whereas no significant differences could be seen for IL-34.

Immunohistochemistry of formalin-fixed, paraffin embedded term placentas from normal controls (n = 15), EOP (n = 9) and LOP (n = 12) was performed. (A-D) Representative pictures of M-CSF staining in placentas from (A) control, (B) EOP, (C) LOP and (D) isotype matched-control. (E-H) Representative pictures of IL-34 staining in placentas from (E) control, (F) EOP, (G) LOP and (H) isotype control. (I) The staining intensity of IL-34 and M-CSF expression in placental villi is given in arbitrary units (A.U.; see Methods). Data were analysed using Kruskal-Wallis and Mann-Whitney tests, graphs show medians. *p ≤ 0.05. EOP, early onset preeclampsia, ctrl, control, LOP, late onset preeclampsia.

Decidual stromal cells induce regulatory M2 macrophages and promote their survival

In paper I we observed production of M-CSF and IL-34 by DSCs and previous studies have indicated a role in immunosuppression for DSCs (28,186). This prompted us to, in paper II investigate the potential role of DSCs in the induction of tolerance at the foetal-maternal interface with regards to the ability of inducing regulatory phenotypes in macrophages and T-cells. When monocytes were cultured with GM-CSF, the addition of DSC-CM was able to over-rule, in a concentration dependant manner, the pro-inflammatory properties of GM-CSF. This was revealed by increased expression of CD163, CD209 and CD14, and a decrease in CD86, *i.e.* a phenotype similar to that induced by M-CSF and that of decidual macrophages

Figure 11. Conditioned medium from DSCs promotes induction of regulatory M2 macrophages. Blood monocytes from healthy women were differentiated into macrophages by culturing them in the presence of (A-D) GM-CSF or GM-CSF in combination with 25% or 50% decidual stromal cell conditioned medium (DSC-CM) (CD14, $n=6$ for the rest $n=7$) or (F-I) in either 100% CM from term DSCs ($n=12$) or (K-N) 1st trim DSCs ($n=8$). Phenotypes were evaluated by flow cytometry. (E) Viability stain of macrophages cultured with GM-CSF + 25 and 50% DSC-CM ($n=7$), (J) viability stain of macrophages cultured with 100% term DSC-CM ($n=12$) and (O) viability of macrophages cultured with 100% 1st trim DSC-CM ($n=7$), shown as proportion of Annexin V and 7AAD double-negative cells. Data were analysed by one-way ANOVA with Sidak's multiple comparison test. Graphs show the mean and SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. CM, conditioned medium, DSC, decidual stromal cell, GM-CSF, granulocyte macrophage-colony stimulating factor, M-CSF, macrophage-colony stimulating factor, MFI, median fluorescence intensity, MΦ, Macrophages.

(Figure 11A-D). In addition to these phenotypic changes, we also observed that cells cultured in DSC-CM had a higher proportion of viable cells compared to GM-CSF alone (Figure 11E), which prompted us to investigate if DSC-CM on its own could promote survival and polarisation of monocytes into macrophages. For this purpose, we cultured monocytes in DSC-CM alone and the phenotype was compared to macrophages cultured with M-CSF, as a representative of M2-like decidual phenotype of macrophages and GM-CSF as a representative of M1-like macrophages. Indeed, macrophages cultured in DSC-CM alone showed a high viability compared to both M-CSF and GM-CSF, as well as a high expression of CD163, CD14 and CD209, similar to that of M-CSF. These findings were observed using DSCs from term placentas (Figure 11F-J) as well as from 1st trimester placentas (Figure 11K-O), indicating that this ability is maintained throughout pregnancy.

