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IMMUNOLOGY OF THE UTERINE MUCOSAE

Dorien Feyaerts
Immunology of the uterine mucosae
PhD thesis, Radboud University, the Netherlands

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IMMUNOLOGY OF THE UTERINE MUCOSAE

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from Radboud University Nijmegen
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CHAPTER 1

General introduction and scope of this thesis
The central role of the human immune system is to recognize self from non-self and dangerous antigens, and to mount an immune response against those dangerous antigens. However, pregnancy is a unique situation where two histo-incompatible organisms peacefully co-exist, even though semi-allogeneic cells of fetal origin come into close contact with maternal immune cells in the uterine mucosa. The uterine mucosa is a unique immunological mucosal barrier that not only provides protection against infections, but also possesses unique tasks that include tissue remodeling during the menstrual cycle and promoting pregnancy success by enabling implantation, placentation, and maintaining tolerance towards fetal antigens. A dysbalanced uterine immune environment has been implicated in the pathophysiology of pregnancy complications, such as pre-eclampsia, recurrent miscarriages, recurrent implantation failure, preterm birth and uterine growth restriction. Unraveling the mechanisms at play at the uterine mucosae will be crucial to our understanding as to why some pregnancies are not successful, and will improve our options for treatment of pregnancy complications.

The focus of this thesis is to increase our insight into the immunology of the uterine mucosae and its dynamic changes during human pregnancy, by examining the phenotype of uterine mucosal immune cells in both the non-pregnant (endometrium) and pregnant state (decidua).

**IMMUNOLOGY OF THE UTERINE MUCOSAE**

The uterine mucosa is highly adaptable and changes its morphology, cell composition, and function depending on the physiological situation. The inner mucosal lining of the non-pregnant uterus is called the endometrium. Each month, under influence of hormones, the endometrium prepares itself for embryo implantation. When pregnancy ensues, the endometrium transforms into what we call the decidua, the maternal part of the placenta. The uterine immune system in the endometrium and the decidua has different specialized functions. In the following chapters, we will address in more detail the adaptations and function of the mucosal immune system of both the endometrium and decidua.

**Immunity in the non-pregnant uterus**

Under influence of ovarian hormones progesterone and estradiol, the morphology and cell composition of the endometrium changes over the course of the menstrual cycle (Fig. 1A). The cyclic rounds of tissue breakdown and regeneration during the menstrual cycle, makes the endometrium unique in comparison with other mucosal
surfaces. The menstrual cycle consists of two phases. The functional layer is regenerated during the proliferative phase, while during the secretory phase, there is an increase in glandular activity, decidualization and influx of immune cells to prepare for a possible embryo implantation. After ovulation, the endometrium transforms into a receptive state suitable for implantation. This process is called decidualization and is independent of the presence of an embryo. Decidualization encompasses vascular remodeling and the transformation of stromal fibroblasts into specialized decidual cells that provide a structural framework for embryo implantation and trophoblast invasion. Next to fibroblast changes, the number of immune cells increases during the decidualization phase, with the greatest abundance of endometrial immune cells at the late secretory phase. These immune cells are important for implantation success, as discussed later. The endometrium is only receptive for blastocyst implantation for a short period of time (2-4 days). This period of receptivity is called the window of implantation. Without fertilization and embryo formation, progesterone and estradiol levels will drop, resulting in the initiation of menstruation and shedding of the endometrium. Hence, a non-invasive way to study endometrial immune cells can be achieved by the collection of menstrual fluid, thereby bypassing the need for endometrial biopsies.

Natural killer (NK) cells, macrophages and T cells are the most abundant immune cells in the secretory endometrium. T cells can be found in lymphoid aggregates present in the basal layer of the endometrium and their cell numbers do not change over the course of the menstrual cycle. T cell numbers do not change over the course of the menstrual cycle. While the majority of peripheral blood T cells are CD4+ T cells, the CD4/CD8 T cell ratio is reversed in the endometrium. Endometrial CD8+ T cells exert cytolytic activity during the proliferative phase but lose this ability during the secretory phase. This absence of cytolytic activity during the window of implantation may support the implantation of the semi-allogeneic blastocyst. However, it may also contribute to the increased susceptibility for HIV infection during this phase of the menstrual cycle. In the endometrium, there is a higher percentage of Th1 T cells compared to Th2 T cells. The Th1/Th2 ratio decreases over the course of the menstrual cycle, possibly to support implantation.

Under the influence of progesterone, stromal cells will produce IL-15. IL-15 supports the proliferation and differentiation of endometrial NK (eNK) cells. eNK cell numbers increase during the menstrual cycle, with around 80% of CD45+ cells being NK cells in the late secretory phase. eNK cells have a distinct phenotype compared to their peripheral counterparts. The majority of eNK cells are CD56bright and CD16, while in peripheral blood the majority of NK cells are CD56+CD16+. eNK cells lack cytotoxic activity and secrete low levels of cytokines, unless activated by IL-15. In addition, eNK cells have a unique NK cell receptor (NKR) repertoire, which is stable over different menstrual cycles. During the menstrual cycle, NK cells are suggested to play a role in mucosal integrity and endometrial
angiogenesis by secreting IL-22 and angiogenic growth factors, respectively \textsuperscript{25,26}. In addition, the IL-15-eNK cell axes has been shown to play a role in endometrial bleeding \textsuperscript{21}.

Figure 1 Immunity in the non-pregnant uterine mucosa. (A) Representation of the different phases of the menstrual cycles. The morphology of the endometrium (i.e. non-pregnant uterine mucosa) changes under influence of hormone fluctuations. After ovulation, decidualization starts and immune cell numbers increase in the endometrium. The endometrium is only receptive for blastocyst implantation 6 to 10 days after ovulation. Without implantation, menstruation is initiated. (B) NK cells, macrophages, neutrophils and mast cells in the endometrium are involved in mediating tissue remodeling during the menstrual cycle via the production of cytokines, angiogenic factors and proteases. Macrophages are also part of lymphoid aggregates where they may participate in antigen presentation towards CD8\(^+\) T cells and B cells. CD8\(^+\) T cells are involved in mucosal defense but the role of B cells is still unclear. The role of dendritic cells is also still unclear but their tolerogenic phenotype suggest they may be involved in tolerance induction.
Density of endometrial macrophages increases in the late secretory phase 27,28. Endometrial macrophages have a M2-like phenotype and they secrete both pro- and anti-inflammatory factors as well as pro-angiogenic factors 28. Next to their role in immune defense 29, endometrial macrophages are involved in tissue homeostasis. They can initiate menstruation by mediating tissue breakdown through expression of enzymes and are involved in tissue repair by secreting angiogenic factors 6.

Mast cells and neutrophils also contribute to initiation of menstruation. The number of mast cells is low within the endometrium but their expression of tryptase increases premenstrually, which can contribute to tissue breakdown 30. Neutrophils are barely detectable during the normal menstrual cycle and are mainly found premenstrually where they are localized at sites of tissue breakdown 31. Stretching of stromal cells by contractions during menstruation can attract neutrophils 32, and endometrial neutrophils further assist in tissue breakdown by releasing proteases 31. However, VEGF expression suggests they can also promote angiogenesis 33.

Other immune cells present in the non-pregnant endometrium are dendritic cells (DC) and B cells. However, the presence of these cells is rather scarce in comparison with NK cells, macrophages and T cells 34,35. B cells can be found in lymphoid aggregates in the endometrium but their phenotype, function and antibody-producing capability is still unknown. The majority of endometrial DCs on the other hand, have a CD1c+ immature and thus tolerogenic phenotype 35,36, but their exact role in the menstrual cycle is unclear as well. However, it has been shown that DCs can modulate T cells responses and contribute to tolerance of the fetal allograft during the implantation phase, which will be discussed below.

Overall, immune cells in the endometrium have unique tissue-specific phenotypes compared to their peripheral counterparts, and they play a key role in immune defense of the uterine mucosa, and in tissue remodeling and repair (Fig. 1B) 37.

Immunity in the pregnant uterus

The uterine mucosa during pregnancy is called the decidua, and the fetal-derived placenta is embedded within the decidua. The decidua comprises three distinct decidual layers 37. The decidua basalis is the site of implantation and forms the maternal part of the placenta. The decidua capsularis covers the implanted embryo on the side of the uterine cavity, and the decidua parietalis lines the remainder of the uterine cavity. The decidua capsularis and parietalis fuse mid—gestation as the fetus grows to form the placental membranes that surround the fetus (Fig. 2A).

During early pregnancy, there is an influx of immune cells into the uterine mucosa, which is crucial for creating a suitable immune environment to support the process of implantation and placentation 38,39. Moreover, maternal decidual immune cells interact with foreign fetal
cells, thus immunomodulatory processes must take place to prevent rejection of fetal tissues.

**Uterine immunity during implantation and placentation**

Pregnancy starts with the fertilization of an ovum. After fertilization, the dividing conceptus travels down the fallopian tube, and when it comes into contact with the decidualized endometrium, implantation is initiated. For implantation to be successful, it is critical that it occurs during the window of receptivity. During this window of receptivity, the epithelial layer of the decidualized endometrium expresses adhesion molecules such as integrins and L-selection ligands, which allow the attachment of the embryo. After implantation, placentation starts. This involves the differentiation of embryo-derived trophoblasts, the invasion of fetal trophoblast cells in the decidualized endometrium, and remodeling of the spiral arteries in order to establish the connection with the maternal blood supply to support nutrient, gas and waste exchange with the developing fetus. Tissue remodeling, angiogenesis and inflammatory processes are essential for successful implantation and placentation, and are actively coordinated by uterine immune cells during the first trimester of pregnancy (Fig. 2B).

The most extensively studied decidual immune cell are decidual NK cells. They represent 70% of total decidual leukocytes and can be found in close proximity to invading trophoblast cells and uterine spiral arteries. Decidual NK cells play a significant role in spiral artery remodeling and trophoblast invasion by the secretion of proteases, cytokines, chemokines, and angiogenic growth factors. Changes in decidual NK cell function during placentation has been suggested to play a role in the pathogenesis of pre-eclampsia, a pregnancy complication associated with defective placentaion. In contrast to peripheral blood NK cells, which have a CD56+CD16+ phenotype, decidual NK cells have a CD56 brightCD16- immunomodulatory phenotype and express high levels of killer-immunoglobulin-like (KIR) receptors. Binding of HLA-G on trophoblast cells to KIR2DL4 on decidual NK cells results in the upregulation of pro-inflammatory and angiogenic cytokines by decidual NK cells. In addition, stimulation of the activating receptor KIR2DS1 on decidual NK cells by HLA-C2 expressed on trophoblast cells, stimulated the production of GM-CSF, resulting in enhanced in vitro trophoblast migration. Moreover, the combination of maternal KIR AA genotype (no activating KIR receptors) with fetal HLA-C2 increases the risk for complications such as recurrent miscarriage and pre-eclampsia. Interestingly, a subset of NKG2C+LILRB1+ decidual NK cells with a unique transcriptional profile and enhanced IFN-γ and VEGFα production, is expanded in repeated pregnancies compared to first pregnancies. This subset resides in the endometrium between pregnancies and may increase angiogenesis and placentation during subsequent pregnancy.
Figure 2 Immunity in the pregnant uterine mucosa. (A) Representation of the different decidual layers of the placenta. The pregnant uterine mucosa is called the decidua. The decidua basalis is the maternal part of the placenta while the decidua parietalis is part of the membranes surrounding the fetus. (B) NK cells, macrophages, neutrophils and mast cells in the early decidua basalis are involved in mediating tissue remodeling, trophoblast invasion and angiogenesis via the production of cytokines, angiogenic factors, chemokines and proteases. NK cells and mast cells can be activated by HLA ligands on trophoblast cells, while ILCs can activate neutrophils and mediate immune defense. Macrophages also regulate epithelial glycan-expression to promote blastocyst attachment during the implantation phase. Moreover, they remove cell debris to avoid inflammatory reactions. Next to activating NK cells, dendritic cells promote Treg and inhibit effector T cells, thereby contributing to immunoregulation and tolerance. Treg are also induced by NK cells, trophoblasts and neutrophils. The role of B cells in the decidua is still unclear but they are likely involved in immunoregulation.

Besides NK cells, also other innate lymphoid cells (ILCs) can be found in the first trimester decidua, although cell numbers are low. Their exact role in this tissue is still unclear but production of IL-8 (CXCL8), IL-22, IL-17, IFN-γ and TNF suggests they could be involved...
in tissue remodeling and uterine immune defense. Moreover, neutrophils co-localize with ILC3 in first trimester decidua, and the number of decidual ILC3 directly correlates with decidual neutrophils, suggesting possible interaction. Indeed, ILC3-derived IL-8 and GM-CSF is able to induce neutrophil migration and survival in vitro. In addition, decidual neutrophils express heparin-binding EGF-like growth factor (HB-EGF). HB-EGF is involved in angiogenesis/blastoic implantation, and GM-CSF produced by decidual ILC3 is able to induce HB-EGF expression in peripheral neutrophils. Poor trophoblast invasion and vascular remodeling can be observed following neutrophil depletion during murine pregnancy. Together, this suggests that decidual ILC3s are involved in neutrophil recruitment to the decidua and promote neutrophil-mediated angiogenesis and trophoblast invasion during early pregnancy.

Also decidual DCs can be found in close contact with decidual NK cells, and in vitro studies showed that crosstalk between decidual DCs and NK cells can promote decidual NK cell activation and proliferation, thereby contributing to enhanced placentation. In mice, DC depletion results in severe impairment of implantation and pregnancy failure by dysregulated tissue remodeling and angiogenesis. Human decidual DCs mainly have an immature phenotype and are shown to induce regulatory T cells (Treg) and immunosuppression.

20% of decidual leukocytes are decidual macrophages, which are also found in close proximity to invading trophoblast cells and the uterine spiral arteries. Various phenotypes have been described for decidual macrophages, predominantly an activated and immunosuppressive M2-like phenotype. Decidual macrophages play various roles during pregnancy. They are enriched at the implantation site, creating a suitable environment for blastocyst attachment by regulating surface glycan structures on epithelial cells. In addition, decidual macrophages are present near the spiral arteries, where they induce tissue breakdown and promote vascular remodeling by secreting cytokines, chemokines, proteases, and angiogenic factors. Decidual macrophages also remove apoptotic cell debris formed during tissue remodeling, thereby preventing detrimental inflammatory conditions. Pregnancy complications marked by defective placentation, such as pre-eclampsia, have been associated with altered macrophage composition and function.

Other innate immune cells present in the decidua during early pregnancy are mast cells. Chymase-expressing mast cells can be found in the early human decidua, close to invading trophoblast cells. Chymase is a protease that is able to degrade extracellular matrixes, suggesting that decidual mast cells might be involved in tissue degradation during placentation. Indeed, mast cells enhance human trophoblast migration in vitro by chymase secretion or by KIR2DL4-dependent interaction. Moreover, mast cell depletion during murine pregnancy impaired spiral artery remodeling.
Next to innate immune cells, cells of the adaptive immune system (B cells and T cells) can be found in the decidua during early pregnancy. Decidual B cells are scarce during normal human pregnancy and it is unclear whether they play a role during implantation and placentation. B cells with a regulatory phenotype have been suggested as potential guardians of tolerance during pregnancy but their exact role has not been fully investigated. Recently, B cells with distinct phenotypes have been characterized in term choriodecidua. B cells were shown to be dysregulated in the choriodecidua of preterm labor subjects. More B cells with reduced expression of IL-10 and progesterone-induced blocking factor 1 (PIBF1) were present in the choriodecidua of women undergoing spontaneous preterm labor compared to spontaneous term labor. In mice, PIBF1 administration suppressed uterine induction of proinflammatory factors and lowered the rates of inflammation-induced preterm labor. This suggests that decidual B cells might play a role in tissue homeostasis and protection against preterm labor.

Around 10% of decidual leukocytes are T cells and they are mainly involved in immune regulation at the maternal-fetal interface. The majority of decidual T cells are CD8+ T cells, most of which have an effector-memory phenotype and show reduced levels of perforin and granzyme B expression. Increased percentages of virus-specific CD8+ T cells can be found in the decidua compared to maternal blood, suggesting an accumulation of virus-specific T cells that could protect the fetus from harmful infections. The most extensively studied T cell subset during pregnancy are Treg. About 5% of CD4+ T cells are Treg, and they are present at a higher frequency in the decidua compared to maternal blood. Treg are essential in maintaining tolerance to paternal antigens, but have also been shown to be crucial during implantation. Depletion of CD4+CD25+ Treg before conception or implantation in mice results in abortion of the pups. Transfer of CD4+CD25+ Treg in Treg-deficient mice rescues implantation failure. In addition, Treg-deficient mice show impaired uterine vascular remodeling and poor trophoblast invasion. Ablation of Treg mid-gestation results in fetal resorptions and reduced numbers of live born pups, suggesting that maternal tolerance is not only required for successful implantation but also for maintaining pregnancy. In humans, it is still unclear how Treg contribute to implantation success but endometrial FoxP3 expression (master regulator of Treg differentiation) is low during the window of implantation in women with unexplained infertility, suggesting inadequate immune tolerance during implantation. 5-30% of first trimester decidual CD4+ T cells are Th1 cells, whereas Th17 (2%) are nearly absent in decidua and Th2 frequencies (5%) are similar in decidua and maternal blood. γδ T cells can also be found in human decidua, and are suggested to be involved in skewing responses towards Th2, and enhancing trophoblast growth and invasion. NKT cells and CD4+CD8- γδ T cells have also been found in human decidua, but their exact function has not been fully investigated.
Overall, decidual immune cells have unique tissue-specific phenotypes and they are essential for pregnancy success by enabling implantation, trophoblast invasion, and tissue and spiral artery remodeling (Fig. 2B).

**Immune modulation and tolerance at the uterine mucosa during pregnancy**

At the maternal-fetal interface, maternal immune cells are in close contact with foreign fetal trophoblast cells. To protect these cells from immune mediated damage, immunomodulatory processes must take place. Many immunological mechanisms are suggested to be involved in immune modulation and tolerance of the allogeneic fetus.

Human fetal trophoblast cells show a unique surface expression of HLA that allows them to circumvent maternal immune attack 127. The syncytiotrophoblast layer, the barrier through which nutrient, gas and waste exchange occurs, interacts with maternal blood in the intervillous space 39,128. This fetal cell layer is completely devoid of HLA-expression, thereby avoiding allogeneic responses by circulating T cells 2,39. Invading extravillous trophoblast cells (EVT), on the other hand, express HLA-C, HLA-G, HLA-E and HLA-F and interact with maternal cells in the decidua basalis 39,127,129. Trophoblast cells of the chorion, which are in contact with maternal immune cells of the decidua parietalis, express HLA-C, HLA-E and HLA-G. The absence of the classical HLA I molecules HLA-A and HLA-B, and HLA class II molecule (HLA-DR, HLA-DP and HLA-DQ) expression on trophoblast cells prevents maternal T cell activation and T cell-mediated attack 39,122.

Since NK cells are abundantly present in close proximity to EVTs at the maternal-fetal interface, a lot of research has been focused on these cells. Interactions between HLA-ligands on invading EVTs and NK cell receptors on decidual NK cells appear to be involved in inhibiting NK-cell mediated trophoblast killing and regulating trophoblast invasion 48,130-133. NK cell cytotoxic function is inhibited by binding of NK cell receptors to self-HLA and they will show cytotoxicity against cells missing self-HLA, such as MHC-null target cells 134-136. Hence, the missing self-response 136. However, unlike peripheral blood NK cells, decidual NK cells have a weak cytolytic activity against standard target cell lines and trophoblast cells in vitro 130-133, even though they contain cytotoxic granules 137,138. Decidual NK cells express high levels of NKG2A, which binds to HLA-E and results in inhibition of NK cell cytotoxicity 139,140. Moreover, inhibition of NK cell cytotoxicity is also mediated by binding of HLA-G and CD48 to LILRB1 and CD244 on NK cells respectively 141-145. However, decidual NK cells can produce pro-inflammatory cytokines and become cytotoxic upon stimulation of NKG2D, Nkp30, Nkp46, and NKG2C by infection-induced cellular stress signals and presentation of viral proteins by HLA-E, thereby providing protection against intrauterine viral infections 146-150. HLA-C is the only known polymorphic HLA molecule expressed by the trophoblast cells and
is a major ligand for KIR2D receptors. Based on a dimorphism at position 80 of the α1 domain of the HLA-C protein, HLA-C is divided into HLA-C1 and HLA-C2. KIR2DL1 and KIR2DS1 can recognize and bind to HLA-C2, while KIR2DL2/L3 can recognize and bind mainly to HLA-C1. Both endometrial and decidual NK cells are biased towards KIR2D expression and HLA-C recognition. As mentioned earlier, mothers with a KIR AA genotype (no activating KIR receptors), in combination with fetal HLA-C2 derived from the father, are at increased risk for pre-eclampsia, recurrent miscarriage and fetal growth restriction. Presence of an activation KIR receptors (KIR2DS1 and KIR2DS5) provides protection against these complications by enhancing placentation.

Next to NK cells, also the potential alloreactive function of T cells needs to be regulated during pregnancy. To date, there is no clear evidence that maternal effector T cells would ever cause damage to trophoblast cells during normal pregnancy. This absence of allograft rejection is mainly mediated by the absence of HLA-A and HLA-B expression on trophoblast cells. HLA-C is the only classical HLA molecule expressed by trophoblast cells to which allograft responses can take place. Increased frequencies of decidual CD4+CD25^{dim} T cells and CD4+CD25^{hi} T cells could be observed in HLA-C mismatched but not HLA-DR or HLA-DQ mismatched pregnancies. Increased frequencies of effector memory CD4 and CD8 T cells in decidua compared to maternal blood and endometrium suggests that T cells become antigen-experienced over the course of pregnancy. Indeed, decidual T cells showed increased specificity towards fetal antigens compared to T cells in maternal blood. So why is there no T cell-mediated attack of the fetal tissues by fetal-specific T cells? Firstly, chemokine gene silencing in decidual stromal cells might limit infiltration of effector T cells into the decidua. Secondly, effector memory CD8+ T cells show a mixed transcriptional signature of T cell dysfunction, activation and effector functions. These decidual effector memory CD8+ T cells show a high expression of coinhibitory molecules TIM-3 and PD-1, and reduced expression of perforin and granzyme B, which allows for immune tolerance. However, they still retain their capacity to respond to proinflammatory situations. Expression of coinhibitory molecules TIM-3 and PD-1 are also upregulated on CD4+ effector memory decidual T cells. Thirdly, T cell activation is hindered by the production of immune modulating factors such as IDO, FASL, TRAIL, PD-L1, and TGF-β. These factors can be expressed by EVTs and maternal decidual immune cells. Decidual macrophages have predominantly an immunosuppressive M2-like phenotype, which can be induced by soluble factors secreted by EVTs and trophoblast-derived debris. High levels of IL-10, TGF-β and IDO, low levels of IL-1β, and low expression of costimulatory molecules CD80 and CD86 by decidual macrophages will inhibit T cell activation in the decidua. In addition, decidual macrophages phagocyte dying trophoblast cells, thereby preventing release of paternal antigens and triggering of a maternal immune response. Therefore, it is suggested that decidual macrophages may be essential in maintaining tolerance.
against fetal antigens. Moreover, human decidual DCs have an immature phenotype, and show reduced capacity to stimulate T cells. In addition, they skew T cells responses towards regulatory Th2 responses, leading to maintenance of immune tolerance at the maternal-fetal interface. Lastly, increased Treg frequencies can be found at the maternal-fetal interface, which can suppress effector T cells responses and induce tolerance. EVTs, decidual macrophages, tolerogenic DCs, NK cells and neutrophils all induce the generation of decidual FoxP3+ Treg. Studies in mice showed that Treg expansion during pregnancy is driven by paternal antigens. Also, in humans, increased Treg frequencies can be observed in HLA-C mismatched pregnancies. Interestingly, exposure to seminal fluid, which contains paternal antigens and immunomodulatory molecules, induced memory T cells and Treg in the preimplantation decidua in mice. In humans, it is still unclear whether seminal fluid can induce Treg generation in the decidua. However, seminal fluid did recruit memory T cells into the human cervix. Moreover, long periods of exposure to semen of the future father reduces the risk of pre-eclampsia development, and treatment with seminal fluid during IVF improves clinical pregnancy rates. This suggests that immunomodulatory factors in seminal fluid can skew the uterine immune environment towards a tolerogenic and fetal-receptive state.

In conclusion, next to their role in implantation and placentation, immunoregulatory mechanisms mediated by decidual immune cells protects the fetal tissue from immune mediated damage. However, the complex interplay and dynamic changes of immune cells at the uterine mucosae are not fully understood. Improving our understanding of the immunological mechanisms at play at the uterine mucosae will improve our insight into pregnancy success and failure.


Chapter 1

SCOPE AND OUTLINE OF THIS THESIS

The work outlined in this thesis is dedicated to understanding the immunology of the uterine mucosae and its dynamic changes during human pregnancy. After the general introduction into the immunity of the uterine mucosae in chapter 1, chapter 2 investigates the immunological changes the uterine mucosae experiences from pre-pregnancy endometrium towards term decidua. The main focus of this chapter is on T cells. The next three chapters, chapter 3, chapter 4 and chapter 5, are dedicated to uterine NK cells since they are important players in the uterine mucosae. Chapter 3 examines the NK cell receptor repertoire of endometrial NK cells, and investigates whether the repertoire is different from peripheral NK cells of paired samples. In chapter 4, the phenotypic modifications that NK cells in the uterine mucosae undergo under the influence of pregnancy-specific factors are examined by comparing the NK cell receptor repertoire of endometrial and early pregnancy decidual NK cells. Moreover, recently, pregnancy-induced trained immunity of uterine NK cells has been described, i.e. a previous pregnancy insult induces long-lasting phenotypic and epigenetic changes in uterine NK cells. Chapter 5 delves deeper in this matter and examines the influence of cytomegalovirus seropositivity on pregnancy-induced trained immunity of uterine NK cells. Consequently, Chapter 6 reviews the available literature on trained immunity and discusses trained immunity of the uterine mucosae in more detail. Furthermore, women with a transplanted kidney need to continue their immunosuppressive drug treatment during pregnancy, which could dysregulate their uterine immune system and expose their offspring to immunosuppressive drugs. Chapter 7 investigates whether the immunosuppressive drug tacrolimus is able to cross the placenta and enter the fetal circulation in both a placenta-perfusion model and ex vivo placental samples. Next, chapter 8, the effect of immunosuppressive drug use during pregnancy on the decidual and neonatal immune system is assessed. Finally, chapter 9 provides a summary, discussion and general conclusion of the chapters of this thesis and future perspectives are outlined.
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Chapter 1


General introduction and scope


Chapter 1


General introduction and scope


Chapter 1


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Chapter 1

General introduction and scope


General introduction and scope
CHAPTER 2

Human uterine lymphocytes acquire a more experienced and tolerogenic phenotype during pregnancy

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Uterine lymphocytes acquire an experienced and tolerogenic phenotype

ABSTRACT

Pregnancy requires a delicate immune balance that nurtures the allogeneic fetus, while maintaining reactivity against pathogens. Despite increasing knowledge, data is lacking on the transition of pre-pregnancy endometrial lymphocytes to a pregnancy state. Here, we immunophenotyped lymphocytes from endometrium (MMC), term decidua parietalis (DPMC), and PBMC for direct comparison. We found that the immune cell composition of MMC and DPMC clearly differ from each other, with less NK-cells, and more NKT-cells and T-cells in DPMC. An increased percentage of central memory and effector memory T-cells, and less naive T-cells in DPMC indicates that decidual T-cells are more experienced than endometrial T-cells. The increased percentage of CD4^+CD25^{high}CD127^- Treg in DPMC, including differentiated Treg, is indicative of a more experienced and tolerogenic environment during pregnancy. The Th cell composition of both MMC and DPMC was different from PBMC, with a preference for Th1 over Th2 in the uterine environment. Between MMC and DPMC, percentages of Th cell subsets did not differ significantly. Our results suggest that already before pregnancy a tightly controlled Th1/Th2/Th17 balance is present. These findings create opportunities to further investigate the underlying immune mechanism of pregnancy complications using menstrual blood as a source for endometrial lymphocytes.
INTRODUCTION

Pregnancy requires a complex interplay of immune cells. Maternal lymphocytes need to accommodate the semi-allogeneic fetus and still maintain robust immune reactivity against pathogens. The barrier between the semi-allogeneic fetus and the maternal immune system is the placenta. At this fetal-maternal interface, maternal lymphocytes of the decidua come into close contact with cells of fetal origin, i.e. trophoblast cells. This contact occurs at two different sites, between invading trophoblast cells and the decidua basalis, which is the site of implantation, and chorionic trophoblast cells and the decidua parietalis, which are part of the membranes surrounding the fetus 1. These trophoblast cells have restricted HLA expression (HLA-C, HLA-E, and HLA-G). Direct response to fetal allogeneic HLA is primarily via HLA-C, but also indirect presentation of fetal antigens by maternal APCs can elicit an anti-fetal maternal leukocyte response 2-6. This restricted immune recognition makes that the uterine immune cell composition and phenotype is different from other mucosal sites 1.

Each month, during the menstrual cycle, the uterus prepares itself for pregnancy by a large influx of leukocytes in the endometrium. When implantation takes place, the number of leukocytes increases even further. Without implantation, the endometrial lining and its leukocytes are shed during menstruation 7. Natural killer (NK) cells are abundantly present in the human endometrium 8,9. Endometrial NK cells increase in number during the menstrual cycle, reaching a peak in the late secretory phase. If implantation occurs, endometrium will transform into decidua and the number of endometrial NK cells will increase even further and will make up 70% of the decidual leukocytes during the first trimester. These uterine NK cells are different from NK cells found in peripheral blood. They are characterized as being CD56brightCD16-, while NK cells found in peripheral blood are mainly CD56dimCD16+ 8,10. Decidual NK cells produce specific cytokines and angiogenic factors to regulate invasion of fetal trophoblast cells and spiral artery remodeling 7,10.

