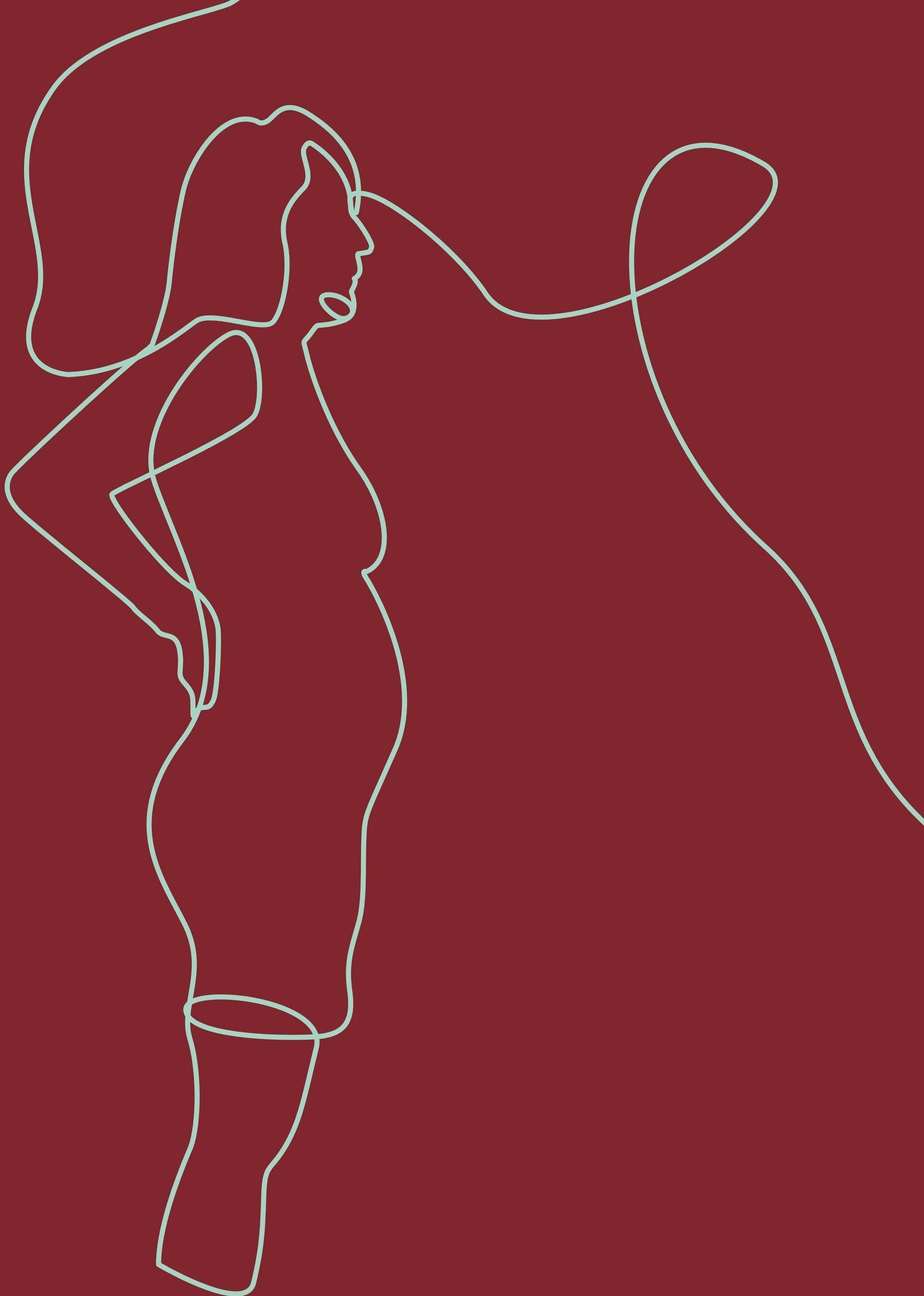
Changing *perspectives* on uterine immunity

Marilen Benner



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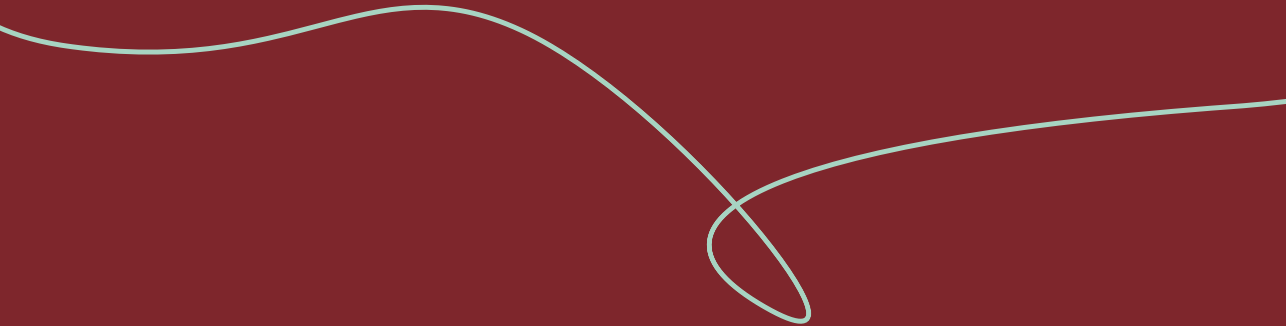

Colophon
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Changing perspectives on uterine immunity

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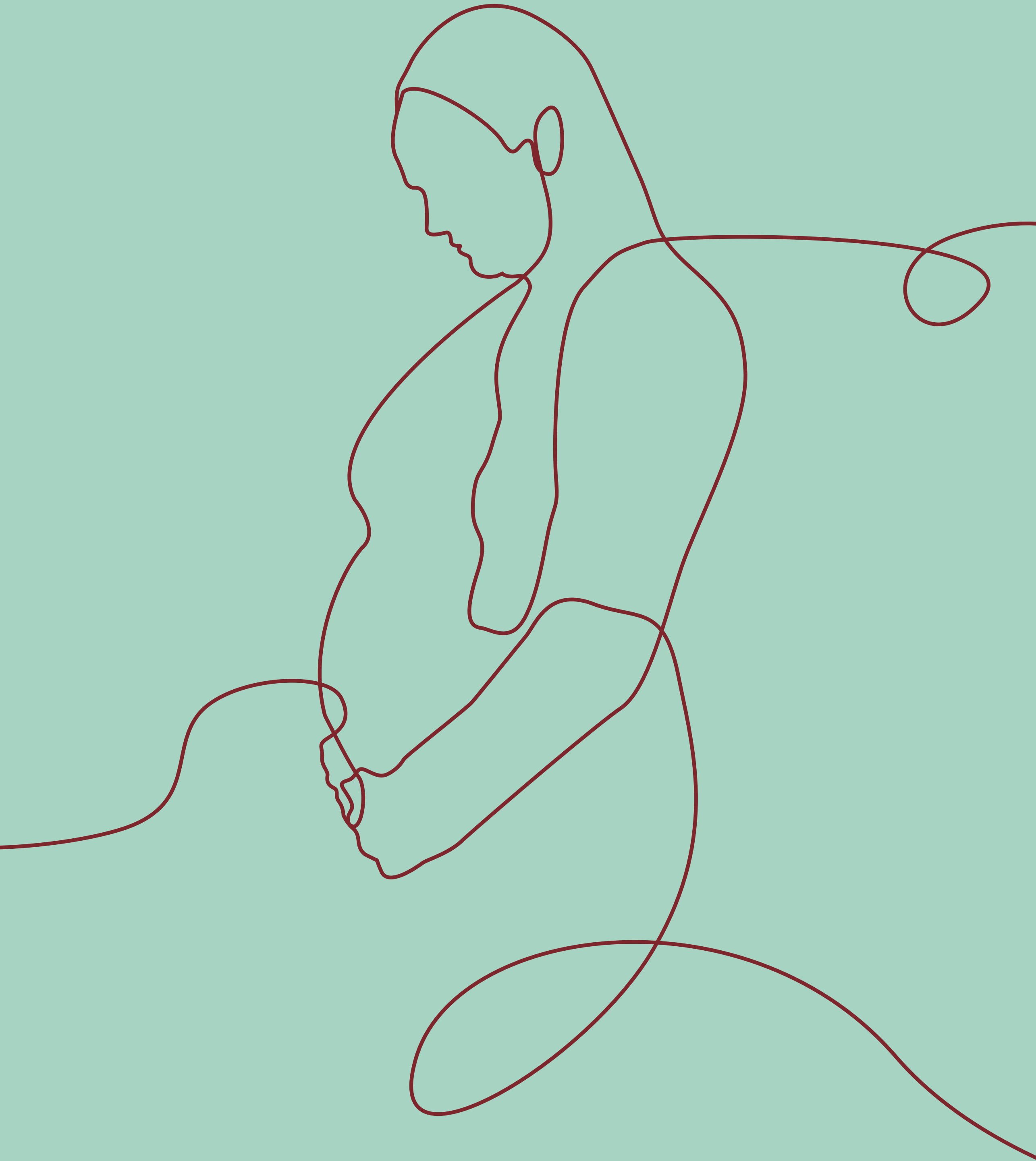
To the strong women
who enabled me to write this thesis

CONTENTS

Chapter 1	General introduction	09
Part I	**Local immune regulation**	**23**
Chapter 2	Human uterine lymphocytes acquire a more experienced and tolerogenic phenotype during pregnancy *(Scientific reports, 2017)*	25
Chapter 3	Three types of functional regulatory T cells control T cell responses at the human maternal-fetal interface *(Cell Reports, 2019)*	51
Chapter 4	Clusters of tolerogenic B cells feature in the dynamic immunological landscape of the pregnant uterus *(Cell Reports, 2020)*	83
Chapter 5	A combination of immune cell types, identified through an ensemble machine learning strategy, detects altered profile in recurrent pregnancy loss	109
Part II	**Microbial contribution to reproductive health**	**135**
Chapter 6	How uterine microbiota might be responsible for a receptive, fertile endometrium *(Human Reproduction Update, 2018)*	137
Chapter 7	Antibiotic intervention affects maternal immunity during gestation in mice	187
Part III	**Connecting the pieces**	**215**
Chapter 8	Summary and Discussion	217
Chapter 9	Graphical summary	243
Chapter 10	Samenvatting (Dutch summary)	255
Chapter 11	Zusammenfassung (German summary)	265
Chapter 12	Afterword	275
Part IV	**Appendix**	**279**
Chapter 13	FAIR principles	283
	Acknowledgements – Dankwoord - Danksagung	285
	Curriculum Vitae	291
	RIMLS PhD portfolio	293
	List of publications	297



CHAPTER 1

A HISTORIC VIEW OF PREGNANCY AS MYSTERY –THE WOMB AT THE CENTER OF ATTENTION

Throughout history, mankind has been in awe of pregnancy. The ability to bear an heir came not only with great power, but also with an enormous responsibility that, for many centuries, has defined a woman's worth. The lack of power to control reproductive success inspired some of the earliest medical investigations known, at times when female health was otherwise underrepresented in medicine. Already around 2000 B.C., the Egyptian Kahun Gynaecological Papyrus documents the desire to understand the mysterious process of fertility, representing the first evidence of the special role that has been ascribed to the womb throughout ancient times (1, 2). Many of these Egyptian concepts depicting the womb as cause for many (if not all) issues of women's health were reinforced by the ancient Greeks (3). Theories of the womb (ὑστέρᾱ - hystera) as origin also for mental (and thus gender specific) illness were prominent until the twentieth century[1]. Moral perceptions hampered the extent to which male medical professionals could examine the female reproductive system. A forward leap in the understanding of uterine health could not be made until the role of physician was not exclusive to men anymore, and the taboos around the menstrual cycle were challenged by feminist movements in the 1960s and early 1970s (4). Current globalized clinical practice now permits us to look beyond mythological and religious associations with female health. Still, there is much left to discover to understand the magic of pregnancy.

THE GATEKEEPER FUNCTION OF THE UTERUS

Even when all conditions are optimal, human reproduction is relatively inefficient and each cycle the probability of conception is merely 30-40% (5). In an attempt to answer the question "Where have all the conceptions gone?", Roberts and Lowe (1975) hypothesized that the relatively low incidence of birth defects amongst the population results from a natural selection of fit embryos (6). A large part of pregnancies are lost before women are aware of it, as up to a third of "occult pregnancies" go unnoticed (Figure 1) (5, 7). This window of high fragility continues until mid-gestation (8, 9). Up to 15-20% of pregnancies end in a loss before 28 weeks of gestation (10). About half of these cases are explained by cytogenetic abnormalities of the embryo (11). These miscarriages result not only from the embryo's failure to interact with the uterine wall, but from an inability on the maternal side to receive the incoming cells (12, 13). The uterine mucosa, termed *endometrium*, or *decidua* once it is at its receptive stage towards the end of the menstrual cycle, actively senses quality of the embryo (14). This selection during implantation, a first checkpoint, has to be extremely well-regulated. On the one hand, failure of the decidua to allow implantation

[1] It was not until 1980 that "Hysteria" was removed from Diagnostic and Statistical Manual of Mental Disorders (15).

of high-quality embryos is seen in recurrent implantation failure (RIF), mostly perceived as infertility. On the other hand, failure to exclude erroneous embryos in the peri-implantation period likely leads to miscarriage later on during gestation. Thus, the decidua holds a key role. Even after correct implantation is achieved, this highly specialized tissue continues to determine the faith of pregnancy in a second checkpoint: through enabling formation of the placenta.

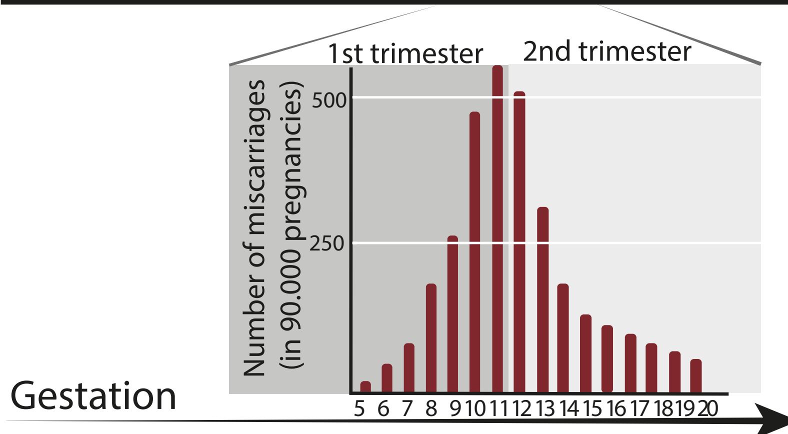


Figure 1. Only a third of all conceptions lead to the birth of a living baby.

The majority of conceptions is lost prior to recognition of the pregnancy. A third of all conceptions are carried to live birth. The majority of recognized miscarriages occurs in the first trimester. *After 28 weeks of gestation, pregnancy loss is termed "stillbirth". Adapted from Macklon et al. 2002, Nilsson et al. 2014.

THE ENDOMETRIUM - FERTILE GROUND ENABLING GROWTH OF A HEALTHY PLACENTA

The placenta connects the fetus to the maternal circulation; a vital bond that forms the basis for all further development of the child. The placental membranes form a safe cocoon with a well-regulated internal environment for the baby to thrive in (Figure 2). Correct placenta formation is not only an essential prerequisite to avoid pregnancy loss, but also determines fetal and neonatal health. Structural impairments of the placenta cause pregnancy complications such as intrauterine growth restriction (IUGR) and pre-eclampsia (PE), with possible long-term effects on health of mother and child (16). Placenta formation depends on a complex interplay of maternal and fetal cells. Trophoblast cells of fetal origin have to invade the spiral arteries and replace their endothelium to successfully attach to the vascular system of the mother. These fetal cells are semi-allogeneic, a hybrid of half paternal and half maternal genes. The field of reproductive immunology aims to understand how these partially foreign cells evade the maternal immune system, the reactive mechanism to eliminate unfamiliar antigens. It is now established that, contrary to the initial hypothesis of Sir Peter Medawar in 1953, maternal immunity is not ignorant to the fetal cells, but tolerant (17, 18). How is this tolerance achieved?

PUZZLING IMMUNITY OF THE PREGNANT UTERUS

The human decidua is abundantly equipped with immune cells that may react to the semi-allogeneic fetal cells. In the peri-implantation period, Natural Killer (NK) cells (~60%), macrophages (10-20%), and T (3-10%) cells represent the most prominent local immune cell types (19-23). Upon conception, immune cells infiltrate the implantation site and levels of proinflammatory cytokines leukemia inhibitory factor (LIF), tumor necrosis factor (TNF)-α, and interleukin (IL)-6 rise in the endometrium (24). These local reactions are more than a mere side effect to the invading foreign antigens, as they belong to the physiological adaptations needed during this period. As such, increasing evidence shows that provoking a local immune reaction, by deliberately scraping the endometrial lining during in vitro fertilization (IVF), increases success rates in assisted reproductive technology (ART)(25-27). However, any inflammatory reaction of implantation, even physiological, needs to be well-contained. Excessive inflammation would threaten the baby and progression of pregnancy, leading to implantation failure, pregnancy loss, or pre-term labor (PTL). To maintain the balance between immunity and tolerance, decidual immune cells have to be equipped with extremely fine-tuned regulatory mechanisms.

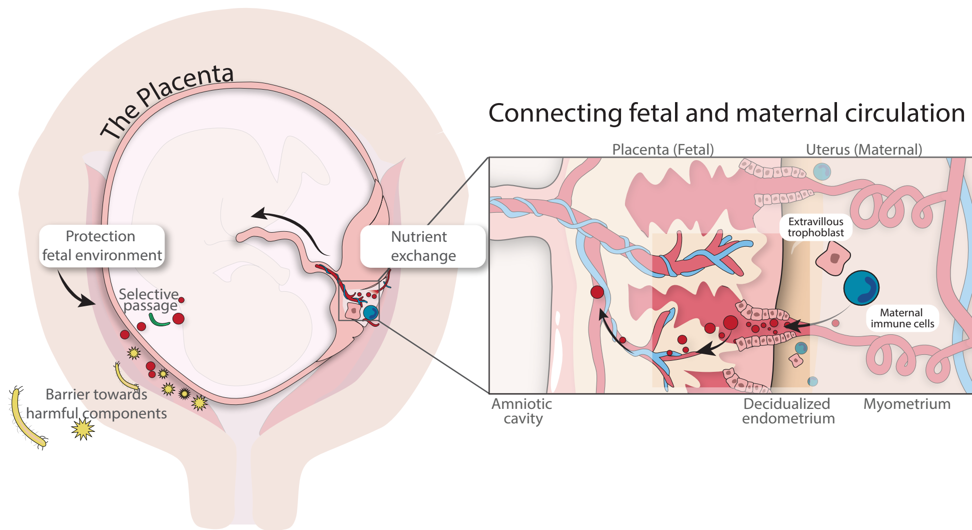


Figure 2. Healthy pregnancy depends on a well-formed placenta.

It maintains fetal growth by providing the essential connection with the maternal circulation and it creates a protective environment.

KNOWN PARTS OF AN UNKNOWN PICTURE

For a number of cell subsets, specialized characteristics of uterine immunity are known. Many of these underline the independence of decidual cells from systemic immunity. The most studied cell types in reproductive immunology are NK cells and T cells.

NK cells

Decidual NK cells (dNK) display functional and phenotypic characteristics different from peripheral blood NK cells (pbNK) (21, 28). The two major subpopulations of NK cells are composed of $CD56^{dim}$ $CD16^{+}$ (NKdim) and $CD56^{bright}$ $CD16^{-}$ (NKbright) cells. With their task as innate effector cells to eradicate virally infected and tumor cells, NKdim cells dominate the systemic pool of NK cells (~90%). NKdim cells are of potent cytotoxicity, containing many cytolytic granules such as granzymes and perforin (29). In the uterus, these major *killers* are almost absent where NKbright cells comprise around 90% of the local NK cells (30). NKbright cells are less cytotoxic but mainly secrete immunoregulatory cytokines, such as IL-10, IL-13, granulocyte – macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, and TNF-β (31). Asides from contributing to the local immune environment, dNK cells also directly influence implantation. By secreting angiopoietin (Ang)-1, Ang-2, IFN-γ, and vascular endothelial growth factor (VEGF), they loosen vascular smooth muscle cells, and degrade extracellular matrix components (32, 33). Moreover, decidual NK cells enhance trophoblast migration through GM-CSF secretion (34). Both processes facilitate spiral artery remodeling. This leads to the notion

that correct placenta formation and thus fetal development depends on the local actions of dNK cells (35).

T cells

Building up on Medawar's initial question "How does the pregnant mother contrive to nourish within itself, for many weeks or months, a fetus that is an antigenically foreign body", research on T cells in pregnancy started from a transplantation point of view. In a transplantation setting, if unmatched to the recipient, cells expressing allogeneic major histocompatibility complex (MHC) class I antigens fail to provide a self-signal (signal 1). In the presence of co-stimulatory signals (signal 2), cytolytic killing by CD8+ T cells is elicited (36). Additionally, MHC class II molecules may present the foreign antigens to CD4+ T cells, causing naïve T cells to differentiate into T helper (Th) cells. Th cells can in turn interact with B cells to initiate antibody-mediated killing, or directly promote the local immune reaction by secretion of pro-inflammatory cytokines (signal 3). Based on this key role in rejection, local T cells were initially suspected to pose a danger to the "fetal transplant" and thus it seemed logical that they need to be silenced after conception.

However, during pregnancy, decidual T cells are responsive and can eradicate infections. In practice, maternal blood is merely in contact with syncytiotrophoblast and extravillous trophoblast cells (EVT), both of which have extraordinary MHC characteristics. Syncytiotrophoblast do not express any MHC rendering them inert to T cell-mediated response (37). EVT express human leucocyte antigen (HLA)-C,-E, and -G and of these, only HLA-C is highly polymorphic (HLA-A, and -B are the major polymorphic inducers of allograft rejection). Decidual T cells are indeed capable of reacting to an HLA mismatch but this does not lead to killing of trophoblast cells as shown in co-culture experiments (38, 39). On the contrary, upon encounter of an HLA-C mismatch, T cell activation was shown to induce increased abundance of regulatory T cells (Treg) (39). Additionally, rather than stimulating Th cells of pro-inflammatory cytokine profiles (Th1: IFN-γ, TNF-α, TNF-β; Th17: IL-17, IL-22, IL-23), implantation provides signaling that allows for differentiation of Th cells that contribute to a tolerogenic environment (Th2: IL-4, IL-5, IL-10, IL-13)(40, 41). Thus, with our improved understanding of T cell mediated immunity, the framework of inert T cells is set aside.