Protein screening of DSC-CM

To establish a protein profile of DSC-CM from 1st trimester and term pregnancies we screened 24 h supernatants for 92 different pro- and anti-inflammatory proteins using PEA, and for 26 proteins using multiplex bead assay. In general, CM from 1st and term DSCs showed similar expression levels. Interestingly, DSCs produced large amounts of M-CSF, while being low producers of other growth factors, such as GM-CSF, G-CSF and IL-3, the latter not being detected. Furthermore, we found that TGF- β was highly expressed by DSCs as well. Both M-CSF and TGF- β were shown to be involved in the induction of M2-like macrophages and Treg cells by foetally-derived trophoblasts (14). Besides M-CSF and TGF- β , DSCs were found to produce a variety of regulatory proteins including LIF, CCL2 and hepatocyte growth factor, as well as pro-inflammatory cytokines like CXCL8 and IL-6. In addition, DSCs also secreted MMP-1 and MMP-10, proteases involved in tissue remodelling, and VEGF, which is a major angiogenic factor, all together showing the multipotency of DSCs. Of note, we did not detect the major suppressive cytokine IL-10 in our supernatants, nor did we detect the Th2-associated proteins IL-4, IL-13, IL-33 and TSLP or the Th17-associated IL-17A and CCL20. However, the Th1-associated CXCL10 was detected whereas CXCL9, CXCL11 and IFN- γ were not. Pregnancy has been described as a Th2-phenomenon, but the lack of Th2 cytokine production by the largest cell population in the decidua, does not support this. Also in support of this notion, mice deficient for the Th2-related cytokines IL-4, IL-5 IL-13 and IL-9 were shown to have a normal pregnancy (187). Rather, the production of M-CSF, TGF- β and LIF would suggest a more regulatory state. In addition, IL-10, which is not a Th2 cytokine, is important at the foetal-maternal interface, albeit it is not produced by DSC but by for example trophoblasts (14) and macrophages (66).

M-CSF is partially responsible for the M2-inducing and viability-promoting effects of DSCs

In order to determine which factors were responsible for the observed M2 induction and high viability, we added neutralising antibodies towards M-CSF, which is a known M2-inducing factor. We have previously shown that M-CSF is involved in trophoblast induction of regulatory M2 macrophages (14) and we observed in paper I that DSCs stained positive for M-CSF, and as shown by the PEA, it was secreted by DSCs. When we added M-CSF neutralising antibodies during the polarisation process with DSC-CM, either with GM-CSF as a basal growth factor or with DSC-CM alone, a significant decrease in the protein expression and cell density of the regulatory marker CD163 was observed (Figure 12A-B and D-E). Inconsistent changes were seen for the other markers. The reduced expression of CD163 was coupled with a reduced viability, at least for cells cultured with GM-CSF and DSC-CM (Figure 12C). No differences were seen in the viability for cells cultured in DSC-CM alone (Figure 12F). Collectively, these findings provide evidence that M-CSF, at least in part, is responsible for the induction of the M2 phenotype and viability-promoting effects. However, since M-CSF blocking only partially reversed the CD163 induction and viability, and since only CD163 was affected, other factors must contribute as well. One such factor is of course IL-34, since it has similar polarisation properties to that of M-CSF and is expressed by DSCs (paper I). However, as stated above, we were unable to measure secreted IL-34 and could not neutralise the effects of IL-34 using IL-34 neutralising antibodies, thus this was not further pursued in paper II. Another factor to take into consideration is IL-10, which is an important immune-modulating cytokine with the ability to induce regulatory M2 macrophages (14,50). However, IL-10 cannot on its own promote survival of macrophages, and as shown by the PEA screening as well as by multiple bead assay, IL-10 is not produced by DSCs, which is in line with previous observations (186). Interestingly, VEGF has been suggested to be able to promote M2 induction (188) and was found to be a highly abundant protein produced by DSCs. However, it is doubtful if VEGF has any effect on viability. Other growth factors that could be involved include GM-CSF, IL-3, and G-CSF, but these factors were either not secreted or secreted at a very low level by DSCs. Thus, M-CSF seems to have an important role in DSC-mediated tolerance induction, a mechanism that is shared with trophoblasts that are also high producers of M-CSF (14). Additional mechanisms of DSC-induced M2 induction and viability promotion remain to be determined.