Besides NK cells, also T cells are a major cell population in the endometrium and decidua 8,11. Decidual T cells differ from peripheral T cells by expression of activation markers such as CD45RO, CD69, HLA-DR, and CD25 12, but their function and mechanism of fetus-specific immune recognition remains poorly defined 13. It has long been thought that maternal tolerance towards fetal alloantigens was established by a predominance of T helper type 2 (Th2) immunity over Th1 immunity during pregnancy. However, this Th1/Th2 paradigm was found insufficient, since both Th1 and Th2 dominant immunity was observed in pregnancy complications 14. Th17 cells produce IL-17 and mediate the induction of inflammation 15. Higher levels of Th17 cells were found in women suffering from recurrent pregnancy loss and preterm delivery 16-18. In contrast, mouse studies revealed that regulatory T cells (Treg) are essential for promoting immune tolerance towards the fetus, and activation of Treg is needed for pregnancy success, while depletion
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of Treg was associated with pregnancy failure \(^{19-22}\). Also, in humans, pregnancy complications, like recurrent pregnancy loss and pre-eclampsia, were found to be associated with lower numbers of Treg \(^{23-26}\). Altogether, this suggests that a tightly regulated balance between Th1, Th2, Th17, and Treg cells is required for successful pregnancy.

Although much effort has been put in elucidating how the immune system contributes to pregnancy, particularly in mice, knowledge on human placentation is scarce. Especially little data is available on early implantation and placentation compared to term pregnancy decidual tissue. As local decidual immune regulation is paramount to successful pregnancy, immune phenotypic changes in the uterine immune environment that fit the notion of a well balanced Th1/Th2/Th17/Treg environment might be expected. In the present study, we made a detailed phenotypic and functional analysis of immune cells in both pre-implantation endometrium and in term decidua, with a focus on T cell subsets. We used menstrual blood as a source of endometrial cells because we showed previously, that with respect to cell composition and phenotypic characteristics, menstrual blood is very similar to biopsy-derived material \(^{27}\). The results of this study will provide us with a more profound insight into which adaptations of the uterine immune system during pregnancy are important for pregnancy success.

**MATERIALS AND METHODS**

**Blood and tissue sampling**

Paired peripheral blood and menstrual blood was collected from 17 healthy women with regular menstrual cycles. Hormones can modulate immune cell responses and change the natural menstrual cycle \(^{28,29}\). To avoid any artificial effect on the hormonal balance, none of the menstrual blood donors used any hormonal contraceptives like birth control pill or an intra-uterine device. See Supplementary Table S1 for donor characteristics. 10 ml of peripheral blood was collected in ACD-A tubes. Menstrual blood was collected during the first 36 hours of menstruation using a menstrual cup (Femmecup Ltd, London, UK). Every 12 hours, the sample was decanted from the cup in a 30 ml tube containing 8 ml 10% human pooled serum (HPS) medium (RPMI 1640 medium supplemented with pyruvate (1 mM), glutamax (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml) (Thermo Fisher Scientific, Waltham, USA), 10% HPS (manufactured in-house), and 0.3% sodium citrate (Merck, Darmstadt, Germany)) and stored at room temperature. After 1.5 days, three tubes containing different fractions of menstrual blood were processed immediately to assess lymphocyte composition. Decidua parietalis, the maternal side of the fetal-maternal interface, was obtained from 19 healthy women after uncomplicated term pregnancy. Decidual samples were obtained after delivery by planned elective cesarean section and processed immediately. The study was approved by the institutional review board
Isolation of lymphocytes
One ml of peripheral blood was lysed with 25 ml lysis buffer [NH₄CL + KHCO₃/Na₂EDTA (Merck, Darmstadt, Germany) diluted in H₂O (Versol, Lyon, France)] for 10 min and washed 3x times with PBS (Braun, Melsungen, Germany). These cells were used for surface staining. For intracellular staining, peripheral blood mononuclear cells (PBMC) were isolated by means of density gradient centrifugation (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway). After isolation, cells were washed twice with PBS. Menstrual blood was washed with PBS and passed through a 70 µm cell strainer (Falcon, Durham, USA) to remove clots and mucus. Granulocytes were depleted by use of a granulocyte depletion kit according to the manufacturer’s instructions (STEMCELL Technologies, Vancouver, Canada). After isolation, cells were washed twice with PBS containing 2% HPS. Decidua parietalis was collected as described previously 30. Briefly, after removing the amnion, the decidua parietalis was carefully scraped from the chorion. The obtained tissue was washed thoroughly in PBS before mincing with scissors. The resulting pulp was washed again until the supernatant became transparent. The tissue was enzymatically incubated with 1% collagenase I (Gibco Life Technologies, Waltham, USA) and 1% DNAse (Roche Diagnostics, Risch-Rotkreuz, Switzerland) in a water bath at 37°C while shaking for 60 minutes. After washing with RPMI medium, the suspension was passed through a 70 µm cell strainer (Greiner, Frickenhausen, Germany) and washed again with RPMI. Lymphocytes were obtained after density gradient centrifugation (Lymphoprep). Analysis was done immediately on fresh material to exclude the influence of cryopreservation on the expression of certain markers. For optimal analysis of the chemokine receptors CD183 (CXCR3), CD194 (CCR4), and CD196 (CCR6) on decidual cells, cells were put to rest at 37°C in a humidified 5% CO₂ incubator for 16 hours before staining for flow cytometry. Typically 93%, 95%, and 81% of respectively peripheral, menstrual, and decidual lymphocytes were viable cells (Supplementary Fig. S1).

Flow cytometry
Samples were phenotypically analyzed using the 10-color Navios™ flow cytometer (Beckman Coulter, Fullerton, CA, USA). Briefly, cells were washed twice with PBS + 0.2% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, USA) and labeled for 20 min at RT in the dark with the fluorochrome-conjugated mAbs of interest. Samples were washed twice with PBS + 0.2% BSA. For cell surface staining of B cells, monocytes/macrophages, T cells, Treg, NKT cells and NK cells, the following conjugated mAbs were used: CD3-PE/ECD/PB (Beckman Coulter; UCHT1), CD4-PC5.5/PB (Beckman Coulter; 13B8.2), CD4-AF700
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(eBioscience, San Diego, USA; RPA-T4), CD8-APC-AF700/APC-AF750 (Beckman Coulter; B9.11), CD14-ECD (Beckman Coulter; RMO52), CD16-FITC (Beckman Coulter; 3G8), CD19-APC-AF750 (Beckman Coulter; J3-119), CD25-PC7/APC (BD Biosciences, New Jersey, USA; M-A251 and 2A3), CD45-KO (Beckman Coulter; J33), CD45RA-FITC/ECD (Beckman Coulter; ALB11 and 2H4LDH11LD89), CD45RO-ECD (Beckman Coulter; UCHL1), CD56-APC (Beckman Coulter; N901), CD62L-FITC/ECD (eBioscience and Beckman Coulter; DREG-56), CD69-PE (Beckman Coulter; TP1.55.3), CD103-FITC (eBioscience; B-Ly7), CD127-APC-AF700 (Beckman Coulter; R34.34), CD183-PC5.5 (CXCR3; Biolegend, San Diego, USA; G025H7), CD194-PC7 (CXCR4; BD Biosciences; 1G1), CD196-PE (CCR6; BD Biosciences; 11A9), CD197-BV421 (CCR7; BioLegend; G043H7), and Fixable Viability Dye-eFluor780 (eBioscience). For intracellular staining, samples were permeabilized and fixed according to manufacturer’s instructions (eBioscience). Cells were incubated with the conjugated mAbs of interest for 30 min at 4°C in the dark. The following conjugated mAbs for intracellular staining were used: IFN-γ-PC7 (4S.B3) and IL-17-APC-AF780 (eBioscience; eBio64DEC17). A minimum of 200,000 cells per staining was applied. Fluorescence minus one (FMO) and isotype controls were used for gate settings. The data were analyzed using Kaluza V1.1 software (Beckman Coulter). A typical gating strategy used for analysis of T cells and NK cells is depicted in Supplementary Fig. S1.

Functional analysis
Peripheral blood (PBMC), menstrual blood (MMC), and decidua parietalis (DPMC) mononuclear cells were stimulated with phorbol-12-myristate-13-acetate (PMA), ionomycin and brefeldin A (respectively, 12.5 ng/ml, 500 ng/ml and 5 µg/ml; Sigma-Aldrich, St. Louis, USA) for 4 hours at 37°C in a humidified 5% CO₂ incubator. Functionality was determined by measuring the intracellular production of IFN-γ and IL-17 by flow cytometry as described above.

Differentiation assay
MMC were stimulated in vitro with medium as a control, with anti-CD3/anti-CD28 mAb-coated microbeads in a 1:10 bead-to-cell ratio (Invitrogen, Bleiswijk, The Netherlands) alone, rhIL-15 (100 ng/ml; Gibco Life Technologies) alone, and beads together with rhIL-15 in 96-well U-bottom plates. The rationale for using IL-15 is because IL-2 is hardly detected in decidua and after implantation, endometrial NK cells start to differentiate as a result of local IL-15 production 9. After 5 days of culture at 37°C in a humidified 5% CO₂ incubator, cells were harvested and the presence of T cell subsets and differentiation was measured with flow cytometry.
**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 5 (Graphpad software Inc., La Jolla, CA, USA). Non-parametric Kruskal-Wallis with Dunn’s post-hoc test was performed to compare PBMC, MMC, and DPMC samples. Statistical significance was denoted as values of P < 0.05. All indicated values are mean percentages ± SD.

**RESULTS**

**The lymphocyte composition of term decidua differs from pre-pregnancy endometrium**

Previously, comparisons between endometrium and decidua had to be inferred from separate studies since data on direct comparison of immune cell changes between endometrium and decidua are scarce. The recently designed method, whereby endometrial lymphocytes can be isolated from menstrual blood, allows for easier access to this material and opens up the opportunity to study pre-pregnancy endometrium together with decidual samples in the same set of experiments. Here, we directly compared the immune cell composition of menstrual blood (MMC), term decidua parietalis (DPMC), and peripheral blood (PBMC) mononuclear cells by using flow cytometric analysis (Fig. 1). Since cell yield from decidua basalis was too low for the extensive analysis we did here, maternal lymphocytes in the decidua parietalis are in close contact with chorionic trophoblast cells, and active immune regulation seems to place at the decidua parietalis as well 31,32, prompted our decision to opt for isolation of cells from decidua parietalis to study the fetal-maternal interface.

In accordance with previous studies, MMC and DPMC clearly differ from PBMC in percentages of lymphocytes, T cells, NK cells and NKT cells 7-9,27,33,34, and contained primarily CD56⁺CD16⁻ NK cells, while the majority of NK cells in PBMC were CD56⁺/-CD16⁺. In a direct comparison between MMC and DPMC, DPMC revealed a higher percentage of NK cells (46.9%±16.4% and 24.3%±10.6% respectively). MMC (43.9%±15.10%) contained a significant lower percentage of T cells, with an increased CD4⁺/CD8⁺ ratio, as compared to DPMC (67.1%±10.66%). In addition, MMC contained significantly less NKT cells (1.2%±0.9% versus 2.5%±1.1%) compared to DPMC. No significant difference in percentages of B cells between MMC and DPMC could be observed. Also, important to note is that MMC and DPMC derived lymphocytes are from mucosal origin since more CD69⁺ and CD103⁺, and less CD62L⁺ T and NK cells can found compared to PBMC (Supplementary Fig. S2). After showing that MMC and DPMC clearly differ in immune cell composition, we investigated T cells in more depth for differences between pre-pregnancy endometrium and decidua.
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Decidual T cells are more experienced than endometrial T cells

We next examined endometrial and decidual T cell subsets in more detail to know if the observed phenotype of more differentiated memory T cells in decidua compared to PBMC would also apply to endometrium. Compared to MMC derived T cells, DPMC derived T cells revealed a significantly lower percentage of CD45RA+ (53.4%±8.8% and 17.2%±7.4%, respectively) and a higher percentage of CD45RO+ cells (28.9%±8.0% and 66.4%±11.7% respectively) (Fig. 2B). In addition, we subdivided CD4+ and CD8+ T cells into naive T cells (CD45RA+CCR7+), effector T cells (Teff, CD45RA+CCR7-), effector memory T cells (EM, CD45RA-CCR7-), and central memory T cells (CM, CD45RA-CCR7+). Both CD4+ and CD8+ T cells present in DPMC were significantly less naive compared to MMC, while a significantly higher percentage of EM and CM T cells was present in DPMC (Fig. 2C). Thus, over the course of pregnancy, decidual T cells appear to acquire an experienced and
differentiated phenotype.

**Figure 2** CD4⁺ and CD8⁺ T cells subsets in peripheral blood (PBMC), menstrual blood (MMC) and term decidua (DPMC). (A) Representative staining for CD45RA and CD45RO on CD3⁺ T cells and CD45RA and CCR7 on CD4⁺ and CD8⁺ T cells from PBMC, MMC, and DPMC. (B) Expression of CD45RA and CD45RO on CD3⁺ T cells (PBMC n=17, MMC n=16, and DPMC n=12. (C) CD4⁺ and CD8⁺ T cells are separated into four subsets based on the expression of CD45RA and CCR7. Naive T cell (CD45RA⁺CCR7⁺); effector T cell, Teff (CD45RA⁺CCR7⁻); effector memory T cell, EM (CD45RA CCR7⁻); central memory T cell, CM (CD45RA⁺CCR7⁺) (PBMC n=17, MMC n=11, and DPMC n=8). Lines indicate mean ± SD. * P < 0.05, **P < 0.01, and ***P < 0.001 (non-parametric Kruskal-Wallis with Dunns post-hoc test)
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To strengthen the claim that endometrial T cells are primed over the course of pregnancy and can differentiate towards a phenotype found in the decidua, we performed in vitro experiments whereby endometrial lymphocytes were stimulated with either rhIL-15 or anti-CD3/anti-CD28 mAb microbeads alone, or in combination. The rationale for using IL-15 is because IL-2 is hardly detected in decidua and after implantation, endometrial NK cells start to differentiate as a result of local IL-15 production. After 5 days of culture, the distribution of T cell subsets was measured with flow cytometry in the same way as above. Endometrial T cells stimulated with anti-CD3/anti-CD28 mAb microbeads reveal a similar subset division as found in term decidual T cells, i.e. less naive T cells and more EM and CMT cells compared to control (medium) and day 0 (Supplementary Fig. S3). Whereas stimulation with IL-15 alone was not sufficient to lead to a similar differentiation, IL-15 did appear to have an effect on T cell differentiation when administered together with anti-CD3/anti-CD28 beads and as compared to the bead alone condition, i.e. more EM and less CM T cells. This may suggest that T cells need an additional TCR trigger before they can differentiate towards a more mature phenotype. These in vitro data showed that endometrial lymphocytes can be differentiated towards a phenotype reminiscent of term decidual T cells, which lends further support for the notion that over the course of pregnancy decidual T cells appear to acquire an experienced and differentiated phenotype.

The decidual immune environment is marked by a tolerance signature

The fetal-maternal interface is the major site where maternal immune cells come into contact with cells of fetal origin. Treg are important for regulation of the decidual immune environment and pregnancy success. DPMC contained significantly more CD4^+CD25^{high}CD127^- Treg than MMC (9.5%±3.5% versus 5.2%±1.9%) (Fig. 3B). We further subdivided Treg based on expression of CD45RA and CD25. DPMC contained significantly more CD4^+CD25^{high}CD45RA^- activated and differentiated Treg than MMC (8.4%±5.9% versus 2.4%±1.6%), while the percentage of CD4^+CD25^CD45RA^ naive Treg did not differ significantly (1.3%±1.2% versus 1.9%±1.3%) (Fig. 3C). Treg percentages in MMC did not differ from PBMC, but DPMC contained significantly less naive Treg compared to PBMC (1.3%±1.2% versus 3.4%±2.0%). This suggests, that different from the pre-implantation endometrium, the human decidual environment is marked by a immune signature that includes the activation and differentiation of Treg.
The Th1, Th2, and Th17 cell profile is similar between endometrium and term decidua

It is suggested that successful pregnancy requires a delicate Th1/Th2/Th17/Treg balance \(^{14}\). To investigate this balance, we classified CD4\(^+\) cells as Th1, unconventional Th1 (Th1-like), Th2, or Th17 cells, based on the expression of the chemokine receptors CCR6, CXCR3, and CCR4 \(^{37}\). Blood Th1-like cells were reported to have a mixed Th1/Th17 phenotype, i.e. production of RORC mRNA and IL-17, together with a higher production of TBX21 mRNA and IFN-\(\gamma\) than conventional Th1 cells, indicating higher Th1 activity \(^{37}\). Th cell composition of MMC and DPMC were both different from PBMC, with significantly less Th2, and more Th1 cells in the uterine environment (Fig. 4B). Between MMC and DPMC, percentages of Th1, Th2, Th17, and Th1-like cells did not differ significantly (Fig. 4B). When investigating the intracellular cytokine expression profile after PMA/Ionomycin/Brefeldin stimulation, we observed a higher intrinsic capacity to express IFN-\(\gamma\) and IL-17 by DPMC CD4\(^+\) T cells (27.8\%\pm 15.4\% and 4.4\%\pm 1.3\% respectively) compared to CD4\(^+\) T cells from MMC (7.4\%\pm 3.3\% and 2.6\%\pm 1.0\% respectively), and also PBMC (7.5\%\pm 6.7\% and 2.3\%\pm 1.3\% respectively) (Fig. 4D).
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Figure 4 Distribution of CD4+ Th cell subsets in peripheral blood (PBMC), menstrual blood (MMC), and term decidua (DPMC) and production of IFN-γ and IL-17 by CD4+ T cells. (A, C) Representative gating for CCR6, CXCR3, and CCR4 on CD4+ T cells and for IFN-γ and IL-17 by CD4+ T cells and for IFN-γ and IL-17 by CD4+ T cells from PBMC, MMC, and DPMC. (B) Th1, Th2, Th17, and nonconventional Th1 (Th1-like) CD4+ T cells can be classified based on the expression of CCR6, CXCR3, and CCR4. Th1; CCR6+CCR4-CCR6-CXCR3-CCR4-; Th2; CCR6+CCR3-CCR4-; Th17 (PBMC n=17, MMC n=11, DPMC n=7). (D) Production of IFN-γ and IL-17 by CD4+ T cells from PBMC (n=12), MMC (n=15), and DPMC (n=7). *P < 0.05, **P < 0.01, and ***P < 0.001 (lines indicate mean, non-parametric Kruskal-Wallis with Dunns post-hoc test).
In summary, a direct comparison between pre-pregnancy endometrium and term decidua reveals a activated immune signature, with more experienced conventional T cells and Treg. The distribution of Th subsets, defined by chemokine receptor expression patterns, did not differ significantly between endometrium and term decidua. However, the intrinsic capacity to produce IFN-γ and IL-17 was highest in term decidual CD4+ T cells, suggesting a possible role for both cytokines in the uterine environment during pregnancy.

**DISCUSSION**

Local decidual immune regulation is paramount to successful pregnancy. Previous studies investigating the phenotype of immune cells in endometrium and decidua are fragmented, and limited to comparing immune cells in either endometrium or decidua with their counterparts in peripheral blood \(^7-9,27,33,34\). Here, we performed a cross-sectional study, directly comparing the immune cell composition and functional capacity of pre-pregnancy endometrium and term decidua using flow cytometric analysis. We show clear differences in immune cell composition, suggestive of immune cell differentiation over the course of pregnancy, i.e. more experienced and less naive T cells and Treg in term decidua compared to pre-pregnancy endometrium. Analysis of the Th1/Th2/Th17/Treg subset composition showed that, while the distribution of Th1, Th2, and Th17 cells did not differ between term decidua and endometrium, there was a preference for Th1 over Th2 cells in the uterine environment compared to peripheral blood.

The role of T cells in pregnancy has been subject to various studies. T cells were shown to be a major cell population in both endometrium and decidua, but their function and antigen-specificity remains poorly defined \(^13\). We observed mainly effector memory (EM) and central memory (CM) T cells at the end of pregnancy, and they were thus more experienced and differentiated than T cells from pre-pregnancy endometrium. This suggests that over the course of pregnancy, T cells at the fetal-maternal interface may differentiate. After implantation of a blastocyst, endometrium will modify to decidua, and cells of fetal origin will come in to contact with lymphocytes at the maternal-fetal interface. This contact might differentiate endometrial T cells towards a phenotype as seen in decidual tissue, as suggested by our in vitro assay. The target specificity of the T cells and exact trigger for this differentiation is unclear, but multiple triggers have been suggested to play a role, including fetal alloantigens like major histocompatibility complex antigens (MHC) (HLA-C in humans) \(^2,4,38\), minor histocompatibility antigens (mHags) \(^6,39\), and/or pathogen-derived antigens \(^40\). In accordance, Tilburgs et al. previously reported that in addition to the lower numbers of naive T cells in decidual tissue, mainly EM and few CM CD8+ T cells were present in term decidua \(^35\).
Pregnancy induces local enrichment of Treg at the fetal-maternal interface. These Treg play an important role in tolerance to the semi-allogeneic fetus and pregnancy in mice, since mouse studies showed that depletion of CD25+ Treg resulted in gestation failure in allogeneic pregnancies. We showed that the percentage of CD4+CD25highCD127− Treg was higher in decidua than in endometrium, suggesting a more tolerogenic environment during pregnancy. As previously reported by our group, no difference in the percentage of Treg in MMC and PBMC samples was observed. The increased percentage of Treg we found in term decidual tissue is comparable to percentages found in other studies. Based on the expression of CD45RA and CD25, human CD4+ T cells can be separated into differentiated Treg and naive Treg. We showed that term decidua contained more differentiated Treg than endometrium, while the presence of naive Treg was lower. In peripheral blood, these differentiated Treg were shown to be derived from recently activated naive Treg and were suggested to be the main effectors of suppression. In mice, it was shown that during the course of pregnancy Treg will acquire a protective regulatory memory phenotype to fetal antigen. These memory Treg persist after pregnancy and re-accumulate rapidly in a subsequent pregnancy. A similar phenomenon thus may take place in the uterus during human pregnancy.

Not only Treg, but the overall balance between Th1, Th2, Th17, and Treg cells is suggested to be of relevance for successful pregnancy. Th subsets can be classified by the production of particular cytokines by T cells, or by the expression of different chemokine receptors on T cells. Using a classification based on chemokine receptor expression patterns, we found that both term decidua and pre-pregnancy endometrium held relatively more Th1 cells, and less Th2 cells compared to peripheral blood. Our results thus showed that in the uterine environment, there is a preference of Th1 over Th2 cells. The same trend was also seen in first trimester decidua. This suggests that this preference is already present in pre-pregnancy endometrium and stays over the course of pregnancy until term. Between term decidua and endometrium, the distribution of Th1, Th2, Th17, and Th1-like cells did not significantly differ. The similar Th cell distribution between endometrium and term decidua suggests that the pre-pregnancy endometrium might already be prepared for pregnancy. It can therefore be envisaged that women who experience recurrent miscarriages already have an imbalance in their endometrium affecting a successful pregnancy outcome. For instance, Shimada et al. showed that in the endometrium of women with recurrent miscarriages less CD4+IFN-γ− T cells were present. In-depth analysis of the Th1/Th2/Th17/Treg balance in the endometrium of women with fertility issues might give us more insight into the underlying pathogenesis of pregnancy complications and could potentially predict subsequent pregnancy outcome. Interestingly, when looking at the actual intrinsic capacity of CD4+ T cells to express IL-17 and IFN-γ intracellular, we found more expression of IFN-γ and IL-17 by decidual T cells compared to peripheral and
endometrial CD4+ T cells. In first trimester decidua, the percentage of IL-17+ T cells was also found to be higher compared to peripheral blood\textsuperscript{16,45}, while the expression of IFN-γ was lower\textsuperscript{46}. Although IFN-γ and IL-17 have been related to pregnancy complications, and an excess of inflammation was associated with a negative impact on pregnancy outcome\textsuperscript{16,18,45,47,48}, the presence of IFN-γ+ and IL-17+ T cells in healthy term decidua suggests that these cytokines may play an important role during pregnancy. IFN-γ for instance, was shown to be essential for implantation, decidual integrity and placentental growth in mice\textsuperscript{47,49}. IL-17 plays an important role in host defense against pathogens\textsuperscript{15}, but it was also shown that IL-17 increases the production of progesterone by JEG-3 cells and supports the survival, proliferation and invasive capacity of trophoblast cells\textsuperscript{50-52}. The presence of normal, balanced levels of IFN-γ and IL-17 could play a role during placentation and/or prevention of intrauterine infection, but this is still far from understood. While we did not show a difference in the capacity of peripheral and endometrial CD4+ T cell to express IL-17, Hosseini et al.\textsuperscript{53} showed more IL-17+CD3+ T cells in menstrual blood compared to peripheral blood. This difference can be explained by a different gating approach since we looked at percentage of CD4+ T cells, while they looked at CD3+ T cells. When we would gate our data in a similar way, we found a similar, although not significant, difference between peripheral and menstrual blood. This can be explained by the higher percentages in our peripheral blood samples (average 4.5%) compared to theirs (average 0.9%). Several reasons could explain this discrepancy: duration of stimulation (4h vs 6h) or differences in stimulus and/or concentration used (PMA (12.5 ng/ml)/Ionomycin (500 ng/ml)/Brefeldin A (5 µg/ml) by us versus PMA (25 ng/ml)/Ionomycin (500 ng/ml)/Monensin (1 µM/ml) by them).

A limitation of our study is that we were not able to study decidual tissue at several time-points during pregnancy, to actually show the changes that occur with time. We could only infer this from the data collected from pre-pregnancy endometrium (implantation stage) and term decidua.

In conclusion, we showed that the immune cell composition of pre-pregnancy endometrium differs from term decidua, but with a similar distribution of Th1, Th2, and Th17 cells. At the end of pregnancy, the uterine immune environment appears to be marked by a tolerogenic phenotype with more experienced T cells and Treg with a potential beneficial phenotype. How exactly the phenotype of the uterine immune cells is shaped and how this differentiation is maintained is unclear. Therefore, in follow-up studies, we aim to explore the influence of different immune triggers on immune cells in the uterine environment before and during pregnancy. This may add insight to our understanding of the pathogenesis of pregnancy complications.
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Author contributions
D.F. coordinated and designed the study, analyzed the data and drafted the manuscript. M.B. and B.C. participated in data collection, and assisted in interpretation of the data. O.W.H.H., I.J., and R.G.M. participated in the conception and design of the study, and provided critical discussion. All authors evaluated the manuscript and contributed to its content.

Additional information, competing financial interest
None of the authors have a competing financial interest to declare.
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CHAPTER 3

Endometrial natural killer (NK) cells reveal a tissue-specific receptor repertoire

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Chapter 3

ABSTRACT

**Study question:** Is the natural killer (NK) cell receptor repertoire of endometrial NK (eNK) cells tissue-specific?

**Summary answer:** The NK cell receptor (NKR) expression profile in pre-pregnancy endometrium appears to have a unique tissue-specific phenotype, different from that found in NK cells in peripheral blood, suggesting that these cells are finely tuned towards the reception of an allogeneic fetus.

**What is known already:** NK cells are important for successful pregnancy. After implantation, NK cells encounter extravillous trophoblast cells and regulate trophoblast invasion. NK cell activity is amongst others regulated by C-type lectin heterodimer (CD94/NKG2) and killer cell immunoglobulin-like (KIR) receptors. KIR expression on decidual NK cells is affected by the presence of maternal HLA-C and biased towards KIR2D expression. However, little is known about NKR expression on eNK cells prior to pregnancy.

**Study design, size, duration:** In this study, matched peripheral and menstrual blood (a source of endometrial cells) was obtained from twenty-five healthy females with regular menstrual cycles. Menstrual blood was collected during the first 36 hours of menstruation using a menstrual cup, a non-invasive technique to obtain endometrial cells.

**Participants/materials, setting, methods:** KIR and NKG2 receptor expression on eNK cells was characterized by 10-color flow cytometry, and compared to matched pbNK cells of the same female. KIR and HLA-C genotypes were determined by PCR-SSOP techniques. Anti-CMV IgG antibodies in plasma were measured by chemiluminescence immunoassay.

**Main results and the role of chance:** KIR expression patterns of eNK cells collected from the same female do not differ over consecutive menstrual cycles. The percentage of NK cells expressing KIR2DL2/L3/S2, KIR2DL3, KIR2DL1, LILRB1, and/or NKG2A was significantly higher in eNK cells compared to pbNK cells, while no significant difference was observed for NKG2C, KIR2DL1/S1, and KIR3DL1. The NKR repertoire of eNK cells was clearly different from pbNK cells, with eNK cells co-expressing more than 3 NKR simultaneously. In addition, outlier analysis revealed 8 and 15 NKR subpopulation expansions in eNK and pbNK cells, respectively. In contrast to the pbNK cell population, the expansions present in the eNK cell population were independent of CMV status and HLA-C genotype. Moreover, the typical NKG2C imprint induced by CMV infection on pbNK cells was not observed on eNK cells from the same female, suggesting a rapid local turnover of eNK cells and/or a distinct licensing process.
Limitations, reasons for caution: Based on our previous work and the parameters studied here, menstrual blood-derived eNK cells closely resemble biopsy-derived eNK cells. However, sampling is not done at the exact same time during the menstrual cycle, and therefore we cannot exclude some, as yet undetected, differences.

Wider implications of the findings: Our data reveals that NK cells in the pre-implantation endometrium appear to have a dedicated tissue-specific phenotype, different from NK cells in peripheral blood. This may indicate that eNK cells are finely tuned to receive an allogeneic fetus. Studying the endometrial NKR repertoire of women with pregnancy related problems could provide clues to understand the pathogenesis of pregnancy complications.

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Trial registration number: NA

Key words: NK cells/KIR/killer immunoglobulin receptor/endometrium/menstrual blood/HLA-C/NK cell receptors/NKG2A/NKG2C/CMV
INTRODUCTION

Natural killer (NK) cells have an important function in immune defense and reproduction. They are specialized in killing virus-infected and tumor transformed target cells by the balanced action of both activating and inhibitory receptors \(^1\). The main families of NK cell receptors (NKR) are the natural cytotoxicity receptors (NCR), the immunoglobulin-like transcripts (LILRB), the C-type lectin heterodimer family (CD94:NKGs), and the killer-immunoglobulin-like receptors (KIRs). The inhibitory NKG2A and activating NKG2C receptor, members of the CD94:NKGs family, can recognize and bind HLA-E \(^2\). The inhibitory receptor LILRB1 can recognize HLA-G \(^3\). KIRs are classified into inhibitory (I) and activating (S) receptors. Members of the KIR2D subfamily recognize and bind HLA-C. Based on a polymorphism at the α1 domain of the HLA-C protein, HLA-C is divided into HLA-C1 and HLA-C2. KIR2DL1 and KIR2DS1 can recognize and bind to HLA-C2, while KIR2DL2/L3 can recognize and bind mainly to HLA-C1 \(^4\).