Beyond NK- and T cell mediated immunity

Still, also regarding the most studied cell types, we are still just beginning to collect the pieces of an unknown bigger picture. Many studies have pointed out that seeking to understand reproductive immunology goes beyond NK- and T cell mediated immunity. Interest in decidual myeloid cells (macrophages, dendritic cells, and granulocytes) is rising (42-47). As obtaining samples of the target site is challenging, few studies are available and research is still in its infancy.

CONSIDERING DIFFERENT ANGLES

Not only maternal immune cells of the decidua have specialized regulatory functions. Previous studies suggest a number of different angles from which to investigate the balance between maternal and fetal cells:

1) Trophoblasts possess mechanisms to facilitate immune evasion.

EVT express immune-modulatory receptors, which match ligands of dNK cells (HLA-G – LILRB1), macrophages (IL10R – IL10) , and T cells (PDL1- PD1) (48). Functionally, this for example translates to the ability of EVT to inhibit proliferation of T cells, and to induce differentiation of CD4+ T cells towards Treg cell in vitro (38, 49). Trophoblasts secrete cytokines that strongly impact the local immune environment. Examples are IL-34, which polarizes decidual macrophages towards a tolerogenic phenotype, and IL-35, which converts naïve T cells to IL-35-producing regulatory T cells and, in mice, thereby prohibiting pregnancy loss (49, 50).

2) Cells of the uterine tissue influence the local immune environment.

Like trophoblast cells, cells of the uterus are equipped with surface molecules to facilitate tolerogenic ligand-receptor interactions with decidual immune cells (48). Through their secretion of indoleamine 2,3-dioxygenase (IDO) and prostaglandin E (PGE), decidual stromal cells (DSC) were shown to reduce NK cell proliferation and cytolytic activity, and inhibit dendritic cell (DC) differentiation (51). Additionally, DSC hamper local T cell accumulation through their reduced expression of Th1 cell - attracting chemokines as a result of epigenetic silencing (52).

3) External factors contribute to a tolerance-favoring milieu (e.g. seminal fluid, microbiota).

Adding to the complexity, there are even more contributors to consider than maternal and fetal cells. Seminal fluid was shown to regulate inflammatory gene expression in cervical and vaginal epithelial cells (53, 54). It contains soluble factors, such as transforming growth factor- β (TGF-β) and PGE, with the ability to modify the induction of Treg (55). Even though direct experimental evidence of uterine contribution of this immune-modulatory action is yet to be exposed, a contribution of seminal fluid to successful implantation is likely; contact with seminal fluid during the course of ART increased clinical pregnancy rates (56). Another external contributor able to enter the uterus, and modify immunity, are microbiota; a topic of heavy debate as the uterus was previously regarded sterile. The discussion on the contribution of bacteria to pregnancy success lifted up with the discovery of commensal colonization of the placenta, and with 16S rRNA sequencing techniques becoming more accessible, studies on endometrial and placental microbiota are rising (57-60).

The factors on this list are of a diverse nature; and it is near impossible to give a full account of them. Still, we have to consider more than a single piece of the puzzle, with an open mind, if we wish to understand the bigger picture.

AIM AND OUTLINE OF THIS THESIS

In this thesis, we aim to introduce new perspectives on the regulation of uterine immunity.

In **Part I** of this thesis, we explore the highly specialized local immune adaptations induced by pregnancy. We focus on tissue of the uterine mucosa. In **Chapter 2**, we give an overview of the immune composition of the uterus. We examine how local immunity, especially NK and T cells of the endometrium, prior to implantation compare to the end of pregnancy. We dive deeper into the importance of specialized T cells in **Chapter 3**, highlighting the presence of distinct types of regulatory T cells in human decidua. In reproductive immunology, most studies revolve around cell types of large abundance in the uterus. Even though small in numbers, we consider the possible contribution of B cells to the local immune interplay in **Chapter 4**. To do so, we compare mucosal tissue of the non-pregnant uterus, during gestation, and upon delivery. We translate this knowledge on immune composition considering a broad spectrum of immune cells to patients suffering from recurrent miscarriages in **Chapter 5**.

In **Part II**, we examine yet another contributor of small abundance but possibly far-reaching impact: microbiota of the uterus. Due to their low biomass, uterine microbiota are difficult to detect. While presence of uterine microbiota is thus debated, microbes of the gut have shown how immunity cannot be fully understood without taking microbiota into account. We describe the possible contribution of microbes and their compounds on uterine immune cells in **Chapter 6**. Manipulating the microbial balance by antibiotics during gestation might have an impact on the direct environment of the growing baby. In **Chapter 7** we investigate if disrupting the natural microbial composition translates to immune adaptation during pregnancy. Finally, we summarize and discuss the findings of this thesis in **Chapter 8**.

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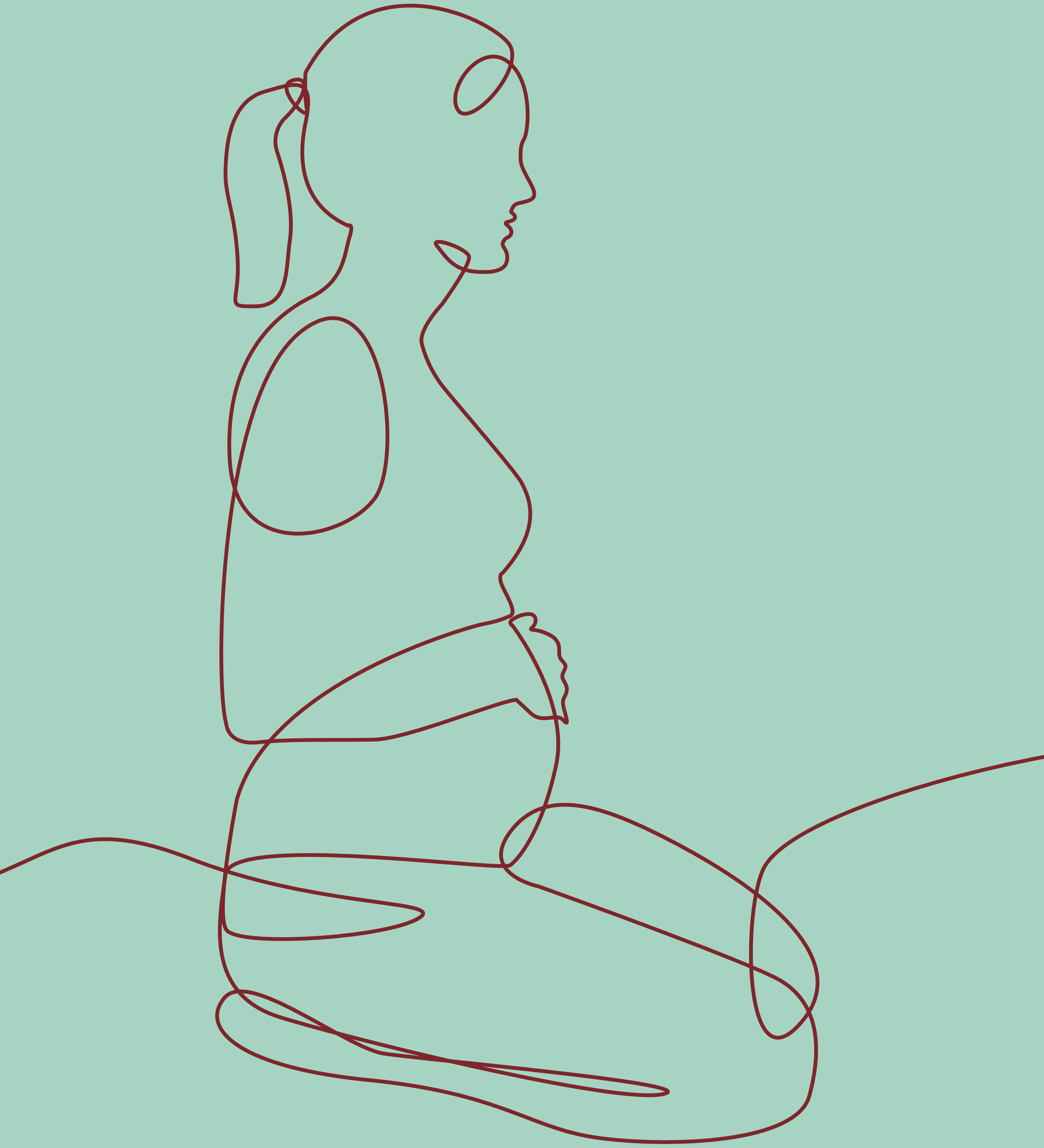
Part 1

Local immune regulation



CHAPTER 2

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

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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 $CD56^{bright}CD16^{-}$, while NK cells found in peripheral blood are mainly $CD56^{dim}CD16^{+}$ (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 of Treg was associated with pregnancy failure (19-22). Also, in humans, pregnancy complications, like recurrent pregnancy loss and preeclampsia, 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.

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 (Figure 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

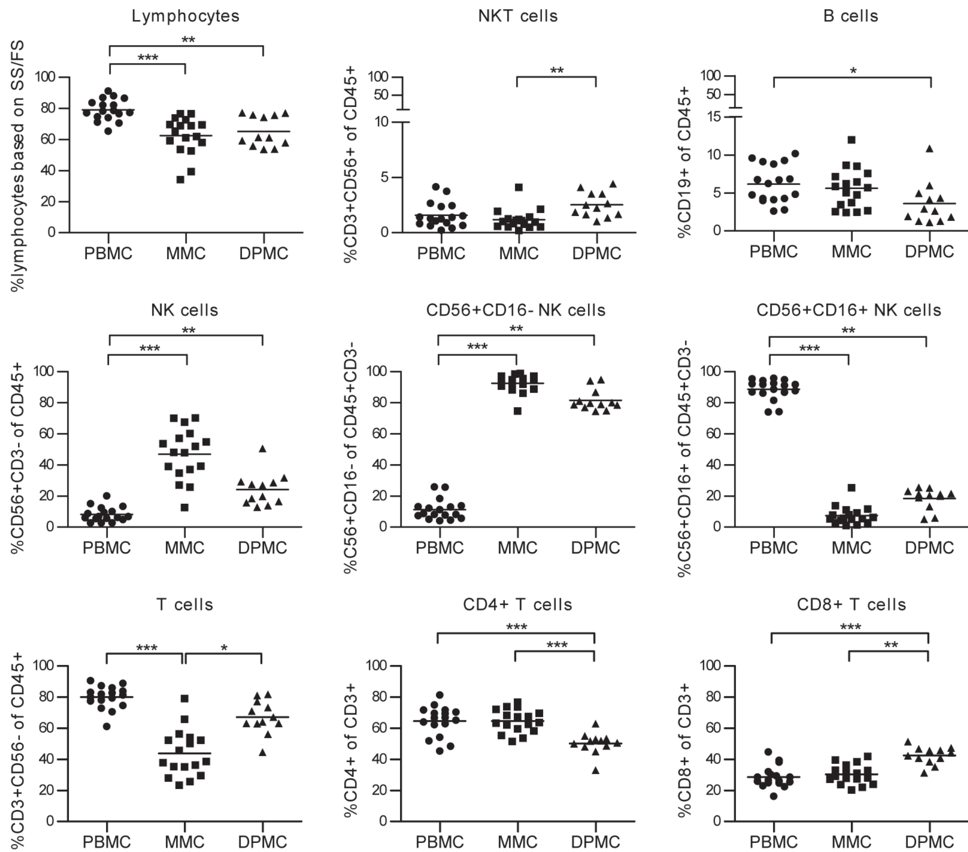

Figure 1. Lymphocyte composition of peripheral blood, menstrual blood, and term decidua.

Mononuclear cells were isolated from peripheral blood (PBMC, n=17), menstrual blood (MMC, n=17), and decidua parietalis (DPMC, n=12). Percentages of lymphocytes (CD45+) based on side scatter (SS) and forward scatter (FS), T cells (CD56-CD3+; CD4+ and CD8+), NKT cells (CD3+CD56+), NK cells (CD3-CD56+; CD56+CD16+ and CD56+CD16-), and B cells (CD19+) were obtained by flow cytometric analysis. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (lines indicate mean, non-parametric Kruskal-Wallis with Dunns post-hoc test).

(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 Figure 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.

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 (35) 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) (Figure 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 (Figure 2c). Thus, over the course of pregnancy, decidual T cells appear to acquire an experienced and differentiated phenotype.

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 (9). 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 CM T cells compared to control (medium) and day 0 (Supplementary Figure 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.

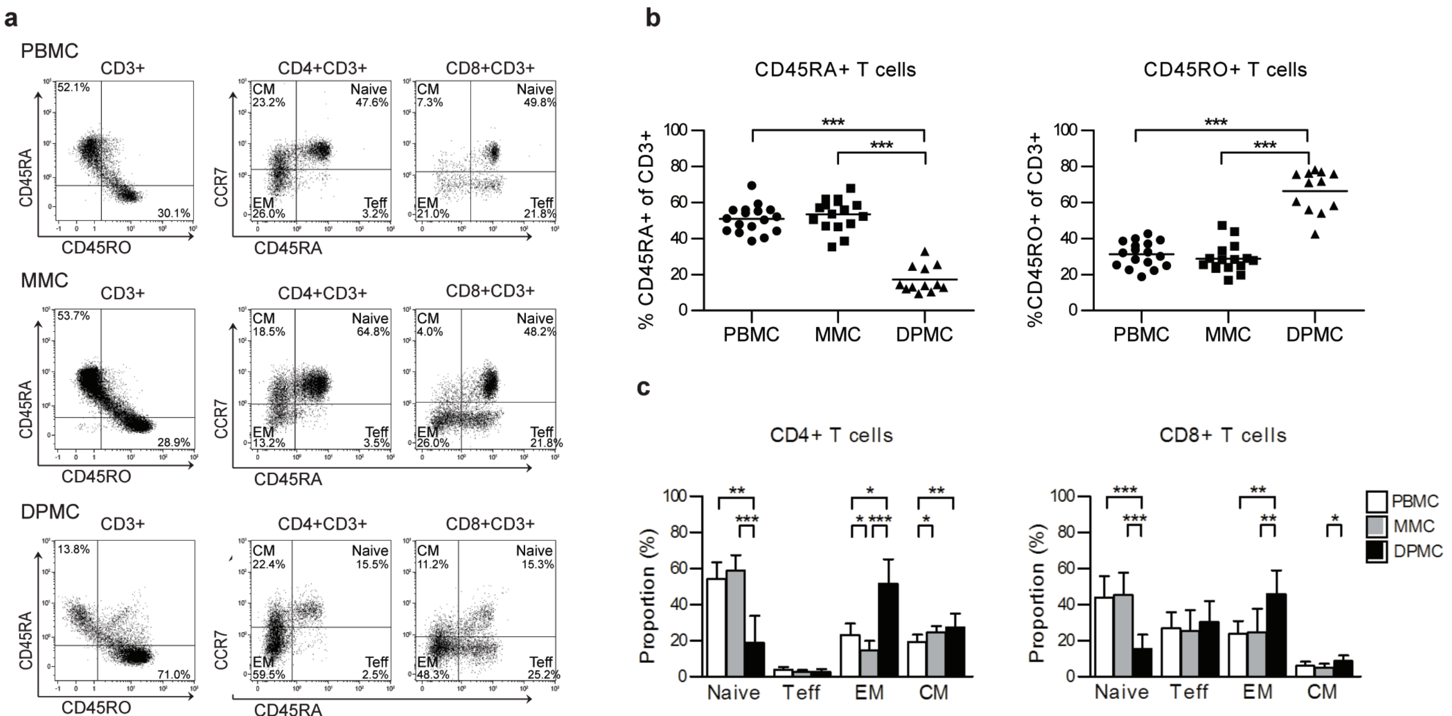

Figure 2. CD4+ and CD8+ T cells subsets in peripheral blood (PBMC), menstrual blood (MMC) and term decidua (DPMC).

(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)

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 (19). DPMC contained significantly more $CD4^{+}CD25^{high}CD127^{-}$ Treg than MMC (9.5%±3.5% versus 5.2%±1.9%) (Figure 3b). We further subdivided Treg based on expression of CD45RA and CD25 (36). 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%) (Figure 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.

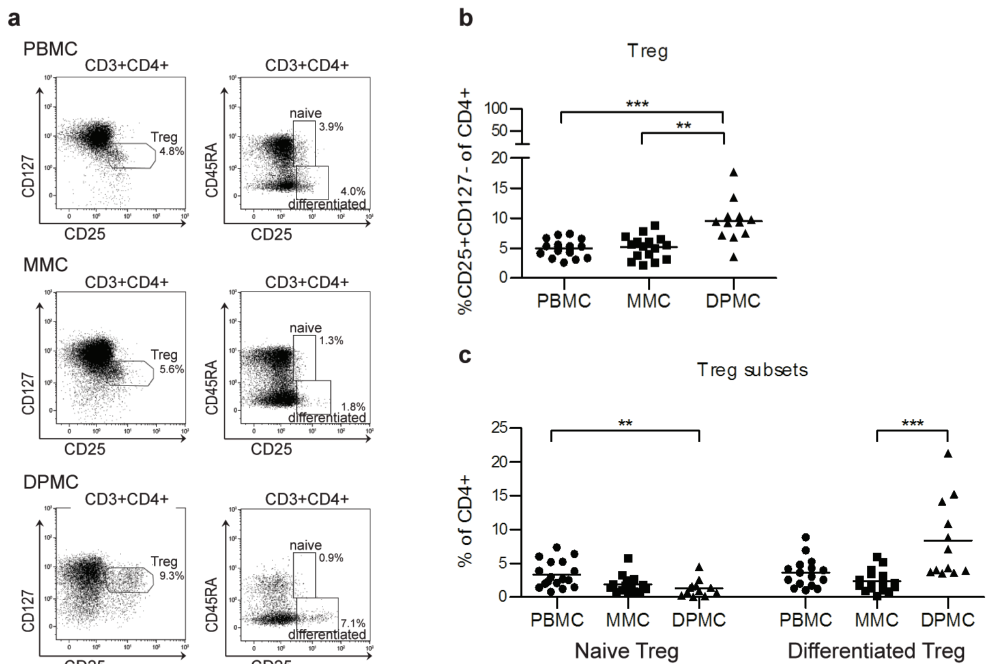

Figure 3. Distribution of regulatory T cells.

(A) Representative staining of CD25, CD127, and CD45RA on CD4+ T cells from peripheral blood (PBMC, n=17), menstrual blood (MMC, n=16), and term decidua (DPMC, n=12). **(B)** Percentage of CD25highCD127- Treg within CD4+ T cells in PBMC, MMC, and DPMC. (C) Distribution of naive Treg (CD45RA+CD25+) and differentiated Treg (CD45RACD25++) in CD4+ T cells. **p < 0.01, and ***p < 0.001 (lines indicate mean, non-parametric Kruskal-Wallis with Dunns post-hoc test).

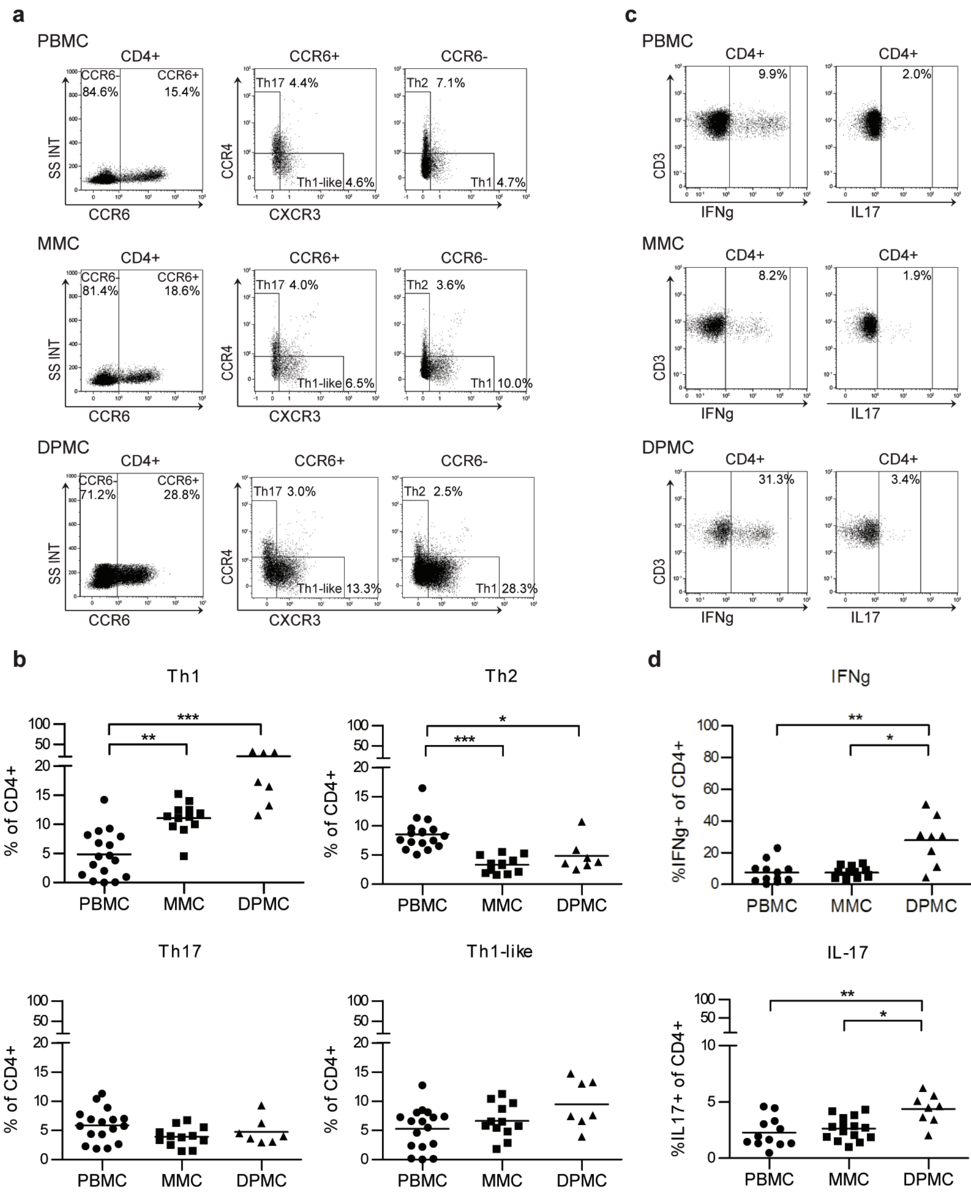

Figure 4. Distribution of CD4+ Th cell subsets in peripheral blood (PBMC), menstrual blood (MMC), and term decidua (DPMC) and production of IFN-γ and IL-17 by CD4+ T cells.