Figure 12: Neutralizing M-CSF in cultures with conditioned medium from term decidual stromal cells reduces the expression of the M2 marker CD163. Macrophages were generated by culturing blood monocytes from healthy women, for 6 days in the presence of GM-CSF and 50% conditioned medium (CM) from term decidual stromal cells, with either isotype control antibodies or αM-CSF antibodies (A-C). In (D-F), macrophages were generated by CM from term decidual stromal cells alone, with the addition of isotype control antibodies or with αM-CSF antibodies. Expression of CD163 and viability markers (Annexin V and 7AAD) were evaluated by flow cytometry. (A-C n=10, D-E n=7, F n=5). Data were analyzed by one-way ANOVA with Sidak's multiple comparison test. Graphs show mean and SD. * p < 0.05, ** p < 0.01, *** p < 0.001. αM-CSF, anti-macrophage-macrophage colony stimulating factor, CM, conditioned medium, ctrl, control, GM, granulocyte macrophage-colony stimulating factor, MFI, median fluorescent intensity.

Decidual stromal cells induce Treg cells in a TGF-β dependent manner

DSCs have previously been shown to induce Treg cells in a contact-dependent manner and reduce the proliferation of alloreactive T-cells (28). We therefore wanted to investigate if DSCs also possess the ability to induce Tregs by production of soluble molecules. For this, we cultured CD4⁺ T-cells in DSC-CM for 5 days. CD4⁺ T-cells incubated together with DSC-CM acquired a higher proportion of CD25^{bright} Foxp3⁺ Treg cells compared to the CD4⁺ T-cells cultured in control culture medium. This was true for CM from both term DSCs and from 1st trimester DSCs (Figure 13A and B). We have previously shown that Treg induction by trophoblast cells is mediated by TGF-β, IL-10 and TRAIL, as shown by neutralisation experiments (14). However, when using neutralising antibodies towards these factors during

polarisation with DSC-CM, no difference in phenotype was observed (data not shown). Accordingly, IL-10 and TRAIL were found to not be produced or produced at low levels. TGF- β was found to be highly expressed, and we therefore targeted intracellular SMAD3 using a blocking agent called SIS3 (specific inhibitor of SMAD3). SIS3 gets phosphorylated upon TGF- β receptor activation and translocates to the nucleus (189) where it binds to the *Foxp3* enhancer element CNS1 (190).

Addition of SIS3 almost completely abolished the induction of Treg cells (Figure 13), indicating a major role for TGF- β signalling in DSC-mediated Treg induction. It has been suggested that the ability to induce peripheral Treg cells is an evolutionary consequence of eutherian placentation and evolved as a mechanism to alleviate the foetal-maternal conflict (99). The authors reported that only placental mammals expressed the enhancer element CNS1, which was shown to be necessary for induction of peripheral Treg cells. Mice lacking this enhancer had increased inflammation and aberrant spiral artery remodelling, as well as higher rates of resorptions in allogeneic pregnancies, which was attributed to a lack of foetus specific pTreg cells (99). Also in human pregnancies, aberrations in the Treg cell compartment have been implicated in complications, among them, lower frequencies and improper suppressive ability have been reported in women with uRPL (147), which might be due to an increase in the proportion of Th17 cells (148,149). Similar observations have been done in preeclamptic women (191,192), supporting a major role for Treg cells in promoting a successful pregnancy. In our study, induction of Treg cells was evaluated by phenotypic features, thus it is a limitation that we did not evaluate their suppressive function. Another limitation is that TGF- β is not the only molecule that can activate SMAD3 (193), so we cannot rule out that other members of the TGF- β family are responsible for the inductions of Treg cells. Interestingly, we observed similar levels of TGF- β and Treg induction from 1st trimester and term DSCs, suggesting that the ability to induce Treg cells is maintained throughout pregnancy. This challenges the concept that 1st trimester is defined as a pro-inflammatory state, which in the second trimester becomes an anti-inflammatory state, followed by a pro-inflammatory state in the third trimester (68). Thus, it is likely that both pro-and anti-inflammatory mechanisms prevail in the 1st and 3rd trimesters. Taken together, we have shown that DSCs induce Treg cells by activation of SMAD3 and that this ability is maintained throughout pregnancy.