During reproduction and pregnancy, NK cells play an important role in ensuring correct placentation, and normal development and growth of the fetus \(^5\). In first trimester decidua, 70% of all lymphocytes are NK cells, which are in close contact with extravillous trophoblast cells (EVT) at the site of implantation. Together with macrophages and stromal cells, these decidual NK (dNK) cells, phenotypically and functionally different from peripheral blood NK (pbNK) cells \(^6\), will promote EVT invasion and spiral artery remodeling, thereby ensuring an adequate blood supply to the fetus \(^7\). NKRs on dNK cell are capable of recognizing the unique array of ligands on EVT cells, i.e. HLA-C, HLA-E, and HLA-G \(^7\). Since both KIR and HLA-C haplotypes are highly polymorphic, each pregnancy will be characterized by their own specific combination of KIR and HLA-C. The combination of maternal KIR AA genotype and fetal HLA-C has been associated with recurrent miscarriage and pre-eclampsia \(^8,9\).

Up till now, most research focused on dNK cells using first trimester abortion material, showing that dNK cells express various KIRs and are biased towards KIR2D expression \(^10,11\). However, little data is available on expression and composition of NK cell receptors (NKR) on endometrial NK (eNK) cells, with one study investigating the composition of uterine NK cell KIR repertoire in menstrual blood \(^12\). It has been suggested that eNK cells are immature precursors that differentiate and proliferate into dNK cells after implantation of an embryo \(^13,14\). As NK cell reactivity is particularly relevant at the time of and early after implantation, analysis of NKR expression around this time may yield insight in the role of NKR\(^+\) eNK cells for successful pregnancy.

Here, eNK cells, obtained from menstrual blood \(^15,16\), were immunophenotyped for NKR expression with 10-color flow cytometry, and compared to pbNK cells of the same female. Our aim was to determine differences in NKR expression between eNK and matched pbNK cells. In addition, we investigated the stability of NKR expression on eNK cells over different menstrual cycles, and the influence of CMV seropositivity and HLA-C genotype.
on NKR expression. We hypothesize that, in order to be prepared for successful pregnancy, eNK cells will have a tissue-specific NKR expression pattern, different from pbNK cells.

MATERIALS AND METHODS

Human subjects
25 healthy female volunteers (Table I), with regular menstrual cycle were included upon written informed consent with regard to scientific use, according to the Dutch Medical Research Involving Human Subject Act (WMO). Exclusion criteria were known autoimmune diseases, smoking, and current use of hormonal contraceptives or a copper intra uterine device. Menstrual blood was collected during the first 36 hours of menstruation, in three 12-hours intervals, using a menstrual cup (Femmecup Ltd, London, UK). Collected menstrual blood was stored in a 30 ml tube, containing 10 ml of RPMI 1640 medium, supplemented with pyruvate (1mM), glutamax (2mM), penicillin (100 U/ml), streptomycin (100 µg/ml) (Thermo Fisher Scientific, Waltham, USA), 10% v/v human pooled serum (HPS, manufactured in-house), and 0.3% v/v sodium citrate (Merck, Darmstadt, Germany) at room temperature. Isolation of mononuclear cells was performed within 24 hours of collection. Peripheral blood was obtained from all women participating.

Table I Donor characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Donors (N=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range)</td>
<td>29 years (20-46)</td>
</tr>
<tr>
<td>Sampled longitudinally (3 menstrual cycles)</td>
<td>4/25 (16%)</td>
</tr>
<tr>
<td>Previous pregnancy</td>
<td>11/25 (44%)</td>
</tr>
<tr>
<td>Previous miscarriage</td>
<td>5/25 (20%)</td>
</tr>
<tr>
<td>Natural conception</td>
<td>10/11 (91%)</td>
</tr>
<tr>
<td>Previous unprotected heterosexual contact</td>
<td>23/25 (92%)</td>
</tr>
<tr>
<td>Menstruation duration, median (range)</td>
<td>28 days (23-35)</td>
</tr>
<tr>
<td>Length of menstrual cycle, median (range)</td>
<td>5 days (4-7)</td>
</tr>
<tr>
<td>CMV positive</td>
<td>10/21 (48%)*</td>
</tr>
<tr>
<td>KIR and HLA-C genotyped</td>
<td>22/25 (88%)*</td>
</tr>
</tbody>
</table>

* Information not available for 4 donors.  
** Information not available for 3 donors.

Isolation of lymphocytes
Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Lymphoprep (1.077± 0.001 g/ml, 290 ± 15 mOsm, Axis-Shield PoC AS, Oslo, Norway). Menstrual blood was first washed with PBS (Braun, Melsungen, Germany), and mucus and blood clots were removed using a 70 µm cell strainer (Falcon®,
Corning Inc., NY, USA). The human granulocyte depletion cocktail RosetteSep™ (Stemcell technologies Inc, Vancouver, Canada) was used, according to manufacturer's instructions, to deplete granulocytes. After density gradient centrifugation (Lymphoprep), menstrual blood mononuclear cells (MMC) were isolated. The remaining cell pellet after isolation of MMC, i.e. red blood cells and granulocytes, was used for DNA isolation. Typically 94% and 96% of respectively PBMC and MMC were viable cells (Supplementary Fig. 1).

**Flow cytometric analysis**
The following fluorochrome-conjugated monoclonal antibodies were used to phenotypically characterize MMC and PBMC samples: CD3-APC-AF750, CD16-FITC, CD45-KO, CD56-ECD (Beckman Coulter, Fullerton, CA, USA), CD85j-PC5.5 (LILRB1;Beckman Coulter, custom made), CD158a-AF700 (KIR2DL1; R&D systems, Abingdon, UK), CD158a/h-PC5.5/ APC-AF700 (KIR2DL1/S1; Beckman Coulter, custom made), CD158b2-FITC (KIR2DL3; R&D systems), CD158b1/b2-PC7 (KIR2DL2/L3/S2; Beckman Coulter), CD158e1/e2-APC/BV421 (KIR3DL1; BioLegend, San Diego, USA), CD158e1/e2-APC (KIR3DL1/S1; Beckman Coulter), CD159a-APC/PB (NKG2A; Beckman Coulter, custom made), CD159c-PE (NKG2C; R&D systems), and Fixable Viability Dye-eFluor780 (eBioscience, San Diego, USA). Custom made antibodies were kindly provided by Beckman Coulter. Briefly, cells were washed twice with PBS-bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, USA) (0,2 % v/v), followed by staining with the antibodies for 20 minutes at room temperature in the dark. After washing twice with PBS-BSA (0,2 % v/v), cells were analysed using the 10-color flow cytometer Navios™ (480 nm argon blue laser, 405 nm solid state violet laser, 636 nm solid state laser, Beckman Coulter). A minimum of 250,000 cells per staining was applied. Unstained samples and samples without the presence of the specific KIR gene were used for gate settings. Data analysis was performed using Kaluza 1.5a software (Beckman Coulter). A typical gating strategy used for analysis of NK cells in menstrual and peripheral blood is depicted in Supplementary Fig. 1.

**KIR and HLA genotyping**
Genomic DNA was isolated from the remaining cell pellet after MMC isolation using silica gel membrane technology (Qiagen, Hilden, Germany). KIR genotype and HLA-C allelic variants were typed by PCR-SSOP, using Luminex 100 (Sanbio, Uden, Netherlands) for the following genes: KIR2DL1/S1, KIR2DL1/L2/L3/L4/L5, KIR3DL1/L2/L2, KIR2DS1/S2/S3/S4/S5, KIR3DS1, KIR2DP1, KIR3DP1, HLA-C1 and HLA-C2.

**CMV seropositivity**
Plasma was obtained from peripheral blood to determine anti-CMV IgG antibodies by chemiluminescence immunoassay (CMV IgG II, Liaison XL, DiaSorin, Saluggia, Italy).
Endometrial NK cell receptor repertoire

**SPADE analysis**
Unsupervised analysis of all PBMC and MMC flow cytometry data was performed with the SPADE algorithm (stand-alone version 3.0). After compensation and preliminary gating with Kaluza 1.3, CD45+CD56+CD3- NK cells (gating strategy Supplementary Figure 1) were isolated from each individual data file by processing with R software (www.r-project.org) using scripts, as developed and kindly provided by S. Schlickeiser and B. Sawitzki (Institute of Medical Immunology, Charité - University Medicine Berlin, Berlin, Germany). These NK cells were used as input during SPADE analysis, while CD16, CD56, NKG2C, NKG2A, LILRB1, KIR2DL2/L3/S2, KIR2DL1/S1, and KIR3DL1/S1 were used as clustering markers.

**Statistical analysis**
Statistical analyses were performed using GraphPad Prism 5 (GraphPad software Inc., La Jolla, CA, USA). For comparison of paired observations a non-parametric Wilcoxon-Signed Ranked test was used. Non-parametric Mann-Whitney test was used for unpaired observations. Results were shown as mean ± SD and \( P < 0.05 \) was considered significant.

Co-expression was calculated with the “Tree” function in Kaluza software. A Tukey’s range test was used to identify outliers in NK cell populations with a distinct NKR expression pattern. As described before, two additional criteria were used: outliers should represent at least 5% of the total NK cell population and at least 20% of the NKG2A+NKG2C+/-, NKG2A- NKG2C-, or NKG2A NKG2C- expressing cells.

**RESULTS**

**Endometrial NK cells contain more NKR expressing cells compared to peripheral blood NK cells**
We first examined the NK cell receptor (NKR) expression patterns of endometrial NK (eNK) cells from four females over three consecutive menstrual cycles. NK cells were defined as CD45+CD56+CD3- lymphocytes (gating strategy Supplementary Fig. 1). Analysis showed that single NKR expression and KIR expression patterns on eNK cells were similar between the different menstrual cycles, even when sampled 15 months apart. This indicates that NKR expression on eNK cells does not differ over different menstrual cycles (Supplementary Fig. 2).

Subsequently, we compared NKR expression of eNK cells from 25 females with matched peripheral blood NK (pbNK) cells. We observed that the percentage of eNK cells expressing NKG2A (86.6%±7.6% versus 39.5%±12.2%), LILRB1 (65.6%±13.2% versus 38.2%±20.6%), KIR2DL2/L3/S2 (64.9%±14.5% versus 32.7%±11.9%), KIR2DL3 (36.3%±16.9% versus 12.4%±10.1%), and KIR2DL1 (23.4%±12.1% versus 15.5%±8.5%) was significantly higher compared to matched pbNK cells (Fig.1). No significant difference was observed in the
frequency of NK cells expressing NKG2C (18.3%±9.4% versus 19.2%±20.3%), KIR2DL1/S1 (37.8%±14.5% versus 30.5%±14.9%), and KIR3DL1 (19.7%±13.9% versus 16.0%±11.4%). Overall, eNK cells contained more NKR expressing NK cells compared to pbNK cells, which might suggest that there is skewing of expression by eNK cells towards HLA recognition.

During differentiation, pbNK cells will first acquire KIR2DL2/L3/S2 expression before acquiring KIR2DL1/S1. Our data showed that the percentage of eNK cells expressing KIR2DL2/L3/S2 alone, and expressing both KIR2DL1/S1 and KIR2DL2/L3/S2 was significantly higher compared to pbNK cells, while very few eNK cells were positive for KIR2DL1/S1 alone (Supplementary Fig. 3A). This phenomenon may suggest that eNK cells are only recently matured.

Uterine NK cells can recognize HLA-E on trophoblast cells by the activating NKG2C and inhibitory NKG2A receptor. The percentage of NKG2A+NKG2C- and NKG2A+NKG2C+ eNK cells was significantly higher compared to pbNK, while the frequency of NKG2A- NKG2C+ and NKG2A NKG2C- eNK cells was significantly lower (Supplementary Figure 3B). Remarkably, the majority of eNK cells were NKG2A+NKG2C+, which might suggest that recognition of HLA-E on trophoblast cells by an inhibitory receptor is preferred.

Figure 1 Continued on next page.
Endometrial NK cells have a distinct NKR expression pattern and co-express multiple NKR

By combining the expression of KIR2DL2/L3/S2, KIR2DL1/S1, KIR3DL1/S1, LILRB1, NKG2A, and NKG2C, we identified 64 phenotypically distinct populations of NK cells (hereafter referred to as subpopulations, although this term does not infer that the phenotypes of these populations are fixed). We observed that the NKR repertoire of eNK was different from pbNK cells and has distinct features (Fig. 2A and Supplementary Fig. 4). Although the majority of eNK cell subpopulations were NKG2A⁺, the percentage of NKG2A single positive eNK cells appeared lower compared to pbNK cells. The frequency of NK cells negative for any of the analysed NKR was lower for eNK compared to pbNK cells (Fig. 2B). Interestingly, the majority of eNK cells co-expressed NKG2A and KIR2DL2/L3/S2 together with other NKR. This may suggest that eNK cells are biased...
towards HLA-E and HLA-C1 recognition. The NKR repertoire on eNK cells is more diverse than on pbNK cells, as is visible by the high frequency of eNK cells co-expressing 3 or more NKR (Fig. 2C). Analysis of matched pbNK and eNK cells of the same female gave similar results (Supplementary Fig. 4). A Tukey’s outlier analysis of the 64 NKR subpopulations revealed expansions of 8 subpopulations within the eNK cell pool and 15 expansions within the pbNK cell pool (Table II and Fig. 2A-B, outliers marked as diamond shapes). Only 4 out of 12 females showed expansions both in eNK and pbNK cells. These expansions were tissue-specific, since different expansions were observed in eNK versus pbNK cells of the same female.

Béziat et al. showed that CMV infection induced expansion and differentiation of self-specific inhibitory KIR-expressing pbNK cells [18]. Our data indeed showed that in pbNK cells 60% of CMV$^{\text{pos}}$ females (6/10) displayed NKR subpopulation expansions, while only 18% of CMV$^{\text{neg}}$ females (2/11) (Table I and Table II; CMV status unknown for 4 females). However, in eNK cells, only 20% of CMV$^{\text{pos}}$ (2/10) and 45% of CMV$^{\text{neg}}$ (5/11) females displayed expansions. Interestingly, in pbNK cells, 73% of expansions (11/15) expressed self-specific KIR, while this was only the case for 25% (2/8) of eNK cells (Table II). Hence, the data suggests that, in contrast to peripheral blood, NKR subpopulation expansions in endometrium are not associated with CMV seropositivity nor express self-KIR.

In addition, unsupervised clustering of our dataset with SPADE analysis showed a higher diversity within the eNK cell population, with 19 additional subpopulations not present in the pbNK cell population. In contrast, pbNK cells revealed only one subpopulation that was not found in the eNK cell population (missing subpopulations indicated with arrows and node number in CD56 plot of Fig. 3). The majority of the 19 additional subpopulations present in the eNK cell population are CD56$^{\text{bright}}$ NK cells with high expression of NKG2A and KIR (Supplementary Fig. 5). The one subpopulation absent in eNK cells is CD16$^+$, and LILRB1$^+$ with low CD56, KIR, and NKG2 expression.
Natural killer (NK) cell receptor (NKR) expression patterns on NK cells are different in menstrual blood compared to peripheral blood (N=25). Frequency of each NKG2A positive NK cell subpopulation (KIR2DL2/L3/S2 +/- KIR2DL1/S1 +/- KIR3DL1/ S1 +/- LILRB1 +/- NKG2C +/- NKG2A) is shown as a percentage of total NK cells for paired endometrial NK (eNK) and peripheral blood (pbNK) cells. Lines indicate mean. Outliers are marked with a diamond shape. *P < 0.05, **P < 0.01, ***P < 0.001. 

Figure continued on next pages.
Figure 2 B Natural killer (NK) cell receptor (NKR) expression patterns on NK cells are different in menstrual blood compared to peripheral blood (N=25). Frequency of each NKG2A negative NK cell subpopulation (KIR2DL2/L3/S2⁻/⁻ KIR2DL1/S1⁻/⁻ KIR3DL1/S1⁺/⁺ LILRB1⁺/⁺ NKG2C⁺/⁺ NKG2A⁻/⁻) is shown as a percentage of total NK cells for paired endometrial NK (eNK) and peripheral blood (pbNK) cells. Lines indicate mean. Outliers are marked with a diamond shape. *P < 0.05, **P < 0.01, ***P < 0.001. Figure continued on next page.
In contrast to peripheral blood NK cells, CMV has no imprint on the endometrial NK cell phenotype

Guma et al. reported that CMV$^{\text{pos}}$ individuals have higher frequencies of NKG2C$^{+}$ pbNK cells compared to CMV$^{\text{neg}}$ individuals. Our data showed that the CMV imprint on pbNK cells, i.e. higher percentage of NKG2C$^{+}$ NK cells, is not observed on eNK cells of the same female (Fig. 4). This suggests that, in contrast to pbNK cells, CMV seropositivity does not affect NKG2C expression of eNK cells.

**HLA-C genotype does not influence KIR expression on endometrial NK cells**

HLA-C genotype has been shown to influence expression of cognate KIR receptors on pbNK cells. In order to study the influence of KIR ligands on self-KIR expression by eNK cells, HLA-C and KIR genotypes of 22 females were determined. In pbNK cells, the presence of the HLA-C2 epitope resulted in increased percentage of KIR2DL1/S1$^{+}$ NK cells (Fig. 5A,B). This effect was not observed in eNK cells. No significant effect of HLA-C genotype was observed for KIR2DL1$^{+}$ and KIR2DS1$^{+}$ pbNK and eNK cells. The HLA-C1 epitope did not affect the percentage of KIR2DL2/L3/S2$^{+}$ pbNK and eNK cells. In addition, independent of the HLA-C genotype, more KIR2DL2/L3/S2$^{+}$ eNK cells could be observed compared to the pbNK cell population.

Based on the combined expression of KIR2DL2/L3/S2, KIR2DL1/S1, and KIR3DL1/S1, we identified 8 different NK cell subpopulations. Four females with the same KIR and HLA-C genotype showed similar KIR expression patterns on eNK and pbNK cells (Fig. 5C). Moreover, 5 females with the same KIR but different HLA-C genotype also had similar KIR expression patterns on eNK cells, while the pattern on pbNK cells was more diverse (Fig. 5D). This suggests that the HLA-C genotype does not have an influence on KIR expression of eNK cells.
Table II Summary table of natural killer receptor (NKR) subpopulation expansion of NK cells in (A) endometrium and (B) peripheral blood.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Expansion</th>
<th>CMV seropositivity</th>
<th>HLA-C</th>
<th>Self-specific KIR expansion?</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A: Endometrium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBC14</td>
<td>NKG2A+LILRB1+KIR2DL2/L3/S2+</td>
<td>-</td>
<td>C2C2</td>
<td>No</td>
</tr>
<tr>
<td>MBC31</td>
<td>NKG2A+LILRB1+KIR2DL2/L3/S2+</td>
<td>-</td>
<td>C1C2</td>
<td>Yes</td>
</tr>
<tr>
<td>MBC33</td>
<td>LILRB1+KIR2DL1/S1+NKG2C+</td>
<td>+</td>
<td>C2C2</td>
<td>Yes</td>
</tr>
<tr>
<td>MBC34</td>
<td>NKG2C+</td>
<td>+</td>
<td>C1C2</td>
<td>No</td>
</tr>
<tr>
<td>MBC40</td>
<td>null</td>
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<td>C1C1</td>
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</tr>
<tr>
<td>MBC46</td>
<td>NKG2A+LILRB1+</td>
<td>-</td>
<td>C1C1</td>
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<tr>
<td>MBC50</td>
<td>null</td>
<td>-</td>
<td>C2C2</td>
<td>No</td>
</tr>
</tbody>
</table>

| **B: Peripheral blood** | | | | |
| MBC14 | LILRB1+ | LILRB1+KIR2DL2/L3/S2+ | - | C2C2 | No |
| MBC16 | NKG2A+NKG2C+ | LILRB1+KIR2DL2/L3/S2+ | + | C1C2 | Yes |
| MBC19 | LILRB1+KIR2DL1/S1+NKG2C+ | NKG2A+LILRB1+NKG2C+ | + | C1C1 | Yes |
| MBC27 | KIR2DL2/L3/S2+NKG2C+ | LILRB1+KIR2DL2/L3/S2+ | ? | C1C2 | Yes |
| MBC33 | LILRB1+KIR2DL1/S1+NKG2C+ | | + | C2C2 | Yes |
| MBC34 | LILRB1+KIR2DL1/S1+NKG2C+ | | + | C1C2 | Yes |
| MBC35 | KIR2DL2/L3/S2+KIR2DL1/S1+ | | + | C1C2 | Yes |
| MBC48 | KIR2DL2/L3/S2+KIR2DL1/S1+ | LILRB1+KIR2DL2/L3/S2+ | + | C2C2 | Yes |
| MBC50 | KIR2DL2/L3/S2+KIR2DL1/S1+ | | - | C2C2 | Yes |
Figure 3 SPADE analysis of natural killer cell receptor (NKR) expression pattern on endometrial NK (eNK) cells and peripheral blood NK (pbNK) cells (N=25). Each tree depicts the expression levels of one marker. SPADE is a visualization method that organizes data in a 2D-plot based on similarities in expression of the selected markers. The nodes of the tree represent clusters of cells with similar marker expression and the size of the node is a representation of the amount of cells with these characteristics. The color red and blue represents respectively high or low expression of a particular marker. The orientation of a node is inferred from cellular hierarchies, i.e. more similar subpopulations are adjacent to each other. Arrows and representative node numbers in CD56 figure indicate which subpopulations are absent in endometrium or peripheral blood while still present in the other.
Figure 4 No increased percentage of NKG2C+ natural killer (NK) cells in menstrual blood of cytomegalovirus (CMV) positive females. Frequencies of NK cells expressing NKG2C are shown for paired endometrial NK (eNK) and peripheral blood NK (pbNK) cells from females seropositive (CMV+, N=10) or seronegative for CMV (CMV-, N=11). Lines indicate mean. *P < 0.05, **P < 0.01.

Figure 5 No influence of HLA-C genotype on the expression of cognate KIR2D receptors on menstrual blood derived natural killer (NK) cells (N=22). (A) The frequency of NK cells expressing KIR2DL1/S1, KIRDL1, and KIR2DS1 is shown as mean ± SD for peripheral blood NK (pbNK) and endometrial NK (eNK) cells of females with presence (C2Cx, HLA-C2C2 and HLA-C1C2 genotype) or absence (C1C1, HLA-C1C1 genotype) of C2 epitope. (B) The frequency of NK cells expressing KIR2DL2/L3/S2 is shown as mean ± SD for pbNK and eNK cells of females with presence (C1Cx, HLA-C1C1 and HLA-C1C2 genotypes) or absence (C2C2, HLA-C2C2 genotype) of C1 epitope. Lines indicate mean. *P < 0.05. Figure continued next page.
Endometrial NK cell receptor repertoire

**Figure 5** Figure continued from previous page. No influence of HLA-C genotype on the expression of cognate KIR2D receptors on menstrual blood derived natural killer (NK) cells (N=22). (C, D) Frequency of each NK cell subset, expressing different KIR combinations (KIR2DL2/L3/S2"KIR2DL1/S1"KIR3DL1/S1") is shown as a percentage of total NK cells. (C) eNK and pbNK cells of 4 females with the same KIR genotype and the same HLA-C genotype. (D) eNK and pbNK cells of 5 females with the same KIR genotype but a different HLA-C genotype. Lines indicate mean. *P < 0.05.

**DISCUSSION**

NK cells play an important role in ensuring correct placentation and normal development and growth of the fetus. It has been implied that eNK cells are immature precursors that differentiate into dNK cells after implantation of an embryo. In order to be prepared for implantation, we hypothesize that eNK cells will have a unique NK cell receptor (NKR) expression pattern compared to pbNK cells. In this study, we directly compared the NKR receptor repertoire of NK cells present in menstrual (a source of endometrial cells) and matched peripheral blood of the same females using 10-color flow cytometry. We showed that, within the same female, the NKR expression profile of eNK cells does not differ between consecutive menstrual cycles. The NKR expression pattern of eNK cells clearly differs from pbNK cells, with most eNK cells expressing ≥3 NKR simultaneously, while pbNK cells mainly express <3 different NKR. In addition, the observed NKR subpopulation expansions of eNK cells were tissue-specific, and in contrast to pbNK cells, independent of HLA-C genotype or CMV status. This unique, tissue-specific NKR repertoire of eNK cells might be important to prepare eNK cells towards the reception of an allogeneic fetus.
The frequency of NKG2A, LILRB1, KIR2DL2/L3/S2, KIR2DL3, and KIR2DL1 positive NK cells was significantly higher for eNK cells, similar to previously reported data. The higher frequency of NKR+ eNK cells implies that the bias towards HLA recognition is already present before pregnancy, and is not only a feature of pregnancy. In addition, while the HLA-C genotype of an individual is of influence on the expression of cognate KIR receptors on pbNK cells, our data showed that maternal HLA-C did not seem to affect cognate KIR2D expression on eNK cells. A role for maternal HLA-C in selecting self-KIR expression on dNK and eNK cells has only been found by Sharkey et al. but not by others.

eNK cells can recognize HLA-E on trophoblast cells by the activating NKG2C and inhibitory NKG2A receptor. We observed that almost all eNK cells are NKG2A+ (~90%), a feature of uterine NK cells also observed by many others. Moreover, the majority of eNK cells expressed NKG2A alone or together with NKG2C, while very few eNK cells expressed NKG2C alone. In peripheral blood, NKG2A regulates the response of NKG2C+ NK cells against target cells expressing HLA-E. This might suggest that recognition of HLA-E on trophoblast cells by NKG2A is preferred to avoid uterine NK cell mediated cytotoxicity towards trophoblast cells.

By combining the expression of KIR2DL2/L3/S2, KIR2DL1/S1, KIR3DL1/S1, LILRB1, NKG2A, and NKG2C we identified 64 phenotypically distinct populations of NK cells. We observed that the NKR repertoire of eNK and pbNK cells are different and have distinct features. Interestingly, the majority of eNK cells co-expressed NKG2A and KIR2DL2/L3/S2 together with other NKR, a feature also observed on dNK cells. It may suggest that eNK and dNK cells are biased towards HLA-E and HLA-C1 recognition. Both KIR2DL2 and KIR2DL1 can recognize HLA-C2 but during KIR acquisition, NK cells will first acquire KIR2DL2/L3 before they can acquire KIR2DL1. Our results showed that the percentage of eNK cells positive for KIR2DL2/L3/S2 alone, and expressing both KIR2DL1/S1 and KIR2DL2/L3/S2 was significantly higher compared to pbNK cells, while the percentage of eNK cells expressing KIR2DL1/S1 alone was significantly lower, which is in line with previous results on eNK and dNK cells. This may suggest that eNK cells are only recently matured. In addition, preference for KIR2DL2/L3/S2 on eNK cells may decrease the influence of KIR2DL1, which could be a protective mechanism for successful pregnancy since the combination of the KIR2DL1 and HLA-C2 genotype has been associated with pre-eclampsia and recurrent miscarriage.

pbNK cells were shown to mainly express up to three receptors simultaneously, while eNK cells also co-expressed more receptors, suggesting that the NKR repertoire on eNK cells is more diverse. KIR repertoire acquisition is strongly modulated by HLA class molecules TAP-deficient patients, whose surface expression of HLA class I is significantly reduced, show a higher frequency of NKG2A+KIR+ NK cells and KIR co-expression. A more diverse NKR co-expression on eNK cells could suggest that eNK
Endometrial NK cell receptor repertoire

cells still need to mature, possibly this will take place only after implantation and might depend on fetal HLA. This diverse NKR repertoire could be necessary for eNK cells to successfully encounter an embryo which could have any possible fetal HLA phenotype.

A Tukey’s outlier analysis of our 64 NKR subpopulations identified 8 subpopulation expansions in eNK cells, and 15 expansions in pbNK cells. These expansions were tissue-specific since different expansions were observed in eNK and pbNK cells. In accordance with Béziat et al., we showed that CMV infection leads to expansion of self-specific KIR-expressing pbNK cells. In endometrium, the observed expansions were not associated with CMV infection and not biased towards self-KIR expression. This is in contrast with Ivarsson et al., who reported NKG2C+ self-KIR+ NK cell expansions in endometrium. However, in their study it was not possible to investigate the association between CMV and self-KIR due to a low percentage of CMVneg females. CMV viral infection has also been correlated with a higher proportion of NKG2C+ pbNK cells, which was also observed in our study for pbNK cells but not for eNK cells. Overall, this suggests that the imprinting observed on pbNK cells is not found on eNK cells. Since the origin of eNK cells is still unclear, there are several possibilities for this discrepancy between expansions in endometrium and peripheral blood. It may reflect selective recruitment of phenotypically distinct populations from peripheral blood to endometrium, induction of distinct populations under influence of the uterine microenvironment after recruitment of immature NK cells, or local proliferation from progenitor cells which are not influenced in the same way by viral infection as pbNK cells.

Although we previously showed that menstrual blood lymphocytes are very similar to endometrial biopsy-derived lymphocytes, and show similar expression patterns over consecutive cycles, it is unknown if NKR expression will change over the course of the menstrual cycle or how expression of menstrual blood NK cells will relate to eNK cells at time of implantation. Nevertheless, it could be a valuable tool to investigate the immune cell composition of women with pregnancy complications and fertility issues. A limitation of our study is that no additional markers were used to identify innate lymphoid cells type 3 (ILC3s), which can also be CD56+. However, in our hands ILC3s constitute only 0.04% and 0.002% of total lymphocytes in menstrual and peripheral blood respectively (data not shown). Therefore, we consider the contribution of ILC3s to the NK cell pool to be limited. In addition, in our previous work we showed that eNK cells are from mucosal origin. Therefore, we didn’t use a tissue resident marker to differentiate pbNK from eNK cells.

In summary, this study shows that the NKR expression profile of eNK cells appears truly unique, co-expressing multiple NKR. The repertoire does not differ over consecutive menstrual cycles, and is independent of CMV status or HLA-C genotype. This unique, tissue-specific NKR repertoire might be important to prepare the endometrium towards reception of an allogeneic fetus and successful pregnancy. Therefore, it would be
interesting to study the NKR repertoire of eNK cells in more detail in the future. Studying the endometrial NKR repertoire of women with pregnancy related problems could provide clues to understand the pathogenesis of pregnancy complications.