(A, C) Representative gating for CCR6, CXCR3, and CCR4 on CD4+ T cells and for IFN-γ and IL-17 by CD4+ T cells 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 37. CCR6-CXCR3+CCR4-, Th1; CR6+CXCR3+CCR4-, Th1-like; CCR6-CXCR3-CCR4+, Th2; CCR6+CXCR3-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, nonparametric Kruskal-Wallis with Dunns post-hoc test).

The Th1, Th2, and Th17 cell profile is similar between endometrium and term deciduas

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, nonconventional 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-γ 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 (Figure 4b). Between MMC and DPMC, percentages of Th1, Th2, Th17, and Th1-like cells did not differ significantly (Figure 4b). When investigating the intracellular cytokine expression profile after PMA/Ionomycin/Brefeldin stimulation, we observed a higher intrinsic capacity to express IFN-γ and IL-17 by DPMC $CD4^+$ T cells (27.8%±15.4% and 4.4%±1.3% respectively) compared to $CD4^+$ T cells from MMC (7.4%±3.3% and 2.6%±1.0% respectively), and also PBMC (7.5%±6.7% and 2.3%±1.3% respectively) (Figure 4d).

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 (30, 41). 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 (20-22). We showed that the percentage of $CD4^+CD25^{high}CD127^-$ Treg was higher in decidua than in endometrium, suggesting a more tolerogenic environment during pregnancy. As previously reported by our group(27), 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 (30). Based on the expression of CD45RA and CD25, human $CD4^+$ T cells can be separated into differentiated Treg and naive Treg (36). 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 (36). 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 (42). 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 (14). 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 (37). 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 (43). 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. (44) showed that in the endometrium of women with recurrent miscarriages less $CD4^{+}IFN\text{-}\gamma^{+}$ 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 $IL\text{-}17^{+}$ T cells was also found to be higher compared to peripheral blood (16, 45), while the expression of IFN-γ was lower (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 (16, 18, 45, 47, 48), the presence of $IFN\text{-}\gamma^{+}$ and $IL\text{-}17^{+}$ 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 placental growth in mice (47, 49). IL-17 plays an important role in host defense against pathogens (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 (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. (53) showed more $IL\text{-}17^{+}CD3^{+}$ 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 or 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.

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 (Commissie Mensgebonden Onderzoek region Arnhem-Nijmegen, CMO nr. 2009/004) and was performed in accordance with the relevant guidelines and regulations. Samples were obtained from each participant upon written informed consent.

Isolation of lymphocytes

One ml of peripheral blood was lysed with 25 ml lysis buffer [NH_4CL + $KHCO_3$/Na_4EDTA (Merck, Darmstadt, Germany) diluted in H_2O (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 37^0C 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% CO_2 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 Figure S1).

Flow cytometry

Samples were phenotypically analyzed using the 10-color NaviosTM 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 (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 2H4LDH11LDB9), CD45RO-ECD (Beckman Coulter; UCHL1), CD56-APC (Beckman Coulter; N901), CD62L-FITC/ECD (eBiosience 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 Figure 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% CO_2 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% CO_2 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 Dunns 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.

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SUPPLEMENTAL INFORMATION

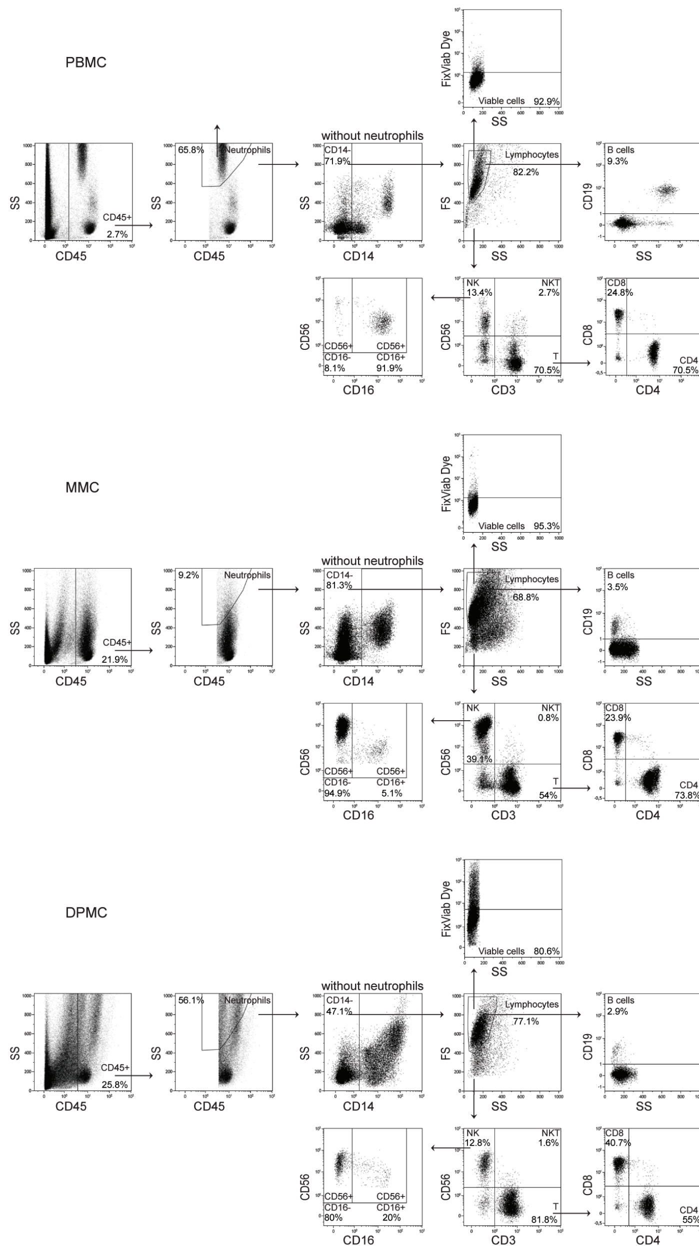
Supplementary Table S1. Donor characteristics

Variable	Menstrual and peripheral blood donors (n=17)	Placenta donors (n=19) (information not available for 3 donors)
Age, median (range)	30 (19-46)	31 (23-37)
Contraceptives	0/17 (100%)	NA
Previous pregnancy	9/17 (53%)	15/16 (94%)
Previous miscarriage	6/17 (35%)	4/16 (25%)
Length of menstrual cycle, median (range)	28 (22-35)	NA
C-section	NA	19/19 (100%)
Duration of pregnancy, median (range)	NA	38 weeks (38-40 weeks)
Natural conception	NA	15/16 (94%; 1 intrauterine insemination)
Birth weight, median (range)	NA	3340 (2948-4325)

NA, not applicable

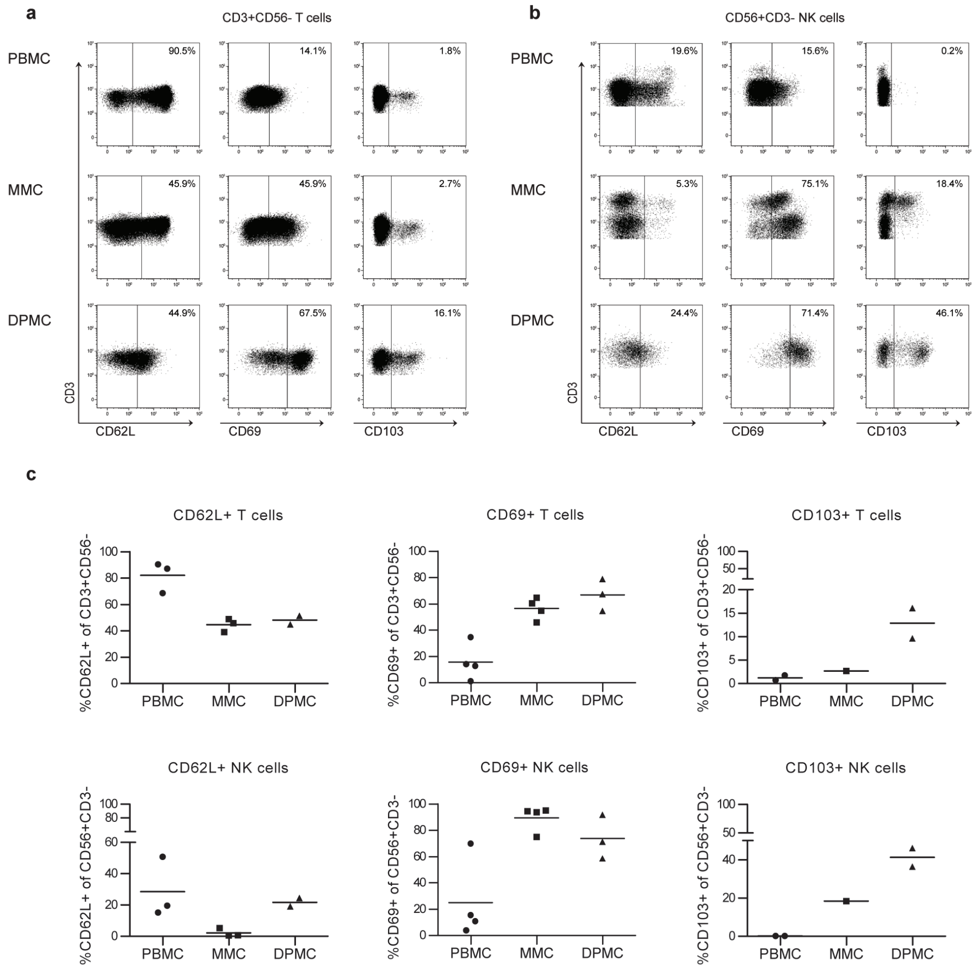
Supplementary Table S2. Mean % ± SD is provided for lymphocyte populations in the three study groups as used in the figures.

	Peripheral blood	Menstrual blood	Decidua
Lymphocytes	79.1±6.9	62.5±12.1	65.2±9.8
T cells ($CD3^+CD56^-$)	80.0±7.3	43.9±15.1	67.1±10.6
$CD45RA^+$	50.9±7.7	53.4±8.8	17.2±7.4
$CD45RO^+$	31.2±7.1	28.9±8.0	66.4±11.7
$CD4^+$ T cells	64.6±9.7	64.7±7.4	50.3±7.0
Naive ($CD45RA^+CCR7^+$)	54.1±9.4	58.7±8.6	18.6±15.0
Teff ($CD45RA^+CCR7^-$)	3.8±1.5	2.6±0.9	2.5±1.6
EM ($CD45RA^-CCR7^-$)	23.0±6.5	14.4±5.3	51.6±13.4
CM ($CD45RA^-CCR7^+$)	19.1±4.1	24.3±3.8	27.4±7.6
Treg ($CD25^{high}CD127^-$)	5.0±1.4	5.1±1.9	9.5±3.5
Naïve Treg ($CD45RA^+CD25^+$)	3.4±2.0	1.9±1.3	1.3±1.2
Differentiated Treg ($CD45RA^-CD25^{++}$)	3.7±2.1	2.4±1.6	8.4±5.9
Th1 ($CCR6^-CXCR3^+CCR4^-$)	4.8±4.0	11.1±2.7	21.3±8.7
Th2 ($CCR6^-CXCR3^-CCR4^+$)	8.6±2.7	3.3±1.4	4.9±2.8
Th17 ($CCR6^+CXCR3^-CCR4^+$)	5.9±2.8	3.9±1.7	4.8±2.3
Th1-like ($CCR6^-CXCR3^+CCR4^-$)	5.3±3.6	6.6±2.9	9.5±4.1
IL-17^+ T cells	2.3±1.3	2.6±1.0	4.4±1.3
IFN-γ^+ T cells	7.5±6.7	7.4±3.3	27.8±15.4
$CD8^+$ T cells	28.6±6.9	30.3±6.3	42.5±5.6
Naive ($CD45RA^+CCR7^+$)	43.6±11.9	45.3±12.6	15.2±8.0
Teff ($CD45RA^+CCR7^-$)	26.6±9.1	25.3±11.5	30.4±11.5
EM ($CD45RA^-CCR7^-$)	23.7±7.0	24.5±13.1	45.8±13.2
CM ($CD45RA^-CCR7^+$)	6.0±2.2	5.0±1.9	8.7±3.0
NK cells ($CD56^+CD3^-$)	8.2±4.8	46.9±16.4	24.3±10.6
$CD56^+CD16^-$ NK cells	11.4±6.7	92.5±5.8	81.6±6.8
$CD56^+CD16^+$ NK cells	88.7±6.7	7.5±5.8	18.5±6.8
MFI CD56 of $CD56^+CD16^-$ NK cells	52.6±15.5	81.2±19.0	18.1±10.8
NKT cells ($CD3^+CD56^+$)	1.6±1.1	1.2±0.9	2.5±1.1
B cells ($CD19^+$)	6.2±2.5	5.6±2.7	3.6±2.8



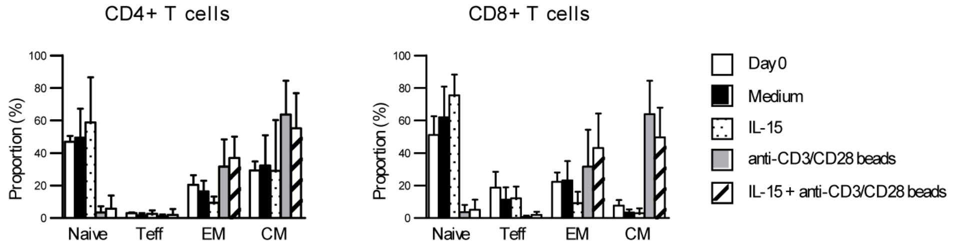
Supplementary Figure S1. Representative gating strategy for immunophenotyping of peripheral blood (PBMC), menstrual blood (MMC), and term decidua (DPMC).

Gate settings for PBMC were copied to menstrual and decidual samples. Leukocytes were first gated on CD45 positivity. Neutrophils and $CD14^{+}$ cells were then excluded. The remaining dot plots represent the gating strategy for lymphocytes (FS-SS scatter), B cells ($CD19^{+}$), NK cells ($CD3^{-}CD56^{+}$), NKT cells ($CD3^{+}CD56^{+}$), T cells ($CD3^{+}CD56^{-}$), $CD4^{+}$ T cells ($CD3^{+}CD56^{-}CD4^{+}$), $CD8^{+}$ T cells ($CD3^{+}CD56^{-}CD8^{+}$), and viable lymphocytes ($FixViabDye^{-}$).



Supplementary Figure S2. Uterine lymphocytes are of mucosal origin.

(a) Representative flow cytometry plots for the expression of CD62L, CD69, and CD103 on T cells (CD3+CD56-) from peripheral blood (PBMC), menstrual blood (MMC), and decidua (DPMC). **(b)** Representative flow cytometry plots for the expression of CD62L, CD69, and CD103 on NK cells (CD56+CD3-) from PBMC, MMC, and DPMC. **(c)** Percentage CD62L$^+$, CD69$^+$, and CD103$^+$ T cells and NK cells in PBMC (n=2-4), MMC (n=1-4), and DPMC (n=2-3).



Supplementary Figure S3. CD4+ and CD8+ T cell subsets after in vitro differentiation of endometrial lymphocytes towards a decidual-like phenotype (n=4).

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+). Lines indicate mean ± SD.

CHAPTER 3

Three Types of Functional Regulatory T Cells Control T Cell Responses at the Human Maternal-Fetal Interface

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ABSTRACT

During pregnancy, maternal regulatory T cells (Tregs) are important in establishing immune tolerance to invading fetal extravillous trophoblasts (EVTs). $CD25^{HI}FOXP3+$ Tregs are found at high levels in decidual tissues and have been shown to suppress fetus-specific and non-specific responses. However, limited data are available on additional decidual Treg types and the mechanisms by which they areinduced. This study investigated three distinct decidual CD4+ Treg types in healthy pregnancieswith a regulatory phenotype and the ability to suppress T cell responses: $CD25^{HI}FOXP3+$, $PD1^{HI}IL\text{-}10+$, and TIGIT+ $FOXP3^{dim}$. Moreover, co-culture ofHLA-G+ EVTs or decidual macrophages with bloodCD4+ T cells directly increased the proportions of $CD25^{HI}FOXP3+$ Tregs compared to T cells cultured alone. EVTs also increased $PD1^{HI}$ Tregs that could be inhibited by HLA-C and CD3 antibodies, suggesting an antigen-specific induction. The presence of distinct Treg types may allowfor the modulation of a variety of inflammatory responses in the placenta.

INTRODUCTION

During pregnancy, $CD4^{+}CD25^{HI}FOXP3^{+}$ regulatory T cells (Tregs) are found at high levels in decidual tissue and have the ability to suppress fetus-specific and non-specific responses (1, 2). Most interestingly, HLA-C mismatched pregnancies (where the fetus and extravillous trophoblasts (EVTs) express an HLA-C allotype that the mother does not have) had increased levels of functional $CD4^{+}CD25^{HI}$ Tregs in decidua, compared to HLA-C matched pregnancies (3). Furthermore, *in vitro* co-culture of naive $CD4^{+}$ T cells with EVTs directly increased the proportion of $CD4^{+}FOXP3^{+}$ Tregs, compared to $CD4^{+}$ T cells cultured alone (4-6). This suggests that maternal T cells may specifically recognize fetal HLA-C, but its expression on EVTs promotes immune tolerance. The importance of maternal immune tolerance for fetal HLA-C is further illustrated by a recent study suggesting that HLA-C antibodies may contribute to the etiology of miscar riage (7). The proportion of circulating $FOXP3^{+}$ Tregs was shown to be diminished in maternal blood obtained after spontaneous preterm birth (SPTB) (8-10)preeclampsia (PE)(11, 12) and in decidual tissue obtained after recurrent spontaneous miscarriage (13, 14). Furthermore, clonally expanded $CD4^{+}CD25^{HI}CD127^{-}CD45RA^{-}$ Treg populations were observed in healthy term pregnancy decidua, and failure of this clonal expansion may be related to development of preeclampsia (15). The importance of Tregs was also demonstrated in murine pregnancy models (16-19). Depletion of $CD25^{+}$ Tregs during allogeneic matings, but not syngeneic matings, resulted in an increased resorption rate (16). Besides highlighting the role for Tregs, this also demonstrated that in the absence of Tregs, effector cells cause immunologic rejection of allogeneic fetal or placental tissues. A more recent murine study demonstrated that $FOXP3^{+}$ Tregs with specificity to paternal antigens were generated extrathymically and accumulated in the placenta. In this study, females with impaired ability for extrathymic Treg induction showed increased fetal resorption rates and had increased influx of immune cells to the placenta in allogeneic matings, but not syngeneic matings (20).Other pathways and molecules have been demonstrated to play a role in Treg induction during pregnancy, including the blockade of the PD1-PDL1 pathway that led to reduced decidual $CD25^{+}FOXP3^{+}$ Treg numbers and increased embryo resorption in mice, which could be abrogated by adoptive $CD25^{+}FOXP3^{+}$ Treg transfer (21). This demonstrates the importance of PD1-PDL1 for decidual Treg induction. However, specific factors that contribute to diminished decidual Treg numbers or function during pregnancy complications have not been identified in human pregnancy.

Thus far, research on the role of Tregs in human pregnancy has mainly focused on $FOXP3^{+}$ Tregs (2, 8-10, 13-15, 22-24), while other types of $FOXP3^{-}$ Tregs have not been studied in as much detail. Most importantly, only a handful of studies provide functional analysis of peripheral blood (11, 12) and decidual Tregs (2, 3)during human pregnancy. FOXP3, in combination with high expression of CD25 and HELIOS and the absence of CD127 expression, primarily identifies natural Tregs (nTregs), although whether HELIOS is a defining marker for human nTregs remains

controversial. The nTregs are generated in the thymus, are specific for self-antigens, and are responsible for preventing anti-self (autoimmune) responses (25, 26). In contrast, induced Tregs (iTregs) are generated in the periphery and can be specific for a large variety of antigens, including allo-antigens and viral-antigens (27-29). A well-characterized type of iTregs are Tr1 cells that secrete high levels of IL-10; express PD1; co-express CD49b and LAG3, but do not express FOXP3; and are important in the control of alloimmune responses (30, 31). Other iTregs include TIGIT^{+} cells that modulate antigen-presenting cells (APC) through interaction with CD155 on APCs and Tr35 cells that function through secretion of IL-35, an immune suppressive cytokine (32, 33). A large variety of other markers have been used to identify distinct iTreg populations (including but not limited to FOXP3, CD25, GITR, TIM3, CD39, LRRC32 (also known as GARP), LAP, and CCR8) (34-37). None of these markers are truly specific for iTregs, as they can also be expressed on activated T cells. Thus, to identify iTregs, functional assays are required to demonstrate their capacity to suppress immune responses such as proliferation, cytokine secretion, and cytotoxicity (29, 38). In this study, we provide extensive phenotypic and functional characterization of three types of decidual CD4^{+} Tregs in uncomplicated human pregnancies and investigate the ability of HLA-G^{+} HLA-C^{+} EVTs and decidual macrophages, the main APCs at the maternal-fetal interface, to increase Treg proportions.

RESULTS

Distinct CD4+ T cell types with a regulatory phenotype are present in decidual tissue

FACS analysis on freshly isolated peripheral blood CD4^{+} T cells (CD4^{+} pTs) and decidual CD4^{+} T cells (CD4^{+} dTs) isolated from first-trimester decidua (gestational age 6–12 weeks) and term placenta decidua basalis (d.basalis) and decidua parietalis (d.parietalis) (gestational age > 37 weeks) was performed to determine cell surface expression of CD45, CD4, CD25, PD1, TIGIT, CD127, CD45RA, CD49b, and LAG3 and intracellular expression of FOXP3 and HELIOS. A clear population of activated nTregs was identified in all tissues based on the high expression of CD25, FOXP3, and HELIOS and the lack of CD45RA and CD127 (Figure 1; Figures S1A-S1D) (25). While the percentage of FOXP3^{+} and HELIOS^{+} cells within this CD25^{HI} population significantly decreased in term pregnancy decidua, the proportion of HELIOS^{+} cells within CD25^{HI}FOXP3^{+} cells remained relatively stable (Figure 1D, right panel). A second T cell population was identified based on the high expression of PD1, the lack of FOXP3 and HELIOS, and low CD25. LAG3 and CD49b were not expressed by CD4^{+} dTs. While both CD25^{HI} and PD1^{HI} cells co-expressed high levels of TIGIT, a third population of CD4^{+} dTs also expressed high levels of TIGIT and low levels of FOXP3, HELIOS, PD1, and CD25 (Figure 1; Figures S1A S1D). FOXP3 expression in TIGIT^{+} cells was significantly lower than in CD25^{HI} cells (Figure S1E). The t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis confirmed the separation of these three T cell populations (Figure S2) that hereafter will be named (1) CD25^{HI}FOXP3^{+}, (2) PD1^{HI}, and (3) TIGIT^{+}.