Figure 13. Conditioned medium from 1st trimester and term decidual stromal cells induce expansion of regulatory T cells, which is reversed when adding the SMAD3 blocker SIS3. CD4⁺ T cells were isolated from healthy, non-pregnant women and cultured for 5 days in either (A) 1st trimester decidual stromal cell conditioned medium (CM), with or without vehicle control or 7.5µM SIS3 (n=6); or in (B) CM from term decidual stromal cells with or without vehicle control or 7.5µM SIS3 (n=6). Expression of Foxp3 and CD25 were analysed by flow cytometry. Data were analysed with one-way ANOVA with Sidak's multiple comparison test. Graphs show the mean and SD. * p < 0.05, ** p < 0.01. CM, conditioned medium, ctrl, control, DSC, decidual stromal cell, SIS3, specific inhibitor of SMAD3, Treg, regulatory T-cell, VC, vehicle control.

The placenta retains its ability to promote expansion of tolerogenic leukocytes in women with a history of uRPL

In paper III, we wanted to investigate if the placenta from women with a history of uRPL showed a compromised ability to induce M2-like macrophages and Treg cells compared with placentas from healthy controls. For this purpose, we utilised leftover materials from CVS, from which we produced CM that was used to polarise macrophages and T-cells. Blood monocytes from healthy women were cultured together with GM-CSF and CVS-CM from either controls or from uRPL patients. Notably, both control and uRPL CM was associated with a high expression of the M2 markers CD163, CD14 and CD209 (Figure 14A-C) and a low expression of CD86 (shown in paper III), compared to macrophages cultured in GM-CSF alone. Similarly, when culturing peripheral blood CD4⁺ T-cells together with control CVS-CM and uRPL CVS-CM there was an expansion of Foxp3⁺CD25^{bright} Treg cells compared to cells cultured in medium alone. Also here, we did not observe any differences between control

CVS-CM and uRPL CVS-CM (Figure 14D). In line with this, no deviation of secreted proteins was found in CVS-CM from women with a history of uRPL compared to healthy controls, suggesting a normal placental function.

It is important to consider that all women (those with a history of uRPL as well) had a normal term pregnancy, which indicates that no dysregulation took place, and hence no differences could be detected. The majority of miscarriages occur early in the pregnancy, around GW 6-8 (194), whereas CVS sampling can only be done after GW 11. If no underlying cause for RPL can be found, the general conception is that the chance of a successful subsequent pregnancy is good (195), which might be attributed to the immunological memory of pregnancy that is imprinted in the maternal immune system (100). Furthermore, we only investigated the role of the foetal part of the placenta in this study and cannot rule out the importance of other compartments. For example, emerging evidence suggests that dysregulation of the decidua contributes to pregnancy complications, including RPL (196,197), as well preeclampsia (198). With the prominent role of DSCs in promoting induction of tolerogenic macrophages and Treg cells, DSCs are an interesting target to investigate in pregnancy complications. Our proteomic screening revealed that the chorionic villous favoured production of regulatory cytokines, such as M-CSF, IL-10, LIF and TGF- β over that of Th2 and Th17 cytokines such as IL-2, IL-4, IL-5, IL-13, IL-17A and IL-17C, which is in line with what we have previously shown (14).

Figure 14. (a-c) Blood monocytes from healthy non-pregnant women were differentiated to macrophages for 6 days with GM-CSF in the absence or presence of 12% or 25% CVS-CM from control (ctrl) women (n=9) and from women with recurrent pregnancy loss (RPL, n=9). d) CD4+ T-cells isolated from healthy non-pregnant women were cultured 5 days in tissue culture medium (TCM) in the absence or presence of 12% or 25% chorionic villous sampling conditioned medium (CVS-CM) from control (ctrl) women (n=9) and from women with recurrent pregnancy loss (RPL, n=9). Phenotypic characterizations were done by flow cytometry. Data are shown as mean and S.D. One-way ANOVA and Sidak' s test: *p<0.05; **p<0.005. ctrl, control, CVS, chorionic villous sampling, GM-CSF, granulocyte macrophage-colony stimulating factor, RPL, Recurrent pregnancy loss, TCM, T-cell culture medium, Treg, regulatory T-cell.