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Author’s roles
D.F. and T.K. coordinated and designed study, analyzed data and wrote manuscript. R.G.M and I.J. conceived and designed study, analyzed data, and wrote manuscript. B.C. and S.Z. participated in data collection, and assisted in interpretation of data. O.W.H.H. and A.M participated in design of study, and provided critical discussion. All authors evaluated the manuscript and contributed to its content.

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Conflict of interest
None of the authors have a conflict of interest.
REFERENCES


Endometrial NK cell receptor repertoire
CHAPTER 4

Uterine natural killer cells change their receptor repertoire upon pregnancy

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Natural killer (NK) cells, with a unique NK cell receptor phenotype, are abundantly present in the non-pregnant (endometrium) and pregnant (decidua) human uterine mucosa. It is hypothesized that NK cells in the endometrium are precursors for the decidual NK cells present during pregnancy. Microenvironmental changes can alter the phenotype of NK cells, but it is unclear whether pregnancy-specific factors would alter the NK cell receptor repertoire of decidual NK cell precursors in the endometrium. In order to examine whether decidual NK cell precursors reveal phenotypic modifications upon pregnancy, we immunophenotyped the NK cell receptor repertoire of both endometrial and decidual NK cells. We showed that NK cells in the pre-pregnancy endometrium have a different phenotype compared to the NK cells in the pregnant decidua. The frequency of KIR2DS1, KIR2DL2L3S2, KIR2DL2S2 and KIR3DL1 expressing NK cells was lower in decidua, while the frequency of NK cells expressing activating receptors NKG2D, NKp30, NKp46 and CD244 increased compared to endometrium. Furthermore, co-expression patterns showed a decrease in NK cells co-expressing KIR3DL1S1 and KIR2DL2L3S2 upon pregnancy. Our results provide new insights into the NK cell receptor repertoire adaptations NK cells in the uterine mucosa undergo during pregnancy.
Chapter 4

INTRODUCTION

Uterine natural killer (NK) cells are one of the most abundant immune cell types in the non-pregnant endometrium, as well as in the first trimester decidua. The number of NK cells in the endometrium increases over the course of the menstrual cycle, preparing the uterus for implantation of the embryo. After fertilization, the number of NK cells further increases up to 70% of all lymphocytes in first trimester decidua. Both endometrium and decidua-derived NK cells have a CD56brightCD16- phenotype, lack cytotoxic activity and secrete cytokines such as IFN-γ, IP-10 and VEGF. During the normal menstrual cycle, NK cells in the pre-pregnancy endometrium play a role in mucosal integrity, endometrial angiogenesis and endometrial shedding. NK cells in the decidua on the other hand, regulate trophoblast invasion and spiral artery remodeling. NK cell function is regulated by NK cell receptors (NKR), such as killer-immunoglobulin-like receptors (KIR), immunoglobulin-like transcripts (LILRB), C-type lectin heterodimer family (NKG2) and natural cytotoxicity receptors (NCR). The interaction between NKR on decidual NK cells and their respective ligands on trophoblast cells plays an important role in correct placentation. Both endometrial and decidual NK cells have a unique NKR repertoire and are biased towards KIR2D expression. It is speculated that tissue-resident NK cells in the endometrium contribute to the pool of decidual NK cells present during pregnancy, an observation confirmed in parabiosis studies in mice, where decidual NK cells developed from proliferating tissue-resident NK cells in the non-pregnant uterus. In addition, after human pregnancy, precursors for pregnancy-induced memory decidual NK cells were found to be present in the endometrium. Microenvironmental changes can alter the phenotype of NK cells and it has been suggested that pregnancy-specific factors can influence the receptor repertoire of decidual NK cells. However, it is still unclear to what extent the phenotype of decidual NK cells differs from its precursors in the endometrium. Previous studies only compared endometrial or decidual NK cells with their peripheral counterparts, and consequently a direct and thorough assessment of the phenotypic differences and similarities of endometrial and decidual NK cells is lacking. To address whether phenotypic modifications of decidual NK cell precursors occurs under the influence of pregnancy-specific factors, we evaluated the NK cell receptor repertoire on endometrial and decidual NK cells. The results of this study will provide us with more insight into the essential adaptations decidual NK cell precursors undergo for pregnancy success. Failure to adequately change the receptor expression of decidual NK cell precursors may increase the risk of developing pregnancy complications such as recurrent miscarriages, recurrent implantation failure, and pre-eclampsia.
MATERIALS AND METHODS

Tissue sampling
Menstrual blood was collected from 26 healthy women during the first 36 hours of menstruation, as described earlier. Decidual tissue from 14 women undergoing elective termination of normal pregnancy between 6 and 15 weeks of gestation was collected at the Mildred Clinic Arnhem. Use of menstrual blood and abortion material was approved by the institutional review board (Commissie Mensgebonden Onderzoek region Arnhem-Nijmegen, CMO nr. 2009/004 and 2017-3253) and was performed in accordance with the relevant guidelines and regulations. Samples were obtained from each participant upon written informed consent.

Isolation of decidual lymphocytes
Placental tissue was rinsed with PBS to remove blood clots. Trophoblast tissue was removed and decidual tissue was cut into small pieces. 5 ml of tissue was transferred to a C-tube and digested with 10 ml of Accutase (Thermo Fisher Scientific) for 60 minutes in a 37°C water bath while shaking. Tissue was processed twice with a gentleMACS dissociator (program h-tumor 02, Miltenyi Biotech) before and after 30 minutes of digestion. The obtained supernatant was passed through a 100 µm, 70 µm and 40 µm cell strainer to obtain a single cell suspension. After washing of the collected cell suspension with supplemented RPMI [RPMI 1640 medium supplemented with pyruvate (1 mM), glutamax (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml)], cells were layered on a discontinuous Percoll gradient (1,050 g/ml, 1,056 g/ml and 1,084 g/mL; GE Healthcare) for density gradient centrifugation (801 x g for 15 minutes, no brake). Lymphocytes were isolated from the 1,084-1,056 g/ml interface.

Isolation of endometrial lymphocytes
Endometrial immune cells were isolated from menstrual blood as described before. In brief, menstrual blood was washed with PBS and passed through a 70 µm cell strainer to remove mucus and blood clots. Granulocytes were depleted by using the human granulocyte depletion cocktail RosetteSep™ according to manufacturer’s protocol, (Stemcell technologies Inc). After Ficoll density gradient centrifugation (Lymphoprep), menstrual blood mononuclear cells (MMC) were collected and washed twice with 2%HPS-PBS (HPS, human pooled serum, manufactured in-house).

Flow cytometry of decidual and endometrial lymphocytes
Cells were washed twice with PBS-bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, USA) (0.2% v/v), followed by staining for 20 min at room temperature in the dark. After washing twice with PBS-BSA (0.2% v/v), cells were analyzed using the 10-color flow
cytometer Navios™ (480 nm argon blue laser, 405 nm solid state violet laser, 636 nm solid state laser, Beckman Coulter). Data analysis was performed using Kaluza 2.1 software (Beckman Coulter).

The following fluorochrome-conjugated antibodies were used to phenotypically characterize NK cells in decidua and endometrium: CD3-APCAF750, CD16-FITC, CD45-KO, CD56-ECD (Beckman Coulter, Fullerton, CA, USA), CD85j-PC5.5 (LILRB1; Beckman Coulter, custom made), CD158a-AF700 (KIR2DL1; R&D systems, Abingdon, UK), CD158a/h-PC5.5/APC-AF700 (KIR2DL1/S1; Beckman Coulter, custom made), CD158b2-FITC (KIR2DL3; R&D systems), CD158b1/b2-PC7 (KIR2DL2/L3/S2; Beckman Coulter), CD158e1-BV421 (KIR3DL1; BioLegend, San Diego, USA), CD158e1/e2-APC (KIR3DL1/S1; Beckman Coulter), CD159a-APC/PB (NKG2A; Beckman Coulter, custom made), CD159c-PE (NKG2C; R&D systems), CD161-PB, CD244-APC-AF700 (2B4; Beckman Coulter, custom made), CD314-APC (NKG2D), CD335-PC7 (NKp46), CD336-PE (NKp44; Beckman Coulter), and CD337-PC5.5 (NKp30; Beckman Coulter, custom made). The gating strategy used for analysis of NK cells in decidua and menstrual blood is depicted in Supplementary Fig. S1.

**Statistical analysis**

Non-parametric Mann-Whitney test was used to compare decidual and endometrial NK cells. Statistical analyses were performed using GraphPad Prism 5 and p-values < 0.05 were considered significant. All values shown are mean ± SD. Co-expression of NK cell receptors was calculated with the ‘Tree’ function in Kaluza software.
RESULTS AND DISCUSSION

NK cell receptor expression of decidual NK cells is different from endometrial NK cells

At the maternal-fetal interface, decidual NK cells are important players for correct placentation during the first trimester of pregnancy. NK cells in the non-pregnant endometrium have been implied to be precursors of decidual NK cells. It is unclear how the phenotype of decidual NK cells compares to their precursors and whether the phenotype of the decidual NK cell precursors alters under the influence of pregnancy-specific factors. Therefore, we collected pre-pregnancy endometrial NK cells derived via menstrual blood and compared those to decidual NK cells obtained from placental tissue of elective pregnancy terminations between 6 and 15 weeks of gestation. To characterize the NK cell receptor repertoire, we evaluated the expression of 17 NK cell receptors suggested to play a role at the maternal-fetal interface by flow cytometry.

Our results show that KIR2DS1, KIR2DL2L3S2, KIR2DL2S2 and KIR3DL1-expressing NK cells are present at a significantly lower frequency in decidua compared to endometrium. No significant difference was found for NK cells expressing LILRB1, KIR2DL1S1, KIR2DL1, KIR2DL3, and KIR3DL1S1. Interestingly, previous studies showed that the phenotype of first trimester decidual NK cells is dynamic, marked by a decrease of KIR2DL1S1 and KIR2DL2L3S2 expressing decidual NK cells throughout the first trimester of pregnancy. This decrease in receptor expression might be due to increased interactions of these receptors with their respective MHC-ligands on trophoblast cells. However, previous studies showed that the fetal HLA-C genotype did not affect KIR expression on decidual NK cells. Instead, the frequency of KIR-expressing decidual NK cells was influenced by the maternal HLA-C genotype. Stromal cells, which are in close contact with decidual NK cells at the maternal-fetal interface, are also known to express KIR ligands and their expression increases after fertilization. This might imply that stimulation through ligands on maternal-derived stromal cells is more likely to change the KIR receptor expression of decidual NK cells than stimulation through trophoblast cells. Next to ligand binding, also chemokines or cytokines secreted by trophoblast cells or stromal cells could account for the changes observed. Indeed, culturing of endometrial NK cells with trophoblast-conditioned medium decreased expression of KIR2DL1S1, KIR2DL2L3S2 and KIR3DL1. These phenotypic changes could have an impact on the functional capacity of decidual NK cells. For example, activating KIR2DS1+ decidual NK cells by HLA-C2 increased the production of GM-CSF, which enhanced the migration of trophoblast cells. Decreasing the expression of this receptor might limit excessive activation of decidual NK cells and too extensive trophoblast invasion.
Next to KIR receptors, it has been shown that endometrial and decidual NK cell also express NCR and other NK cell receptors.\(^5,18,21,27\). We observed that the percentage of decidual NK cells expressing NKG2D, Nkp30, Nkp46 and CD244 was significantly higher compared to endometrial NK cells (Fig. 2). We did not find a significant difference for NKG2A, NKG2C, and Nkp44 expression. This increase in receptor expression could be mediated by increasing IL-15 levels upon fertilization\(^5\) since IL-15 stimulation can enhance the expression of these receptors on NK cells.\(^5,28\). Stimulation of these receptors results in increased production of chemokines, cytokines and angiogenic factors, which in turn can promote vascular growth and trophoblast invasion.\(^6,11,29\). Therefore, increased receptor expression on
decidual NK cells could have an impact on the functional capacity of these NK cells and might be necessary for correct initiation of placentation. Moreover, MICA and MICB, the ligands for NKG2D, are induced upon cellular stress during pathogenic infection. NKG2D on decidual NK cells is involved in cytotoxic killing of virus-infected decidual fibroblasts. Therefore, increased NKG2D expression may help protect the fetus from intrauterine viral infections. Overall, this suggests that the microenvironment during pregnancy changes the NK cell receptor repertoire of uterine NK cells, either by secreted soluble factors or cell-cell contact.

![Graphs showing different NK cell receptor expression on NK cells in endometrium and decidua](image)

**Figure 2** Different NK cell receptor expression on NK cells in endometrium and decidua. Percentages of NK cells expressing NKG2A, NKG2C, NKG2D, NKp30, NKp44, NKp46, CD244 and CD161 for endometrial (eNK) and decidual (dNK) NK cells. P-values are indicated on the graphs.
Different NK cell subpopulations are present in decidua versus endometrium

After showing that the expression of NK cell receptors differs between decidual and endometrial NK cells, we next assessed co-expression patterns. NK cell function is tightly controlled by the co-expression of a variety of activating and inhibitory receptors. This receptor co-expression results in a high degree of NK cell diversity, with an estimate of 6000 to 30000 different phenotypic NK cell subpopulations. Priming by viral infections can skew the NK cell subpopulation composition. We and others have shown that both endometrium and decidua contain a unique profile of NK cell subpopulations compared to peripheral blood. To address whether pregnancy induces a change in the NK cell subpopulation composition in the uterus, we identified 64 distinct NK cell subpopulations based on the co-expression of KIR2DL1S1, KIR2DL2L3S2, KIR3DL1S1, LILRB1, NKG2A and NKG2C in both endometrium- and decidua-derived NK cells. We observed that different NK cell subpopulations were present in decidua versus endometrium (Fig. 3 A-B). For example, while almost absent in decidual tissue, the endometrial NK cell population clearly contained KIR2DL2L3S2+KIR3DL1S1+, KIR2DL2L3S2+KIR3DL1S1+LILRB1+, and KIR2DL2L3S2+KIR3DL1S1+NKG2C+ NK cells. The majority of NK cell subpopulations that were significantly different between decidua and endometrium co-expressed KIR3DL1S1 and KIR2DL2L3S2 together with other NK cell receptors. These subpopulations were found at a lower percentage in the decidual NK cell population. This suggests that upon pregnancy the uterine NK cell population changes, with a decrease of NK cells co-expressing KIR3DL1S1 and KIR2DL2L3S2. Few studies have examined NK cell subpopulations in human tissues and it remains poorly understood which factors determine the decrease or increase of certain NK cell subpopulations. We and others speculate that both ligand stimulation and soluble factors could contribute to the observed changes.

Future research should aim to determine the contribution of these NK cell subpopulations to their role in the uterus.

FUTURE PERSPECTIVES, LIMITATIONS AND CONCLUSION

It has become clear that the receptor repertoire of decidual NK cells during pregnancy is different compared to the non-pregnant situation, which may have important consequences for correct placentation and protection during intrauterine infection. It can be envisaged that complications such as implantation failure or miscarriage could result from dysregulated changes in NK cell receptor expression. Several reports have highlighted a different NK cell phenotype on decidual NK cells from complicated pregnancies compared to non-complicated pregnancies. Therefore, investigating whether the decidual NK cell precursors in the endometrium of women with pregnancy...
complications have difficulties to change into decidual NK cells could improve our understanding of the underlying pathogenesis.

A limitation of our study is that we had no access to paired samples, i.e. endometrial and decidual NK cells from the same individual. This would have provided us with more insight into the specific changes NK cells undergo in a single individual. However, this poses inconceivable logistic and ethical barriers.

In conclusion, in this study we have demonstrated that decidual NK cell precursors change their receptor repertoire upon pregnancy. We showed that the phenotype and cell composition of pre-pregnancy endometrial NK cells is different from decidual NK cells. The frequency of several KIR+ NK cells decreases upon pregnancy, while the frequency of NK cells with activating receptors such as NKp30, NKp46 and NKG2D increases. In addition, less NK cells co-expressing KIR3DL1S1 and KIR2DL2L3S2 are present in the decidual NK cell population. This suggests that the uterine environment during pregnancy, either through secretion of soluble factors or by ligand stimulation, changes the phenotype of uterine NK cells. This change in receptor expression may be important in controlling placentation. Further research should be aimed at investigating the factors involved in the phenotypic changes and the role of each of the NK cell subpopulations in pregnancy success.
Figure 3 A NK cell receptor co-expression pattern is different for endometrial (eNK) and decidual (dNK) NK cells. Percentage of each NKG2A+ NK cell subpopulation (KIR2DL1S1+/-KIR2DL2L3S2 +/-KIR3DL1S1+/-LILRB1+/-NKG2C+/-NKG2A+) is shown as a percentage of total endometrial (eNK) and decidual (dNK) NK cells. *P < 0.05, **P < 0.01, ***P < 0.001. Figure continued on next page.
Figure 3 B  Figure continued from previous page. NK cell receptor co-expression pattern is different for endometrial (eNK) and decidual (dNK) NK cells. Percentage of each NKG2A NK cell subpopulation (KIR2DL1S1+/-KIR2DL2L3S2+/-KIR3DL1S1+/-LILRB1+/-NKG2C+/-NKG2A-) is shown as a percentage of total endometrial (eNK) and decidual (dNK) NK cells. *P < 0.05, **P < 0.01, ***P < 0.001
REFERENCES


Supplementary figure 1 A Gating strategy and FACS plots of NK cell receptor expression. Representative staining for NK cell receptors on endometrial NK cells, as depicted in the graphs of figure 1 and 2. Figure continued on next page.
B - Decidua

Supplementary figure 1 B Gating strategy and FACS plots of NK cell receptor expression. Representative staining for NK cell receptors on decidual NK cells, as depicted in the graphs of figure 1 and 2. Figure continued from previous page.
CHAPTER 5

Selective expansion and CMV-dependency in pregnancy trained human endometrial NK cells

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Chapter 5
Natural killer (NK) cells are innate lymphocytes that specialize in discriminating and killing altered self-cells, such as malignant and virus-infected cells. However, during pregnancy, local uterine NK cells do not exhibit this killing capacity and display an important regulatory role by promoting trophoblast invasion and spiral artery remodeling and ensuring correct placentation. Over the past decade, it has become clear that NK cells possess adaptive properties and innate immune memory. In humans, memory NK cells have mainly been described as expansions of NKG2C+ and LILRB1+ peripheral blood NK cells (pbNK), and pbNK cell subpopulation expansions expressing self-specific killer-immunoglobulin-like receptors (KIR) following cytomegalovirus (CMV) infection.

It is known that pregnancy complications such as pre-eclampsia and intrauterine growth restriction affect subsequent pregnancies less frequently compared to first pregnancies. Pregnancy in repeated pregnancies is also enhanced in terms of more extensive and earlier trophoblast invasion, vascularization, and angiogenesis. Since 70% of first trimester lymphocytes in the placenta are NK cells and play such a crucial role in proper placentation, it is plausible that pregnancy could induce NK cell memory. The induction of such pregnancy trained NK cells could play a beneficial role in a more efficient placentation in future gestations.

Recently, Gamliel et al. described the presence of pregnancy trained NK cells in the non-pregnant uterus, i.e. endometrium, of women who have experienced pregnancy before. In this article, all samples were derived from women who were CMV positive (CMV pos). From studies reporting expansions of memory NK cells in response to viral infections such as hantavirus, HIV, and hepatitis C virus, it became clear that these expansions were in part driven by underlying CMV co-infection. This raises the question as to whether pregnancy trained NK cells also exist in the endometrium of CMV negative (CMV neg) women. To address this, we phenotyped NK cells from the non-pregnant endometrium of both nulli- (n = 10; women who have never been pregnant) and multigravidae (n = 25; women who have been pregnant before) women. We also determined anti-CMV IgG antibodies in the plasma (n = 11 CMV pos, n = 13 CMV neg, and n = 11 not determined; methods described in ref. 11). Interestingly, we did not see an increase in the percentage of NKG2C+ endometrial NK (eNK) cells and the percentage of LILRB1 expression on NKG2C+ eNK cells in multigravidae compared to nulligravidae women, irrespective of CMV status (Fig. 1A). However, when separating our cohort based on CMV status, we indeed observed a trend towards a higher percentage of LILRB1 expression on NKG2C+ eNK cells in the multigravidae samples of CMV pos women (Fig. 1B). This increase was not observed in the CMV neg multigravidae women. Additionally, no difference in the percentage of NKG2C+ eNK cells was observed when comparing nulli- and multigravidae CMV pos samples.

These results imply that the increase of pregnancy-induced LILRB1 expression on NKG2C+ eNK cells only occurs in CMV pos and not in CMV neg women, suggesting that CMV
seropositivity might be a prerequisite for the induction of these pregnancy-induced memory eNK cells. Although CMV seropositivity itself does not lead to an induction of memory-like eNK cells, the possible molecular changes (epigenetic and transcriptional) induced by CMV on eNK cells could make them more receptive towards pregnancy-induced training.

In addition to the expansion of LILRB1-expressing NKG2C⁺ NK cells, it is also known that CMV infection can induce the expansion of pbNK cell subpopulations that express KIR receptors specific for self-HLA-C. We questioned whether pregnancy would induce such expansions of eNK cell subpopulations. We performed an outlier analysis on eNK cell subpopulations derived from all our nulli- and multigravidae women (methods described in ref. 11). We found that 5 out of 25 multigravidae endometrial samples (20%) show eNK cell subpopulation expansions, while none of the nulligravidae samples showed expansions (Figure 1C). Notably, the majority of the expansions in the multigravidae samples express KIR receptors specific for the woman’s own HLA-C. Our outlier analysis data suggests that the expansions in multigravidae eNK cells was not restricted to CMV seropositivity since these expansion were present in both CMV⁺ and CMV⁻ donors (Fig. 1C; 1 CMV⁺, 2 CMV⁻, 2 not determined), suggesting that CMV might not be a prerequisite for pregnancy-induced eNK cell subpopulation expansions.

In conclusion, our data on eNK cells from CMV⁺ and CMV⁻ women suggests that priming by CMV infection might be a prerequisite for the presence of pregnancy-induced LILRB1-expressing NKG2C⁺ eNK cells in multigravidae women. However, our results on pregnancy-induced self-HLA-C specific eNK subpopulation expansions imply that pregnancy can also shape the eNK cell population regardless of CMV seropositivity. Further research is needed to determine how CMV primes the induction of pregnancy trained LILRB1-expressing NKG2C⁺ eNK cells and whether the observed expansions expressing self-specific KIR would alter NK cell function in a manner beneficial for future gestations.
Figure. 1 Pregnancy trained endometrial natural killer cells. (A, B) Percentage of NKG2C expression on CD56bright endometrial natural killers (eNK) cells and percentage of LILRB1 expression on CD56brightNKG2C+ eNK cells. (A) Comparison of eNK cells from women with (>1) and without (0) previous pregnancies. (B) Comparison of eNK cells from CMV+ and CMV− women, with (>1) and without (0) previous pregnancies. P-values determined with the Mann-Whitney one-tail test. (C) Characteristics of eNK subpopulation expansions in multigravidae samples. ND not determined.
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Pregnancy trained endometrial NK cells
CHAPTER 6

A pregnancy to remember: trained immunity of the uterine mucosae

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ABSTRACT

Natural killers cells and macrophages are abundantly present in the uterine mucosae, regulating tissue remodeling, angiogenesis and placentation. Several pregnancy complications, associated with defective placentation, are more common in first pregnancies. Increasing evidence shows that innate immune cells can develop immunological memory. This innate immune memory, termed trained immunity, manifests itself as a modulated immune response upon a second stimulus. It is hypothesized that the enhanced placentation observed in subsequent pregnancies, is modulated by pregnancy-trained innate immune cells in the uterine mucosae. In this review, we discuss pregnancy-induced trained immunity of natural killer cells and macrophages in the uterine mucosae, and its potential role in pregnancy success. Outstanding questions for future research to obtain new insight into pregnancy-induced trained immunity are also highlighted.
INTRODUCTION

Pregnancy is a unique situation, where two histo-incompatible organisms live together. Sir Peter Medawar was the first to describe the so-called immune paradox of pregnancy in 1953. Since then, it has become clear that immune cells in the uterus play an important role in pregnancy success by enabling implantation, placentation, and maintaining tolerance. A dysfunctional immune system has been implicated in the pathophysiology of pregnancy complications, such as pre-eclampsia, recurrent miscarriages, recurrent implantation failure, preterm birth and uterine growth restriction.

One of the hallmarks of the adaptive immune system, is the development of immunological memory. Immunological memory is the ability of the immune system to respond more effectively and rapidly to a previously encountered antigen, and is the basis for vaccination. This memory response requires the presence of specific memory immune cells. During reproduction, the maternal immune system is exposed to paternal and paternal-fetal antigens. This exposure occurs by seminal fluid during coitus, by contact with fetal trophoblast cells in the placenta during pregnancy, and by fetal microchimerisms that are present in the maternal circulation after pregnancy. It is well established that memory T cells will be formed during pregnancy, which are fetal-specific and are suggested to contribute to fetal tolerance (reviewed by Kieffer et al.). However, over the last decade, it has become apparent that immunological memory is not only a feature of the adaptive immune system, but also cells of the innate immune system can remember a previous immune insult.

In this review, we will discuss the innate immune cells present in the uterine mucosae, the possibility of pregnancy-induced innate immunological memory, and its potential role in pregnancy success.

WHAT IS TRAINED IMMUNITY?

Immunological memory formation is the foundation of the adaptive immune system and is considered to be the fundamental basis of long term protective immunity. Adaptive immunological memory is mediated through antigen-specific activation of T cell receptors (TCR) and B cell receptors (BCR). However, it has become clear that also cells of the innate immune system can develop immunological memory. The term “trained immunity” has been used to describe the modulated immune response of innate immune cells towards a second stimulation. Trained immunity manifests itself as increased responsiveness and increased production of inflammatory mediators upon a second stimulus, and enhanced elimination capacity of pathogens compared to untrained cells. This reprogramming of innate immune cells occurs through epigenetic modifications,
Trained immunity of the uterine mucosae

and results in phenotypic and metabolic changes. Interestingly, this modulated immune response is not antigen-specific and can occur towards a second encounter with both the same and a pathogen-derived molecule that is unrelated to the primary one. This lack of specificity exemplifies the main difference with the classical adaptive immune memory, which entails a high degree of specificity. Thus far, trained immunity has mainly been investigated in systemic monocytes, macrophages and natural killer (NK) cells, although other innate immune cells such as neutrophils, innate lymphoid cells (ILC), γδ T cells, and dendritic cells (DC) are hypothesized to also display trained immunity. Since NK cells and macrophages are the innate immune cells most abundantly present and studied at the maternal-interface (discussed below), we will briefly discuss the phenotypic and molecular reprogramming occurring upon trained immunity of these innate immune cells.

Monocytes and macrophages

Already in 1986, Bistoni and colleagues showed that previous infection with an avirulent Candida albicans strain protects mice from invasive candidiasis in a T and B cell independent manner. This protection was demonstrated to be mediated by macrophages, shown both after adoptive transfer of plastic-adherent cells and in monocyte-defective ccr2-deficient mice. In vitro priming of both PBMC and monocytes with low dose C. albicans or β-glucan (component of fungal cell wall) enhanced the production of TNF-α and IL-6 after secondary stimulation with the same or other stimuli. These β-glucan trained monocytes showed epigenetic reprogramming of H3K4me3 of innate immunity and glycolysis genes through dectin-1 and mTOR signaling. Blocking of the mTOR pathway abrogated trained immunity. In addition, monocytes derived from dectin-1-deficient patients failed to induce trained immunity. Moreover, also Bacillus Calmette-Guerin (BCG) vaccination of mice induced nonspecific protection against lethal C. albicans infection, which proved to be macrophage-mediated as well. In humans, BCG vaccination enhanced IFN-γ, TNF-α and IL-1β production by PBMCs exposed to Mycobacterium tuberculosis (MTB) and unrelated pathogens 2 weeks and 3 months after vaccination. In addition, in vitro priming of monocytes with BCG increased TNF-α and IL-6 production upon secondary stimulation with MTB and nonmycobacterial ligands. BCG training effects of human monocytes were induced through NOD2 receptor and Akt-mTOR signaling, and mediated by increased H3K4me3 at the promoter of tnfα, il6 and tlr4 genes. This training could be reversed by blocking histone methylation, mTOR signaling and metabolic pathways. Human challenge models showed that BCG vaccination of healthy volunteers can confer protection against yellow fever infection and can modulate the response against malaria infection. Although innate immune cells are short-lived, a long-term role for nonspecific immune memory mediated by innate immune cells has been suggested by the reduced morbidity and mortality against
subsequent infections observed after BCG vaccination. Long-term effects are suggested to be mediated by modulation of hematopoietic progenitors in the bone marrow. Recently, trained immunity has also been observed for long-lived tissue macrophages. On the other hand, immunological imprinting of monocytes and macrophages not only results in enhanced responsiveness (trained immunity), but can also lead to tolerance or immunosuppression. A common complication of sepsis is immunoparalysis, where systemic monocytes enter a refractory state, incapable of producing proinflammatory cytokines. This immunoparalysis or hypoinflammatory state strongly influences the susceptibility to secondary infections. In vitro stimulation of monocytes with LPS mimics this tolerance state, displayed as a decreased production of proinflammatory cytokines IL-6 and TNF-α. Taken together, these studies demonstrate that priming of monocytes and macrophages induces epigenetic changes, thereby altering their responsiveness to secondary, unrelated antigens.