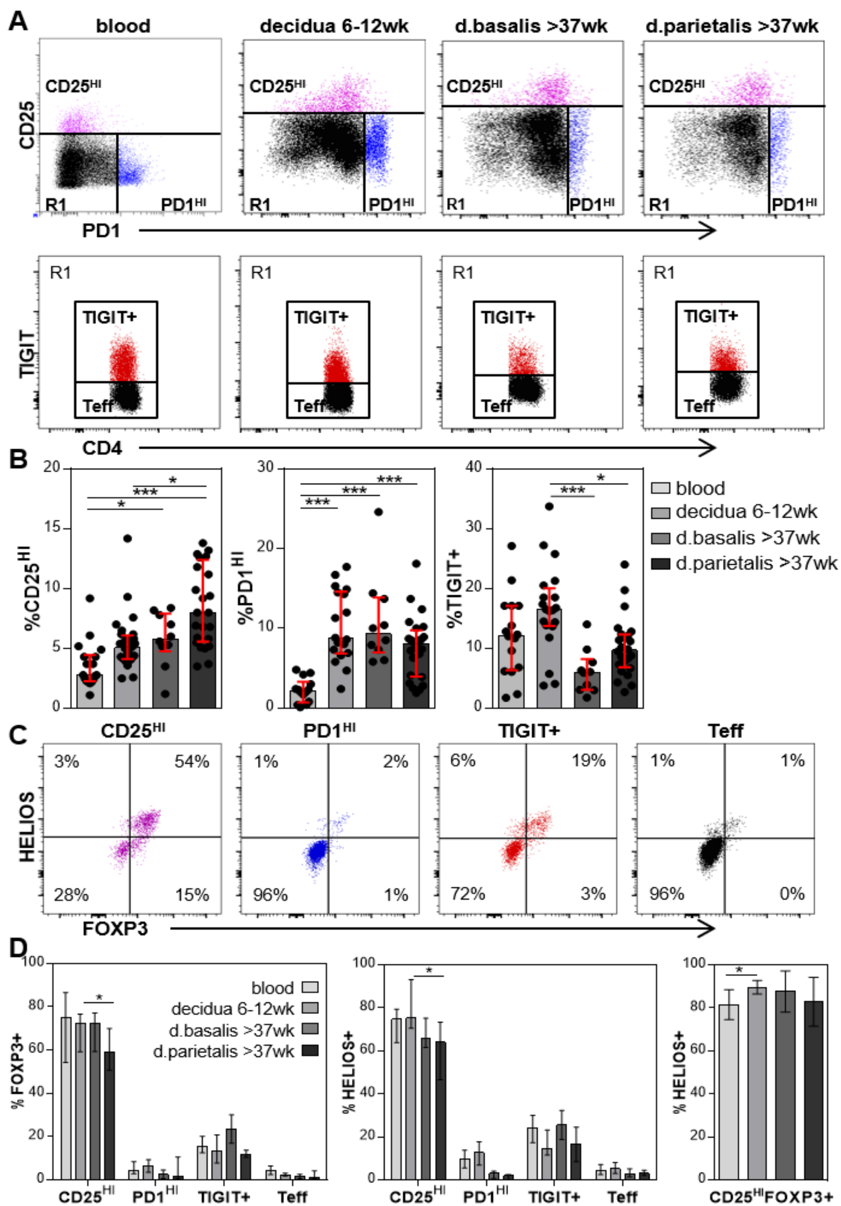

Figure 1. Three distinct CD4+ T cell populations express Treg-associated markers.

(A and B) Representative FACS plots **(A)** and percentages **(B)** of $CD25^{HI}$, $PD1^{HI}$, and TIGIT+ cells within CD4+ T cells in blood, decidua 6–12 weeks, d.basalis >37 weeks, and d.parietalis >37 weeks. **(C and D)** Representative FACS plots **(C)** and percentages **(D)** of FOXP3+ and HELIOS+ cells within the four CD4+ T cell types and HELIOS+ cells within $CD25^{HI}$FOXP3+ cells in blood, decidua 6–12 weeks, d.basalis >37 weeks, and d.parietalis >37 weeks (n = 8–10). Bars represent median and interquartile range; *p < 0.05, **p < 0.01 and ***p < 0.001. See also Figures S1-S3.

These three $CD4^+$ T cell types were purified by FACS sort and analyzed for their ability to produce the pro- and anti-inflammatory cytokines IFNγ, IL-2, and IL-10 upon phorbol 12-myristate 13-acetate (PMA)/Ionomycin stimulation. $PD1^{HI}$ T cells from all tissues expressed the highest levels of IL-10 and coexpressed IFNg (Figure S3), suggesting a resemblance to Tr1 (30). $CD25^{HI}FOXP3^+$ cells of all tissue compartments expressed the lowest levels of IL-10, IFNγ, and IL-2, whereas $TIGIT^+$ cells expressed high levels of IFNγ and IL-2 and low levels of IL-10. A limitation of this study is the lack of clinical information on the blood and tissues used for experiments that may have impacts on Treg phenotypes and contribute to the observed variation in Treg proportions. However, a previous report did not find an influence of clinical variables such as mode of delivery and fetal sex on the presence of $CD4^+CD25^{dim}$-activated T cells and CD4+$CD25^{HI}$FOXP3+ Tregs in decidual tissues of term pregnancy (3).

$CD25^{HI}$FOXP3+, $PD1^{HI}$, and TIGIT+ suppress proliferation of CD4+ and CD8+ Teff cells

To determine the capacity of $CD25^{HI}$FOXP3, $PD1^{HI}$, and $TIGIT^+$ T cells to suppress proliferation of effector T cells (Teffs), carboxyfluorescein succinimidyl ester (CFSE)-labeled $CD4^+$ or $CD8^+$ Teffs were stimulated with anti-CD3 and -CD28 beads in the presence or absence of sample-matched Tregs for 4 days in a 1:2 Treg to Teff ratio. CFSE profiles were analyzed to determine the percentage of undivided cell (generation 0) and the average number of divisions per cell (division index) within the $CD4^+$ and $CD8^+$ Teffs cultured with and without Tregs (Figure 2A). All three $CD4^+$ Treg types in decidual samples of 6–12 weeks suppressed proliferation of $CD4^+$ Teffs. This is illustrated by the significant increase in the percentage of undivided cells (Figure 2B), and a significant decrease of the division index (Figure 2E) upon co-culture of Tregs and Teffs, compared to stimulated Teff cultured alone. Additionally, purified $PD1^{dim}$ cells from 6-12 week decidual tissues did not suppress proliferation of $CD4^+$ T cells (Figure S4A). $CD25^{HI}$ $FOXP3^+$ T cells also suppressed proliferation of sample-matched $CD8^+$ Teffs, whereas $PD1^{HI}$ and $TIGIT^+$ cells did not consistently reduce $CD8^+$ Teff proliferation (Figures 2C and 2F). Analysis of the three T cell populations from term placenta d.parietalis demonstrated that these cells had a reduced capacity to suppress $CD4^+$ Teff proliferation, compared to their first-trimester counterparts (Figures 2D and 2G), whereas peripheral blood $CD25^{HI}$ and $PD1^{HI}$, but not TIGIT+ cells, suppressed proliferation of $CD8^+$ Teffs (Figures S4B-S4D). To determine if IL-10 secretion by $PD1^{HI}$ Tregs is their predominant mechanism to suppress proliferation of $CD4^+$ Teffs, as was previously shown for Tr1 cells (30, 31), anti-IL-10R blocking antibodies or immunoglobulin G (IgG) control antibodies were added to anti-CD3 and -CD28 stimulated CFSE- labeled $CD4^+$ Teffs cultured alone or with sample-matched $CD25^{HI}$ or $PD1^{HI}$ cells. Indeed, addition of IL-10R antibodies abrogated the suppressive capacity of $PD1^{HI}$ cells but not of the $CD25^{HI}$ cells that were added in parallel control cultures (Figure 2H).

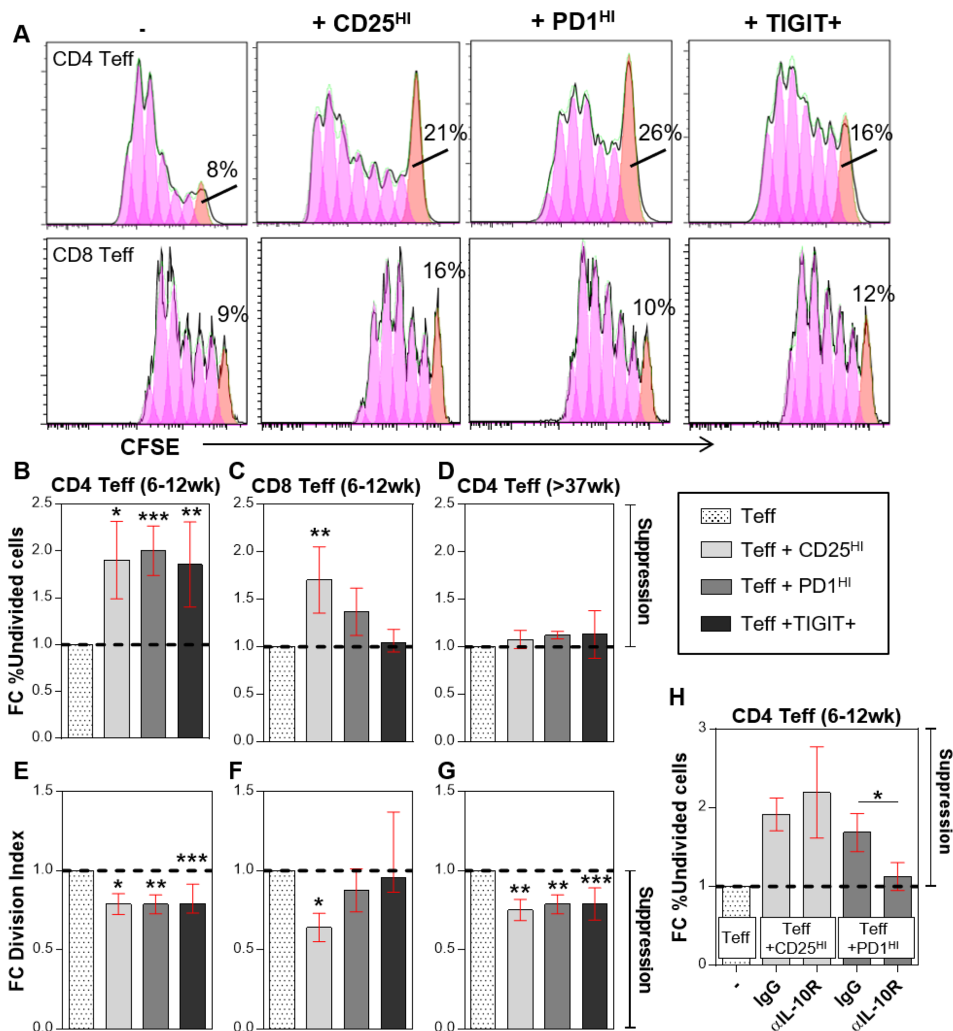

Figure 2. Decidual Tregs suppress proliferation of Teffs.

(A) CFSE dilution of 6-12-week decidual CD4+ Teffs (upper panel) and CD8+ Teffs (lower panel) stimulated with anti-CD3/28 and co-cultured with or without CD25HI, PD1HI, or TIGIT+ Tregs during 4 days in a 1:2, Treg: Teff ratio. Percentages of the undivided Teffs are shown. **(B–G)** Fold change **(FC)** in the percentage of undivided cells **(B–D)** and in the division index (**E–G)** after the addition of $CD25^{HI}$, $PD1^{HI}$, or TIGIT+ Tregs, compared to CD4+ Teffs 6–12 weeks (B **and E)**, CD8+ Teffs 6–12 weeks **(C and F)**, and CD4+ Teffs >37 weeks (**D and G)** cultured alone. **(H)** Addition of IL-10R blocking antibodies to Teffs $+PD1^{HI}$ but not Teffs + $CD25^{HI}$ cultures inhibit suppression of proliferation, measured by FC in percentage of undivided cells, compared to isotype control cultures. Bars represent median and interquartile range; In **(B)**–**(G)**: n = 7–11; (H): n = 3–4; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$; values > 1 for FC % undivided cells and < 1 for FC division index represent suppression of proliferation. Division index reflects the number of divisions per cell as calculated in FlowJO v7.6.5. See also Figure S4.

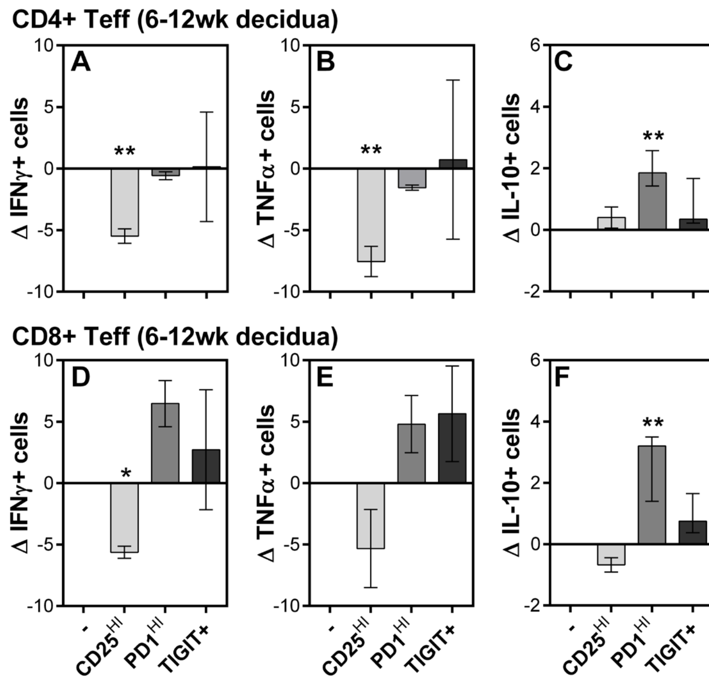

Figure 3. Decidual Tregs influence cytokine production by CD4+ and CD8+ Teffs.

(A–F) Graphs depict absolute change **(D)** in percentage of IFN-γ+ (A and D), TNF-α+ **(B and E)**, and IL-10+ **(C and F)** decidual CD4+ Teffs (top panels) and decidual CD8+ Teffs (bottom panels) upon addition of sample-matched CD25^{HI}, PD1^{HI}, or TIGIT+ T cells, compared to CD4+ Teffs and CD8+ Teffs cultured alone. Bars represent median and interquartile range; n = 4–5; $^{*}p < 0.05$, $^{**}p < 0.01$. See also Figure S5.

CD25^{HI}FOXP3+ and PD1^{HI} Tregs influence cytokine production of CD4+ and CD8+ Teffs

Next, the capacity of decidual CD25^{HI}FOXP3^{+}, PD1^{HI}, and TIGIT^{+} Tregs to influence production of pro- and anti-inflammatory cytokines by CD4^{+} and CD8^{+} Teffs was investigated. CFSE- labeled CD4^{+} Teffs and CD45-Alexa700 labeled CD8^{+} Teffs were cultured with or without sample matched Tregs in a 1:1:1 CD4 Teff: CD8 Teff: Treg ratio and stimulated with anti-CD3 and -CD28. At day three, the cells were restimulated with PMA and Ionomycin for 6h in the presence of GolgiStop for the last 4h. CD4^{+} Teffs, CD8^{+} Teffs, and Tregs were identified (Fig ure S5A) and analyzed for the presence of intracellular cytokines IFNɣ, TNFɑ, and IL-10 (Figure S5B). Addition of CD25^{HI} cells significantly decreased the percentage of IFNɣ$^{+}$ and TNFɑ$^{+}$ CD4^{+} and IFNɣ$^{+}$ CD8^{+} Teffs (Figures 3A,3B, and 3D). Addition of PD1^{HI} cells significantly increased the percentage of IL-10^{+} CD4^{+} and CD8^{+} Teffs (Figures 3C and 3F) but also increased the percentage of IFNɣ$^{+}$ CD8^{+} Teffs (Figure 3D). TIGIT^{+} cells did not consistently change the production of cytokines by CD4^{+} or CD8^{+} Teffs (Figure 3). Analysis of cytokine production by Tregs themselves confirmed the overall

low expression of cytokines by $CD25^{HI}$ cells and high expression of IL-10 by $PD1^{HI}$ cells (Figure S5C). Thus, besides suppressing Teff proliferation, both $CD25^{HI}$ and $PD1^{HI}$ Tregs also modulated the production of cytokines by Teffs.

$CD25^{HI}FOXP3+$, $PD1^{HI}$, and TIGIT+ Tregs from decidua and blood express distinct transcriptional profiles

To further investigate the molecular mechanisms the three Treg types may utilize to modulate T cell proliferation and cytokine production, RNA was isolated from $CD25^{HI}$ $FOXP3^{+}$, $PD1^{HI}$, and $TIGIT^{+}$ Tregs purified from peripheral blood, first-trimester decidua (6–12 weeks), and term pregnancy d.parietalis (>37 weeks). The BioMark Fluidigm 96x96 QPCR chip was used to detect gene expression. Sixty-six primer pairs had detectable CT values and melting curves (gene and primer list are included in Table S1). CT values were normalized against GAPDH (ΔCT), and the fold change (FC) was calculated relative to the median ΔCT of the blood $CD4^{+}$ Teffs. Expression levels of CD25, FOXP3, PD1, and TIGIT confirmed the purity of the Treg fractions (Figure S6A). K-means cluster analysis of gene expression in the four T cell populations visualized two separate gene clusters in blood and decidua. Cluster I identified a set of genes, including CD25, FOXP3, TIGIT, CD39, LRRC32 (GARP), ST2, BATF, and CCR8, which are highly expressed by $CD25^{HI}$ $FOXP3^{+}$, low in $PD1^{HI}$, and intermediate in $TIGIT^{+}$Tregs (Figure 4A). Cluster II identified a set of genes, including PD1, IFNɣ, IL-10, CCR5, and CXCR3, that are upregulated by $PD1^{HI}$ Tregs (Figure 4B). Heatmaps depicting the differentially expressed genes identified in these clusters are shown (Figures 4C - 4E). These data further support that $CD25^{HI}FOXP3^{+}$, $PD1^{HI}$, and $TIGIT^{+}$ are separate Treg types that may utilize distinct molecular mechanisms of immune modulation.

Additional K-means cluster analysis revealed several key regulatory genes that differ throughout gestation and between decidual and blood Tregs (Figures S6B and S6C). Decidual $CD25^{HI}FOXP3^{+}$ Tregs show increased expression of CCR5, ST2, CD25, BATF, IL10, GITR, LRRC32 (GARP), and CCR8, compared to blood $CD25^{HI}FOXP3^{+}$ Tregs, while decidual $PD1^{HI}$ Tregs had increased expression of IL-10, IFNɣ, and CCR5, compared to blood $PD1^{HI}$ Tregs. Interestingly, first- trimester decidual $TIGIT^{+}$ Tregs increased expression of IL- 10, IFNɣ, LRRC32, and GZMA, while term pregnancy $TIGIT^{+}$ Tregs had increased expression of, for example, VEGFA, IFNɣ, GITR, and CD39 (Figures S6B and S6C). Flow cytometric analysis of freshly isolated Tregs from all tissues confirmed differential protein expression of key mRNAs identified here (Figure S7). The increased expression of a variety of cytokines (e.g., IL-10, IFNɣ), chemokines (e.g., CCR5, CCR8), activation markers (e.g., HLA-DR, GZMA), and co-inhibitory genes and molecules (e.g., CD39, TIM3, LRRC32) on decidual Tregs may suggest increased Treg activation and suppressive function in decidual tissue to regulate inflammatory responses at the maternal fetal interface.

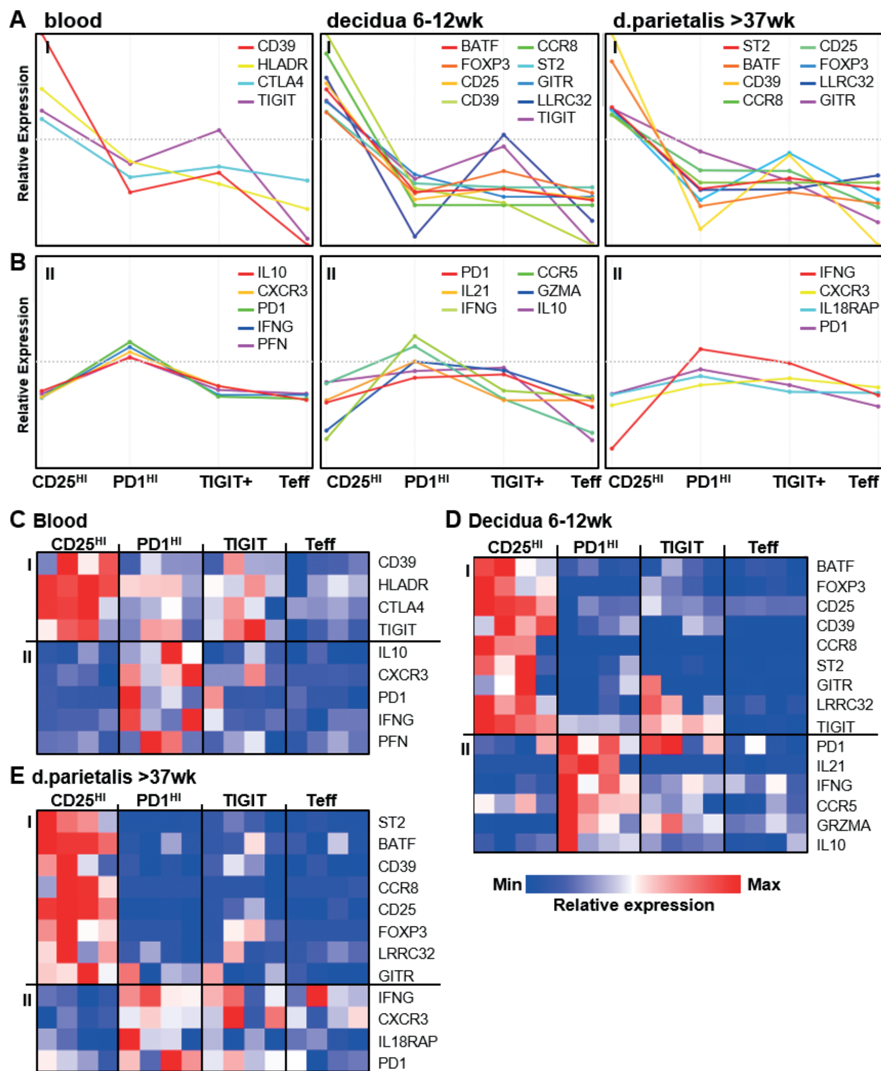

Figure 4. Gene expression profiles of CD4+ Tregs and Teffs.