Summary and conclusion

In paper I, we investigated the role of the second ligand to the CSF-1 receptor, IL-34, in promoting foetal tolerance. We showed that IL-34 is expressed by both the foetal placenta (STBs, CTBs and EVT_s) and by the maternal decidua (DSC_s) at the foetal-maternal interface. Macrophages cultured in the presence of IL-34 acquired high expression of the M2-related markers CD163, CD209 and CD14, produced high amounts of IL-10 and low amounts of the pro-inflammatory TNF, which is similar to that of M-CSF-polarised macrophages. However, a few discrepancies between the two were found, with a higher cell density of HLA-DR and CD14 in IL-34 macrophages. However, combining IL-34 and M-CSF induced macrophages that were phenotypically closer to M-CSF than IL-34 macrophages. Furthermore, since IL-34 and M-CSF macrophages share phenotypic characteristics of decidual macrophages, important for tolerance at the foetal-maternal interface, we wanted to investigate if there was a compromised ability to produce these cytokines in preeclampsia. However, we found no evidence of an aberrant abundance of IL-34 levels in preeclampsia, neither in plasma, nor in the chorionic villous in the placenta. Taken together, we showed that IL-34 is expressed at the foetal-maternal interface and that it has the ability to induce macrophages with a phenotype similar to that of M-CSF and decidual macrophages.

In paper I we showed that:

- IL-34 is expressed by both the foetal placenta and the maternal decidua.
- IL-34 is able to polarise macrophages into a regulatory phenotype, similar to M-CSF and decidua macrophages.
- IL-34 expression locally and systemically is not altered in preeclampsia.

Thus, IL-34 is present at the foetal-maternal interface and seems to have a regulatory role similar to that of M-CSF.

In paper II, we investigated the role of DSC_s in promotion of foetal tolerance by induction of regulatory M2 macrophages and Treg cells. We again utilised our established macrophage polarisation model where monocytes are polarised to an M2-like phenotype using M-CSF and to an M1-like phenotype using GM-CSF. The addition of DSC-CM to GM-CSF polarisation enables us to evaluate the ability of DSC derived factors to promote M2-polarisation.

We showed that CM from DSC_s was able to, in a concentration dependent manner, over-rule the pro-inflammatory properties of GM-CSF, in favour of induction of macrophages with the M2-related markers CD163, CD14 and CD209 and a low expression of the co-stimulatory CD86. In this setting, we also observed that cells cultured with DSC-CM had higher viability than cells cultured with M-CSF or GM-CSF alone. The higher viability prompted us to polarise macrophages using DSC-CM only, without any additional growth factors. Indeed, culturing of macrophages in CM alone resulted in macrophages expressing high levels of CD163, CD209 and CD14, while having low CD86 expression and high viability. This was true for DSC_s from both 1st trimester and term placentas. Next, blocking experiments showed that M-CSF is in

part responsible for induction of the M2-phenotype, which is in line with the previously observed trophoblast-mediated induction of regulatory M2 macrophages (14). Furthermore, blocking experiments showed that M-CSF was in part responsible also for the viability-promoting effect of DSCs. In addition, DSC-CM promoted expansion of Foxp3+CD25^{bright} Treg cells during *in vitro* culture. This expansion was dependent on TGF- β family-signalling and subsequent SMAD3 activation. We also showed that DSCs produce a variety of cytokines and chemokines, including the regulatory TGF- β and M-CSF, while low to no production of Th17- and Th2-associated proteins was found. These data demonstrate that DSCs are able to promote local tolerance induction during pregnancy by inducing regulatory macrophages and Treg cells.

In paper II we showed that:

- DSCs produce factors that over-rule the pro-inflammatory effects of GM-CSF and induce regulatory M2 macrophages.
- DSCs produce factors that promote survival of macrophages.
- M-CSF is partially responsible for the polarising effects on macrophages and the viability.
- DSCs induce expansion of Treg cells, which is dependent on TGF- β signalling and SMAD3 activation.
- DSC produce a spectrum of proteins including M-CSF and TGF- β , as well as several pro- and anti-inflammatory proteins

Thus, decidual stromal cells are able to contribute to foetal tolerance by producing factors, including M-CSF and TGF- β , that shape immune regulatory phenotypes of macrophages and T-cells.