NK cells

In 2006, O’Leary and colleagues described immunological memory in mouse NK cells. They showed that NK cells were responsible for hapten-specific contact hypersensitivity responses in RAG-deficient mice (lack T and B cells) after repeated exposure to the same hapten. In addition, contact hypersensitivity was acquired in mice after transfer NK cells from sensitized donors. The concept of immunological memory by NK cells was reinforced by the study of Sun et al. were long-lived memory NK cells were found in mice infected with cytomegalovirus (CMV), which showed enhanced function and protection against subsequent CMV exposure. In humans, memory NK cells have best been studied in cases of CMV infection, which has a striking imprint on the NK cell repertoire. CMV seropositive humans harbor an expanded population of long-lived peripheral NKG2C+ NK cells. This has been confirmed in in vitro studies where exposure of peripheral NK cells to CMV-infected fibroblasts expanded NKG2C+ NK cells. These NKG2C+ NK cells are characterized by LILRB1 expression and biased towards expression of self-specific KIR receptors. In addition, memory features of NK cells include lack of the signaling molecules FcRγ, SYK, and EAT-2, reduced expression of the transcription factor PLZF, epigenetic remodeling of the IFNG locus, SYK, FCER1G and SH2D1B promoter, enhanced response to target cells in an antibody-dependent manner, and metabolic reprogramming. Several reports on other viral infections such as HIV, hantavirus, hepatitis B and C virus, Epstein-Barr virus, and Chikungunya virus, also describe expansions of peripheral NKG2C+ NK cells in these virus-infected individuals. However, in studies where also CMV seropositivity was assessed, it became clear that the observed expansions were confined to CMV positive individuals. Hence, underlying CMV infection might prime the NK cells, and thereby influence the expansion of memory NK cells upon other viral infections.
emphasizes the importance of considering underlying CMV status when investigating NK cell memory in other settings. Training of NK cells has also been found after BCG vaccination. In mice, BCG vaccination has been shown to induce memory NK cells that provide protection against subsequent \textit{M. tuberculosis} challenge. Peripheral NK cells from BCG-vaccinated healthy volunteers showed increased pro-inflammatory cytokine production and IFN-\(\gamma\) expression by NK cells upon stimulation with both MTB and nonspecific \textit{C. albicans}, even up until 1 year after vaccination. While no phenotypic changes associated with memory NK cell characteristics were observed in these studies, increased expression of CD69 on NK cells was observed after \textit{in vitro} stimulation of blood from BCG-vaccinated infants. Notably, in these studies the CMV status of the participants was not taken into account. Next to viral and BCG-induced NK cell memory, it has been reported that cytokines can induce NK cells bearing features of immunological memory in an antigen-independent manner. Collectively, these studies indicate that NK cells show both characteristics of trained immunity (antigen-independent) and classical immunological memory (antigen-dependent).

\section*{INNATE IMMUNITY OF THE UTERINE MUCOSAE}

Innate immune cells are present both in the non-pregnant uterine mucosa (i.e. endometrium) and in the uterine mucosa during pregnancy (i.e. decidua), where they exhibit dedicated functions. Although innate immune cells such as ILCs, neutrophils, DCs, and mast cells are present in the uterine mucosae as well, in this review we will focus on those innate immune cells for which trained immunity has been described, which are NK cells and macrophages.

\section*{Endometrium}

The inner lining of the non-pregnant uterus is called the endometrium. Under the influence of the ovarian hormones progesterone and estradiol, the morphology and cell composition of the endometrium changes over the course of the menstrual cycle. After ovulation, the endometrium transforms into a receptive state suitable for embryo implantation. This process is called decidualization, and during this phase the number of immune cells increases to prepare the endometrium for a possible blastocyst implantation. Without fertilization, progesterone and estradiol levels will drop, resulting in the initiation of menstruation and shedding of the endometrium. Immune cells in the endometrium have unique tissue specific phenotypes compared to their peripheral counterparts and they play a key role in immune defense of the uterine mucosa, in tissue remodeling and repair, and in uterine receptivity.
Natural killer (NK) cells and macrophages are the most abundant innate immune cells in the secretory endometrium. The density of endometrial macrophages increases in the late secretory phase. Endometrial macrophages have a M2-like phenotype and they secrete both pro- and anti-inflammatory factors as well as pro-angiogenic factors. Next to their role in immune defense, endometrial macrophages are involved in tissue homeostasis. They can initiate menstruation by mediating tissue breakdown through expression of enzymes and are involved in tissue repair by secreting angiogenic factors.

Endometrial NK cell numbers increase as well during the menstrual cycle. Endometrial NK cells have a distinct phenotype (CD56<sup>hi</sup>CD16<sup>-</sup>) compared to their peripheral counterparts, and lack cytotoxic activity and secrete low levels of cytokines, unless activated by IL-15. In addition, endometrial NK cells have a unique NK cell receptor repertoire which is stable over different menstrual cycles and is biased towards KIR2D expression. During the menstrual cycle, NK cells are suggested to play a role in mucosal integrity and endometrial angiogenesis by secreting IL-22 and angiogenic growth factors respectively. In addition, the IL-15-endometrial NK cell axes has been shown to play a role in endometrial bleeding.

Decidua
Pregnancy starts with the fertilization of an oocyte, and its success will depend on successful implantation of the blastocyst into the decidua (decidualized endometrium) and subsequent placentation. Decidual immune cells are responsible for these critical stages by creating a suitable environment for blastocyst implantation and by promoting placentation during the first trimester of pregnancy. During early pregnancy, there is an influx of immune cells into the uterine mucosae. The most abundant decidual leukocytes present are NK cells and macrophages, 70% and 20% of total leukocytes respectively, and they can be found in close proximity to invading trophoblast cells and the uterine spiral arteries.

Decidual macrophages play various roles during pregnancy. They are enriched at the implantation site and create a suitable environment for blastocyst attachment by regulating surface glycan structures in epithelial cells. In addition, decidual macrophages are present near the spiral arteries where they induce tissue breakdown and promote vascular remodeling by secreting cytokines, chemokines, proteases, and angiogenic factors. Decidual macrophages also remove apoptotic cell debris formed during tissue remodeling, thereby preventing detrimental inflammatory conditions. Various phenotypes have been described for decidual macrophages, predominantly an activated and immunosuppressive M2-like macrophage phenotype. High levels of IL-10, TGF-β and IDO, low levels of IL-1β, and low expression of costimulatory molecules CD80 and CD86 by decidual macrophages will inhibit T cell activation in the decidua. Therefore, it is suggested that decidual...
macrophages may be essential in maintaining tolerance against fetal antigens. In addition, pregnancy complications with defective placentation, such as pre-eclampsia, have been associated with altered macrophage composition and function.

Decidual NK cells have a CD56<sup>hi</sup>CD16<sup>-</sup> immunomodulatory phenotype and express high levels of KIR receptors. Although decidual NK cells contain cytotoxic granules, they have a weak cytolytic activity against fetal trophoblast cells. It has been demonstrated that decidual NK cells play a significant role in spiral artery remodeling and trophoblast invasion by the secretion of proteases, cytokines, chemokines, and angiogenic growth factors. Cross-talk between decidual NK cells and decidual macrophages can prevent NK cell mediated killing of trophoblast cells, and it results in the induction of regulatory T cells. Moreover, decidual NK cells provide protection against intrauterine viral infections. Changes in decidual NK cell function during placentation have been suggested to play a role in the pathogenesis of pre-eclampsia. Overall, decidual macrophages and NK cells are essential in coordinating tissue remodeling and tolerogenic immune responses during human pregnancy.

**PREGNANCY-INDUCED TRAINED IMMUNITY**

Previous reports indicate significant differences between first and subsequent pregnancies. Epidemiological studies have shown that pregnancy complications are more common in first pregnancies, i.e. first pregnancies (primiparity) are a risk factor for pre-eclampsia development and stillbirth. Primiparity is also associated with a greater risk of small-for-gestational-age infants and first-born babies have a lower birth weight compared to the second-born. Many of these pregnancy complications have been associated with defective placentation. Interestingly, evidence for enhanced placentation in subsequent pregnancies can be observed. First trimester decidua from parous women shows more extensive endovascular trophoblast invasion compared to decidua from nulliparous women. In addition, pregnancy induces structural changes in the uterine anatomy that do not disappear completely after parturition, i.e. a decrease in the muscular component of the spiral arteries can be found with increasing parity in the non-pregnant uterus. This may allow for more effective spiral artery remodeling in subsequent pregnancies. Enhanced placentation and vascular development in subsequent pregnancies is further presented as an increased placental flow index and higher levels of circulating angiopoietin-2 during the early first trimester in multiparous women. Moreover, altered fetoplacental hemodynamics in parous compared to nulliparous women are indicative of enhanced placental function. Overall, this indicates that placental development is more effective in subsequent pregnancies. Given the importance of decidual macrophages and decidual NK cells in successful
placentation, and the ability of macrophages and NK cells to develop immunological memory, it is plausible that pregnancy could induce innate immunological memory, which in turn could contribute to enhanced placentation in repeated pregnancies.

**Macrophages**

Recently, it has been suggested that the uterine environment might not be sterile, but contains a distinct microbiome \(^ {138,139}\). Continuous exposure to microbial ligands such as LPS, could induce endotoxin tolerance in monocytes and macrophages, and lower their pro-inflammatory response \(^ {40}\). It is hypothesized that constant exposure to LPS during pregnancy could lower the production of pro-inflammatory cytokines to protect the fetus from maternal inflammatory responses and contribute to immune tolerance of the fetus \(^ {140}\). Decidual macrophages could play a role in this.

Trained immunity is not only a feature of circulating systemic innate immune cells. Long-lived tissue macrophages in the lung mucosa and the brain can acquire trained immunity as well \(^ {35,36}\). This suggests that it is plausible that macrophages of the uterine mucosae are able to develop trained immunity. At the moment, trained immunity of decidual macrophages, induced by previous pregnancy, has not been described. Future studies should investigate whether macrophages in the decidua of multigravida (multiple pregnancies) women have different phenotypic and functional characteristics compared to decidual macrophages from nulligravida decidua that could enhance placentation in subsequent pregnancies.

**NK cells**

Gamliel et al. recently described a distinct population of uterine NK cells that remember a previous pregnancy \(^ {141}\). While assessing the first trimester decidua of primigravida (first pregnancy) and multigravida women, they observed an expanded population of decidual NK cells with a NKG2C\(^+\)LILRB1\(^+\) memory phenotype in the decidua of multigravida women. These NKG2C\(^+\)LILRB1\(^+\) decidual NK cells in multigravida women exhibited a distinct transcriptional profile. In addition, epigenetic profiling showed that the IFNG and VEGFA loci were more accessible in NKG2C\(^+\) decidual NK cells from multigravida women compared to their NKG2C NK cells. Moreover, decidual NK cells from multigravida women showed enhanced IFN-\(\gamma\) and VEGF\(\alpha\) secretion compared to decidual NK cells from primigravida women. Supernatant derived from decidual NK cells from multigravida women, as compared to primigravida-derived decidual NK cells, enhanced vascularization in *in vitro* and *in vivo* models. A recent single-cell transcriptomics study of the maternal-fetal interface identified three main decidual NK cell subsets named dNK1, dNK2, and dNK3 \(^ {122}\). The dNK1 subset was characterized by high expression of NKG2C, LILRB1 and KIR, showed an active glycolytic metabolism, and it was suggested that dNK1 cells interact with trophoblast cells. These findings suggest that a specific subset of decidual NK cells,
the dNK1 subset, represent pregnancy-trained decidual NK cells.

Interestingly, pregnancy-trained NK cells appear to reside in the endometrium between pregnancies. A significant expansion of NKG2C⁺LILRB1⁺ NK cells with an altered epigenetic signature could also be found in the endometrium of multigravida women. Importantly, in the aforementioned study of Gamliel et al., all decidual and endometrial samples that were used, were derived from women who were CMV seropositive (CMV⁺) women. As mentioned earlier in this review, an underlying CMV infection might prime NK cells, thereby influencing the expansion of memory NK cells by other stimuli. To address whether pregnancy-trained NK cells also exist in the endometrium of CMV seronegative (CMV⁻) women, we investigated the presence of pregnancy-trained endometrial NK cells in a cohort of CMV⁺ and CMV⁻ nulli- and multigravida women. We showed that pregnancy-trained NKG2C⁺LILRB1⁺ NK cells are indeed expanded in the endometrium of CMV⁺ multigravida women. However, this expansion was not present in the endometrium of CMV⁻ multigravida women. These results imply that an underlying CMV infection might be a prerequisite for the expansion of pregnancy-trained NK cells.

To further support this notion, it would be of interest to examine whether pregnancy-trained decidual NK cells would appear during pregnancy in decidual tissue of CMV⁻ multigravida women or rather stay absent. Taken together, these studies suggest that a pregnancy leads to induction of the expansion of uterine NK cells bearing characteristics of trained immunity. This result may provide an explanation as to why placentation is improved and complications of defective placentation are less common in subsequent pregnancies.

As CMV has an extensive imprint on peripheral blood NK cells and the observed pregnancy-trained NKG2C⁺LILRB1⁺ endometrial NK cells are only observed in CMV⁺ multigravida women, could it be possible that the expansion of trained NKG2C⁺LILRB1⁺ uterine NK cells is solely mediated by CMV exposure in the decidua rather than by pregnancy-specific factors? Pereira et al. showed that latent CMV infection of cells at the maternal-fetal interface is common. 31 out of 35 (89%) first trimester decidual samples, collected from healthy and uncomplicated pregnancies, showed detectable CMV DNA. CMV infection during pregnancy can cause pregnancy complications (intrauterine growth restriction, pre-eclampsia and spontaneous pregnancy loss) and severe fetal morbidity when the fetus is infected. Interestingly, co-culture of decidual NK cells with CMV-infected fibroblasts increased the percentage of NKG2C⁺ expressing decidual NK cells, while LILRB1 expression decreased. Upon exposure to CMV-infected cells, decidual NK cells become cytotoxic to clear intrauterine CMV infection during pregnancy. Especially during the first trimester of pregnancy, when NK cells are abundantly present, the rate of congenital CMV infection is low. In addition, epidemiological studies showed that a positive CMV status during previous pregnancies reduced the risk of congenital CMV infections in future pregnancies. The risk of vertical transmission is 30-40% in case of
primary infection during pregnancy, while transmission risk is only 2-4% in case of a non-primary maternal infection. This may suggest that the observed pregnancy-trained decidual NK cells are induced during pregnancy because of decidual CMV exposure, which could provide protection against intrauterine CMV infections in subsequent pregnancies.

In conclusion, trained NK cells with enhanced functional capacity, epigenetic reprogramming, and a distinct phenotype can be found in the endometrium and decidua of multigravida CMVpos women. Further research is needed to investigate pregnancy-induced decidual NK cells in the decidua of CMVneg versus CMVpos women in more detail. This might unravel whether pregnancy-specific signals and/or decidual CMV exposure contribute to the reprogramming of decidual NK cells, and whether the expansion of pregnancy-trained decidual NK cells is to enhance placentation in subsequent pregnancies, to protect against intrauterine CMV infection or possibly both.

**Figure 1** Proposed model for pregnancy-trained NK cells in the uterine mucosae. During first pregnancy, decidual NK cells get primed. Which signals contribute to priming remains unclear, but we hypothesize that next to HLA-E and HLA-G expressed on trophoblast cells, priming by CMV-infected decidual cells will be crucial to induce pregnancy-trained NK cells. Trained NK cells with enhanced NKG2C and LILRB1 expression reside in the endometrium between pregnancies. During subsequent pregnancies, trained decidual NK cells (i.e. NKG2C+LILRB1+ NK cells) expand rapidly. These trained decidual NK cells secrete greater amounts of IFN-γ and VEGFα, thereby enhancing trophoblast invasion and placentation. In addition, we hypothesize that these trained decidual NK cells could provide improved protection against intrauterine CMV infection.
CONCLUDING REMARKS AND FUTURE DIRECTIONS

Trained immunity of innate immune cells in the uterine mucosae provides another piece in the complex immunological puzzle needed for pregnancy success, and offers a compelling explanation as to why placentation is enhanced and complications of deficient placentation are less frequent in subsequent pregnancies. Despite clear evidence supporting the induction of pregnancy-trained decidual and endometrial NK cells with enhanced functional capacity and signs of epigenetic reprogramming, our observation that pregnancy-trained endometrial NK cells are only present in CMV pos women highlights the need for further research (see also Box Outstanding Questions). One of the most critical questions to be investigated is whether pregnancy-trained decidual NK cells can be found in the decidua of multigravida women who are CMV seronegative. So far, it is unclear which signals are involved in the induction of pregnancy-trained uterine NK cells and we hypothesize that next to pregnancy-specific factors, priming of decidual NK cells by decidual CMV exposure during pregnancy will be essential in the induction of pregnancy-induced uterine NK cells (Fig. 1). An interesting future research avenue would be to investigate whether women with recurrent miscarriages, despite having multiple pregnancies, lack the ability to induce pregnancy-trained uterine NK cells. In addition, next to NK cells, trained immunity has also been shown for macrophages, which are also important players at the maternal-fetal interface. It will be intriguing to investigate whether macrophages in the decidua and endometrium of multigravida women show pregnancy-trained characteristics. Recent advances in cutting-edge technologies such as single-cell RNA sequencing, mass cytometry (CyTOF) and epigenomics will allow to tackle the major questions listed here. Unraveling the properties of trained immunity at the maternal-fetal interface will open up new possibilities for therapeutic interventions to treat complications of inadequate placentation by manipulating and expanding trained immune cells in the uterine mucosae, or on the other hand, might contribute to the generation of a maternal vaccine that could control congenital CMV infection.
Outstanding questions

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<th>Question</th>
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<tr>
<td>Does pregnancy induce decidual NK cells with characteristics of trained immunity in women who are CMV&lt;sup&gt;−&lt;/sup&gt; or are pregnancy-trained decidual NK cells only an attribute of pregnancy in CMV&lt;sup&gt;+&lt;/sup&gt; women?</td>
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<td>Is priming by CMV essential for the induction of pregnancy-trained decidual and endometrial NK cells?</td>
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<td>Which signals contribute to the development of pregnancy-trained decidual NK cells?</td>
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<td>Is there an association between the presence of CMV DNA in the decidua and the presence of pregnancy-trained decidual NK cells?</td>
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<td>Are NKG2C&lt;sup&gt;+&lt;/sup&gt;LILRB1&lt;sup&gt;+&lt;/sup&gt; trained decidual NK cells found in close proximity to invading trophoblast cells and/or CMV-infected decidual cells?</td>
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<tr>
<td>Do NKG2C&lt;sup&gt;+&lt;/sup&gt; pregnancy-trained decidual NK cells from CMV&lt;sup&gt;+&lt;/sup&gt; multigravid women show enhanced functional capacity to clear CMV-infected decidual cells compared to NKG2C&lt;sup&gt;+&lt;/sup&gt; decidual NK cells from CMV&lt;sup&gt;−&lt;/sup&gt; multigravid and CMV&lt;sup&gt;−&lt;/sup&gt;/CMV&lt;sup&gt;+&lt;/sup&gt; nulligravid women?</td>
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<td>Do pregnancy-trained uterine NK cells play a role in the pathogenesis of recurrent miscarriages?</td>
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<tr>
<td>Do uterine macrophages in multigravid women show properties of trained immunity, such as increased IFN-γ production and epigenetic reprogramming?</td>
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Author contributions

D.F. wrote the article. R.G.M. and I.J. reviewed and edited the article. All authors approved the final version of the article.
REFERENCES


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CHAPTER 7

Placental disposition of the immunosuppressive drug tacrolimus in renal transplant recipients and in ex vivo perfused placental tissue

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ABSTRACT

Currently, tacrolimus is the most potent immunosuppressive agent for renal transplant recipients and is commonly prescribed during pregnancy. As data on placental exposure and transfer are limited, we studied tacrolimus placental handling in samples obtained from renal transplant recipients. We found transfer to venous umbilical cord blood, but particularly noted a strong placental accumulation. In patient samples, tissue concentrations in a range of 55 – 82 ng/g were found. More detailed ex vivo dual-side perfusions of term placentas from healthy women revealed a tissue-to-maternal perfusate concentration ratio of 113 ± 49 (mean ± SEM), underlining the placental accumulation found in vivo. During the 3 hour ex vivo perfusion interval no placental transfer to the fetal circulation was observed. In addition, we found a non-homogeneous distribution of tacrolimus across the perfused cotyledons. In conclusion, we observed extensive accumulation of tacrolimus in placental tissue. This warrants further studies into potential effects on placental function and immune cells of the placenta.
INTRODUCTION

The incidence of pregnancy in the renal transplant patient population is relatively high and increasing. Treatment with immunosuppressive drugs needs to be continued during pregnancy to prevent graft rejection. Maintenance treatment usually consists of a calcineurin inhibitor combined with a glucocorticoid and/or an antimetabolite drug. The optimal use of the calcineurin inhibitors tacrolimus and cyclosporine A in pregnancy has been discussed in literature.

Although >90% of all kidney transplant recipients in the US receive tacrolimus as part of their immunosuppressive regimen, resulting in a considerable use during pregnancy, data on placental handling of tacrolimus are scarce. Since pregnant women are generally excluded from randomized controlled clinical trials because of ethical, legal, and practical considerations, there is a paucity of studies with respect to safety of drug use during pregnancy. Data on fetal drug exposure are therefore limited to either case reports or pharmacoepidemiological studies describing pregnancy outcome after kidney transplantation under tacrolimus or cyclosporine A, as for instance performed by the National Transplantation Pregnancy Registry (NTPR). This large voluntary registry included almost 2000 pregnancies in female kidney recipients in the period 1990-2010 in North America and reported that of all neonates born, 53% was premature and 46% had a low birthweight (<2500 g), which is significantly higher than the general prevalence in the US in 2010 of approximately 12% and 8%, respectively. In addition, 50-60% of all neonates were delivered by caesarean section, compared to approximately 33% for general pregnancies. In a large systematic review and meta-analysis of 4706 pregnancies in kidney transplant recipients, an increased rate of pregnancy complications was also observed compared to the general population. Nevertheless, the limited available literature does not report any clinically relevant differences in pregnancy outcome when comparing immunosuppressive drugs.

Particularly in the absence of conclusive evidence on drug safety from clinical trials, mechanistic pharmacological knowledge regarding placental drug handling can help to decide which calcineurin inhibitor may be preferred during pregnancy. Tacrolimus (MW 804 g/mol, logP 3.3) and cyclosporine A (MW 1202 g/mol, logP 2.9) have been reported to cross the placental barrier to a similar extent. For both drugs venous umbilical cord concentrations have been reported to be lower than maternal blood concentrations. However, when considering the safety of tacrolimus during pregnancy, one should not only be concerned about placental drug transfer, but also take a possible interference with placental function into account. It is known that placental insufficiency may lead to the pregnancy outcomes associated with the use of calcineurin inhibitors. Therefore, it possible that tacrolimus may indeed affect placental function. As placental drug exposure will drive such potential adverse effects, particularly more information on
Tacrolimus accumulation in placenta of kidney transplant patients is needed.

To obtain more insight into the placental handling of tacrolimus during pregnancy, we studied tacrolimus concentrations in placental tissue samples from 6 renal transplant patients as well as in available corresponding umbilical cord blood samples. In addition, we determined placental accumulation and transfer of tacrolimus in an ex vivo model using isolated dual-side perfusions of cotyledons from healthy females.

**MATERIALS AND METHODS**

**Collection of whole blood and placental tissue from renal transplant patients**

Pregnant renal transplant recipients (n = 6) signed informed consent, according to the Dutch Medical Research Involving Human Subjects Act (WMO file number: 2014-232). Maternal peripheral blood was collected in EDTA tubes during routine visits to the clinic to monitor tacrolimus trough levels during pregnancy. Venous umbilical cord blood samples were available from 2 patients. Placentas were collected immediately after delivery and pieces of approximately 1 cm³ of the central part of the placental bed were stored at -80 °C until processing.

**Conduction of placenta perfusion experiments**

The conduction of ex vivo placenta perfusion studies with human tissues was approved by the local ethics committee (file number: 2014-1397) and an informed consent was signed which allowed use of placental tissue for ex vivo studies, but no reporting of clinical data or pregnancy outcomes. Placentas were collected from healthy pregnant women, who were not infected with HIV, hepatitis B or hepatitis C. Women who had a retained placenta or with a multiple pregnancy were also excluded. The perfusion procedure was based on the method described by Schneider et al. with minor modifications as reported previously. In brief, placentas were collected immediately after delivery and time until perfusion was kept below 45 minutes. The perfusion medium consisted of Krebs-Henseleit buffer, supplemented with 11.1 mM glucose, 30 g/L human serum albumin (Albuman®, Sanquin, Nijmegen, the Netherlands) and 2500 IU/L heparin (LEO Pharma, Amsterdam, The Netherlands). The medium was oxygenated with 95% O₂/5% CO₂, while being kept on 37 °C at a pH between 7.2 and 7.5. An intact cotyledon was selected and a closed fetal circulation was reestablished by cannulation of the matching vein and artery (6 mL/min). Subsequently, four cannulas were inserted through the maternal decidual plate into the intervillous space, resulting in an inflow of 12 mL/min. Medium flowing from the intervillous space into the perfusion chamber was recirculated via the maternal reservoir ensuring a closed system. In a pre-perfusion period, both circulations were initially left...
open in order to wash out residual blood. Subsequently, at the start of the experiment, maternal and fetal circulations were closed and 10 ng/mL tacrolimus (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to the closed maternal circulation, which had a volume of 200 mL. After 10, 30, 45, 60, 90, 120, 150 and 180 minutes of perfusion, samples were taken from both the maternal and fetal reservoir and stored at -20 °C until analysis. At the end of the experiment, the perfused cotyledon was excised from the remainder of the placenta, snap frozen in liquid nitrogen and stored at -80 °C until further use. To test for non-specific adherence of tacrolimus to our perfusion system a control perfusion was conducted in the absence of placental tissue.

For every placental perfusion, control substances were included to determine whether the placental capillary bed remained intact throughout the period of perfusion and to confirm overlap of the fetal and maternal circulation. Because of its size, FITC-dextran (molecular weight: 40 kDa) is expected not to cross the placental barrier after addition to the fetal circulation (36 mg/L), and therefore served as a marker for integrity of the fetal capillary bed. Antipyrine (molecular weight: 188 Da, logP: 0.38) was added to the maternal circulation (100 mg/L). It undergoes rapid passive diffusion across the placental barrier and was used to confirm overlap of the maternal and fetal circulation. These control markers have been used previously in placental perfusion studies by Myllynen et al., Mathiesen et al. and Schalkwijk et al. 15-17.

Analysis of antipyrine and FITC-dextran concentrations in perfusate samples
Samples were processed according to the method of Brodie et al. after which antipyrine levels could be measured at 350 nm using a plate reading spectrophotometer 18. A Fluorescence Multi-well Plate Reader (PerkinElmer, Ex 485 nm / Em 530 nm) was used to determine the FITC-dextran level in the maternal and fetal reservoir over time. The experiment was considered successful when the antipyrine maternal-to-fetal concentration ratio at the end of the perfusion was <1.25 (i.e. good overlap of maternal and fetal circulation), the maternal-to-fetal concentration ratio of FITC-dextran was <0.03, and volume loss from the fetal circulation did not exceed 3 mL/h during 180 min of perfusion (confirmed integrity of placental capillary bed).

Analysis of tacrolimus concentration in whole blood, tissue and perfusate samples
The tacrolimus assay in whole blood is based on validated UPLC–tandem mass spectrometry 19 and is used for therapeutic drug monitoring purposes in our medical center. To determine the tacrolimus concentration in placental tissue of renal transplant patients and in placental tissue after 180 min of perfusion, homogenates were made to allow subsequent LC-MS/MS analysis, by homogenizing 1 part of tissue in 4 parts of
Krebs-Henseleit buffer. Rapamycin (Sigma-Aldrich, Zwijndrecht, The Netherlands) was used as internal standard. To precipitate proteins, methanol was added to perfusate and homogenate samples. Following ultrasonification (5 min) and subsequent centrifugation (12,000 g for 3 minutes), supernatant was used to quantify tacrolimus. Tacrolimus levels were determined by LC-MS/MS analysis, using an Acquity UPLC system (Waters, Milford, MA, USA) coupled to a Xevo TQ-S (Waters) triple quadrupole mass spectrometer. The elution gradient was as follows: 0 min, 55% B; 0.6–0.7 min, 70% B; 0.7–0.8 min, 90% B; 0.8–2.3 min 100% B and 2.3–2.5 min 55% B. The mobile phase consisted of solvent A (1 mM NH₄F + 0.1% formic acid in H₂O) and solvent B (1 mM NH₄F + 0.1% formic acid in MeOH). The following MRM transitions were used: for tacrolimus m/z 821.8 (parent ion) to m/z 768.7 and 786.8 (both product ions), for rapamycin m/z 931.9 (parent ion) to m/z 864.9 and 882.8 (both product ions). The detection limit of the assay was 0.24 nM. Data were analyzed using Graphpad Prism 5.03 (Prism, Graphpad software Inc, San Diego, USA) and results are presented as mean ± SEM.

**RESULTS**

**In vivo placental handling of tacrolimus in renal transplant patients**

Patient characteristics of the 6 women participating in this part of our study can be found in Table 1. Based on analysis of venous umbilical cord concentrations as well as corresponding placental tissue concentrations, placental handling of tacrolimus could be assessed in vivo. The maternal C_{trough} level at the last sampling time point before delivery varied between 2.9 – 7.6 ng/mL. In 2 cases, venous umbilical cord blood concentrations were recorded in patient records, being 4.8 and 6.2 ng/mL, which is indicative of placental transfer of tacrolimus. A placental tissue concentration ranging between 55 – 82 ng/g demonstrated tacrolimus accumulation within the organ (Table 2).