(A and B) K-means clustering identified two clusters of genes **(A and B)** that significantly correlate their mRNA expression pattern among CD25^{HI}, PD1^{HI}, TIGIT+, and Teffs. Blood (left panels), decidua 6–12 weeks (middle panels), and d.parietalis >37 weeks (right panels) are depicted. **(C–E)** Heatmaps depict differentially expressed genes identified in these clusters in blood **(C)**, decidua 6–12 weeks **(D)**, and d.parietalis >37 weeks **(E)**. n = 4 for all T cell types. See also Figures S6 and S7.

EVTs and decidual macrophages increase Treg proportions during co-culture

Previous studies demonstrated that EVTs directly increased FOXP3 levels in $CD4^+$ $CD25^{HI}$ Tregs during co-culture (5, 6) and that decidual macrophages favored Treg differentiation (39). To investigate the capacity of EVTs and decidual macrophages to increase the three Treg types, EVTs and decidual macrophages isolated from first-trimester placental tissue were co- cultured with naive $CD4^+$ pTs for 3 days as described previously (6). Co-culture of EVTs or decidual macrophages with $CD4^+$ pTs significantly increased the proportion of $FOXP3^+$ and $HELIOS^+$ Tregs (Figure 5A). EVTs, but not decidual macrophages, also increased the proportion of $PD1^{HI}$ Tregs, while neither EVTs nor decidual macrophages changed the $TIGIT^+$ population (Figure 5A). To deter mine whether EVTs and decidual macrophages increased Treg proportions through cell-cell contact or by secretion of soluble factors, EVTs or decidual macrophages and $CD4^+$ pTs were co-cultured in a transwell system. Separation of EVTs or decidual macrophages from $CD4^+$ pTs by a transwell membrane resulted in a small but not significant decrease in the induction of $FOXP3^+$ and $HELIOS^+$ cells by EVTs and decidual macrophages (Figures 5B and 5C), suggesting that both cell-cell contact and soluble factors may play a role in the induction of $FOXP3^+$ cells by EVTs and decidual macrophages. In contrast, when EVTs and $CD4^+$ pTs were separated by a transwell mem brane, the increase in the proportion of $PD1^{HI}$ cells was abrogated, demonstrating that cell-cell contact is required here (Figure 5B).

To further investigate the mechanisms by which EVTs and decidual macrophages increase Treg proportions, additional cell cultures were established where EVTs or decidual macrophages were co-incubated with $CD4^+$ pTs in the presence of IgG controls and blocking antibodies for a panel of co-inhibitory molecules and anti-inflammatory cytokines. The increase in $FOXP3^+$ cells was not reversed by addition of any of the blocking antibodies tested in EVT co-cultures (TCR coreceptor CD3; HLA-C; HLA-G receptor ILT2; HLA-G; coinhibitory molecule PDL1; and the immune modulatory cytokine TGFβ) (Figure 5D) and decidual macrophage co-cultures (ILT2, HLA-DR, PDL1, TGFβ, IL-10 receptor (IL-10R)) (Figure 5E). Interestingly, addition of HLA-C and CD3 antibodies signifi cantly inhibited the induction of $PD1^{HI}$ cells by EVTs (Figure 5F), while blocking HLA-G interactions resulted in a small but not significant reduction. Blocking ILT2, PDL1, and TGFβ in the EVTs and $CD4^+$ T cell co-cultures did not significantly affect induction of $PD1^{HI}$ cells by EVTs (Figure 5F). The question of whether placental viral infections alter Treg induction or Treg stability and thereby exacerbate placental inflammation is clinically important. EVTs were infected with human cytomegaloviruses (HCMVs), the most common pathogen to infect the placenta and a major cause of congenital disease (40). Interestingly, no differences were observed in the capacity of healthy or HCMV-infected EVTs to increase $CD25^{HI}FOXP3^+$ and $PD1^{HI}$ Treg proportions (Figure 5G). Collectively, these results suggest that Tregs can locally be induced by EVTs and decidual macrophages and that antigen-specificity may be involved.

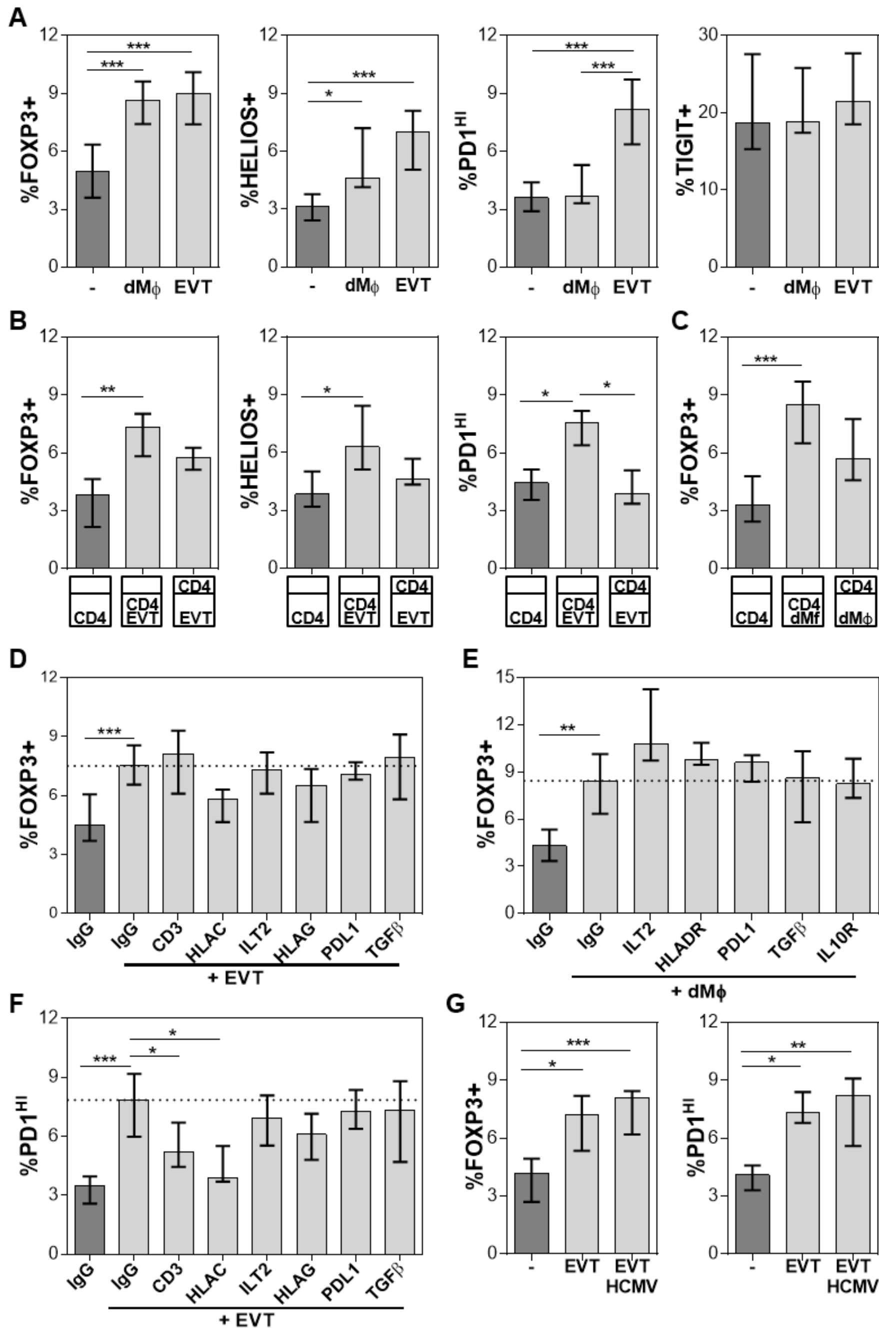

Figure 5. EVTs and Decidual Macrophages Increase Treg Proportions

(A) CD4+ T cells were cultured alone or in the presence of EVTs or decidual macrophages for 3 days. Cells were analyzed for the percentage of FOXP3+, HELIOS+, PD1HI, and TIGIT+ cells as described in Figures S1A and S1B. **(B)** CD4+ T cells were cultured alone, with EVTs, or with EVTs separated by a transwell membrane for 3 days and analyzed for the percentage of FOXP3+, HELIOS+, and PD1HI cells. **(C)** CD4+ T cells were cultured alone, with decidual macrophages, or with decidual macrophages separated by a transwell membrane for 3 days and analyzed for the percentage of FOXP3+ cells. **(D–F)** CD4+ T cells were cultured alone or with EVTs or decidual macrophages in the presence of IgG, or blocking antibodies for CD3, HLA-C, ILT2, HLA-G, PDL1, TGFb, and IL-10R and analyzed for the percentage of FOXP3+ **(D and E)** and PD1HI **(F)** cells. **(G)** CD4+ T cells were cultured alone or with EVTs or HCMV-infected EVTs and analyzed for the percentage of FOXP3+ and PD1HI cells. Bars represent median and interquartile range; n = 8–14; *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

DISCUSSION

Modulation of co-inhibitory molecules and Tregs function has exceptional therapeutic potential for treatment of a wide variety of inflammatory disorders, including cancer, chronic infection, autoimmune disease, and pregnancy complications. This study has refined our view of Treg populations at the maternal-fetal interface by presenting phenotypic and functional data of three Treg populations, CD25^{HI}FOXP3^{+}, PD1^{HI}FOXP3^{-}IL-10^{+}, and TIGIT^{+}FOXP3^{dim} Tregs, found in decidual tissues of human first-trimester and term pregnancy. Functional suppression assays confirmed that decidual CD25^{HI}FOXP3^{+} Tregs suppress prolifer ation and production of IFNγ and TNFα by CD4^{+} and CD8^{+} Teffs. Decidual PD1^{HI} Tregs were shown to suppress proliferation of CD4^{+} (but not CD8^{+}) Teffs in an IL-10-dependent manner. PD1^{HI} Tregs also increased expression of IL-10 in Teffs, possibly resulting in a positive feedback loop sustaining Teff suppression while inducing additional IL-10 secreting Tregs.

Decidual TIGIT^{+} cells significantly inhibited CD4^{+} T cell proliferation but did not influence CD8^{+} Teff proliferation or cytokine production, suggesting TIGIT^{+} Tregs only have limited capacity to suppress T cells, but their ability to suppress other cell types (e. g. APCs) was not investigated here. Further analysis of gene and protein expression of all Treg types in blood and decidua clearly separated the three Treg types and revealed that many immune regulatory molecules (e. g. CTLA4, ST2, LRRC32, GITR, IFNγ, and IL-10) have increased expression in decidua compared to blood. Thus, decidual Tregs are highly activated and have the potential to influence immune responses through a variety of molecular pathways and cellular targets. Of importance here is further investigation into the contribution of decidual Treg types on the modulation of decidual CD8^{+} effector-memory T cells, which were shown to have signatures of T cell activation and dysfunction (41).

Most interesting here is the discovery of the role of HLA-C in the induction of PD1^{HI} Tregs by EVT. EVTs, but not decidual macrophages, have the capacity to directly increase PD1^{HI} Tregs through cell-cell contact. Blocking of the TCR co-receptor CD3 or HLA-C in these EVT and CD4 T cell co-cultures significantly reduced this increase, suggesting that antigen-specificity may be involved.

Although it is well established that $CD4^+$ T cells recognize major histocompatibility complex (MHC) class II molecules, MHC class I-restricted $CD4^+$ T cells have been reported (42). Decidual $PD1^{HI}$ Tregs somewhat resemble Tr1 cells that have been extensively characterized in murine tissues (43) and human peripheral blood (30, 44). Their similarities include the high expression of PD1, IL-10, IFNγ, and granzymes and the lack of FOXP3 expression. In contrast to Tr1 cells that were shown to depend on the ILT2-HLA-G pathway for their induction (45), induction of $PD1^{HI}$ cells by EVTs was not dependent on the HLA-G receptor ILT2, while directly blocking HLA-G during the EVT and CD4 T cell co-culture resulted in a small but not significant decrease of $PD1^{HI}$ Tregs induction. Tr1 cells were also shown to be induced by DC-10 through IL-10 secretion and interaction of HLA-G and ILT4 (46). Thus, other decidual cell types such as $HLA\text{-}G^+$ DC-10, that are found in decidual tis sues may contribute to induction of the $PD1^{HI}$ Tregs described here (47). The main mechanisms of Tr1-mediated suppression are the secretion of IL-10 and killing of APCs by granzyme B (48). The expression of perforin and granzymes in first-trimester decidua $PD1^{HI}$ Tregs may suggest they can also diminish APC activity using this pathway. Tr1 cells were identified in HLA-mismatched fetal liver hematopoietic stem cell transplant (HSCT) patients and were shown to suppress allogeneic responses in transplant patients and prevent autoimmune responses (31, 49). While the specificity of decidual $PD1^{HI}$ Tregs remains unknown, the requirement for direct cell-cell contact with EVTs for their induction, as well as the role for the TCR co-receptor CD3, may provoke speculation that decidual $PD1^{HI}$IL-10 Tregs have specificity for fetal allo-antigens expressed by EVTs (e.g., HLA-C). In comparison with decidual $PD1^{HI}$ Tregs, decidual $CD25^{HI}FOXP3^+$ Tregs had an increased capacity to suppress proliferation and a more potent capacity to suppress IFNγ and TNFα production by $CD4^+$ and $CD8^+$ Teffs. Thus, the mechanisms of suppression utilized by $CD25^{HI}FOXP3^+$ Tregs and $PD1^{HI}$ Tregs as well as the effects on their cellular targets are inherently different. The increased levels of co-inhibitory proteins and/or mRNAs (e.g., CTLA-4, GITR, CD39, ST2, LRRC32) expressed by decidual $CD25^{HI}FOXP3^+$ Tregs compared to blood $CD25^{HI}$ $FOXP3^+$ Tregs suggests increased Treg activation and suppressive function in decidual tissue to regulate inflammation at the maternal-fetal interface. Furthermore, it demonstrates that decidual Tregs may utilize distinct molecular mechanisms of immune modulation against a variety of cellular targets beyond the inhibition of $CD4^+$ and $CD8^+$ Teff responses.

The question of whether decidual Tregs are nTregs generated in the thymus with specificity for self-antigens or iTregs generated in the periphery with specificity for paternal antigens is immunologically interesting and of clinical relevance. It was previously reported that HELIOS is a marker of thymic-derived Tregs, while $HELIOS^-$ Tregs were induced from $FOXP3^-$ T cells in the periphery (50). HELIOS and FOXP3 double-positive Tregs had increased suppressive capacity, more stable FOXP3 expression, and dissimilar TCR repertoire compared to $HELIOS^-FOXP3^+$ Tregs (51, 52). Instability of HELIOS-deficient Tregs was also associated with conversion to a T-effector phenotype and enhanced antitumor immunity (53), and HELIOS was shown to control

certain aspects of Treg-suppressive function, differentiation, and survival (51). However, the lack of HELIOS expression does not exclusively identify human iTregs (54). Here, we demonstrate that human decidual $CD25^{HI}FOXP3^{+}$ Tregs have a high expression of HELIOS, which reduces in term pregnancy decidua as well as in cases of miscarriage, as was shown previously (14). Furthermore, we demonstrate that co- culture of $CD4^{+}$ T cells with EVTs or decidual macrophages both significantly increased the expression of FOXP3 and HELIOS, advocating for either a local expansion of $FOXP3^{+}$ and $HELIOS^{+}$ nTregs or a possible *de novo* induction of $FOXP3^{+}$ and $HELIOS^{+}$ iTregs. The increased suppressive capacity of first-trimester $CD25^{HI}$ Tregs compared to term pregnancy $CD25^{HI}$ cells aligns with the observation that HELIOS expression increases and/or stabilizes Treg function. Clonally expanded $CD4^{+}CD25^{HI}CD127^{-}CD45RA^{-}$ Treg populations were observed in term pregnancy decidua (15), and preferential recruitment of fetus-specific $CD25^{HI}FOXP3^{+}$ Tregs from the maternal peripheral blood to the maternal-fetal interface has been suggested to occur in human pregnancy (2). Furthermore, $CD4^{+}CD25^{HI}$ Tregs had increased suppressive function when they were isolated from decidual tissue with a maternal-fetal HLA-C mismatch compared to an HLA-C match (3). In neither of these studies was HELIOS expression investigated, but an explanation to align these observations may be that $CD25^{HI}FOXP3^{+}$ Tregs are a mixed population of self-specific nTregs and fetus-specific iTregs. Another explanation may suggest that an increased induction of other iTreg types (e.g., $PD1^{HI}$ Tregs) induced in response to fetal allo-antigens may indirectly increase or enhance $CD25^{HI}FOXP3^{+}$ Tregs stability and/or function through secretion of immune-suppressive IL-10 and/or other factors. Because of the intracellular nature of HELIOS and the lack of other markers to distinguish $CD25^{HI}FOXP3^{+}$ nTregs from iTregs, it is technically not possible to investigate functional differences between these cells from human tissues. A further focus on the mechanisms by which both $CD25^{HI}$ $FOXP3^{+}$ and $PD1^{HI}$IL-10 Tregs are induced by EVTs and decidual macrophages at the maternal-fetal interface as well as the effects of their mutual interactions will contribute to answering burning questions on their origin and antigen specificity.

In contrast to our expectations, infection of EVTs with HCMVs did not diminish the ability of EVTs to increase $CD25^{HI}$ $FOXP3^{+}$ and $PD1^{HI}$ Tregs, suggesting that HCMV infection does not alter the capacity of EVTs to promote immune tolerance. This is in line with a previous observation that decidual natural killer (NK) cells degranulated in response to HCMV-infected maternal decidual stromal cells, but not in response to HCMV-infected EVTs, suggesting that immune tolerance is maintained at the expense of efficient clearance of HCMV infection (40). Relevance of the distinct Treg populations should be addressed by studying their presence and functionality in cases of pregnancy complications. Decreased proportions of decidual $CD4^{+}CD25^{HI}$ $FOXP3^{+}$ and $HELIOS^{+}$ Tregs were associated with spontaneous recurrent miscarriage (13, 14) and preeclampsia (11, 12, 55). The detailed phenotypic characterization, combined with gene expression and extensive functional analysis of multiple Treg populations as presented here,

provides a strong platform for guiding analysis of altered Tregs in clinical conditions for which no systematic functional Treg characterization has been performed.

This study provides strong evidence that multiple types of decidual Tregs, including nTregs and iTregs, play a key role in maintaining maternal-fetal immune tolerance during pregnancy. Moreover, the decidual microenvironment contains cell types, particularly EVTs and decidual macrophages, which have mechanisms to stabilize and expand Treg populations. Of importance here is the data demonstrating that blocking the TCR co-receptor CD3 as well as HLA-C during EVT- CD4 T cell co-cultures inhibited the increase in $PD1^{HI}$ Tregs, suggesting antigen specificity. Further, characterization of decidual Tregs and Teffs in placental materials obtained after spontaneous preterm birth, preeclampsia, and intrauterine infections will accelerate discovery of therapeutic targets to prevent and cure these severe pregnancy complications, as is underway for treatment of many types of cancer and autoimmune diseases.

MATERIALS AND METHODS

Discarded human placental and decidual materials (gestational age 6-12 weeks) were obtained from women undergoing elective pregnancy termination at a local reproductive health clinic. Term placental tissues (gestational age > 37 weeks) were obtained from healthy women after uncomplicated pregnancy at term delivered by elective cesarean section or uncomplicated spontaneous vaginal delivery at Tufts Medical Center. All tissues were visually inspected for signs of excessive inflammation (including discoloration, large infarctions and foul odor) and only healthy tissues were used for further processing. Peripheral blood leukocytes were isolated from discarded leukopacks from healthy volunteer blood donors at the Massachusetts General Hospital in Boston, MA.

All human tissue used for this research was de-identified, discarded clinical material. No clinical information including the fetal sex and sex of blood donors was available for analysis. The Committee on the Use of Human Subjects (the Harvard IRB) determined that this use of placental and decidual material is Not Human Subjects Research. Term placental tissue was collected under a protocol approved by Tufts Health Sciences IRB. All procedures to process these human blood and tissues materials are described in method details.

Isolation of T cells and decidual macrophages

The procedures to isolate lymphocytes and EVT have recently been described (6) and are also described in detail hereafter. To isolate 1st trimester decidual lymphocytes, villous and decidual tissues from elective pregnancy terminations were macroscopically identified and separated. Decidua parietalis from term pregnancy was collected by removing the amnion and delicately

scraping the decidua parietalis from the chorion. Decidua basalis was macroscopically dissected from the maternal side of the placenta. Collected decidual tissues were washed with PBS, minced and thereafter digested with 0.1% collagenase type IV and 0.01% DNase I (Sigma-Aldrich) gently shaking in a water bath for 75 min at 37°C. After digestion, released lymphocytes from 1st trimester and term placenta decidua were washed with RPMI 1640 (Life technologies) containing 10% FBS (Atlanta Biologicals) for 8 min at 1800 rpm and filtered through 100 mm, 70 mm and 40 mm sieves (BD, Labware; NJ). Lymphocytes were dissolved in 20 mL 1.023 g/ml Percoll (GE Healthcare) and layered on a Percoll gradient (10 mL 1.080 g/ml; 15ml 1.053 g/ml) for density gradient centrifugation (25min, 2000rpm). Decidual lymphocytes were isolated from the 1.080 – 1.053 g/ml interface, and decidual macrophages were isolated from the 1.053 – 1.023 g/ml interface. Cells were washed twice with RPMI and directly stained for flow cytometric analysis on a BD LSR-II or for FACS sort on a BD FACS ARIA-II. Peripheral blood CD4+ T cells were isolated using a RosetteSep human CD4+ enrichment cocktail (Stem Cell Technologies) followed by Ficoll (GE Healthcare) density gradient centrifugation (20 min, 2000 rpm).