In paper III, we used surplus chorionic villous material from CVS to generate CM. These samples were taken from women with a history of uRPL and from control women. We wanted to investigate if the placenta from women with a history of uRPL had a compromised ability to induce regulatory M2 macrophages and Treg cells compared to healthy controls. In line with what we have previously shown (14), the chorionic villous induced macrophages with high expression of the M2 markers CD163, CD14 and CD209 and expanded the Treg cell population during *in vitro* cultures. However, no differences were seen in uRPL CVS compared to control CVS, neither in macrophage polarisation and Treg induction, nor in the secreted protein profile. Taken together, these data indicate that the placenta retains its regulatory properties in women who has a history of uRPL, at least when there is a successful pregnancy.

In paper III we showed that:

- Placenta from uRPL patients retains its ability to induce Treg cells and regulatory M2 macrophages
- No aberrations in cytokine production is present in placentas from uRPL patients.

Figure 15. DSC-derived factors (M-CSF, IL-34, TGF-β) and trophoblast derived factors M-CSF, IL-34, TGF-β, IL-10 and TRAIL may promote foetal tolerance by inducing regulatory M2 macrophages and Treg cells. With additions from Svensson-arvelund *et al.* J Immunol. 2015

Future perspectives

The data presented in this thesis provides further insight into how the intricate network at the foetal-maternal interface promotes survival and induction of regulatory immune cells, important for inducing and maintaining tolerance.

Although we showed that the maternal tissue resident stromal cells were able to promote expansion of Treg cells and regulatory M2 macrophages, some questions are still left unanswered. One of these questions is which other DSCs-derived factors, besides M-CSF, are involved in induction of M2 macrophages and viability. One way to investigate this would be to fractionate the DSC-CM, pinpoint which fraction induce these effects, narrow down the search, and possibly identify the responsible protein. In addition, we found a prominent ability of DSCs to produce a variety of chemokines, which warrant further investigations into their role in the recruitment of, not only leukocytes into the decidua, but also their role in recruiting and directing trophoblast invasion. Another aspect worth investigating is the functionality of our *in vitro* generated Tregs and macrophages, how these cells interact with other decidual cell populations, their suppressive capacity etc. Furthermore, it would be interesting to perform a wider array of phenotypic/genomic screening on the *in vitro* generated Tregs and macrophages and compare the phenotypes with the corresponding cells isolated from decidual tissue.

Another worthwhile pursuit would be to further investigate the role of the decidual stromal cells in pregnancy complications. Emerging evidence (196–198) suggests alterations in the decidual response during pregnancy complications. Since we know that DSCs from normal pregnancies contribute to homeostasis, it would be interesting to perform similar experiments from DSCs isolated from complicated pregnancies. However, this raises the question, if a dysregulation is present, is this a consequence of the complication or the cause of it? If there was a pre-existing problem, it is possible that it is present in the pre-decidualised endometrium as well, thus investigation of the function and characteristics of endometrial stromal cells from women with a history of pregnancy complications might provide further insights. However, obtaining this material might prove hard. One interesting possibility would be to collect menstrual blood from women with a history of pregnancy complications and follow up on the development of subsequent complications. This would provide an opportunity to not only characterise stromal cells, but also the leukocyte populations. This might also provide a tool to predict if a pregnancy complication will manifest so that early interventions can be started.

CSFs are not only important for haematopoiesis, but also major drivers of macrophage polarisation and can thus drive a pro-inflammatory or homeostatic response (51). A balance of CSFs is important for reproductive success (199), and imbalance of CSFs has been found in preeclampsia (140). Also, aberrantly activated macrophages have been found in RPL decidua (155,156), implicating a skewed CSF response. Restoring the balance of CSFs has the potential of being the source of possible future treatment options for pregnancy complications. The balance could be restored by neutralising GM-CSF or its receptor. In mouse models of inflammatory diseases like MS (200) and collagen induced arthritis (201) it

has been shown that mice lacking GM-CSF are resistant to disease development, implying that GM-CSF might be a possible target in other inflammation related diseases. Furthermore, in paper II we observed that DSCs were able to counteract the pro-inflammatory properties of GM-CSF, which indicates that they have the ability to control inflammation and are a potential source of treatment for inflammatory diseases. Indeed, the immune-modulating properties of DSCs are under investigation as a novel treatment strategy for alleviating inflammation in graft-versus-host disease (202,203). Also, taking into consideration the improvement of autoimmune diseases like MS and RA during pregnancy, DSCs might prove to be a potential treatment strategy for these conditions.

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