**Ex vivo placental perfusion experiments**

A total of four out of eight perfusion experiments with placentas of healthy women met the quality control criteria as explained above; three from vaginal deliveries and one from an elective caesarean section. As can be seen in Fig. 1A, the antipyrine concentrations equilibrate in both circulations. Upon addition to the maternal circulation, a mean maternal-to-fetal concentration ratio of 1.07 ± 0.04 for antipyrine was observed. This is in accordance with extensive passive diffusion across the placental barrier and hence a good overlap of the maternal and fetal circulations. Integrity of villous structures was confirmed by the poor placental passage (maternal-to-fetal concentration ratio of <0.03) of FITC-dextran (Fig. 1B). For both the maternal and fetal reservoir, pH and volume loss were within the predefined required ranges (pH 7.2-7.5, volume loss <3 mL/h).
After addition of tacrolimus (10 ng/mL) to the maternal circulation, maternal perfusate levels decreased to 1.9 ± 0.4 ng/mL at t=180 min, while tacrolimus remained undetectable in the fetal reservoir (Fig. 2). The poor placental transfer from maternal to fetal circulation in the investigated perfusion interval and absence of the drug in the fetal circulation suggested placental retention of tacrolimus. Comparison of the tacrolimus concentration in placental tissue with tacrolimus concentration in maternal perfusate at the end of the experiment, indeed revealed a tissue-to-maternal perfusate concentration ratio of 113 ± 49, as shown in Table 3. Interestingly, our *ex vivo* perfusion studies also demonstrated that tacrolimus was not distributed homogeneously across the perfused cotyledon (Supplementary Table S1). The measured tissue concentrations differed up to a factor 19 in a series of samples taken from different locations in the same cotyledon. Nevertheless, in all samples obtained in the *ex vivo* perfusions, placental tissue concentrations exceeded maternal and fetal perfusate concentrations.
Table 1
Patient characteristics of renal transplant recipients at the time of delivery.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at delivery (yr)</th>
<th>Immunosuppressive regimena</th>
<th>eGFR (mL/min/1.73m²)</th>
<th>Months between transplantation and delivery</th>
<th>Gestational age at delivery (wks + days)</th>
<th>Mode of delivery (vaginal or CS)</th>
<th>Hospital of delivery</th>
<th>Birthweight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>30</td>
<td>AZA (125 mg) + TAC (8 mg)</td>
<td>41</td>
<td>34</td>
<td>36 + 2</td>
<td>CS</td>
<td>Radboudumc, Nijmegen</td>
<td>2342</td>
</tr>
<tr>
<td>#2</td>
<td>30</td>
<td>TAC (10 mg) + PRED (5 mg)</td>
<td>88</td>
<td>37</td>
<td>36 + 3</td>
<td>CS</td>
<td>University Medical Center Utrecht</td>
<td>2405</td>
</tr>
<tr>
<td>#3</td>
<td>38</td>
<td>TAC (7 mg) + PRED (15 mg) + AZA (150 mg)</td>
<td>25</td>
<td>72</td>
<td>33 + 1</td>
<td>Vaginal</td>
<td>Radboudumc, Nijmegen</td>
<td>1735</td>
</tr>
<tr>
<td>#4</td>
<td>30</td>
<td>TAC (6 mg bid) + PRED (10 mg)</td>
<td>46</td>
<td>27</td>
<td>37 + 3</td>
<td>CS</td>
<td>Radboudumc, Nijmegen</td>
<td>3240</td>
</tr>
<tr>
<td>#5</td>
<td>33</td>
<td>TAC (3 mg bid) + AZA (100 mg) + PRED (alternately 5 mg and 7.5 mg)</td>
<td>72</td>
<td>28</td>
<td>38 + 2</td>
<td>Vaginal</td>
<td>Radboudumc, Nijmegen</td>
<td>2400</td>
</tr>
<tr>
<td>#6</td>
<td>30</td>
<td>TAC (5.5 mg bid) + PRED (alternately 5 mg and 7.5 mg)</td>
<td>79</td>
<td>12</td>
<td>38 + 0</td>
<td>Vaginal</td>
<td>Radboudumc, Nijmegen</td>
<td>2615</td>
</tr>
</tbody>
</table>

aIf not specified: once daily.
Abbreviations: AZA = azathioprine; TAC = tacrolimus; PRED = prednisone; bid = twice a day; eGFR = estimated glomeluar filtration rate; CS = caesarian section
Table 2
Tacrolimus concentrations in maternal whole blood, placental tissue and venous umbilical cord blood of renal transplant recipients. The reported maternal tacrolimus concentration is the last $C_{\text{trough}}$ level measured before delivery and derived from patients records. Hence sampling of maternal blood was not at the time of delivery but at varying times prior to delivery.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Maternal $C_{\text{trough}}$ level before delivery (ng/mL)</th>
<th>Placental tissue concentration (ng/g) as mean ± SEM</th>
<th>Venous umbilical cord blood concentration (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>4.2</td>
<td>55 ± 3</td>
<td>–</td>
</tr>
<tr>
<td>#2</td>
<td>7.6</td>
<td>68 ± 19</td>
<td>–</td>
</tr>
<tr>
<td>#3</td>
<td>3.9</td>
<td>82 ± 6</td>
<td>–</td>
</tr>
<tr>
<td>#4</td>
<td>6.6</td>
<td>75 ± 2</td>
<td>–</td>
</tr>
<tr>
<td>#5</td>
<td>2.9</td>
<td>62 ± 2</td>
<td>6.2</td>
</tr>
<tr>
<td>#6</td>
<td>4.0</td>
<td>82 ± 1</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Figure 1 Placental transfer of the control molecules antipyrine and FITC-dextran. Concentration-time profile of (A) antipyrine (100 mg/L) and (B) FITC-dextran (36 mg/L) in the maternal (open circles) and fetal circulation (squares), based on four successful experiments during 180 min of perfusion. Data are depicted as mean ± SEM.

Figure 2 Placental transfer of tacrolimus. Concentration-time profile of tacrolimus (10 ng/mL) in the maternal (open circles) and fetal circulation (squares), based on four successful experiments during 180 min of perfusion. Data are depicted as mean ± SEM.
Tacrolimus accumulation in placenta of kidney transplant patients

**Table 3**

<table>
<thead>
<tr>
<th>Perfusion</th>
<th>Maternal perfusate concentration at t = 180 min (ng/mL)</th>
<th>Placental tissue concentration (ng/g) as mean ± SEM</th>
<th>Placental tissue-to-maternal perfusate concentration ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>1.8</td>
<td>54 ± 8</td>
<td>31</td>
</tr>
<tr>
<td>#2</td>
<td>2.1</td>
<td>138 ± 85</td>
<td>65</td>
</tr>
<tr>
<td>#3</td>
<td>2.8</td>
<td>287 ± 103</td>
<td>102</td>
</tr>
<tr>
<td>#4</td>
<td>0.9</td>
<td>236 ± 76</td>
<td>253</td>
</tr>
<tr>
<td>Mean</td>
<td>1.9</td>
<td>179</td>
<td>113</td>
</tr>
<tr>
<td>SEM</td>
<td>0.4</td>
<td>52</td>
<td>49</td>
</tr>
</tbody>
</table>

**DISCUSSION**

We observed a substantial, > 10-fold accumulation of tacrolimus in placentas obtained from both renal transplant recipients as well as in *ex vivo* perfusion experiments using placental tissue from healthy females. To our knowledge, tacrolimus concentrations in placentas from renal transplant recipients have not been reported before. In a study performed by Jain et al., who focused on liver transplant patients, a 3-fold higher tacrolimus concentration in placental tissue compared to maternal plasma levels was reported. In our study we found a much higher degree of accumulation in placental tissue samples from exposed patients. This could be due to differences in placental disposition between renal and liver transplant patients, but possibly also because of differences in methods of analysis, tissue preservation or sampling, which were only briefly described by the authors and did not allow us to compare these aspects between the studies. As tacrolimus is a drug with a high volume of distribution, it is likely that the tissue accumulation of this drug is not limited to the placenta. Indeed, tissue distribution studies in rats showed accumulation within the heart, lung and spleen, while also accumulation was noted in liver and kidney biopsies obtained from liver and kidney transplant patients. Nevertheless, we now describe that in renal transplant patients, significant exposure of the placenta clearly takes place, indicating that this organ may be prone to tacrolimus-induced toxicity as well.

In contrast to our observations in short term perfusion experiments that did not show detectable tacrolimus levels in the fetal circulation, analysis of venous umbilical cord samples indicated that *in vivo*, fetal exposure to tacrolimus does occur. This discrepancy could mean that the extensive placental binding of tacrolimus that contributes to the strong placental retention in the *ex vivo* studies, may have become saturated upon the
chronic exposure in the clinical setting. Saturation of binding capacity would result in an increase in unbound tacrolimus concentrations within the placenta and therefore a stronger driving force for diffusion from placenta to the fetal circulation. To further study whether saturation of tissue binding plays a role, *ex vivo* placental perfusion experiments could be performed with placentas from renal transplant recipients that have been chronically exposed to tacrolimus *in vivo*. In this case, an increased placental transfer to the fetal circulation would be expected to occur.

Our *in vivo* data demonstrated that the venous umbilical cord blood concentrations were somewhat higher than the maternal $C_{\text{trough}}$ levels. However, because the sampling times of venous umbilical cord blood and maternal blood did not match in our observational study, it was not possible to give an accurate estimate of the maternal tacrolimus level around the time of delivery, and hence calculate an exact placental transfer ratio. Zheng et al. collected maternal blood immediately after delivery and observed venous umbilical cord blood concentrations in exposed pregnant patients that were $71 \pm 18\%$ (range 45–99%) of maternal concentrations. As tacrolimus displays extensive erythrocyte binding *in vivo*, but no erythrocytes were added to the *ex vivo* perfusion buffer, the total tacrolimus concentration of 10 ng/mL used in this study probably results in higher free concentrations than present in whole blood of renal transplant recipients. Still, physiological albumin concentrations were present in the *ex vivo* perfusion buffer, as well as some circulating erythrocytes since they cannot be washed out completely from the placenta. We did not measure the ultimate unbound concentrations in the *ex vivo* experiments, but this could be considered relatively high. On the other hand, it should be noted that the tacrolimus fraction unbound *in vivo* also increases during pregnancy. After addition of tacrolimus to the maternal perfusate, we found that rapid extraction occurred to the placenta, while also some adherence to components of the perfusion system was observed. Despite the high unbound tacrolimus exposure in the maternal perfusate and therefore also a large driving force for passive placental transfer, the amount of drug transported to the fetal circulation still remained negligible during 180 min of perfusion, further pointing to a high tacrolimus binding capacity of placental tissue.

Information about differences in placental accumulation and transfer between different calcineurin inhibitors may aid in the decision-making process of the drug of first choice during pregnancy. Nandakumaran et al. studied *ex vivo* placental transfer of cyclosporine A in a perfusion set-up and reported <5% passage along with placental accumulation of cyclosporine A. This is in line with our findings with tacrolimus. The question now arises to which extent placental accumulation of either cyclosporine A and tacrolimus is causally linked to placental toxicity and how this relates to the adverse pregnancy outcomes reported in literature. Considering the vasoconstrictive effects of calcineurin inhibitors as well as their toxic effects on the kidney and pancreatic...
beta cells, potential cytotoxic effects on the placental level might be expected as well \(^{27,28}\). Placental cytotrophoblasts, syncytiotrophoblasts and endothelial cells may be differentially affected in the face of the high concentrations we described here. Moreover, since the immune system is crucial for proper placentation, placental tissue accumulation of calcineurin inhibitors could also interfere with placental lymphocyte function and maturation, leading to pregnancy complications \(^{29}\). Finally, rat embryo culture experiments showed that tacrolimus was more likely to induce apoptosis and cause more morphological abnormalities than cyclosporine A \(^{30}\). Translation of the data from such studies to a toxicological risk for adverse placental drug effects in human is still difficult, because of species differences and because effects were not linked to the placental drug concentrations that were reached. We propose to combine our data on human placental drug concentrations with dose-response data for effects of tacrolimus on key placental physiological processes measured in vitro or ex vivo in human placental cells and tissues. This will help to further delineate risks of adverse placental effects of calcineurin inhibitors during pregnancy. In this respect, physiologically-based pharmacokinetic modeling approaches can also provide an opportunity to further advance our understanding of the link between placental pharmacokinetics and pharmacodynamics of immunosuppressive drugs \(^{31}\).

We conclude that tacrolimus accumulates in placental tissue of renal transplant recipients treated with this drug, which is in line with observations in the ex vivo perfused human cotyledon. The build-up of a placental reservoir of tacrolimus in renal transplant patients warrants further studies into potential adverse pharmacological and immunological effects on placental function.

**Disclosure**

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

**Acknowledgements**

We thank Gerard Zijderveld for inclusion of participants, all women who donated placenta, and the caretakers who collected them. We also thank Dr. A.T. Lely from the University Medical Center in Utrecht (the Netherlands) for the inclusion of a renal transplant patient.
REFERENCES


Tacrolimus accumulation in placenta of kidney transplant patients


CHAPTER 8

Decidual and cord blood lymphocyte composition is affected in pregnant kidney transplant patients

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Manuscript in preparation
ABSTRACT

Pregnancy after renal transplantation is associated with an increased risk for complications, such as pre-eclampsia and preterm birth. To prevent graft rejection, patients require lifelong immunosuppressive drug treatment. A delicately balanced uterine immune system is essential for pregnancy success and perturbations are associated with pregnancy complications. Little is known about the effect of immunosuppressive drug use throughout pregnancy on the uterine immune system. Moreover, children born to kidney transplant patients are exposed in utero to immunosuppressive drugs. We hypothesized that the use of immunosuppressive drugs during pregnancy may affect both the uterine and neonatal immune system. We characterized decidual immune cells from placentae obtained after delivery as well as neonatal cord blood from 14 kidney transplant patients and 20 controls, i.e. healthy, uncomplicated pregnancies, by flow cytometry.

The immune cell composition in decidua of women with a kidney transplant did not differ from healthy controls with regard to percentages of monocytes, NK cells, T cells, CD25+CD127lowFoxP3+ regulatory T cells (Treg), and B cells. Also, no difference in CD4+ T cells positive for IFN-γ and IL-17 was observed. We did observe a decreased frequency of highly suppressive HLA-DR+ Treg in decidua of women with a kidney transplant, particularly in those treated with tacrolimus versus those that were treated with azathioprine next to tacrolimus, or with azathioprine alone.

In addition, significant differences were observed in the neonatal immune system of children born to kidney transplant patients compared to those born to healthy women. Specifically, altered frequencies of monocyte subsets, and decreased frequencies of NKT cells, CD25+CD127lowFoxP3+ Treg, HLA-DR+ Treg, and B cells were observed at birth. Our findings indicate that immunosuppressive drug use during pregnancy in kidney transplant patients has a significant and distinct impact on the decidual and neonatal immune system. This could have important consequences for the incidence of pregnancy complications, and the offspring’s health. Further large-scale studies are required to better clarify the role of different drug regimens on the decidual and neonatal immune system.
INTRODUCTION

Achieving successful pregnancy in women with advanced chronic kidney disease or end-stage renal disease is clinically challenging. Renal transplantation is the treatment of choice for most of these patients, especially in women of childbearing age as renal transplantation greatly improves fertility and the ability to conceive. Therefore, it is not surprising that the number of pregnancies in patients with a kidney transplant is rising. Unfortunately, kidney transplant patients have a higher risk of developing pregnancy complications. Pre-eclampsia occurs in about one-third (21-38%) of the pregnant kidney transplant patients, while the risk of pre-eclampsia in the general population is only 3-5%. Low birth weight children (< 2500 g; 50%) and preterm delivery (< 37 weeks of gestation; 50%) is also more common in kidney transplant patients compared to the general population. To prevent graft rejection, these patients require a lifelong immunosuppressive drug regimen that needs to be continued during pregnancy. The recommended drugs for use during pregnancy are antiproliferative drugs (azathioprine) and/or calcineurin inhibitors (tacrolimus or cyclosporin), both in combination with low dose steroids (prednisone). It is well established that immunosuppressive drugs have distinct effects on immune cells. Moreover, the immunosuppressive drug tacrolimus accumulates in the placenta of women with a kidney transplant. Therefore, it can be envisaged that the use of immunosuppressive drugs during pregnancy could influence the delicately balanced dynamics in the uterine immune system that are essential for a pregnancy to be successful. This could explain the higher incidence of complications observed in this group of patients, as it is well accepted that immune alterations in the uterus will result in pregnancy complications. For example, pre-eclampsia and preterm birth have been associated with a shift from tolerogenic Th2 and regulatory T cell immunity towards pro-inflammatory Th1 and Th17 immunity.

In addition, azathioprine, tacrolimus and prednisone are able to cross the placenta and enter the fetal circulation. Although no increased incidence of major congenital malformations has been reported for these drugs, it can be hypothesized that in utero drug exposure of the developing fetus could impact the development of the neonatal immune system. Ono et al. indeed showed that infants born to kidney transplant patients had reduced B cell numbers at birth and an increased risk of hospital admission in the first months of life.

To investigate whether the use of immunosuppressive drugs impacts the local uterine immune system, we immunophenotyped maternal immune cells derived from the placenta after delivery of kidney transplant patients and controls (healthy, uncomplicated pregnancies). In addition, we immunophenotyped immune cells in cord blood of neonates born to kidney transplant patients and controls, to assess the effect of in utero
Decidual and neonatal lymphocytes of kidney transplant patients

immunosuppressive drug exposure on the development of the neonatal immune system. In this study, we report that development of the neonatal immune system is affected in the offspring of kidney transplant patients, which could have important consequences for the offspring’s health outcomes. In addition, we show for the first time that immunosuppressive drugs influence the decidual immune environment, and demonstrate that different immunosuppressive drugs have distinct effects. This suggests that the choice of drugs might influence the risk for the development of complications differently.

MATERIALS AND METHODS

Study population
14 pregnant women with a kidney transplant and 20 pregnant healthy controls were included in this study. We collected cord blood (umbilical vein; EDTA tubes) and placentae after delivery. All participants provided written informed consent (CMO nr. 2014-232). Women with a kidney transplant were recruited from the Radboud University Medical Center and University Medical Center Utrecht in the Netherlands. Controls were only recruited from the Radboud University Medical Center. Donor characteristics can be found in Table 1.

| Table I Donor characteristics |
|-------------------------------|------------------|------------------|
|                               | Kidney transplant | Control          |
|                               | (N=14)            | (N=19)           |
| Median age in years (range)   | 30 (22-38)        | 31 (26-39)       |
| Median gestational age at delivery, in weeks (range) | 36 (26-38) | 39 (37-41)** |
| Preeclampsia                  | 5/14              | NA               |
| Immunosuppressive drugs       |                  |                  |
| Azathioprine                  | 7/14              | NA               |
| Tacrolimus                    | 11/14             | NA               |
| Prednisone                    | 12/14             | NA               |

NA, not applicable
** Mann-Whitney test

Isolation of cord blood lymphocytes
One ml of blood was lysed with 25 ml lysis buffer (NH$_4$CL + KHCO$_3$/Na$_4$EDTA diluted in H$_2$O) for 10 minutes and washed 3x times with PBS. Lysed blood samples were used when only surface staining was performed. For intracellular staining protocol, lymphocytes were isolated by density gradient centrifugation (Lymphoprep, Axis-Shield PoC AS). After
centrifugation (801 x g, 15 minutes, no brake), the lymphocyte layer was collected and isolated cells were washed twice with PBS before further analysis.

**Isolation of decidual lymphocytes**

Decidua parietalis was collected from the obtained placentae as previously described. After removing the amnion, the decidua parietalis (i.e. maternal layer of the placental membranes surrounding the fetus) was carefully scraped from the chorionic trophoblast layer. The tissue was washed thoroughly with PBS, minced with scissors and washed again until the supernatant became clear. The tissue was incubated with 0.2% collagenase (Gibco Life Technologies) and 0.04% DNAse (Roche Diagnostics) in a water bath at 37°C while shaking. After 60 minutes, digested tissue was washed with supplemented RPMI (RPMI 1640 medium supplemented with 1 mM pyruvate, 2 mM glutamax, 100 U/ml penicillin, and 100 mg/ml streptomycin; Thermo Fischer) and passed through a 70 µm cell strainer (Greiner). Lymphocytes were obtained after density gradient centrifugation (801 x g for 15 minutes, no brake) on a discontinuous Percoll gradient (1,050 g/ml, 1,056 g/ml and 1,084 g/ml; GE Healthcare). Lymphocytes were isolated from the 1,084-1,056 g/ml interface.

**Flow cytometric analysis**

Supplemental table 1 lists the fluorochrome-conjugated monoclonal antibodies that were used to phenotypically characterize immune cells in cord blood and decidual samples. Samples were analyzed on a 10-color Navios™ flow cytometer (480 nm argon blue laser, 405 nm solid state violet laser, 636 nm solid state laser, Beckman Coulter). In brief, cells were washed twice with PBS-0.2%BSA (bovine serum albumin, Sigma-Aldrich) before staining with surface antibodies for 20 minutes at room temperature, protected from light. After permeabilization and fixation, intracellular staining was performed for 30 minutes at 4°C in the dark. For intracellular cytokine expression, cells were first stimulated for 4 hours with PMA (phorbol-12-myristate-13-acetate; 12.5 ng/ml), ionomycin (500 ng/ml), and brefeldin A (5 μg/ml) at 37°C in a humidified 5% CO2 incubator. 28 immune cell subpopulations (Fig. S1) were identified in decidual and cord blood samples by manual gating using Kaluza software v2.1 (Beckman Coulter). The gating strategy is illustrated in Fig. S1.

**Statistical analysis**

Non-parametric Mann-Whitney test was used to compare immune cell subsets in decidua and cord blood of the control and transplanted group. P-values < 0.05 were considered significant. Percentages in text are depicted as mean ± standard deviation.
RESULTS AND DISCUSSION

Immunosuppressive drug use during pregnancy affects uterine regulatory T cells

A tightly regulated uterine immune system is critical for successful pregnancy and uterine immune perturbations can be found in pregnancy complications. We hypothesize that the lifelong use of immunosuppressive drugs to prevent graft rejection, will have an impact on the delicate uterine immune system, thereby contributing to the increased risk of complications observed in kidney transplant patients. To investigate whether immunosuppressive drug use during pregnancy has an influence on the local uterine immune system, we collected the decidua parietalis from placentae (after delivery) of kidney transplant patients and healthy, uncomplicated control pregnancies and examined the immune cell composition by flow cytometry. With this approach we were able to assess the frequency of monocytes, NK cells, B cells/subsets, T cells/subsets (regulatory T cells (Treg), effector/memory T cells (CD45RA and CCR7 expression)), and T cell cytokine expression (IFN-γ and IL-17) (Fig. S1: gating strategy and assessed immune cell subpopulations).

We observed no difference in the frequency of monocytes, NK cells, T cells, effector/memory T cells, and B cells/subsets in the decidua of kidney transplant patients compared to controls (Fig. S2). In addition, we also did not observe a difference in percentage IFN-γ+ and IL-17+ CD4+ T cells in the decidua of patients and controls (Fig. 1A). This is in contrast to what we would have expected as tacrolimus, azathioprine and prednisone suppress pro-inflammatory cytokine production. However, this could be due to a difference in an ex vivo stimulation method and the in vivo situation, where there is continuous exposure to immunosuppressive drugs.

Treg with a suppressive phenotype are enriched in the decidua during healthy pregnancy. These decidual Treg are necessary to mediate suppression against the fetus-specific antigens they encounter, thereby preventing rejection of the fetal tissues. In this study, we did not observe a significant difference in the percentage of CD25+CD127lowFoxP3+ Treg in the decidua of women with a transplanted kidney compared to controls (Fig. 1B). However, we observed a significant decrease in the percentage of HLA-DR+ Treg within the total decidual Treg pool of kidney transplant patients; i.e. 51.5±16.5% in patients versus 76.4±9.7% in controls (Fig. 1C). These HLA-DR+ Treg are described as being highly suppressive. In women suffering from preterm labor, as well as during acute kidney allograft rejection, circulating HLA-DR+ Treg were found to be strongly reduced, resulting in reduced suppressive activity. In addition, other pregnancy complications have also been associated with altered Treg populations and disturbed Treg tolerance. This may suggest that there is a loss of fetal tolerance at the maternal-fetal interface, which could explain the observed
increase in pregnancy complications in women with a kidney transplant.

Figure 1 T cell cytokine expressing and frequency regulatory T cells in decidua of kidney transplant patients (KT) and controls (CTRL). (A) Percentage IFN-γ and IL-17+ CD4+ T cells, (B) percentage CD25+CD127lowFoxP3+ regulatory T cells (Treg) and (C) percentage HLA-DR+ Treg in decidua from KT and CTRL are shown. (D) Percentage HLA-DR+ Treg is separated based on immunosuppressive drug regimen used, i.e. combination of prednisone (Pred), tacrolimus (Tacrol) and azathioprine (Aza). Lines indicate mean ± standard deviation. P-values are shown on each graph and are calculated against control (non-parametric Mann-Whitney test).
In addition, while the effect of azathioprine on Treg induction is less clear, tacrolimus negatively affects the induction of Treg, and reduced frequencies of circulating Treg can be found in tacrolimus-treated transplant patients. Here, we observed that the decidua of women with a transplanted kidney who used tacrolimus (without azathioprine) showed the greatest decrease in percentage HLA-DR$^+$ Treg; 40.1±12.6% versus 76.4±9.7% in controls (Fig. 1D). This decrease in HLA-DR$^+$ Treg was less when azathioprine was added to the drug regimen (57.2±13.1%), and the highest frequency of HLA-DR$^+$ Treg could be observed when tacrolimus was not used (63±18.3%; azathioprine use only). This shows that different immunosuppressive drugs have distinct effects on uterine immunity. It suggests that azathioprine may have a less detrimental impact on the uterine immune system than tacrolimus, or maybe even be protective, and perhaps could be more favorable to use during pregnancy. In future studies, it would be interesting to investigate in more detail whether the use of immunosuppressive drugs during pregnancy would alter the functional capacity of decidual Treg and whether there would be distinct differences based on the drug regimen.

In this study, we used decidual tissue after delivery to study the effect of immunosuppressive drug use on the decidual immune environment. Pregnancy complications such as pre-eclampsia are a consequence of defective placentation during the first weeks of pregnancy. Therefore, it would be interesting to assess the effects of the different immunosuppressive drugs on the first trimester decidual immune environment. However, this is not feasible due to ethical constraints.

**Immunosuppressive drug use during pregnancy affects the neonatal immune system**

The fetus develops its immune system during pregnancy and increasing evidence suggests that the maternal immune environment will influence the fetal immune system and the offspring’s health in later life. In kidney transplant patients, the fetus develops its immune system under an immunosuppressive drug regimen as immunosuppressive drugs are able to cross the placenta and enter the fetal circulation. To assess whether immunosuppressive drug use during pregnancy has an influence on the development of the neonatal immune system, we collected cord blood (umbilical vein) of neonates born to kidney transplant patients and healthy controls. We immunophenotyped the neonatal immune cell composition by flow cytometry and were able to assess the frequency of monocytes, NK cells, B cells/subsets, T cells/subsets (Treg, Th cells, memory T cells) and T cell cytokine expression (Fig. S1).

A significantly decreased percentage of classical monocytes (CD14$^+$CD16$^-$), and an increased percentage of non-classical monocytes (CD14$^+$CD16$^+$) and intermediate monocytes (CD14$^+$CD16$^+$) monocytes was observed in cord blood of infants born kidney
transplant patients compared to infants born to women with healthy and uncomplicated pregnancies (Fig 2A). Monocyte subsets display divergent biological roles 44-46. Classical monocytes are highly phagocytic while non-classical monocytes are involved in antigen presentation, T cell stimulation and are the primary producers of pro-inflammatory cytokines 44-46. Intermediate monocytes are a transitional subset that displays both phagocytic and pro-inflammatory function 46. Increased frequencies of non-classical and intermediate monocytes, and decreased classical monocyte frequencies can be found in inflammatory disorders such as systemic lupus erythematosus (SLE) and rheumatoid arthritis 46,47. This suggests that the monocyte subset composition of neonates born to kidney transplant patients resemble a pro-inflammatory state. Moreover, also CD56+CD3+ NKT cell percentages were significantly lower in cord blood of children born to kidney transplant patients compared to infants born to controls (Fig 2B). NKT cells play a suppressive role in chronic inflammatory diseases and decreased frequencies are observed in patients with SLE 48,49. The pro-inflammatory monocyte state and altered NKT cell frequencies might predispose children of women with a transplanted kidney to develop inflammatory or autoimmune disorders. Indeed, SLE development is more common in children born to kidney transplant patients compared to controls 50.

Figure 2 A,B: Percentage monocyte subsets and NKT cells in cord blood of neonates born to kidney transplant patients (KT) and controls (CTRL). (A) Percentage classical monocytes (CD14++CD16-), intermediate monocytes (CD14++CD16+), non-classical monocytes (CD14+CD16+), and (B) NKT cells (CD56+CD3+) in cord blood of neonates born to KT and CTRL are shown. Lines indicate mean ± standard deviation. P-values are shown on each graph (non-parametric Mann-Whitney test).
We also observed a significantly higher percentage of T cells and CD4+ effector T cells (CCR7 CD45RA+) in cord blood of children born to kidney transplant patients compared to controls (Fig 3A-B). In addition, the frequency of CD25+CD127lowFoxP3+ Treg, Ki67+ Treg, and HLA-DR+ Treg was decreased in cord blood of children born to patients compared to controls (Fig. 3C). We did not observe a difference in percentage IFN-γ+ and IL-17+ CD4+ T cells (Fig 3D). These results are in agreement with others.22,51-54 No difference was observed between tacrolimus- and azathioprine-based drug regimens on percentage HLA-DR+ Treg in cord blood of the neonates, as we showed before in decidua (Fig. S3). Treg are indispensable in maintaining immunological tolerance to self-antigens and maintaining immune homeostasis.55 Abnormalities of self-tolerant and immunosuppressive Treg are observed in autoimmune diseases and allergies.56,57 Indeed, impaired Treg numbers and function at birth (cord blood) have been associated with an increased risk to develop sensitization to food allergens and atopic dermatitis in the first year of life.58,59 This underscores that reduced Treg frequencies in cord blood of children born to mothers with a kidney transplant could potentially predispose them to an increased risk of developing inflammatory and allergic diseases later in life. However, to date, no long-term and large-scale studies exist that investigate the development of inflammatory and allergic diseases later in life in offspring of kidney transplant patients.