Flow Cytometry

Antibodies used for flow cytometry are listed in the Key Resources Table. For surface staining, cells were stained for 30 min on ice in PBS 1% NCS. For intracellular staining, cells were fixed and permeabilized using the eBioscience FOXP3 staining kit (eBiosciences). For detection of intracellular cytokines, CD4+ T cells were stimulated for 6 hours with phorbol 12-myristate 13-acetate (PMA; 1 mg/ml; Sigma) and Ionomycin (1 mg/ml; Sigma), and Golgistop was added for the last 4 hours (1 ml/ml; BD Biosciences). Cells were fixed and permeabilized using the BD CytoFix/CytoPerm kit (BV Biosciences). Acquisition and analysis was performed on a LSR-II (BD) using FACS Diva software. t-SNE analysis was performed using FlowJo 10 software.

Suppression of T cell proliferation assay

Purified decidual or peripheral blood $CD4^{+}$ T cells were sorted on a BD FACS Aria into four fractions ($CD4^{+}CD25^{HI}$, $CD4^{+}PD1^{HI}$, $CD4^{+}TIGIT^{+}$ and $CD4^{+}$ Teff) according to the gating strategy described in Figure S1. Decidual $CD8^{+}$ T cells were obtained from the same decidual sample. For CFSE labeling $CD4^{+}$ Teff and $CD8^{+}$ cells were resuspended in PBS at a concentration of 0.2 -1.0 x106 cells per ml. CFSE (Invitrogen) was added in a 1:2500 dilution and cells were incubated for 5 min in a water bath at 37°C. Cells were washed with X-VIVO 10 supplemented with 50U IL-2 and 5% human AB serum (Corning) for 8 min at 1800 rpm. Cells were resuspended at 0.4 x106 cells per ml in X-VIVO 10 supplemented with 50U IL-2 and 5% human AB serum. Treg were cultured with the CFSE- labeled Teff in a 1:2 ratio of 20.000 Treg: 40.000 Teff cells, with the addition of Dynabeads Human T-Activator CD3/CD28 (1 ml/ml). After four days, cells were collected and stained for CD4-PerCP, CD8-Alexa700 and CD45-PacificOrange. CFSE dilution of $CD4^{+}$Teff and $CD8^{+}$ T cells was analyzed using the proliferation analysis tool of FlowJo v7.6.5 software. The percentage of undivided cells (generation 0) was calculated based on the total

number of cells. The division index was calculated by FlowJo software and reflects the average number of divisions per cell. Suppression of T cell cytokine production assay Purified decidual $CD4^+CD25^{HI}$, $CD4^+PD1^{HI}$, $CD4^+TIGIT^+$, $CD4^+$ Teff and $CD8^+$ T cells were obtained as described above (Figure S1). $CD4^+$ Teff cells were labeled with CFSE (Invitrogen) and $CD8^+$ cells were labeled with CD45-Alexa700. Treg were cultured with $CD4^+$ and $CD8^+$ Teff in a 1:1:1 ratio (20.000 Treg: 20.000 $CFSE^+$ $CD4^+$ Teff: 20.000 Alexa 700+ $CD8^+$ Teff). Control cultures without Treg contained $CD4^+$ and $CD8^+$ Teff in a 1:1 ratio (30.000 $CFSE^+$ $CD4^+$ Teff: 30.000 $Alexa700^+$ $CD8^+$ Teff). CD3/CD28 Dynabeads (1 ml/ml) were added to all cultures and after three days, cells were re-stimulated with 2.5 ng/mL PMA in combination with 0.1 mg/mL ionomycin for 6h in the presence of 1 mg/mL GolgiStop (BD Bioscience). Cells were collected and stained for cell surface expression of CD4-PerCP, CD8-Alexa700, CD45-Pacific Orange and intracellular expression of IL-10, IFNg and TNFa upon fixation and permeabilization (CytoFix/Cyto PermTM Plus kit (BD)). Acquisition was performed on an LSR-II (BD) using FACS Diva software for analysis following the gating strategy shown in (Figure S4).

RNA isolation and QPCR chip analysis

Purified $CD4^+$ T cells from four samples of blood, decidua 6-12wk and d.parietalis >37wk (Figure S1A) were resorted into four types $CD4^+CD25^{HI}$, $CD4^+PD1^{HI}$, $CD4^+TIGIT^+$ and $CD4^+$ Teff (Figure S1B) and collected directly into 600 mL Trizol reagent (Life technologies) supplemented with 0.5 mL glycogen (20mg/ml; Affymetrix) and stored at 80°C until RNA isolation. Total RNA was isolated using the RNAeasy Micro Kit (QIAGEN) per manufacturer's instruction. RNA was analyzed on a Nanodrop to determine RNA yield and integrity. RNA quality of all samples was further confirmed by performing a QPCR analysis for GAPDH and FOXP3 expression. In short: RNA was reverse transcribed with Stratagene's AffinityScript QPCR. cDNA Synthesis Kit and amplification of specific PCR products for FOXP3 and GAPDH were detected using the PerfeCTa SYBR Green Super Mix with Low ROX (QuantaBio) in duplicates. Subsequently, high quality samples were run in duplicate on the BioMark Fluidigm QPCR 96.96 chip. 1.25ml DNA was pre-amplified in a 96-well plate using the Fluidigm PreAmp Master mix combined with 500nM forward and reverse primer of each primer pair (2min at 95°C, 10 thermal cycles of 15sec at 95°C and 4min at 60°C) (primers are listed in Table S1). Exonuclease treatment to remove unincorporated primers was carried out using the Exonuclease I at 40U/ml (New England BioLabs). 2 ml of the Exonuclease I dilution was added to each pre-amplification reaction and incubated in a thermal cycler for 30min at 37°C and 15min 80°C. A 5-fold dilution was prepared in TE buffer (10mM Tris-HCl, 1.0mM EDTA, TEKnova, PN T0224). Sample pre-mix for the 48 samples was prepared using 2ml of the prepared cDNA with 20X DNA binding dye sample loading reagent (Fluidigm) and 2X SsoFast EvaGreen supermix with low ROX (Bio-Rad). The assay mix was prepared in a separate 96-well plate consisting of 2X assay loading reagent, 1X DNA suspension buffer and 100mM of mixed forward and reverse primers. Chips were primed, loaded with both assay and sample mix and run on BioMark readout instruments as described by the manufacturer, at Harvard University's

core facility. Biomark data were processed using Fluidigm Biomark software. Quality control was based on EvaGreen reagent allowing for detection of unspecific PCR product and formation of primer dimers using the BioMark software. Samples of failed reactions were automatically excluded by the software. The resulting values for 66 primers of 48 samples were normalized by subtracting CT values for GAPDH (ΔCT) and subsequent conversion into fold-change values relative to the median peripheral blood Teff (2ΔΔCT). K-means cluster analysis was performed using the fold-change values of the 66 genes in Express Cluster V1.3 in Genepattern https://cloud. genepattern.org/gp. Differential expression of significantly correlating genes was based on K-means with a minimum of a 1.5-fold change.

Isolation of EVT

Isolation of EVT and co-culture with CD4+ T cells was performed as described previously (6) and are also described in detail hereafter. 1st trimester villous tissue was gently scraped from the basal membrane and the tissue was digested for 8 min at 37°C with a trypsin (0.2%) EDTA (0.02%) solution. Trypsin was quenched with DMEM/F12 medium containing 10% Newborn Calf Serum (NCS) and1%Pen/Strep (all from GIBCO) and filtered over a gauze mesh. Filtrate was washed once and layered on Ficoll (GE Healthcare) for density gradient centrifugation (20 min, 2000 rpm). Cells were collected, washed once and incubated for 20 min at 37°C in a 30mm tissue culture dish for removal of macrophages. To establish untouched (free of antibody staining) EVT cultures, for each sample the percentage $CD45^{-}HLA\text{-}G^{+}$ EVT was determined by FACS analysis for EGFR1, HLA-G and CD45. Samples with > 8% $CD45^{-}HLA\text{-}G^{+}$ cells were stained for CD45 and sorted for viable CD45- large trophoblast cells. 50.000 $CD45^{-}HLA\text{-}G^{+}$ EVT (calculated based on percentage $HLA\text{-}G^{+}$ cells and total cell number) were plated in 48 well cell culture plates (Costar) pre-coated with fibronectin (100 mL 20ng/ml 45min, BD), in DMEM/ F12 (GIBCO) supplemented with 10% NCS, pen/strep and glutamine, insulin, transferrin, selenium (GIBCO), 5ng/ml EGF (Peprotech) and 400 units human gonadotropic hormone (Sigma). Trophoblasts were incubated for 2h at 37°C and thereafter washed 3 times to remove all non-adherent VT. Cultures resulted in 50%–80% HLA-G+ EVT.

Co-culture of EVT, decidual macrophages and CD4+ T cells

50.000 EVT or decidual macrophages were co-incubated with 100.000 $CD4^{+}$ pT from unrelated blood donors in X-Vivo10 medium (Lonza), supplemented with 5% human AB serum and 50 units/ml IL-2 (Biolegend). For blocking experiments, LEAF-purified anti- HLA-G (MoAb 87G; 20 mg/ml), anti-ILT2 (MoAb GHI/75; 20 mg/ml), anti-PDL1 (MoAb 29E.2A3; 20 mg/ml), anti-TGFβ (MoAb 19D8; 25 mg/ml), anti-IL-10R (MoAb 3F9; 20 mg/ml), anti-HLA-DR (MoAb L243; 20 mg/ ml), purified anti-HLA-C (MoAb DT-9; 10 mg/ml) and IgG controls (all from Biolegend), or anti-CD3 (MoAb SPV-T3b; 25 mg/ml) (ThermoFisher Scientific) were added 30 min before addition of $CD4^{+}$ T cells. For transwell assays, 100.000 EVT or decidual macrophages were plated in 24 well cell culture plates pre-coated with fibronectin, non-adherent EVT were removed and 200.000

CD4^{+} pT were added directly to the EVT and decidual macrophage cultures or were added on top of the membrane after insertion of a 0.4 mM trans well membrane (Costar). CD4^{+} pT were harvested after 60 hours and analyzed by Flow cytometry for CD45, CD4, CD25, PD1, TIGIT and intracellular FOXP3 and HELIOS.

HCMV Infection of EVT

High titer virus stocks of HCMV-AD169-GFP (IE-1-GFP) (a gift from Prof. Donald Coen at Harvard Medical School) was obtained by infecting Human Foreskin Fibroblasts (HFF) (ATCC) and collecting supernatants after 7 days. Supernatants were aliquoted and snap frozen in liquid nitrogen until use. Primary EVT were infected with HCMV at an MOI of 2-4 for 12 hours (41). Infected cells were imaged in a Nikon EclipseTi fluorescence microscope at 20x magnification. HCMV infection of EVT reached > 80% at day 2, and no cytopathic effects on EVT were visible after HCMV (41). For co-culture assays, HCMV infected cells were washed twice after 12 hours of infection and 100.000 CD4^{+} pT were added. CD4^{+} pT were harvested after 48 hours and analyzed by Flow cytometry for CD45, CD4, CD25, PD1, TIGIT and intracellular FOXP3.

Quantification and statistical analysis

All data was analyzed using GraphPad Prism version 6.07 software. To determine differences between 2 paired groups a non-parametric Wilcoxon Signed Rank test was performed (Figure S1E). To determine differences among more than 2 unpaired groups, a nonparametric Kruskal-Wallis test with Dunn's multiple comparison post-test was performed (Figure 1, 2, 3, and 5; Figure S3, S4, and S7). For the Dunn's post-test the mean ranks were compared to control column A (Figure 2B 2G and 3; Figure S4 and S7), column B (Figure 5D-5F) or with the mean rank of every column (Figure 1, 5A-5C, and 5G; Figure S3). P values < 0.05 were considered to denote significant differences. *$p < 0.05$; **$p < 0.01$; *** $p < 0.005$ are indicated within each figure. Sample size indicates biological replicates of individual placental of blood cell isolates and are indicated indicted in each figure legend. Sample sizes were not determined beforehand.

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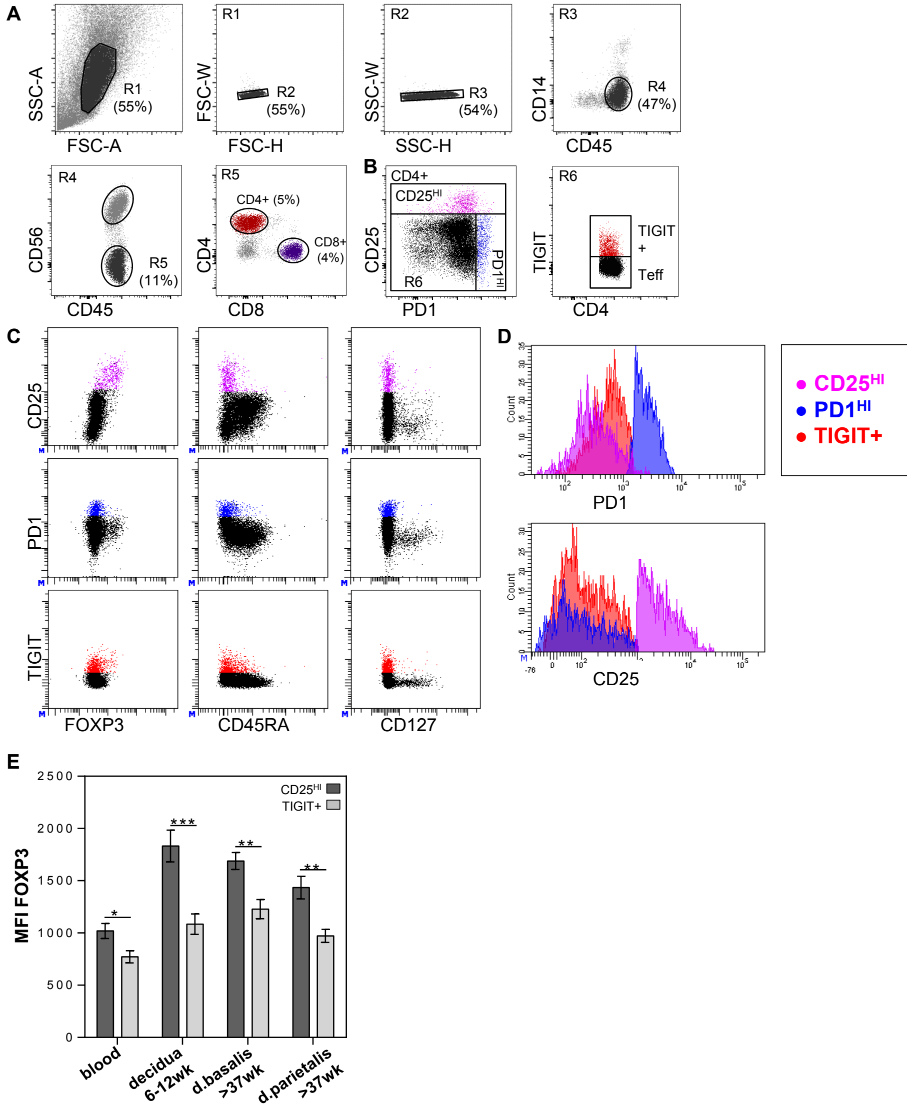
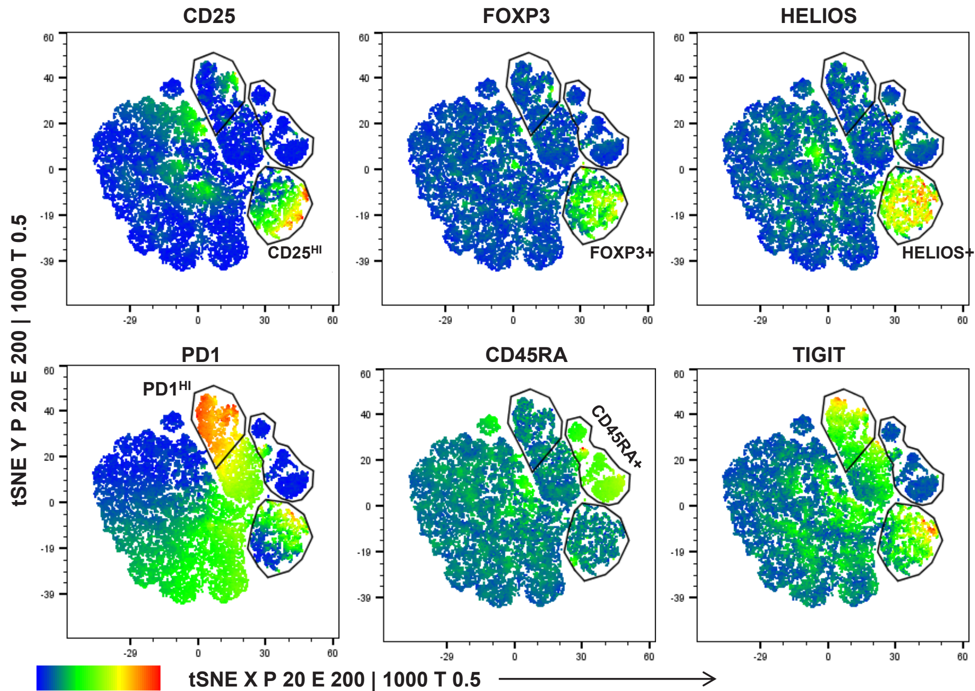
SUPPLEMENTAL INFORMATION



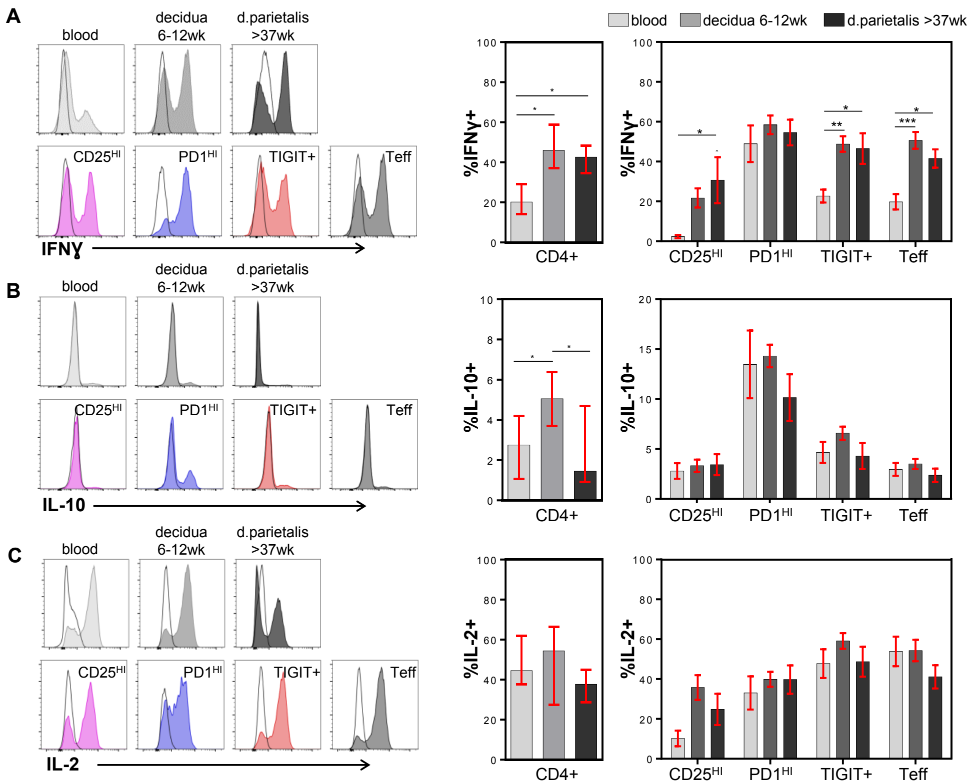
Supplementary Figure S1. FACS gating strategy.

Related to Figure 1. **(A)** CD4+ and CD8+ T cells were identified by selecting live cells (R1), duplicates were excluded (R2, R3), and CD45+CD56−CD3+CD4+ (CD4+) and CD45+CD56−CD3+CD8+ (CD8+) T cells were selected. **(B)** Based on CD25, PD1 and TIGIT expression, CD4+ T cells were further separated into $CD25^{HI}$, $PD1^{HI}$, TIGIT+ and Teff cells. Representative FACS plots of **(C)** FOXP3, CD45RA and CD127 expression and **(D)** PD1 and CD25 expression in $CD25^{HI}$, $PD1^{HI}$ and TIGIT+ cells in a 6-12wk decidual sample. **(E)** Graph depicts the expression of FOXP3 (MFI) within $CD25^{HI}$ and TIGIT+ cells in blood, decidua 6-12wk, decidua basalis >37wk and d.parietalis >37wk. n=8-10. Bars represent median and interquartile range; n= 8-10; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.



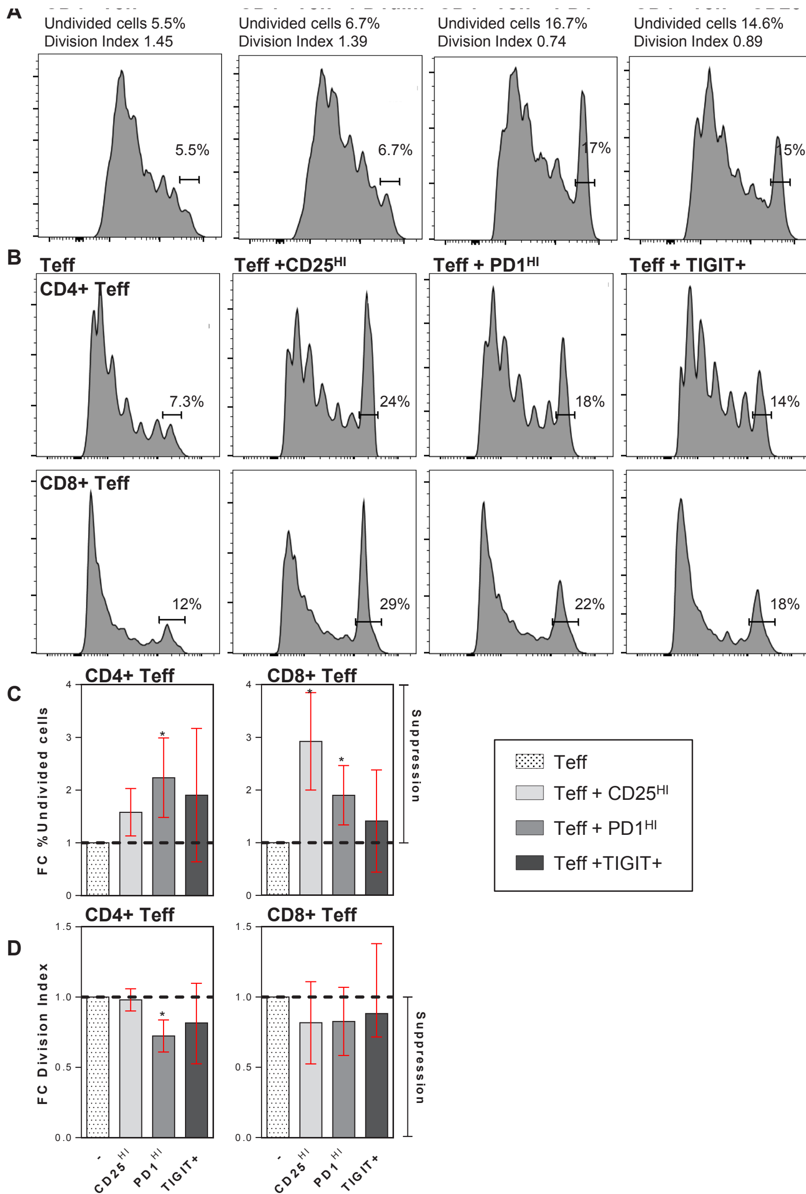
Supplementary Figure S2. tSNE analysis of decidual CD4+ T cells.

Related to Figure 1. Representative tSNE plots of one decidual CD4+ T cell sample (6-12wk), highlighting CD25, FOXP3, HELIOS, PD1, CD45RA and TIGIT. Three separate CD4+ T cell populations are identified i) $CD25^{HI}$FOXP3+HELIOS+, ii) $PD1^{HI}$ and iii) CD45RA+. TIGIT is co-expressed by $CD25^{HI}$ and $PD1^{HI}$ cells but not by CD45RA+ cells. TIGIT is also expressed by a large proportion of the remaining CD4+ T cells, these TIGIT+ cells are clearly identified in Fig 1B bottom panels.



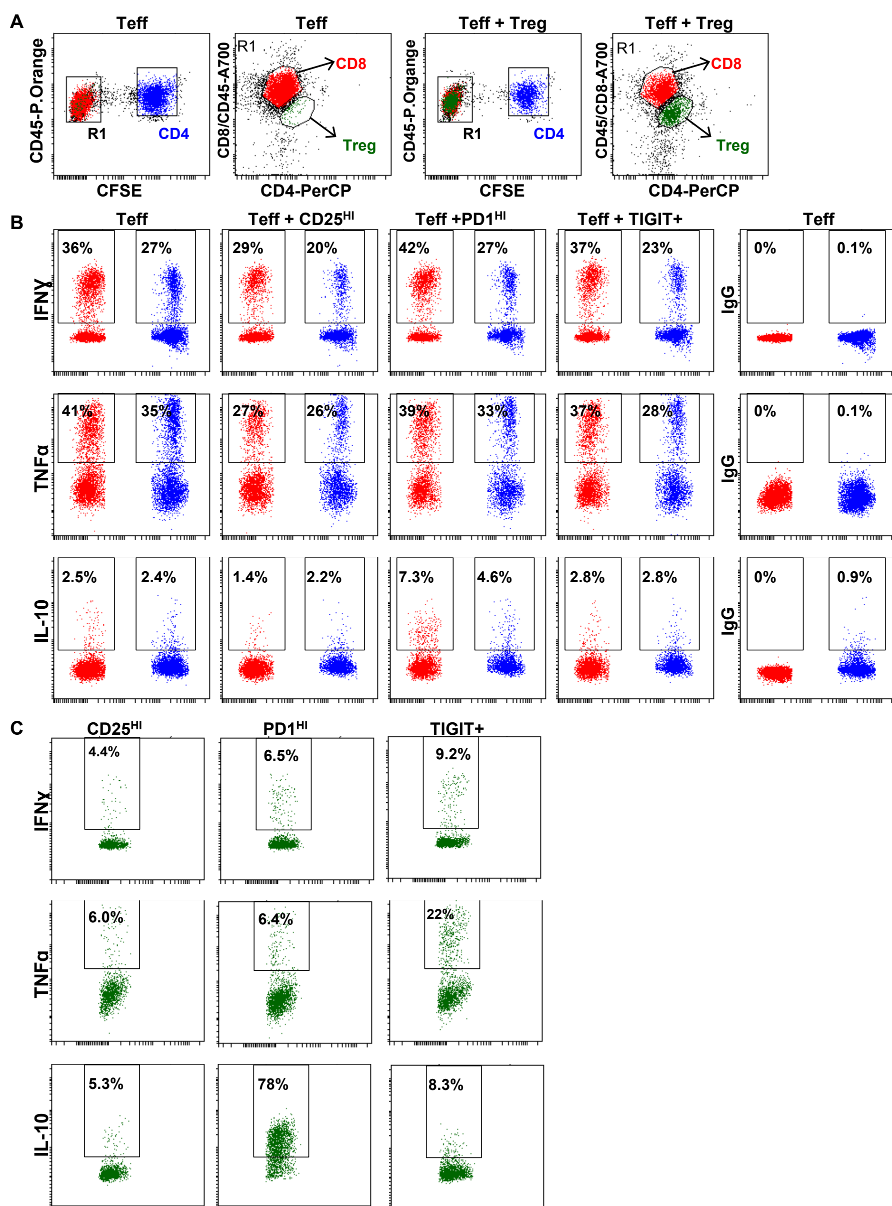
Supplementary Figure S3. IFNγ+, IL-2+ and IL-10+ expression by Treg.

Related to Figure 1. Representative histograms of **(A)** IFNγ, **(B)** IL-10 and C) IL-2 expression by CD4+ T cells in blood, decidua 6-12wk and d.parietalis >37wk and in the four T cell subpopulations (CD25^{HI}, PD1^{HI}, TIGIT+ and Teff) in decidua 6-12wk. Graphs depict percentage of **(A)** IFNγ+, B) IL-10+ and **(C)** IL-2+ cells within CD4+ T cells (left) and the four T cell subtypes (right). Bars represent median and interquartile range; n= 8-12; *P<0.05, **P<0.01, ***P<0.001. Kruskal-Wallis with Dunn's post-test comparing the mean ranks to the mean rank of every other column (column A,B,C) within each of the T cell populations.



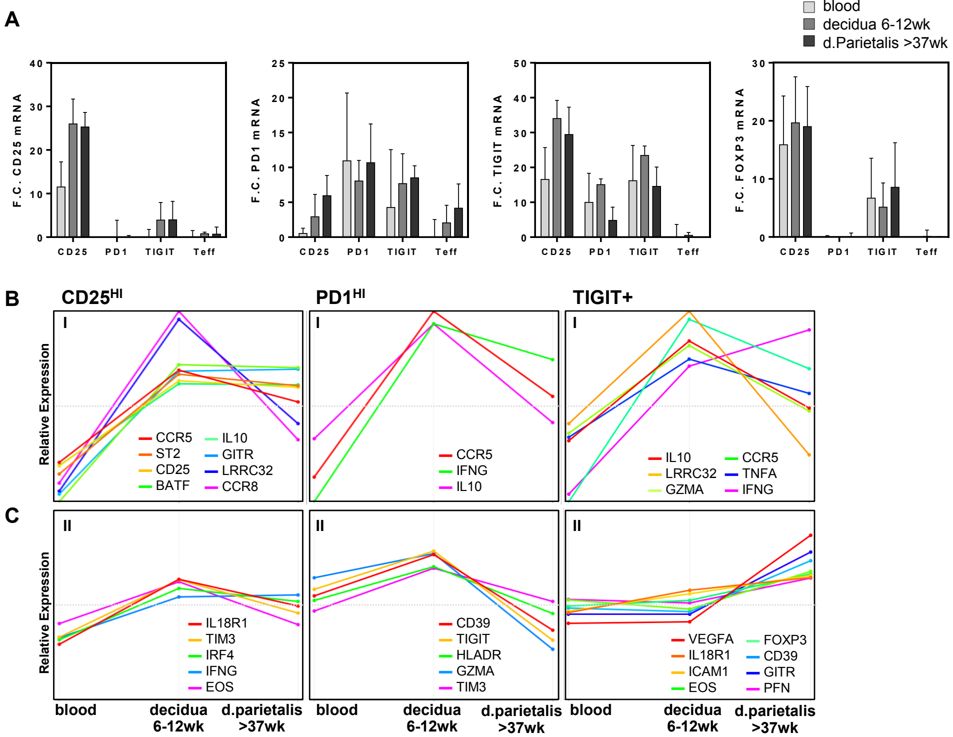
Supplementary Figure S4. Treg suppression of T cell proliferation.

Related to Figure 2. **(A)** CFSE dilution of 6-12wk decidual CD4+ Teff cells stimulated with anti-CD3/28 and co-cultured with or without PD1^{dim}, PD1^{HI} or CD25^{HI} cells and **(B)** CFSE dilution of peripheral blood CD4+ Teff cells (upper panels) and CD8+ Teff cells (bottom panels) stimulated with anti-CD3/28 and co-cultured with or without CD25^{HI}, PD1^{HI} or TIGIT+ cells. Cells were cultured for four days in a 1:2, Treg:Teff ratio. Percentages of the undivided Teff cells are shown. **(C)** Fold Change (FC) in percentage undivided peripheral blood Teff cells and **(D)** FC in division index after addition of CD25^{HI}, PD1^{HI} or TIGIT+ Treg, compared to CD4+ Teff cells (left panels) and CD8+ Teff cells (right panels) cultured alone. Bars represent median and interquartile range; n=5-7; *P<0.05, Kruskal-Wallis with Dunn's post-test comparing columns B-D to column A was performed. Values >1 for FC %undivided cells and <1 for FC division index represent suppression of proliferation. Division index reflects the number of divisions per cell as calculated in FlowJO v7.6.5.



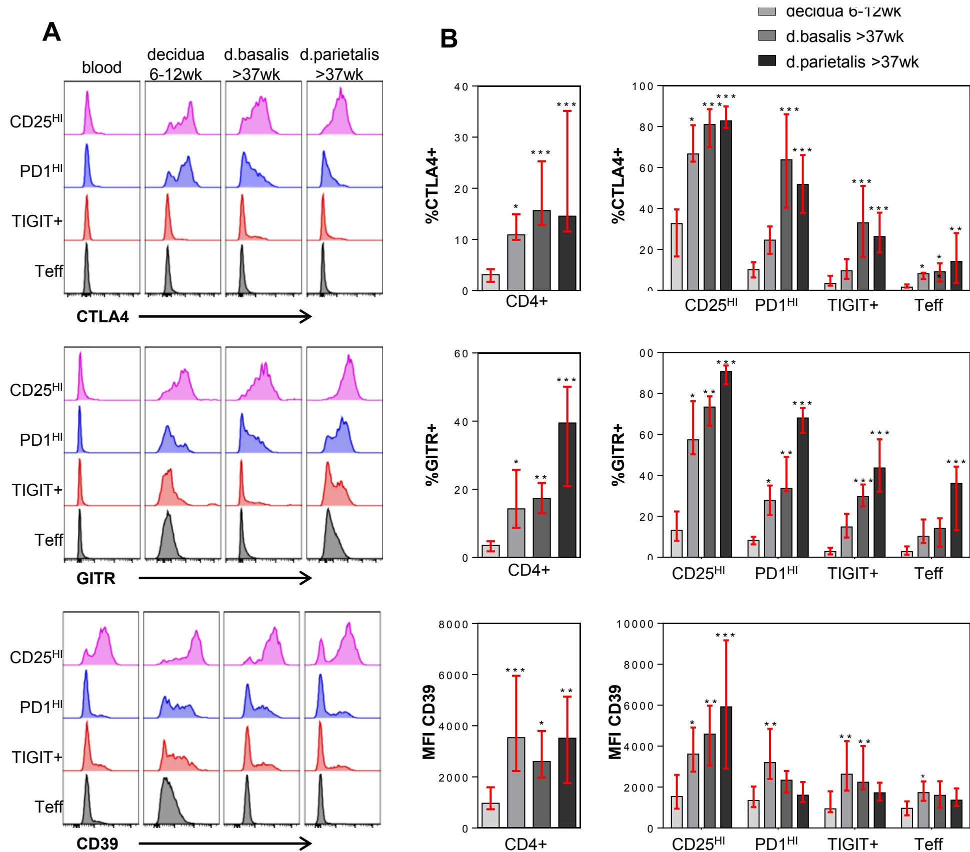
Supplementary Figure S5. Decidual Treg influence cytokine production by CD4+ and CD8+ Teff cells.

Related to Figure 3. **(A)** Gating strategy identifies CD45+CFSE+CD4+ T cells (Blue), CD45+CFSE-CD8+ T cells (Red) and CD45+CFSE-CD8- Treg (green). **(B)** Representative FACS plots of IFNγ, TNFα and IL-10 expression in decidual CD4+ Teff (Blue) and decidual CD8+ Teff (Red) upon addition of CD25^{HI}, PD1^{HI} or TIGIT+ Treg compared to CD4+ Teff and CD8+ Teff cultured alone and IgG controls. **(C)** Representative FACS plots of IFNγ, TNFα, IL-10 expression in decidual CD25^{HI}, PD1^{HI} or TIGIT+ Treg in the same co-cultures.



Supplementary Figure S6. Gene expression profiles of CD4+ Treg.

Related to Figure 4. **(A)** FC mRNA values of CD25, PD1, TIGIT and FOXP3 (n=4). Bars represent median and interquartile range. **(B and C)** K-means clustering identified two clusters of genes that significantly correlate their mRNA expression pattern in blood, decidua 6-12wk and d.parietalis >37wk. CD25^{HI} (left panels), PD1^{HI} (middle panels) and TIGIT+ (right panels) are depicted.



Supplementary Figure S7. Expression of CTLA-4, GITR and CD39 by Treg.

Related to Figure 4. **(A)** Representative histograms of CTLA-4 (top panels), GITR (middle panels) and CD39 (bottom panels) within $CD25^{HI}$, $PD1^{HI}$, TIGIT+ and Teff cells of blood, decidua 6-12wk, d.basalis >37wk and d.parietalis >37wk. **(B)** Graphs depict percentage of CTLA-4+ (top panels), percentage of GITR+ (middle panels) and Mean Fluorescence Intensity (MFI) of CD39 (bottom panels) within the total CD4+ cell population and within $CD25^{HI}$, $PD1^{HI}$, TIGIT+ and Teff cells of blood, decidua 6-12wk, d.basalis >37wk and d.parietalis >37wk. (n= 8-12) Bars represent median and interquartile range. $*P<0.05$, $**P<0.01$ and $***P<0.001$. Kruskal-Wallis with Dunn's post-test comparing all decidual fractions (columns B-D) to blood (column A) was performed.

CHAPTER 4

Clusters of Tolerogenic B Cells Feature in the Dynamic Immunological Landscape of the Pregnant Uterus.

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Cell Reports, 2020

ABSTRACT

Well-timed interaction of correctly functioning maternal immune cells is essential to facilitate healthy placenta formation, as the uterine immune environment has to tolerate the semi-allogeneic fetus, and allow adequate trophoblast invasion. Here, we assess the uterine immune signature before and during pregnancy. Extensive supervised and unsupervised flow cytometry clustering strategies not only show a general increase in immune memory throughout pregnancy, but also reveal a continuous presence of B cells. Contrary to the belief that B cells are merely a consequence of uterine pathology, decidual B cells produce IL-10 and are found to be localized in clusters, together with Foxp3^{+} T cells. Our findings therefore suggest a role for B cells in healthy pregnancy.

INTRODUCTION

The uterus is a highly dynamic compartment. Each cycle, in preparation of pregnancy, the endometrium undergoes decidualization; a complex process of mucosal differentiation and influx of immune cells dictated by changing hormone levels. During the early stages of pregnancy, the well-timed presence of correctly functioning maternal immune cells contributing to both correct trophoblast invasion of semi-allogeneic fetal cells, and spiral artery remodeling, is essential (1-4). Impairment in the recruitment, induction, or activation of immune components at the fetal-maternal interface can be harmful for mother and/or child. Depending on the nature and timing of the erroneous interactions, (recurrent) miscarriages, preeclampsia (PE), intra-uterine growth restriction or pre-term labor (PTL) may occur (3, 5, 6).

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After the initial challenge of implantation, the uterine immune environment faces the task of maintaining an anti-inflammatory state (3). Maternal uterine immune cells have to regulate the response to local or systemic infections, on the one hand counteracting threats to maternal and fetal health while on the other hand preventing a harmful inflammatory cascade. Any disbalance in the local immune response during this crucial window of development may affect the unborn child, with possible long-term effects on its immunity (7, 8) or cognitive function (9, 10). In the last stage of pregnancy, proinflammatory signaling, through infiltration of leucocytes into decidua and myometrium, and stimulation of cytokine secretion, facilitates physiologic uterine contractions (11). In addition, these adaptations may prepare for wound healing and tissue remodeling processes after parturition (12, 13).

Although knowledge on the chronology of the systemic immune states during pregnancy has advanced in recent years, studies on gestational uterine immune cell fluxes are scarce (14, 15). Here, we examined local immune cell dynamics throughout human gestation using endometrial lymphocytes derived from menstrual blood alongside decidual tissue from 1st and 2nd trimester, and term decidua from uncomplicated pregnancies. We provide an overview of lymphocytes present within the uterus throughout gestation with a focus on B cells with IL-10 secreting capacities.

RESULTS

The uterus is a highly dynamic immune compartment

Onset and maintenance of pregnancy elicits both short term, as well as long term adaptations of local immunity to facilitate healthy pregnancy. Thus, we assessed the immune cell composition in human uterine tissues (menstrual blood and decidua) from the non-pregnant phase, to 1st and 2nd trimester, up to term pregnancy by high dimensional immunophenotyping (Figure 1A). We first examined the abundance of the major lymphocyte cell types in the uterine mucosa

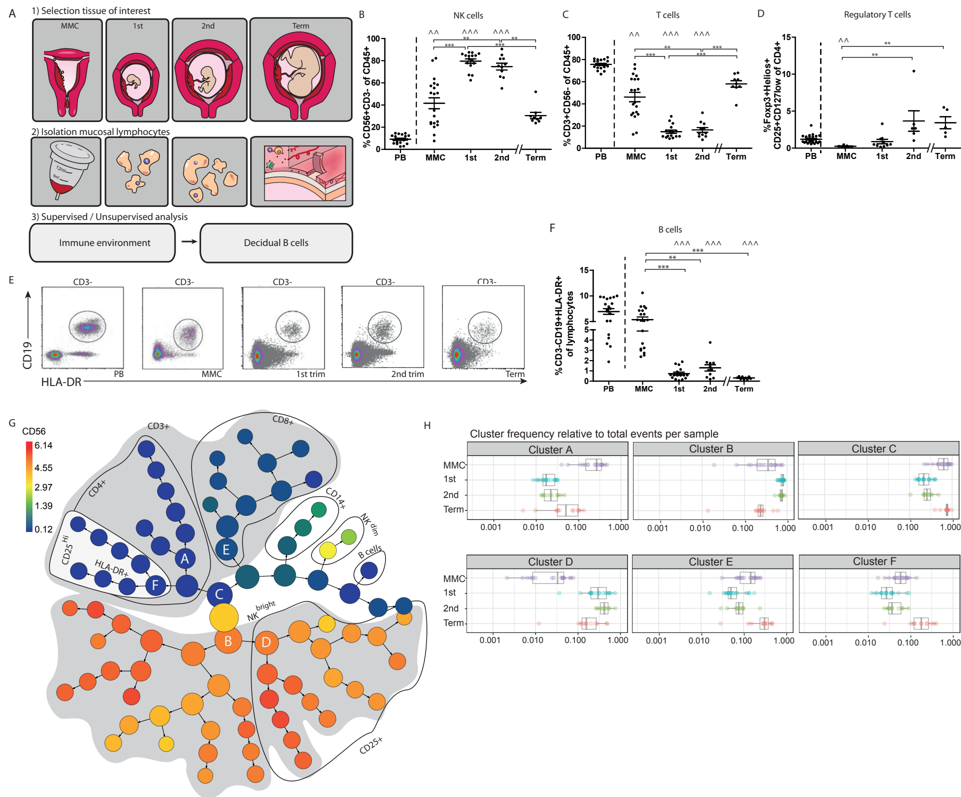


Figure 1. The dynamic immune environment of mucosal tissue from menstrual blood to term decidua

(**A**) Representation of samples chosen and workflow to study decidual lymphocytes of varying time points in absence of, and during, pregnancy.

(**B**-**D**) Frequencies of NK cells (**B**), T cells (**C**), and regulatory T cells (D) of single cell suspensions. Gating strategies are shown in Figure S1A and B. Peripheral blood (PB) n=20, menstrual blood (MMC) n=20, 1st trimester n=16, 2nd trimester n=11, term pregnancy n=9).

(**E** and **F**) $CD19^{+}$ $HLA\text{-}DR^{+}$ B cells could be found in MMC, 1st trimester, 2nd trimester, and term decidua.

(**G** and **H**) Stratification of menstrual blood-derived and lymphocytes based on hierarchical clustering using CD3, CD4, CD8, CD14, CD16, CD19, CD25, CD56 and HLA-DR to identify populations of similar marker expression and their abundance using the CITRUS tool by Cytobank™. 5000 events (gating strategy Figure S2A) were sampled per file and PAM predictive clustering was applied to identify clusters contributing to gestational-age dependent, stratifying signatures (model error rate is shown in Figure S2B). The color scale illustrates the relative marker expression per cluster while size of each node represents event frequency. As an example, CD56 expression is shown (**G**), super-clusters indicates frequency of each marker per cluster (Figure S2C+D). Relative frequency of clusters compared to total lymphocyte count identified as most stratifying between menstrual blood, 1st, 2nd trimester and term decidua samples, ordered from A to F decreasing in their contribution to stratification (**H**). Samples <5000 lymphocytes were excluded from analysis (MMC n=20, 1st trimester n=40, 2nd trimester n=16, term pregnancy n=9).