Figure 3 Percentage T cells and T cell subsets in cord blood of neonates born to kidney transplant patients (KT) and controls (CTRL). (A) Percentage T cells, (B) percentage effector/memory CD4+ and CD8+ T cells defined as CM = central memory (CD45RA−CCR7+); EM = effector-memory (CD45RA−CCR7−); Teff = effector T cell (CD45RA+CCR7−); naive (CD45RA+CCR7+). Lines indicate mean ± standard deviation. P-values are shown on each graph (non-parametric Mann-Whitney test). Figure continued on next page.
Lastly, we observed a decrease in B cell frequency in cord blood of neonates born to kidney transplant mothers compared to newborns of mothers with a healthy and uncomplicated pregnancy (Fig. 4). This observation is in agreement with others. The composition of the B cell population did not differ between our two groups of infants; i.e. no difference in the frequency of naïve (CD27IgD⁺), non-switched (CD27IgD⁻), switched (CD27IgD⁻), plasmablast (CD27IgDCD38⁺), and CD24⁺CD38⁺ B cells was observed at birth (Fig. S3). Ono et al. showed that infants born to kidney transplant patients were more frequently admitted to the hospital compared to the control group, and B cell numbers
at birth were lower in these hospitalized children 22. Another study also reported more hospitalizations and acute bronchitis in children born from kidney transplant patients compared to controls 50. This suggests that the impaired immune system development in infants born to mothers with a kidney transplant could increase the risk for infections during the first year of life. Adequate B cell responses are important for vaccination success 61. Therefore, it is plausible that impaired B cell development in neonates from kidney transplant patients could interfere with vaccination responses. Indeed, Cimaz et al. reported that 5 out of 28 children (17%) exposed to immunosuppressive drugs in utero were not able to develop protective C. Tetani antibody titers after tetanus vaccination 62. However, a Brazilian study with 24 children born to kidney transplant recipients showed that, although B cell numbers were low at birth, vaccine-specific antibody levels were normal at 7-8 months of age (tetanus, H. influenza and S. pneumoniae) and no adverse events for BCG vaccine scar healing was observed (normal Brazilian vaccination schedule) 51. In addition, rotavirus vaccination did not result in more adverse events in 24 children born to kidney transplant patients compared to children not exposed to immunosuppressive drugs in utero 63. These different results concerning vaccination responses could be due to different immunosuppressive drug regimens and drug dosages. In addition, small numbers of children enrolled in the aforementioned studies contributes to low power of these studies. At the moment, there are no clear vaccination guidelines for children born to transplanted mothers as large-scale population studies with long-term follow-up are lacking. Even though there is only a low level of evidence concerning safety, recently published vaccination guidelines for offspring of mothers with immune-mediated disorders (rheumatoid arthritis, inflammatory bowel disease, psoriasis, psoriatic arthritis and autoimmune disease) who are exposed to immunosuppressive drugs during the 3rd trimester of pregnancy, recommends normal vaccination strategies 64. Whether these guidelines can be extrapolated to children born to transplanted mothers is uncertain since transplantation requires higher doses of immunosuppressive drugs compared to immune-mediated disorders. Further studies in significantly larger study populations are required to assess the long-term health outcomes in children born to mothers with a kidney transplant and to define the effect of different drug combinations and dosage regimes on vaccination responses in more detail.

In conclusion, it has become clear that immunosuppressive drug use during pregnancy in kidney transplant patients has a significant impact on the decidual and neonatal immune system development. We showed that the percentage of monocytes, NK cells, T cells, CD25^+CD127^lowFoxP3^+ Treg, and B cells was not affected in the decidua of women with a kidney transplant. Lower percentages of highly suppressive HLA-DR^+ Treg were present in the decidua of kidney transplant patients, especially when tacrolimus was prescribed. In addition, altered monocyte subset frequencies, and decreased NKT cell,
CD25⁺CD127lowFoxP3⁺ Treg, HLA-DR⁺ Treg, and B cell frequencies were observed in cord blood of children born to kidney transplant patients. Dysregulation of the decidual and neonatal immune system development in kidney transplant patients could have important consequences for the risk of pregnancy complications and health outcomes in the offspring. Future studies should include larger cohorts that follow offspring’s health outcomes longitudinally, including vaccination responses and immunological evaluation at later time points, and should be aimed at elucidating the effect of different drug regimens and dosages on the decidual and neonatal immune environment.

Figure 4 Percentage B cells in cord blood of neonates born to kidney transplant patients (KT) and controls (CTRL). Lines indicate mean ± standard deviation. P-values are shown on each graph (non-parametric Mann-Whitney test).
REFERENCES


Decidual and neonatal lymphocytes of kidney transplant patients


SUPPLEMENTARY FIGURES

Supplemental Table I
List of antibodies used for flow cytometry labeling

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A - Decidua

Supplemental Figure 1 Gating strategy and FACS plots of decidua and cord blood. (A) Gating strategy and marker expression of a representative decidua sample, as used in figure 1 and supplemental figure 2. (B) Gating strategy and marker expression of a representative cord blood samples, as used in figures 2-3-4 and supplemental figure 3. Figure continued on next pages.
**A - Decidua continued**

**B - Cord blood**

*Supplementary Figure 1 Continued from previous page.*
Supplemental Figure 1 Figure continued from previous pages. Gating strategy and FACS plots of decidua and cord blood. (A) Gating strategy and marker expression of a representative decidua sample, as used in figure 1 and supplemental figure 2. (B) Gating strategy and marker expression of a representative cord blood samples, as used in figures 2-3-4 and supplemental figure 3.
Supplementary Figure 2 Immune cell composition in decidua of kidney transplant patients (KT) and controls (CTRL). (A) Percentage monocytes and NKT cells, (B) percentage NK cells and NK cell subsets (CD16+ and CD16- NK cells), (C) percentage T cell, CD4+ and CD8+ T cells, CD45RA+ and CD45RO+ T cells, (D) and effector/memory CD4+ and CD8+ T cells defined as CM = central memory (CD45RA−CCR7+); EM = effector-memory (CD45RA−CCR7−); Teff = effector T cell (CD45RA−CCR7+); naive (CD45RA+CCR7+), (E) percentage Ki67+ and CD45RA+ regulatory T cells, (F) percentage B cells and B cell subsets in decidua from KT and CTRL are shown. Lines indicate mean ± standard deviation. P-values are shown on each graph (non-parametric Mann-Whitney test). Figure continued on next pages.
Decidual and neonatal lymphocytes of kidney transplant patients

Supplementary Figure 2 Continued from previous page.
Supplementary Figure 2 Continued from previous pages.
Supplementary Figure 3  Immune cell composition in cord blood of neonates born to kidney transplant patients (KT) and controls (CTRL). (A) Percentage monocytes, (B) percentage NK cells and NK cell subsets (CD16+ and CD16 NK cells), (C) percentage CD4+ and CD8+ T cells, CD45RA+ and CD45RO+ T cells, (D) percentage CD45RA+ regulatory T cells, and percentage CD25+CD127lowFoxP3+ and HLA-DR+ Treg separated based on immunosuppressive drug regimen used, i.e. combination of prednisone (Pred), tacrolimus (Tacro) and azathioprine (Aza). (E) percentage B cell subsets in cord blood of neonates born to KT and CTRL are shown. Lines indicate mean ± standard deviation. P-values are shown on each graph and are calculated against CTRL (non-parametric Mann-Whitney test). Figure continued on next pages.
Supplementary Figure 3 Figure continued from previous page.
Decidual and neonatal lymphocytes of kidney transplant patients

Supplementary Figure 3 Figure continued from previous pages.
CHAPTER 9

Summary, discussion and future perspectives
The uterine mucosa is a unique immunological barrier that is entailed with the important task of enabling successful pregnancy. Immune cells of the uterine mucosae have been shown to be involved in tissue remodeling during the menstrual cycle, blastocyst implantation, placentation, and tolerance towards fetal antigens. A dysbalanced uterine immune system has been suggested to be involved in the pathogenesis of pregnancy complications. Unraveling the mechanisms at play at the uterine mucosae will be essential to understand why some pregnancies are successful and others are not. The work described in this thesis is dedicated to increasing our insight into the immunology of the uterine mucosa and its dynamic changes during human pregnancy. To this end, we focused the work in this thesis on examining the phenotype of uterine mucosal immune cells in both the pre-pregnancy endometrium and the pregnant decidua.

**Uterine T cells acquire an experienced and tolerogenic signature**

The uterine mucosa is highly adaptable. Depending on the physiological situation, it changes its morphology, cell composition and function. The endometrium is the mucosal lining of the non-pregnant uterus. Each month, the endometrium prepares itself to be able to support a possible embryo implantation by an increase in immune cells and transforming stromal fibroblasts into specialized decidual cells. During pregnancy, the uterine mucosa is called decidua, which forms the site of contact with cells of fetal origin. Without fertilization and embryo formation, menstruation will be initiated and the endometrial lining will be shed. The immune cells present in the uterine mucosa play a key role in immune defense, and in coordinating tissue remodeling and repair during both menstruation and placentation. Despite increasing knowledge, data on the transition of immune cells in the pre-pregnancy endometrium towards immune cells in the pregnant decidua is lacking. In the study presented in chapter 2, we examined the phenotype and functional capacity of immune cells in peripheral blood (control), pre-pregnancy endometrium and decidua parietalis of term placentae by flow cytometry. In this study, we bypassed the need for endometrial biopsies by utilizing the fact that the endometrial immune cells are shed during menstruation in the menstrual fluid. We also used this method in chapters 3, 4 and 5 of this thesis. We showed that, in accordance with others, the immune cells composition of endometrium and decidua is clearly different from peripheral blood, with less T cells and more NK cells with a CD56⁺CD16⁻ phenotype found in the uterine mucosa. Moreover, T cells and NK cells in endometrium and decidua are CD69⁺, CD103⁺ and CD62L⁻, while T cells and NK cells in peripheral blood are CD62L⁺, CD69 and CD103⁺. This indicates that the immune cells present in endometrium and decidua are from mucosal origin and not derived from peripheral contamination. In addition, we observed a lower percentage of naïve (CD45RA⁺CCR7⁺) and a higher percentage of effector memory (CD45RA⁺CCR7⁻) and central memory (CD45RA⁻CCR7⁺)
CD4+ and CD8+ T cells in decidua compared to endometrium. This proportion of decidual T cell subsets is in accordance with the study previously reported by Tilburgs et al. 18. *In vitro* stimulation of endometrial T cells with anti-CD3/CD28 mAb microbeads revealed a similar division of T cell subsets as found for term decidual T cells, i.e. less naïve and more effector memory and central memory T cells. This suggests that over the course of pregnancy, uterine T cells become antigen-experienced and acquire a differentiated phenotype. The exact antigens required for uterine T cell differentiation are unclear. Fetal alloantigens like major histocompatibility antigens (MHC) 19-21, minor histocompatibility antigens (mHags) such as male offspring-derived HY mHag 22-24, and/or pathogen-derived antigens 22,23 have been suggested to be involved.

Decidual effector memory T cells are able to respond to fetal antigens 24,25. Therefore, mechanisms need to be in place to control T cell-mediated attack of the fetal tissues by fetal-specific T cells and avoid allograft rejection. At the maternal-fetal interface, increased Treg frequencies can be found compared to peripheral blood, which can suppress effector T cells responses and induce tolerance 24,26,27. We showed that term decidua also contained a higher percentage of CD4+CD25hiCD127- Treg compared to endometrium, indicating that the increase of Treg is pregnancy-specific. Interestingly, higher frequencies of differentiated CD45RA CD25hi Treg were present in decidua compared to endometrium. In blood, differentiated Treg are suggested to be the main effectors of suppression 28. This suggests that in contrast to pre-pregnancy endometrium, the decidual immune environment is marked by an immune signature that includes the activation and differentiation of Treg.

It has long been thought that T cell activity at the maternal-fetal interface was dominated by a Th2-immunity over Th1-immunity 29,30. However, this Th1/Th2 paradigm has been found to be insufficient 30. In chapter 2, we used a classification method based on chemokine receptor expression 31 to examine the presence of Th1, Th2, and Th17 CD4+ T cells in endometrium and decidua. We observed that while the percentage of CCR6+CXCR3+CCR4+ Th17 cells was similar, both endometrium and term decidua contained a higher percentage of CCR6+CXCR3+CCR4+ Th1 cells and a lower percentage of CCR6+CXC3+CCR4+ Th2 cells compared to peripheral blood, suggesting a preference of Th1 cells over Th2 cells in the uterine mucosa. This is in accordance with results from first trimester decidual samples 27. Between endometrium and term decidua, percentages of Th1, Th2, and Th17 cells did not differ significantly. This suggests that the Th cell distribution required for successful pregnancy is already present before pregnancy. However, here we compared endometrium with term decidua. It remains to be elucidated whether the uterine Th distribution would alter from pre-pregnancy endometrium to first, second and term decidua. Nevertheless, in-depth analysis of Th cell distribution in the endometrium of women with fertility
Summary and discussion

problems might give more insight into the underlying pathogenesis. In addition, we observed that term decidual CD4+ T cells showed a higher intrinsic capacity to express IFN-γ and IL-17 intracellularly compared to endometrium. Although excessive production of IFN-γ and IL-17 has been related to pregnancy complications, IFN-γ and IL-17 production has been shown to be essential during implantation, trophoblast invasion and mucosal defense as well.

Overall, in chapter 2, we showed that the immune cell composition of pre-pregnancy endometrium differs from term decidua. While the distribution of Th1, Th2 and Th17 cells was similar, term decidual T cells were marked by a tolerogenic and more experienced phenotype. It remains to be elucidated which (fetal-)antigens induce uterine T cell and Treg differentiation. As this study showed that the uterine immune environment changes from pre-pregnancy endometrium to term decidua, it will be interesting to add first and second trimester decidual tissue obtained from elective abortions to the comparison. This will give the most complete overview of the dynamic changes the uterine mucosa undergoes over the course of pregnancy and will aid us in our understanding of the pathogenesis of pregnancy complications.

Composition and dynamics of NK cells in the uterine mucosae

As highlighted in the introduction of this thesis (chapter 1), NK cells are one of the most abundantly present immune cells in both the non-pregnant endometrium and the pregnant decidua, and are of significant importance for spiral artery remodeling and trophoblast invasion. NK cell receptors (NKR) regulate NK cell function and the interaction between NKR on decidual NK cells with their respective ligands on trophoblast cells contributes to correct placentation. Genetic studies revealed associations between pregnancy complications (pre-eclampsia and recurrent miscarriages) and maternal KIR and fetal HLA-C, revealing the importance of NK cell receptors for successful pregnancy outcomes. It is known that decidual NK cells express various NKR variants and are biased towards KIR2D expression. However, little information is available about the regulation of NKR expression and the repertoire of NKR on endometrial NK cells.

For instance, is KIR expression on endometrial NK cells influenced by HLA-C genotype, does cytomegalovirus (CMV) seropositivity influence NKR expression, and is the same NKR repertoire generated over different menstrual cycles? In chapter 3 of this thesis, we investigated the NKR repertoire of endometrial NK cells and showed that endometrial NK cells have a dedicated tissue-specific NKR repertoire that is clearly different from matched peripheral blood NK cells. Endometrial NK cells contained high levels of NKR-expressing NK cells, are biased towards KIR2D expression, and co-expressed multiple NKR, which is line with the study of Ivarsson et al. The majority of endometrial NK cells co-expressed...
NKG2A and KIR2DL2L3S2 together with other NKR. This may indicate that endometrial NK cells are finely tuned towards HLA-E and HLA-C1 recognition of fetal cells. In addition, in contrast to peripheral blood NK cells, KIR receptor expression on endometrial NK cells was not influenced by cognate HLA-C genotype, nor did prior cytomegalovirus (CMV) infection affect the frequency of NKG2C+ endometrial NK cells. Despite the regeneration of the endometrium with each cycle, within the same female, NKR expression of endometrial NK cells did not differ between different menstrual cycles. This is in agreement with the study of Ivarsson et al. However, it is still unknown whether NKR expression will change within a menstrual cycle. It will be interesting to determine the relationship between the expression of endometrial NK cells derived from menstrual blood and endometrial NK cells derived during the window of implantation, i.e. the period when the endometrium is receptive for implantation. Nevertheless, menstrual blood represents an attractive method to examine the cellular composition of the endometrium in a non-invasive way. The results presented in chapter 3 are an incentive to use menstrual blood as a method to assess endometrial NK cells of women with pregnancy-related problems.

CMV infection not only expands a population of NKG2C+ peripheral blood NK cells, but also promotes a bias towards peripheral blood NK cell subpopulations expressing self-specific KIR. In chapter 3, based on co-expression of KIR2DL2L3S2, KIR2DL1S1, KIR3DL1S1, LILRB1, NKG2A and NKG2C, we also determined phenotypically distinct NK cell subpopulations in matched peripheral and endometrial NK cells. A Tukey's outlier analysis on these subpopulations revealed 8 subpopulation expansions within the endometrial NK cell pool and 15 expansions within the peripheral blood NK cell pool. In agreement with previous reports, peripheral blood NK cell expansions in our study expressed KIR specific for self-HLA-C and were associated with CMV seropositivity. Expansions of endometrial NK cells, on the other hand, were not associated with a positive CMV status and were not biased towards self-specific KIR expression. Although it remains to be elucidated which signals promote the expansion of endometrial NK cells in some individuals and not in others, results presented below, in chapter 5 and 6, suggest that pregnancy-specific factors could be involved.

It is speculated that endometrial NK cells are precursors for decidual NK cells present during pregnancy. Manaster et al. showed that endometrial NK cells express low levels of NKP30 and NKP44, while previous studies showed that decidual NK cells express relatively high NKP30 and NKP44 levels. IL-15 stimulation, present at higher levels in the decidua, increased NKP30 and NKP44 expression on endometrial NK cells. Manaster et al. suggested that endometrial NK cells await pregnancy, and following conception, will differentiate toward decidual NK cells. It is indeed known that microenvironmental changes can alter the phenotype of NK cells. However, it is still
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unclear whether pregnancy-specific factors would alter the unique NK cell receptor repertoire expression on endometrial NK cells. Although we know that endometrial and first trimester decidual NK cells share phenotypic similarities such as a CD56brightCD16 phenotype, expression of various KIR and a bias towards KIR2D expression 16,58,70, previous studies only compared endometrial or decidual NK cells with their peripheral counterparts. A direct and thorough assessment of the phenotypic differences and similarities of endometrial and decidual NK cells is lacking. In chapter 4, we evaluated the expression of 17 NK cell receptors on endometrial and early-pregnancy decidual NK cells by flow cytometry. We showed that pre-pregnancy endometrial NK cells have a different phenotype compared to decidual NK cells during early pregnancy. The frequency of KIR2DS1, KIR2DL2L3S2, KIR2DL2S2 and KIR3DL1 expressing NK cells was lower in decidua compared endometrium, while the frequency of LILRB1, KIR2DL1S1, KIR2DL1, KIR2DL3, and KIR3DL1S1 expressing NK cells did not differ. The observed decrease in KIR receptor expression might be due to either increased ligand interaction or increased production of soluble factors during pregnancy 58,59,71-73. Furthermore, co-expression patterns showed that NK cells subpopulations co-expressing KIR3DL1S1 and KIR2DL2L3S2 were decreased or almost absent in decidua. In addition, the frequency of NKG2D, NKP30, NKP46 and CD244 expressing NK cells increased in the decidua, while percentages of NKG2A, NKG2C and NKP44 expressing NK cells stayed the same. This increase in NKG and NKP receptor expression could be mediated by increasing IL-15 levels following fertilization 16,74. We hypothesize that these phenotypic changes in receptor expression will be important to regulate the functional capacity of decidual NK cells by regulating vascular remodeling and trophoblast invasion, and enhancing protection of the fetus from intrauterine viral infection 49,50,52,75-77. The results of this study provided us with more insight into the essential adaptations decidual NK cell precursors undergo for pregnancy success. Future research should aim to determine which mechanisms, soluble factors or cell-cell contact, contribute to the conversion of endometrial NK cells towards decidual NK cells. This could be especially interesting in the light of pregnancy complications. Are endometrial NK cells of women with pregnancy complications able to convert to fully functional decidual NK cells? Failure to adequately change the receptor expression of decidual NK cell precursors may increase the risk of developing complications such as recurrent miscarriages, recurrent implantation failure, and pre-eclampsia.
Pregnancy-induced trained immunity of the uterine mucosa: a role for CMV-induced priming?

Since the seminal study in 2006 by O’Leary and colleagues, it has become clear that NK cells can develop innate immune memory. The term “trained immunity” has been used to describe the modulated, often enhanced, immune response of innate immune cells towards a second stimulation. Interestingly, epidemiological observations indicate that pregnancy complications are less common in subsequent pregnancies compared to first pregnancies, and enhanced placentation is observed in subsequent pregnancies. Given the importance of decidual NK cells in successful placentation, together with the fact that subsequent pregnancies show enhanced placentation and the ability of NK cells to develop immunological memory, is it possible that pregnancy induces uterine NK cell memory? Recently, Gamliel et al. indeed described the presence of an expanded population of NK cells with a memory phenotype, i.e. NKG2C+ and NKG2C+LILRB1+ NK cells, in both the decidua and endometrium of multigravida women compared to the endometrium of nulli- and primigravida women. These memory NK cells exhibited a distinct transcriptional and epigenetic signature, and increased IFN-γ and VEGFα production. Importantly, in the aforementioned study of Gamliel et al., all decidual and endometrial samples that were used, were derived from women who were CMV seropositive (CMVpos). Several studies reporting expansions of memory NK cells in individuals infected with HIV, hantavirus, hepatitis B and C, Epstein-Barr virus, and Chikungunya virus, showed that the observed expansions are confined to CMV positive individuals, suggesting that these expansion were in part driven by CMV infection. To assess whether the observed pregnancy-trained NK cells are also present in the endometrium of CMV seronegative (CMVneg) women or whether they are restricted to CMVpos women, we immunophenotyped endometrial NK cells from a cohort of CMVpos and CMVneg nulli- and multigravida women. We showed that pregnancy-trained NKG2C+LILRB1+ NK cells were indeed expanded in the endometrium of CMVpos nulligravida women. However, CMVneg nulligravida women did not show an increased frequency of NKG2C+LILRB1+ endometrial NK cells compared to CMVneg nulligravida women. We did not observe a significant difference for NKG2C+ NK cells. This implies that CMV seropositivity might be a prerequisite for the induction of pregnancy-trained NKG2C+LILRB1+ endometrial NK cells. In addition, an outlier analysis of endometrial NK cell subpopulations showed expansions in the multigravida samples, while none of the nulligravida samples showed expansions. Notably, the majority of the observed expansions express self-specific KIR receptors. This, together with the results from chapter 3, suggests that pregnancy-specific factors, and not CMV seropositivity, might induce the expansion of distinct endometrial NK cell subpopulations. It remains to be elucidated what the role these expanded subpopulations play during subsequent pregnancy.
Our observation in chapter 5 that pregnancy-trained NKG2C⁺LILRB1⁺ NK cells are only present at higher frequencies in the endometrium of CMV⁺ women and not in the endometrium of CMV⁻ women, highlights the need for further research to unravel the mechanisms involved. Could it be possible that the expansion of trained NKG2C⁺LILRB1⁺ uterine NK cells is solely mediated by CMV exposure in the decidua during pregnancy rather than by other pregnancy-specific factors? This is discussed in more detail in chapter 6, where we reviewed the current literature on trained immunity and discussed pregnancy-induced trained immunity of the uterine mucosae. It is important to note that CMV seropositivity itself does not lead to a higher frequency of NKG2C⁺ (chapter 3) or NKG2C⁺LILRB1⁺ (data not shown) endometrial NK cells. Thus, the pregnancy environment is necessary for the induction. Latent CMV infection of cells at the maternal-fetal interface is common during the first trimester of pregnancy 101. Co-culture of decidual NK cells with CMV-infected fibroblasts increased the percentage of NKG2C⁺ expressing decidual NK cells 77, and intrauterine CMV infection is cleared by decidual NK cells infection 77,102-104. Especially during the first trimester of pregnancy, when decidual NK cells are abundantly present, the rate of congenital CMV infection is low 105. Epidemiological studies showed that a positive CMV status during pregnancy reduced the risk of congenital CMV infections in future pregnancies 106,107, and the risk of vertical transmission is lower when maternal CMV infection occurs in CMV⁺ women in comparison with a primary infection during pregnancy in CMV⁻ women 107,108. These studies suggest that the observed pregnancy-trained decidual NK cells could be induced because of decidual CMV exposure during pregnancy, to provide protection against intrauterine CMV infections in subsequent pregnancies. We hypothesize that next to pregnancy-specific factors, priming of decidual NK cells by decidual CMV exposure during pregnancy will be essential to induce pregnancy-trained uterine NK cells. It remains to be elucidated whether these pregnancy-trained uterine NK cells play a role in enhancing placenta in subsequent pregnancies, in protecting against intrauterine CMV infection or possibly both. To identify the mechanisms, pregnancy- and/or CMV-specific, involved in the reprogramming and induction of pregnancy-trained uterine NK cells and NK cell subpopulation expansions, future studies should aim at characterizing endometrial and decidual NK cells in a large cohort of women of childbearing age, before and after a first pregnancy, while maternal CMV seropositivity, CMV DNA in decidual material, and maternal and fetal HLA genotype are measured as well.

In chapter 6, we also briefly discussed trained immunity of monocytes/macrophages 80-82,109, as macrophages are also abundantly present in the uterine mucosae 11,41,47,110-115. Their role in spiral artery remodeling might imply that trained decidual macrophages could be involved in the enhanced placenta and decreased complication risk observed in subsequent pregnancies 84-92. So far, trained immunity of
decidual macrophages, induced by previous pregnancy, has not yet been described. It will be intriguing to examine whether macrophages in the decidua and endometrium of multigravida women show characteristics of trained immunity as NK cells do. Unraveling the properties of trained immunity at the maternal-fetal interface will open up treatment possibilities for complications of inadequate placentation, or, on the other hand, it might contribute to the generation of a maternal vaccine that could control congenital CMV infection.

Immunosuppressive drug treatment during pregnancy affects the decidual immune system
Renal transplantation greatly improves fertility in women of childbearing age with advanced chronic kidney disease or end-stage renal disease 116-120. Therefore, it is not surprising that the incidence of pregnancy in women with a kidney transplant is relatively high 121. Unfortunately, pregnancy in kidney transplant patients is associated with an increased risk for pregnancy complications 120. Immunosuppressive drug treatment needs to be continued during pregnancy and could potentially influence the delicately balanced dynamics of the uterine immune system and the development of the neonatal immune system. The recommended drugs for use during pregnancy are antiproliferative drugs (azathioprine) and/or calcineurin inhibitors (tacrolimus or cyclosporin), both in combination with low dose steroids (prednisone) 122. To obtain more insight into the placental handling of tacrolimus during pregnancy, we determined tacrolimus concentrations in placental tissue and transfer to cord blood of samples obtained from kidney transplant patients and ex vivo placenta perfusion models (chapter 7). We showed that tacrolimus accumulated extensively in placental tissues from both kidney transplant patients as well as in ex vivo perfusion experiments, i.e. a more than 10-fold increase in tacrolimus concentration was measured in placenta compared to maternal circulating levels. Analysis of venous umbilical cord blood of kidney transplant patients showed tacrolimus concentrations in the range of maternal Cthrough levels, indicating fetal exposure to tacrolimus, which could influence the neonate’s immune system. These tacrolimus levels in cord blood are in contrast to the ex vivo placental perfusion experiments where tacrolimus could not be detected in the fetal circulation. It is conceivable that saturation of the placental binding capacity of tacrolimus has not yet been reached during the 180 minutes long ex vivo perfusion set-up. Saturation of binding capacity upon continuous exposure for 9 months would result in more unbound tacrolimus and consequently a strong driving force for diffusion over the placenta to the fetal circulation. It would be interesting to use a placenta derived from a kidney transplant patient in the ex vivo perfusion set-up to examine whether saturation of binding capacity in the placenta of a kidney transplant patients would results in detectable tacrolimus levels in the fetal
The accumulation of tacrolimus in placental tissue during pregnancy indicates that this organ might be at considerable risk for tacrolimus-induced toxicity. Moreover, the uterine immune system could be influenced significantly by this continuous high concentration, which we investigated in chapter 8.

In chapter 8, we immunophenotyped decidual immune cells derived from the placenta of kidney transplant patients and controls to examine whether the use immunosuppressive drugs has an impact on the uterine immune system. We reported that the decidual immune cell composition of women with a kidney transplant did not differ from healthy controls with regard to percentages of monocytes, NK cells, T cells, CD25+CD127lowFoxP3+ regulatory T cells (Treg), IFN-γ+ and IL-17+ CD4+ T cells, and B cells. However, decreased frequencies of highly suppressive HLA-DR+ Treg were present in the decidua of kidney transplant patients, particularly in those patients treated with tacrolimus versus those that were treated with azathioprine next to tacrolimus, or with azathioprine alone. This may suggest that immunosuppressive drug use during pregnancy might lead to a loss of fetal tolerance at the maternal-fetal interface, resulting in an increased risk for pregnancy complications. Moreover, azathioprine could potentially be the preferable drug to use or added to the drug regimen during pregnancy. Future larger cohorts should investigate in more detail the effect of different drug regimens on the decidual immune environment and whether pregnancy complications would be less common when azathioprine has been used. It is conceivable that certain drugs would have a less detrimental impact during pregnancy and consequently would be preferable to use, if possible to switch. Moreover, it would be interesting to also investigate the effect of immunosuppressive drugs on the uterine immune system in women with autoimmune or inflammatory diseases. Pregnancy in women with an autoimmune disease is considered high risk, and interestingly, a higher incidence of complications has been observed when these women were treated with immunosuppressive drugs compared to those who were not treated. This indicates that immunosuppressive drugs might also effect the uterine immune system of women with autoimmune diseases.

In addition, immunosuppressive drugs could not only impact the uterine immune system, also the development of the neonatal immune system could be affected by in utero drug exposure. Therefore, we immunophenotyped immune cells in cord blood of neonates born to kidney transplant patients and controls, and showed that altered monocyte subset frequencies, and lower NKT cell, CD25+CD127lowFoxP3+ Treg, HLA-DR+ Treg, and B cell frequencies were present in cord blood of children born to kidney transplant patients. These observations are in accordance with previous studies. This dysregulation of the neonatal immune system might predispose children born to kidney transplant patients to develop allergies, autoimmune or inflammatory disorders later in life. Moreover,
altered B cell frequencies in children born to kidney transplant patients might affect their vaccination responses\textsuperscript{147,148}. Future studies including large cohorts are advised to assess the health outcomes and vaccination responses of these children and to define the effect of different drug regimes.

**Concluding remarks and future perspectives**

The findings presented in this thesis showed that over the course of pregnancy, uterine T cells acquire an antigen-experienced and tolerogenic signature. Moreover, uterine NK cells showed a tissue-specific receptor repertoire that also changed upon pregnancy and NK cells with a memory phenotype can be found in the endometrium of multigravida CMV\textsuperscript{pos} women. Furthermore, immunosuppressive drug treatment during pregnancy affected the decidual and neonatal immune system, and the immunosuppressive drug tacrolimus was shown to accumulate in the placenta of women with a kidney transplant.

As it is hypothesized that a dysbalanced uterine immune system is involved in the pathogenesis of pregnancy complications such as pre-eclampsia, recurrent miscarriage, recurrent implantation failure, preterm birth and uterine growth restriction\textsuperscript{2-4}, it will be of crucial importance to fully understand the dynamic and complex interplay of the uterine immune system. Recent advances in cutting-edge technologies such as single-cell RNA sequencing, mass cytometry (CyTOF) and epigenomics will allow to tackle the complexity of the precisely timed immune dynamics and critical interactions of the uterine mucosa. Recently, a single-cell transcriptomics study of cells isolated from first trimester placenta and decidua revealed a single-cell atlas of the early maternal-fetal interface\textsuperscript{45}. They not only described new subsets of decidual NK cells and stromal cells, but also predicted potential cell-cell communication networks in the decidua and placenta, which can now be explored in more detail. One caveat of this and most studies on human reproduction, is that we tend to treat all first trimester samples, from gestational week 6 to 12, as equivalent. Together with ignoring what happens before week 6, we might be obscuring valuable information. For instance, an immune clock of human pregnancy, i.e. precisely timed immunological events that are able to predict gestational age, can be observed in peripheral blood\textsuperscript{149}. Obviously, longitudinal analysis of human uterine development is not feasible, but reproductive immunologists should aim to understand what happens in the uterine mucosa throughout human pregnancy.

Recurrent miscarriage affects 1\% of couples trying to conceive\textsuperscript{150}. Although a miscarriage can be the result of a myriad of factors\textsuperscript{150}, unfortunately, around half of the recurrent miscarriage cases remains unexplained. This leaves the couples with the burden of uncertainty and clinicians without treatment options\textsuperscript{151}. These couples often seek help...
in fertility clinics where treatment is provided based on NK cell cytotoxicity testing of peripheral blood, as it is shown that NK cells could be involved. As shown on the website of the Alan E. Beer Medical Center (www.repro-med.net): “While these cells differ from those found in the blood, it is fortuitous that we can measure the natural killer activity of the cells in the blood and learn much about the activity of those within the uterus.” Is there value in assessing peripheral immune cells to understand a local, dysregulated uterine immune system? Doubtfully, since this thesis showed that the uterine mucosa harbors a unique composition of tissue-specific immune cells. However, there is without a doubt a place for peripheral blood measurement in reproductive immunology research, as peripheral blood markers measured during pregnancy will be crucial in predicting the development of pre-eclampsia or preterm birth. Nonetheless, there is an urgent need to assess in detail the pre-pregnancy endometrial immune system of women with fertility problems to understand the underlying pathogenesis and to predict future pregnancy outcomes. Moreover, understanding the mechanisms that contribute to the conversion of endometrial immune cells towards decidual immune cells, and whether this is affected in women with complications, will provide valuable information.

Understanding the immunology of pregnancy complications will ultimately, and hopefully, lead to therapeutic options. However, as we have shown in this thesis, immunosuppressive drug treatment during pregnancy can affect the decidual and neonatal immune system. Ethical, legal, practical and medical considerations usually exclude pregnant women from randomized controlled trials. Therefore, information on drug safety during pregnancy is limited to case reports or pharmacoepidemiologic studies describing pregnancy outcomes. It will be crucial to carefully select the preferred intervention for its intended effect, placental accumulation, and possible side-effect on the neonatal immune system. Placental explants of first trimester tissue, placental, decidual and endometrial organoids, and ex vivo placental perfusion experiments will be important models to use during this decision-making process.

In conclusion, the work outlined in this thesis provided new insight into the immunology of the uterine mucosae and its dynamic changes during human pregnancy. It showed that the uterine mucosa harbors a unique composition of tissue-specific immune cells that changes over the course of pregnancy and can be influenced by immunosuppressive drugs. To answer all the questions and remarks mentioned above, more research is needed. Recent technological advances will advance our understanding of the dynamic and complex interplay of the immune system in the uterine mucosa during healthy and complicated pregnancy, and will open the door towards effective treatment.
REFERENCES

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Chapter 9


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De centrale rol van het immuunsysteem is het herkennen en onderscheid maken tussen eigen antigenen en lichaamsvreemde antigenen (bv. bacteriën, virussen en tumorcellen). Het immuunsysteem moet een goede immuunrespons ontwikkelen tegen deze lichaamsvreemde antigenen zodat deze onschadelijk gemaakt kunnen worden. Het immuunsysteem bestaat uit 2 takken: het aangeboren en het verworven immuunsysteem. Wanneer je geïnfecteerd raakt met een pathogeen (bv. bacterie of virus) zullen de immuuncellen in het aangeboren immuunsysteem, waaronder onder ander natural killer (NK) cellen, heel snel en algemeen (niet pathogeen-specifiek) reageren. Voor de immuuncellen van het verworven immuunsysteem duurt het langer voor zij werkzaam zijn. Zij moeten eerst aangezet worden door de immuuncellen van het aangeboren immuunsysteem zodat zij een pathogeen-specifieke respons kunnen maken. De immuuncellen van het verworven immuunsysteem ontwikkelen ook een soort geheugen waardoor ze sneller werkzaam zijn wanneer je weer geïnfecteerd zou worden met hetzelfde pathogeen. De werking van vaccinaties berust op deze geheugen-eigenschap.

Zwangerschap is echter een unieke situatie waarbij een “lichaamsvreemd” organisme in alle rust samen leeft, ook al komt het immuunsysteem van de moeder in contact met de lichaamsvreemde antigenen afkomstig van de foetus. Eén van de plaatsen waar er contact is tussen het immuunsysteem van de moeder en de foetale antigenen is in de uteriene mucosa van de baarmoeder. De uteriene mucosa is het slijmvlies (mucosa) dat de binnenkant van de baarmoeder (uterus) bekleed. Er zijn veel immuuncellen aanwezig in de uteriene mucosa en de morfologie, cel compositie en functie van de uteriene mucosa verandert naargelang de situatie. In een niet-zwangere situatie wordt de uteriene mucosa endometrium genoemd. Dit endometrium bereidt zich elke maand voor op de mogelijke innesteling van een embryo door te transformeren in een mucosa genaamd decidua. De decidua zal later deel uitmaken van de placenta. Het immuunsysteem in de uteriene mucosa heeft niet alleen een belangrijke barrière-functie (i.e. het onschadelijk van infecties) maar het heeft ook andere gespecialiseerde functies. Zo spelen de immuuncellen in de uteriene mucosa een rol in het veranderen van de structuur van de uteriene mucosa tijdens de menstruele cyclus, ondersteunen ze de innesteling van de blastocyst (embryo), zorgen ze mee voor het ontwikkelen van de placenta (placentation), en zorgen ze voor een tolerantie van de foetale antigenen; taken die belangrijk zijn in het volbrengen van een succesvolle zwangerschap. Het is dan ook niet verwonderlijk dat een verkeerd werkend uteriene immuunsysteem wordt voorgesteld als onderliggende oorzaak voor zwangerschaps complicaties zoals zwangerschapsvergiftiging (pre-eclampsie), herhaalde miskramen, herhaaldelijk innestelingsfalen, vroeggeboorte, en groeivertraging. Ontrafelen welke mechanismen aan het werk zijn in de uteriene mucosa zal essentieel zijn om beter te begrijpen waarom sommige zwangerschappen succesvol zijn en andere niet. Bovendien zal dit het mogelijk maken om behandelingen voor
complicaties te ontwikkelen en te verbeteren.

Dit proefschrift is daarom gewijd aan het verbeteren van ons inzicht in het immuunsysteem van de uteriene mucosae en zijn dynamische veranderingen tijdens humane zwangerschap. Om dit te bewerkstelligen legden we de focus in dit proefschrift op het onderzoeken van de karakteristieken (fenotype) van de uteriene immuuncellen uit zowel het endometrium als de decidua.

In hoofdstuk 2, onderzochten we met behulp van flow cytometry het fenotype en de functionele capaciteit van de systemische immuuncellen in het bloed (= controle), en van de lokale immuuncellen in het endometrium en de decidua parietalis (i.e. decidualdeel van de placenta-vliezen) van à term placentas. Voor deze studie, en de studies in hoofdstuk 3, 4 en 5, omzeilden we de noodzaak van het nemen van endometrium-biopsies door gebruik te maken van het feit dat de endometriale immuuncellen uitgescheiden worden in het menstruatiebloed tijdens de menstruatie. In hoofdstuk 2 toonden we aan dat de compositie van de lokale immuuncellen in het endometrium en de decidua heel anders is dan in het bloed, en dat deze uteriene immuuncellen wel degelijk van mucosale oorsprong zijn (CD62L-, CD69+ en CD103+) en geen contaminatie uit het bloed. We toonden aan dat de T cellen in à term decidua gekarakteriseerd zijn door een tolerogeen (regulatoire T cel; Treg) en meer ervaren/geheugen fenotype in vergelijking met T cellen uit het endometrium, welke meer naïef zijn. Het is nog onduidelijk welke (foetale)-antigenen bijdragen tot het tot stand brengen van deze ervaren cellen. Aangezien deze studie aantoont dat het uteriene immuunsysteem verandert van pre-zwangerschap endometrium naar à term decidua, zal het interessant zijn om ook tijdens het eerste en tweede trimester van de zwangerschap de uteriene immuuncellen te bekijken. Dit zal ons een completer beeld geven van de veranderingen die het uteriene immuunsysteem ondergaat tijdens de zwangerschap.

Zoals aangegeven in de inleiding van dit proefschrift (hoofdstuk 1), bevatten zowel het endometrium als de decidua veel natural killer (NK) cellen. In het bloed hebben NK cellen als functie het "killen" van cellen die er niet meer uitzien als je eigen normale cellen, zoals tumorcellen of virus-geïnfecteerde cellen. Ook al komen de NK cellen in de decidua in nauw contact met lichaamsvreemde foetale cellen, ze doden deze cellen niet. Integendeel, ze gaan deze foetale cellen helpen met het vormen van de placenta. NK cellen brengen verschillende NK cel receptoren (bv. KIR, NKG2D, NKp30, etc.) tot expressie op hun celmembranaan. Deze NK cel receptoren regelen de functie van de NK cellen en kunnen in interactie gaan met liganden op de foetale cellen (bv. HLA-C). Genetische studies toonden aan dat bepaalde combinaties van maternale NK cel receptoren en foetaal HLA-C geassocieerd zijn met zwangerschapscomplicaties. Er is nog niet veel
bekend over NK cel receptoren op NK cellen uit het endometrium. In hoofdstuk 3 van dit proefschrift toonden we dan aan dat endometriale NK cellen een weefselspecifiek NK cel receptor repertoire hebben dat heel verschillend is van de NK cellen in het bloed. Ook brengen ze meerdere receptoren samen tot expressie en wordt de expressie niet beïnvloed door het eigen HLA-C genotype of cytomegalovirus (CMV) seropositiviteit, wat wel het geval is voor NK cellen uit het bloed. Door naar de co-expressie van verschillende receptoren te kijken, kunnen we verschillende NK cel subpopulaties beschrijven. Hierdoor tonen we ook aan dat de verdeling van NK cel subpopulaties in het endometrium versus het bloed heel verschillend is. In het bloed brengen geëxpandeerde NK cel subpopulaties NK cel receptoren tot expressie die specifiek zijn voor het eigen HLA-C, en zijn ze geassocieerd met een positieve CMV status. Dit is niet het geval voor NK cel subpopulatie expansies in het endometrium. Welke factoren dan wel verantwoordelijk zijn voor NK cel subpopulatie expansies in het endometrium moet nog verder worden onderzocht, maar resultaten in hoofdstuk 5 suggereren dat zwangerschaps-specifieke factoren een rol zouden kunnen spelen. Verder beschrijven we dat de NK cellen en hun receptor expressie gelijksoortig is tussen de verschillende menstruatiecyci van dezelfde vrouw, ook al wordt het endometrium elke maand opnieuw opgebouwd. Dit maakt dat het gebruik van menstruatiebloed een interessante methode is voor het onderzoeken van NK cellen in het pre-zwangerschap endometrium van vrouwen met bijvoorbeeld herhaaldelijk innestelingsfalen of herhaalde miskramen.

NK cellen in het endometrium zijn mogelijk voorlopers van de NK cellen in de decidua. Eerdere studies hebben voornamelijk NK cellen uit het endometrium en decidua vergeleken met NK cellen uit het bloed, waardoor een directe vergelijking tussen endometrium en decidua ontbreekt. Daarom is in hoofdstuk 4 de expressie van verschillende NK cel receptoren op endometriale NK cellen vergeleken met deciduale NK cellen van eerste en tweede trimester placenta’s. De resultaten laten zien dat endometriale NK cellen er anders uitzien dan NK cellen uit de decidua. De frequentie van verschillende KIR-positieve NK cellen was hoger in endometrium in vergelijking met decidua, terwijl de frequentie van andere receptoren, zoals NKG2D en NKp30, juist hoger was in de decidua. Deze verandering van de NK cel voorlopers wordt waarschijnlijk beïnvloed door de interactie met foetale cellen in de decidua of door een verhoogde productie van allerlei zwangerschaps-specifieke factoren. Deze resultaten geven ons meer inzicht in de essentiële veranderingen die NK cellen moeten ondergaan tijdens de zwangerschap. We denken dat deze veranderingen in receptor expressie belangrijk zullen zijn voor het correct uitvoeren van de deciduale NK cel functie, maar ook voor een goede bescherming van de foetus tegen virale infecties. Vervolgonderzoek zal moeten uitwijzen welke mechanismen zorgen voor de verandering van een endometriaal naar een deciduaal NK cel fenotype. Dit zal vooral interessant zijn in het licht van zwangerschapscomplicaties: zijn NK cellen in het endometrium van
bijvoorbeeld vrouwen met herhaalde miskramen in staat om te veranderen naar volledig functionerende decidual NK cellen? Het niet kunnen veranderen van het NK cel receptor repertoire zou het risico op het ontwikkelen van complicaties namelijk kunnen verhogen.

NK cellen zijn cellen van het aangeboren immuunsysteem en er werd lang gedacht dat immuuncellen uit het aangeboren immuunsysteem geen geheugen hebben. Dit blijkt echter niet zo te zijn 16-18. Het geheugen van de aangeboren immuuncellen noemen we “trained immunity” en wordt gekenmerkt door een gemoduleerde en vaak verhoogde respons tegen een andere stimulus 17,18. Epidemiologische studies geven aan dat complicaties zoals pre-eclampsie minder vaak voorkomen in volgende zwangerschappen in vergelijking met een eerste zwangerschap 19-21. Ook is er een verbeterde placenta-aanleg aanwezig in volgende zwangerschappen 22-24. Aangezien NK cellen belangrijk zijn in het succesvol aanleggen van de placenta, zou zwangerschap een soort geheugen kunnen induceren in de uteriene NK cellen? Recent is er inderdaad aangetoond dat er een verhoogde populaatie NK cellen met een geheugen (memory) fenotype (i.e. NKG2C+ en NKG2C+LILRB1+ NK cellen) te vinden is in zowel de decidua als endometrium van vrouwen met meerdere zwangerschappen (= multigravidae) in vergelijking met vrouwen die nog nooit zwanger zijn geweest (= nulligravidae) of voor de eerste keer zwanger zijn (= primigravidae) 14. Opmerkelijk in deze studie is dat alle deelnemende vrouwen cytomegalovirus (CMV) hebben doorgemaakt (i.e. ze zijn CMV seropositief). In andere studies waar ze memory NK cellen beschrijven, bijvoorbeeld in HIV, hepatitis B of C virus seropositieve personen 25-30, waren deze memory NK cellen altijd beperkt tot diegenen die ook seropositief waren voor CMV. Dit suggereert dat CMV een belangrijke rol speelt in het sturen van deze memory NK cellen. Is dit ook het geval voor de zwangerschaps-getrainde memory NK cellen in het endometrium? Komen zwangerschaps-getrainde NK cellen ook voor in het endometrium van CMV-seronegatieve (CMVneg; nog geen CMV doorgemaakt) vrouwen of zijn ze beperkt tot CMV-seropositieve (CMVpos) vrouwen? Om dit te beantwoorden hebben we in hoofdstuk 5 endometriale NK cellen van zowel CMVpos als CMVneg nulli- en multigravidae vrouwen onderzocht. We toonden aan dat er inderdaad een hoger percentage zwangerschaps-getrainde memory NK cellen (NKG2C+LILRB1+) aanwezig was in het endometrium van CMVpos multigravidae vrouwen. CMVneg multigravida vrouwen daarentegen toonden geen verhoogd percentage van deze memory NK cellen in vergelijking met CMVneg nulligravidae vrouwen. Dit suggereert dat CMV seropositiviteit noodzakelijk lijkt te zijn voor het induceren van zwangerschaps-getrainde endometriale NK cellen. Meer onderzoek is nodig om uit te zoeken welke mechanismen hier nu aan ten grondslag liggen. Is de inductie van zwangerschaps-getrainde NK cellen in de baarmoeder enkel gemedieerd door blootstelling aan CMV tijdens de zwangerschap? Of zijn andere zwangerschaps-specifieke factoren belangrijk? Dit wordt in meer detail bediscussieerd in hoofdstuk 6 waar we een overzicht geven van de huidige literatuur over
“trained immunity” in het algemeen en specifiek in de uteriene mucosae. Zo bespreken we dat CMV latent aanwezig kan zijn in cellen van de placenta tijdens het eerste trimester en dat blootstelling van deciduale NK cellen aan CMV-geïnfecteerde cellen het fenotype van deze deciduale NK cellen verandert. Dit suggereert dat de zwangerschaps-getrainde NK cellen misschien geïnduceerd kunnen worden door blootstelling aan CMV tijdens de zwangerschap. Welke factoren nu exact zorgen voor de inductie van zwangerschaps-getainde NK cellen en welke functie deze NK cellen tijdens de zwangerschap nu vervullen blijft momenteel nog een open vraag en moet verder worden onderzocht.

De laatste twee hoofdstukken van dit proefschrift gaan over zwangerschap na een niertransplantatie. Het transplanteren van een nier verbetert de fertiliteit van jonge vrouwen met chronische nierziekten en ernstig afgenomen nierfunctie. Het is daarom niet verwonderlijk dat zwangerschap na een niertransplantatie steeds vaker voorkomt. Zwangerschap in niertransplantatiepatiënten is jammer genoeg echter geassocieerd met een verhoogd risico op zwangerschapscomplicaties. Omdat het immuunsysteem het getransplanteerde orgaan kan aanvallen en afstoten, is het nodig om levenslang immunosuppressieve medicatie te gebruiken, ook tijdens de zwangerschap. Aangezien het immuunsysteem in de uteriene mucosa belangrijk is voor het succesvol volbrengen van een zwangerschap, is het goed mogelijk dat het gebruik van immunosuppressiva het uteriene immuunsysteem verstoord, wat dan zwangerschapscomplicaties tot gevolg kan hebben. De immunosuppressieve medicijnen aanbevolen voor gebruik tijdens zwangerschap zijn azathioprine en/of calcineurine inhibitoren (bv. tacrolimus), beiden in combinatie met lage dosis prednison. Om meer inzicht te verkrijgen in hoe de placenta omgaat met tacrolimus, hebben we allereerst tacrolimus-concentraties gemeten in de placenta en navelstrengbloed van niertransplantatiepatiënten en na placenta-perfusie experimenten (hoofdstuk 7). De resultaten laten zien dat 10x hogere concentraties van tacrolimus gemeten worden in de placenta in vergelijking met concentraties in de maternale circulatie. Tacrolimus stapelt zich dus op in de placenta. Dit geeft aan dat de placenta een aanzienlijk risico heeft op tacrolimus-geïnduceerde toxiciteit, maar ook dat het uteriene immuunsysteem enorm beïnvloed zou kunnen worden door deze hoge concentraties, met alle gevolgen van dien.

In hoofdstuk 8, hebben we in meer detail onderzocht wat de invloed van immunosuppressiva op het uteriene immuunsysteem is door te kijken naar de deciduale immuuncellen verkregen uit de placenta van niertransplantatiepatiënten en gezonde, ongecompliceerde controles na bevalling. Zo rapporteren we dat de zeer immuunonderdrukkende HLA-DR+ Treg aan een lagere frequentie aanwezig zijn in de decidua van niertransplantatiepatiënten in vergelijking met de decidua van gezonde controles.
We vinden voornamelijk een lager percentage van deze cellen wanneer de patiënten behandeld worden met tacrolimus versus diegenen die behandeld worden met azathioprine naast tacrolimus, of met azathioprine alleen. Dit zou kunnen betekenen dat het gebruik van immunosuppressiva tijdens de zwangerschap zou kunnen leiden tot een verlies van foetale tolerantie, met een verhoogd risico op complicaties als gevolg. Bovendien zou azathioprine mogelijk medicatie van eerste keus kunnen zijn voor gebruik tijdens de zwangerschap. Studies met grote groepen patiënten zijn dus nodig in de toekomst om in meer detail het effect van de verschillende medicatie-regimes op het uteriene immuunsysteem te onderzoeken en om te bestuderen of zwangerschapscomplicaties nu minder vaak voorkomen wanneer bijvoorbeeld azathioprine gebruikt wordt. Het is niet ondenkbaar dat sommige medicijnen minder schadelijk zijn tijdens de zwangerschap, en dus wenselijker zijn om te gebruiken, indien dit mogelijk is.

Naast accumulatie van tacrolimus in de placenta, meten we in het navelstengbloed van niertransplantatiepatiënten concentraties die vergelijkbaar zijn aan concentraties in het bloed van de moeder (hoofdstuk 7). Dit betekent dat de ontwikkelende foetus blootgesteld wordt aan tacrolimus, wat de ontwikkeling van het immuunsysteem van het kind zou kunnen beïnvloeden. Daarom hebben we in hoofdstuk 8 ook gekeken wat het gebruik van immunosuppressiva nu doet op het immuunsysteem van het kind. Hiervoor maakten we gebruik van het navelstengbloed van de kinderen van niertransplantatiepatiënten en van gezonde controles. We beschrijven dat onder andere de frequenties NKT cellen, CD25^+CD127^{low}FoxP3^+ Treg, HLA-DR^+ Treg, en B cellen lager zijn in het navelstengbloed van kinderen van niertransplantatiepatiënten. Deze dysregulatie van het neonatale immuunsysteem zou deze kinderen niet alleen vatbaarder kunnen maken voor het ontwikkelen van allergieën, auto-immuunziekten of inflammatoire ziekten later in hun leven, maar het zou ook hun vaccinatie-respons kunnen beïnvloeden. We adviseren om de gezondheid en vaccinatierespons van deze kinderen op te volgen en te definiëren wat het effect van verschillende medicatie-regimes is.

Concluderend geven de bevindingen in dit proefschrift ons nieuw inzicht in het immuunsysteem van de uteriene mucosae. We toonden aan de uteriene mucosae een unieke compositie van weefselspecifieke immuuncellen heeft dat veranderd tijdens de zwangerschap en kan beïnvloed worden door immunosuppressieve medicijnen. Dit proefschrift laat zien dat het belangrijk is om verder uit te zoeken hoe we de onderliggende oorzaak van zwangerschapscomplicaties willen bestuderen. Wat is de waarde van het onderzoeken van bloed wanneer er lokaal een weefselspecifiek immuunsysteem aanwezig is? Dit wil uiteraard niet zeggen dat er helemaal geen plaats is voor het meten van bloedmarkers voor het bijvoorbeeld voorspellen van de ontwikkeling van pre-eclampsie of vroegegeboorte tijdens de zwangerschap. Ook moeten we er bij stilstaan dat we...
Nederlandse samenvatting

de eventuele medicatie voor zwangerschapscomplicaties zorgvuldig moeten uitkiezen en afwegen voor het beoogde effect, eventuele ophopingen in de placenta, en mogelijk bijwerkingen in het ongeboren kind. Placenta perfusie experimenten, zoals gebruikt in dit proefschrift, maar ook placentale explants en organoids zullen belangrijke modellen zijn tijdens dit beslissingsproces. Het is van cruciaal belang om de complexe interactie en dynamiek van het uterine immuunsysteem beter in kaart te brengen zodat we uiteindelijk zwangerschapscomplicaties beter kunnen behandelen of zelfs voorkomen.
REFERENCES


Chapter 10


CHAPTER 11

Curriculum Vitae
List of publications
RIMLS PhD portfolio
FAIR principles
Acknowledgements | Dankwoord
CURRICULUM VITAE

Dorien Feyaerts was born on February 21st 1989 in Leuven, Belgium. She grew up in Gelrode (Belgium) and in 2009, she graduated from Sancta Maria Institute, Aarschot, with a degree in Science-Mathematics. That same year, she started her Bachelor studies in Biomedical Sciences at the KU Leuven (Leuven, Belgium). In 2010, after graduating cum laude from her Bachelor, she started her Master Biomedical Sciences, Minor Management and Communication at the KU Leuven. During her studies, Dorien developed a keen interest in immunology, which led to her Master thesis at the department of Clinical and Experimental Endocrinology at the KU Leuven. Under the supervision of Prof. dr. Chantal Mathieu and dr. Tom van Belle, she showed that 1,25-Dihydroxyvitamin D3 and its analog TX527 promote a stable regulatory T cell phenotype in T cells from type 1 diabetes patients. Dorien graduated from her Master degree cum laude in 2012. In the same year, she moved from academia to industry to work as a Research Technician at Apitope International NV (Diepenbeek, Belgium). At Apitope, together with scientist dr. Kathleen Vrolix, Dorien contributed to the discovery of tolerogenic peptides that can be utilized as antigen-specific immunotherapy for the autoimmune disease Graves’ Disease. The discovered Apitopes® recently finished phase I clinical trials successfully. In March 2015, Dorien moved to the Netherlands, to pursue a PhD in Reproductive Immunology under the supervision of Prof. dr. Irma Joosten, dr. Renate van der Molen and dr. Olivier van der Heijden at the Radboudumc, Nijmegen. Her PhD research, as described in this dissertation, focused on the immunology of the uterine mucosae. During her PhD, she developed an educational program for secondary schools to promote vaccine education, immunology, programming and robotics, together with colleagues Joshua Gillard and Yessica Alina Rodriguez Rosales. Dorien will continue her research in the field of Reproductive Immunology as a PostDoc in the group of Dr. Brice Gaudillière at Stanford University.
LIST OF PUBLICATIONS

In this thesis

D. Feyaerts, A. van der Meer, I. Joosten, R.G. van der Molen
Selective expansion and CMV-dependency in pregnancy trained human endometrial NK cells
*Cellular and Molecular Immunology* 2019 Apr;16(4):410-411
doi:10.1038/s41423-018-0193-x

Placental disposition of the immunosuppressive drug tacrolimus in renal transplant recipients and in *ex vivo* perfused placental tissue
*European Journal of Pharmaceutical Sciences* 2018 Jul 1;119:244-248
doi:10.1016/j.ejps.2018.04.017

D. Feyaerts*, T. Kuret* B. van Cranenbroek, S. van der Zeeuw-Hingrez, O.W.H. van der Heijden, A. van der Meer, I. Joosten, R.G. van der Molen
Endometrial natural killer (NK) cells reveal a tissue-specific receptor repertoire
doi:10.1093/humrep/dey001

D. Feyaerts, M. Benner, B. van Cranenbroek, O.W.H. van der Heijden, I. Joosten, R.G. van der Molen
Human uterine lymphocytes acquire a more experienced and tolerogenic phenotype during pregnancy
*Scientific Reports* 2017 Jun 6; 7(1):2884
doi: 10.1038/s41598-017-03191-0
Additional publications
E.A. van Erp, D. Feyaerts, M. Duijst, H.L. Mulder, O. Wicht, W. Luytjes, G. Ferwerda, P.B. van Kasteren
Respiratory Syncytial Virus (RSV) infects primary neonatal and adult natural killer cells affects their antiviral effector function
*Journal of Infectious Diseases* 2019 Feb 15;2019(5):723-733
doi:10.1093/infdis/jiy566

1,25-Dihydroxyvitamin D3 and its analog TX527 promote a stable regulatory T cell phenotype in T cells from type 1 diabetes patients
*PloS One* 2014 Oct 3;9(10):e109194
doi: 10.1371/journal.pone.0109194
RIMLS PhD PORTFOLIO

**RIMLS PhD portfolio**

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**TRAINING ACTIVITIES**

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</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td><strong>46.85</strong></td>
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^ oral presentation; # poster presentation
FAIR PRINCIPLES

The work in this thesis is based on samples obtained from human subjects. The studies were approved by the institutional review board (Committee on Research Involving Human Subjects region Arnhem-Nijmegen, the Netherlands; CMO numbers 2009/004, 2017-3253, 2014-232, and 2014-1397) and was performed in accordance with the relevant guidelines and regulations (Declaration of Helsinki). The privacy of the participants in these studies is warranted by using individual and anonymous identification codes that are stored in password protected data files. Only senior staff members involved in the studies have access to these files. Obtained samples will be saved for 10 years after inclusion. Using patient’s data in future research is only possible after renewed informed consent, as stated in the patient’s information letter.

The data obtained during this PhD has been captured and stored in an electronic lab notebook (Labguru) and in paper lab notebooks. Labguru project files are only accessible by the associated technicians and senior staff members, and are backed-up daily. Paper lab notebooks are stored in the second metal file cabinet in room M379.03.167 at the Laboratory Medical Immunology at the Radboudumc (route 469). Location of cryopreserved cells and DNA samples can be found in itemtracker and stored serum samples can be found in Labguru by searching for the identification codes.

Raw data files, analysis files, and password protected metadata files are stored on the department’s Z-drive (Z:\Research_BU\Sectie HCI_kweek\z239158_Dorien Feyaerts\Raw data and Z:\Research_BU\Sectie HCI_kweek\z239158_Dorien Feyaerts\Analysis), in separate project-specific folders. This drive is backed-up on a weekly basis.
Acknowledgments | Dankwoord

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UITNODIGING

Voor het bijwonen van de openbare verdediging van mijn proefschrift

IMMUNOLOGY OF THE UTERINE MUCOSAE

Op woensdag 8 januari 2020 om 14:30 in de Aula van de Radboud Universiteit
Comeniuslaan 2, Nijmegen.

U bent van harte welkom bij deze plechtigheid en de aansluitende receptie

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