^ $p<0.05$, ^ ^ $p<0.01$, ^ ^ ^ $p<0.001$ Kruskal-Wallis and post-hoc Dunn's multiple comparison test to assess difference between mucosa and peripheral blood; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ Kruskal-Wallis; to compare menstrual blood mononuclear cells, 1st trimester, 2nd trimester and term decidua. Data are represented as mean ± SEM.

over time, using supervised flowcytometric analysis. For menstrual blood mononuclear cells (MMC), we observed that the NK cell abundance (CD45^{+}CD3^{-}CD56^{+}) was relatively higher than in peripheral blood (Figure 1B), as previously shown (16, 17). NK cell levels were high in 1st trimester decidua, stayed at that level in 2nd trimester and dropped towards term (Figure 1B). Independent of the time point of gestation, the majority of the decidual NK cells were negative for CD16 and showed high expression of CD56 (NK^{bright}, Figure S1C). Compared to peripheral blood, lower T cell (CD45^{+}CD3^{+}CD56^{-}) frequencies were seen in MMC, decreasing further in 1st and 2nd trimester decidua (Figure 1C), and increasing again towards term. In MMC, the distribution of CD4^{+} and CD8^{+} cells within the total CD3^{+} T cell population was similar to that in peripheral blood (Figure S1D). After initiation of pregnancy, within CD3^{+} T cells, the frequency of CD8^{+} cells increased locally and remained at that level throughout pregnancy. CD4^{+} T cell frequency decreased in 1st trimester decidua (Figure S1D). Overall abundance of CD4^{+} T cells within all CD3^{+} T cells remained relatively low over the course of pregnancy compared to MMC or peripheral blood. Previously, the percentage of naive CD4^{+} (CD45RA^{+}CCR7^{+}) T cells was shown to decrease over the course of pregnancy (Feyaerts et al., 2017). Here, we observed that this switch from naive towards a memory/effector phenotype already takes place at the earliest stage of pregnancy in 1st trimester decidua (Figure S1E,F). A slight drop in CCR4^{-}CCR6^{-}CXCR3^{+}CD4^{+} T cells (Th1) was observed in 2nd trimester samples (Figure S1G). CCR4^{+}CCR6^{+}CXCR3^{-}CD4^{+} (Th17) decreased from 1st trimester to term (Figure S1G). A prominent role in the delicate balance of immunity vs tolerance at the fetal-maternal interface is ascribed to regulatory T cells (Treg (18). Compared to peripheral blood, (CD4^{+}Foxp3^{+}Helios^{+}CD25^{Hi}CD127$^{low/-}$, Figure S1H) Treg levels in MMC were low. In pregnancy, levels rose gradually from 1st trimester to 2nd trimester, and remained at this level up to parturition (Figure 1D). While levels of NKT cells (CD45^{+}CD3^{+}CD56^{+}) in MMC were similar to those in peripheral blood, their abundance in decidual tissue was highest at term (Fig S1I). Samples of all gestational ages contained a small CD3^{-}CD19^{+}HLA-DR^{+} B cell population (Figure 1E, F).

To further explore phenotypic features of the different cell populations, we employed an unsupervised approach, using the CITRUS (cluster identification, characterization, and regression) tool on the Cytobank™ platform. This hierarchical clustering method stratifies cell subtypes based on similar descriptive features (i.e. marker expression) that correlate with the assigned sample type, in this case, gestational age (19). The six most stratifying clusters to predict gestational age are shown in Figure 1G and 1H. Most predictive clusters were CD4^{+} T cells (Cluster A, CD45^{+}CD3^{+}CD4^{+}CD8^{-}), followed by NK^{bright} (Cluster B, CD45^{+}CD3^{-}CD56^{+}CD16^{-}) cells, CD8^{+} T cells (Cluster E, CD45^{+}CD3^{+}CD4^{-}CD8^{+}), and regulatory T cells (Cluster F, CD45^{+}CD4^{+}CD25^{Hi}HLA-DR^{+}). In line with supervised analysis, T cell clusters A, C, E, F decreased in frequency of overall lymphocytes from MMC to 1st trimester decidua and increased towards term; NK cell clusters (B, D) showed the reverse pattern. Decidual NK cells expressing CD25 (Cluster D, CD45^{+}CD3^{-}CD56^{+}CD25^{+}CD16^{-}), an NK cell subtype suspected to be upregulated due to trophoblast contact at the fetal-maternal interface (20) was detected. A

cluster representing $CD45^+CD3^-CD19^+HLA\text{-}DR^+$ B cells was present in both MMC and decidual tissue of all gestational ages (Figure 1G).

Pregnancy is accompanied by a gain in decidual B cell memory

Having established that B cells are present in decidua throughout pregnancy, we investigated their phenotype in more detail. Little is known on their phenotype within the uterine mucosa. Supervised analysis revealed that similar to peripheral blood, MMC derived B cells were mainly naive (IgD^+CD27^-, Figure 2A). Their frequency was lower upon initiation of pregnancy, and further dropped towards the 2nd trimester (Figure 2B). Switched memory B cells (IgD^-CD27^+) increased from MMC throughout gestation (Figure 2C). Abundance of non-switched memory B cells (IgD^+CD27^+) was comparable to peripheral blood and remained steady during gestation (Figure 2D). The frequency of plasmablasts ($CD27^+CD38^{+/hi}IgD^-$) was low in MMC and 1st trimester decidua compared to peripheral blood (Figure 2E). Plasmablast abundance increased from MMC to 2nd trimester and term decidua. The overall frequency of B cells positive for CD27, a memory marker, increased upon pregnancy (Figure 2F) (21, 22).

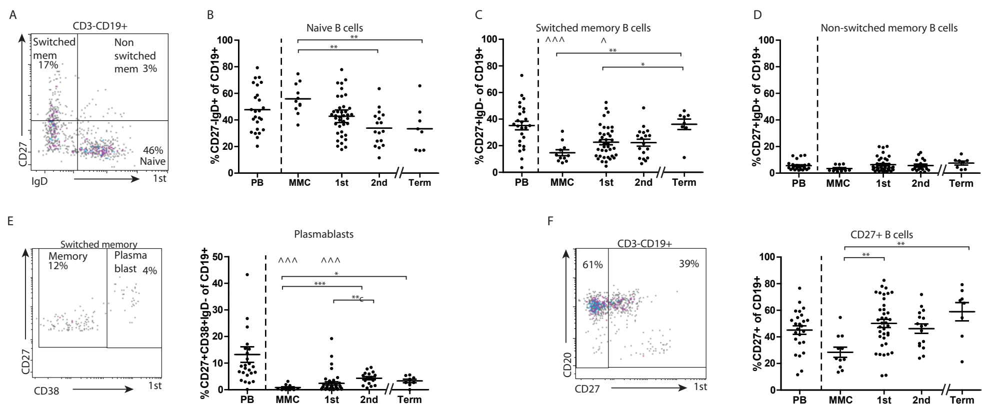


Figure 2. Differential expression of B cell subsets in samples of decidua

(A-D) Gating strategy (A) for characterization of naive **(B)** and switched memory **(C)** and non-switched memory B cells **(D)** based on IgD and CD27 expression (within B cells, gating strategy Figure S3A) in peripheral blood (PB, n=25), menstrual blood (MMC, n=11), 1st (n=39) and 2nd (n=17) trimester as well as term decidua (n=8).

(E) $CD27^+IgD^-$ cells were further classified as plasmablasts in case of additional CD38 staining.

(F) CD27 positivity of $CD19^+$ B cells.

^p<0.05, ^^p<0.01, ^^^p<0.001 Kruskal-Wallis and Dunn's multiple comparison test to assess difference between isolated mucosal tissues and peripheral blood; *p<0.05, **p<0.01, ***p<0.001 to compare MMC, 1st trimester, 2nd trimester and term decidua. Data are shown as mean±SEM.

Decidual B cells are distinct from peripheral blood B cells

Recent studies emphasize the diversity in B cell subsets, including populations with regulatory capacities (Breg), especially when comparing systemic to tissue-specific immunity (23, 24). To gain more in-depth insight in B cell subsets in decidual tissues versus peripheral blood, we employed a CITRUS based multidimensional unsupervised clustering analysis. While considered a common B cell marker (25), we included CD20 in our clustering analysis for assessment of its possible differential expression. The decidual B cell population contained four subsets that were absent in peripheral blood (Cluster I-IV, Figure 3A; marker expression 3C,D); cluster I, comprising $CD24^{hi}CD27^{+}CD38^{-}$ cells, and cluster II, comprising $CD24^{+}CD27^{+}CD38^{+}$ cells (cells of both clusters were further characterized as $IgD^{low}IgM^{low}CD20^{+}$), cluster III, comprising $IgD^{-}IgM^{low}CD20^{-}CD24^{hi}CD27^{-}CD38^{hi}$ cells, and cluster IV, comprising $IgD^{+}IgM^{low}CD20^{+}CD24^{+}CD27^{-}CD38^{+}$ cells (Figure 3A). Cluster III was not observed in term decidua. Two additional clusters were more abundant in peripheral blood: cluster V, resembling plasmablasts ($IgD^{-}IgM^{low}CD20^{-}CD24^{-}CD27^{hi}CD38^{hi}$ cells) and cluster VI ($IgD^{+}IgM^{+}CD20^{+}CD24^{low}$ $CD27^{low}CD38^{low}$ cells) (Figure 3B, marker expression 3C,D).

Decidual B cells have in vitro IL-10 producing capacity and are found in clusters

Mouse studies revealed a role for regulatory B cells (Breg) in healthy pregnancy (26-28). For human B cells, regulatory properties have been ascribed to a number of phenotypically distinct B cell subsets, united in their ability to produce IL-10 (29). To examine the presence of possible Breg in the different decidual tissues, we performed *in vitro* stimulation of decidual and peripheral blood mononuclear cells (PBMC) in the absence or presence of CpG (TLR9 activation) and CD40L (mimicking T cell dependent triggering of CD40) (30, 31). Upon stimulation, higher frequencies of decidua derived IL-10 expressing B cells compared to peripheral blood derived ones were found (Figure 4 A,B). In the absence of additional CpG and CD40L stimulation, 1st and 2nd trimester decidua derived B cells already expressed IL-10 (Figure 4C).

Information on decidual B cell localization is scarce. Immunohistochemical analysis on 1st and 2nd trimester decidua revealed that B cells were located closely together with T cells (Figure 4D). As several studies in humans and mice have pointed out the importance of IL-10 secreting B cells for regulatory T cell induction (32-35), we investigated whether these decidual lymphocyte clusters also harbored Treg. Additional staining of Foxp3, a key transcription factor of Treg, showed the presence of $Foxp3^{+}CD3^{+}$ T cells in close proximity to the B cells (Figure 4E, S3C).

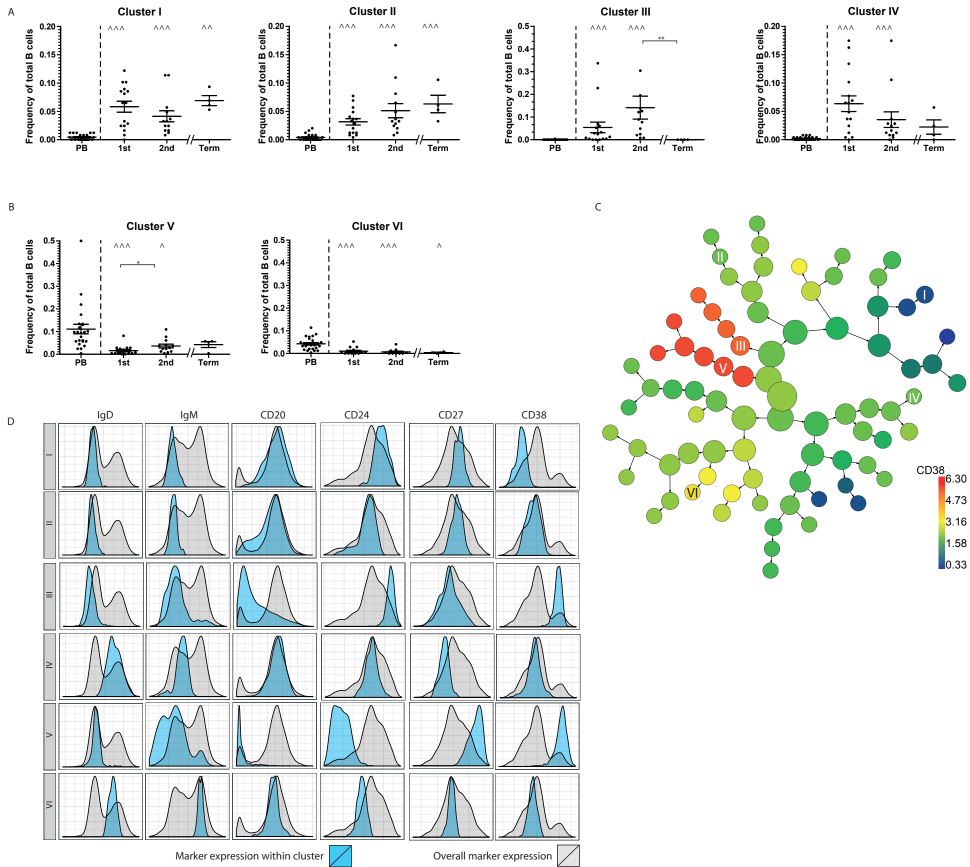


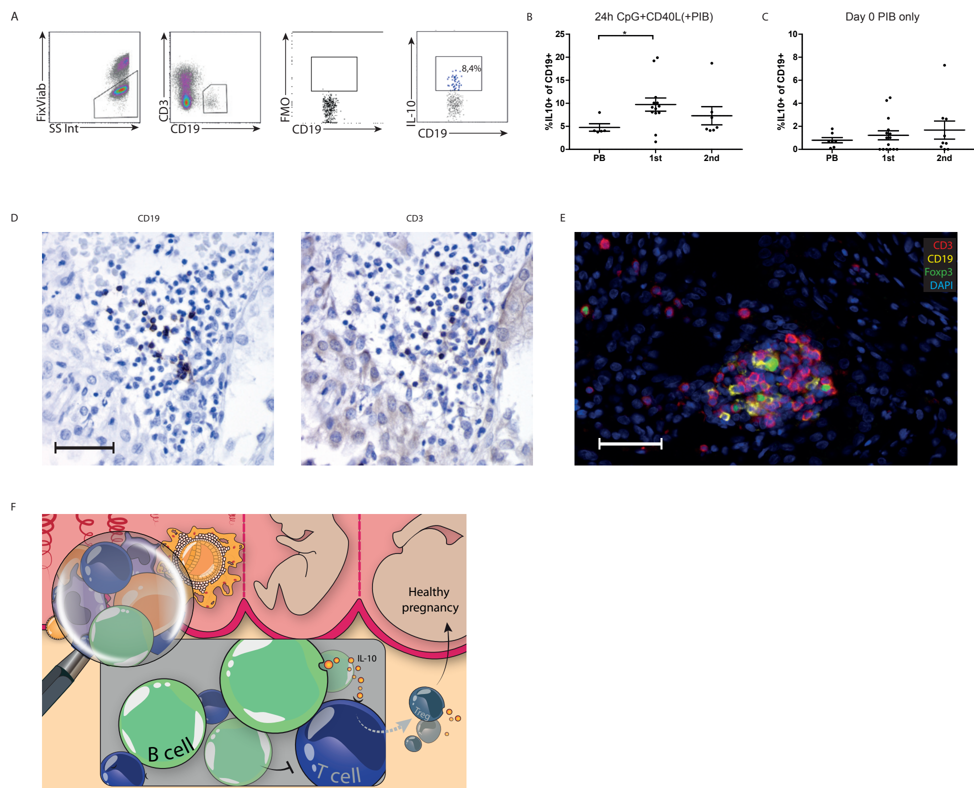
Figure 3. Unsupervised clustering of B cell subsets in decidua

(A and B) Decidual B cells (n=33) were stratified by automatic clustering based on IgD, IgM, CD20, CD24, CD27 and CD38 expression using the CITRUS tool by Cytobank™ and compared to peripheral blood (PB, n= 25). 246 B cells as gated in Figure S3A for each sample were used as input. A minimum cluster size of 2% was handled. Clusters of significantly higher (I-IV, A) or lower (V and VI, B) expression in decidua compared to peripheral blood are shown and abundance was separated into the individual gestational time points. Abundance depicts number of cells within a cluster divided by the total number of cells per sample. Samples of <200 B cells were excluded (1st trimester n=16, 2nd trimester n=13, and term n=4). Data are represented as mean±SEM.

(C) Clustering tree depicting relation of identified clusters. The color scale illustrates relative marker expression per cluster while size of each node represents event frequency. As an example, CD38 expression is shown (remaining markers Figure S3B).

(D) Marker expression of each subset in relation to overall expression.

^p<0.05, ^^p<0.01, ^^^p<0.001 Kruskal-Wallis and Dunn's multiple comparison test; *p<0.05, **p<0.01 to compare 1st trimester, 2nd trimester and term decidua. Data are represented as mean±SEM.



4

Figure 4. B cells in decidua secrete IL-10

(A) Representative gating strategy for assessment of intracellular IL-10 in peripheral blood and decidual B cells. Live, $CD19^{+}$ events were selected. For each IL-10 assessment, a fluorescent minus one (FMO) strategy (using a staining mix excluding IL-10) was applied to cells subjected to the identical conditions for correct positioning of the gate selecting IL-10 positive cells.
(B) 50.000 mononuclear cells were stimulated for 24h with 5μg/ml CpG and 1μg/ml CD40L, or (C) in absence of stimulation (peripheral blood, PB n= 7, 1st trim. n=15, 2nd trim. n=9).
(D) Representative immunohistochemistry of 10 week decidua stained for CD19 (brown) and CD3 (red) of decidual lymphocyte clusters (n=8 in 3 biological replicates, 9wk-14.5wk). Original magnification x40, scale bar (black) indicating 200μm.
(E) Representative 4-color multiplex staining of 10 week decidua. Original magnification x20, scale bar (white) indicating 50μm.
(F) Schematic representation summarizing co-localization of B and T cells. Decidual B cells are able to secrete IL-10, with possible impact on regulatory T cell maintenance and thus healthy pregnancy.
PIB, PMA+ionomycin+Brefeldin A. Treg, regulatory T cell. **$p<0.01$ Kruskal-Wallis and Dunn's multiple comparison test for comparison of PB, 1st trimester and 2nd trimester decidua. Data are shown as mean±SEM.

DISCUSSION

Pregnancy poses an immunological challenge as semi-allogeneic fetal cells have to invade maternal tissue for correct placenta formation. Here, we provide an overview of the dynamic changes in lymphocyte subsets, shaping the gestational immune environment. We highlight a possible role for decidual B cells, whose potential contribution to healthy pregnancy is just starting to be considered. Our data reveal that B cells undergo phenotypic adaptations in the decidual tissues over time, which may be regarded as a physiologic component of healthy pregnancy.

The possible relevance of B cells for healthy pregnancy is gaining attention (36). In mice, it was shown that the lack of mature B cells is associated with reduced litter size, decreased size of the embryos and higher susceptibility to prenatal infections (35). In women with common variable immunodeficiency, a condition associated with reduced frequencies of switched memory B cells in peripheral blood (37), an elevated risk for preterm birth or other pregnancy complications, such as PE, stillbirth or vaginal bleeding was observed (38). Recurrent miscarriage (39, 40) and PE (41) was also linked to alterations in the induction of B cell memory. Although there is no consensus regarding frequencies of systemic B cell subsets in pregnant women, healthy pregnancy, especially in its final stages, was consistently shown to be marked by a decrease in absolute numbers of circulating B cells (42-46). This may be explained by a decrease in B cell lymphopoiesis, as was shown in mice during gestation (47, 48), but it may also be due to a (re) distribution to local sites. Our observation that naive B cells in early decidual tissues increase in abundance and switch to a memory phenotype could be an indication that redistribution occurs.

The question is, what purpose do these decidual B cells serve? Although there are indications that B cells may contribute to the inflammation required for the onset of labor (49), decidual B cells may well serve an additional purpose. By supervised and unsupervised analysis, we observed high frequencies of $CD24^{hi}CD27^{+}$ (cluster I) and $CD24^{hi}CD38^{hi}$ (cluster III) B cells in 1st and 2nd trimester decidua, cell types associated with IL-10 producing capacity, a cytokine involved in immune regulation (24). In our hands, decidual B cells produced IL-10, more than peripheral blood B cells, and even without prior *in vitro* stimulation. The latter may suggest that decidual B cells already received the necessary activation *in utero*. Unsupervised analysis revealed that these cell clusters, together with two additional clusters expressing CD24 and CD38 (clusters II and IV) are unique for decidua and not present in peripheral blood. These cells might represent a specialized tissue-resident B cell subset or they may be induced *de novo* through local interaction with fetal cells. Indeed, recent *in vitro* experiments point out that trophoblast cells have the capacity to upregulate IL-10 in B cells, underlining the impact of fetal cells on B cell stimulation during the peri-implantation period (50). Likewise, upon *in vitro* stimulation, especially B cells derived from 1st trimester decidua produced IL-10. This might support the notion that B cell mediated IL-10 production is induced relatively early on in gestation, shortly after primary

encounter with invading fetal trophoblast cells. So far, there is only scant availability of data regarding decidual IL-10 expression *in vivo* (51). Also, in our hands, using immunohistochemistry, *in vivo* IL-10 producing B cells were not readily detectable. Indeed, IL-10 staining in tissues poses a technical challenge; IL-10 has a short half-life, it is rapidly secreted, and *in vivo* secreted levels in the local micro-environment, although functionally relevant, may likely be too low to yield positive staining results (52). While unsupervised clustering of decidual B cells did not show significant differences in subsets between 1st and 2nd trimester, less IL-10 production upon *in vitro* stimulation of decidual immune cells was observed in 2nd trimester. It may be possible that the potency of IL-10 production declines independent from the unchanged phenotype.

CD20 is a well-known marker expressed by multiple B cell subsets, amongst which are B cells with regulatory function (53). Of note, unsupervised analysis showed that the most abundantly expressed B cell populations lacked CD20. Hasan and colleagues showed the diverse heterogeneity of marker expression by Breg, but unfortunately, they did not include CD20 in their study (24). While the biological function of CD20 remains poorly understood, future studies might benefit from considering CD20 as differentially expressed marker.

We found that decidual B cells colocalized with T cells, similar to what has been described for non-pregnant endometrium (Yeaman et al., 1997). This colocalization supports the possibility of a functional interaction that may go both ways: T cell derived cytokines may facilitate the induction of Breg (Rosser et al., 2014), whereas in turn, through the production of IL-10, Breg may contribute to the induction of Foxp3^{+} Treg (34, 53, 54). In decidua, we detected Foxp3^{+} T cells in clusters of lymphocytes, closely located to B cells. In healthy individuals, peripheral blood Breg convert effector T cells to Treg through IL-10 production, and inhibit development of naive T cells towards Th1 or Th17 cells (34), thereby mediating suppression in case of a local inflammatory reaction (54). It is thus tempting to speculate that the IL-10 secreted by nearby B cells contributes to placental Treg induction and maintenance; these latter cells are well recognized for their contribution to successful pregnancy (55, 56). So, besides the idea that colocalization of decidual T and B cells is a phenomenon exclusive to pathologies (57), the interaction between Breg and T cells may just as well contribute to the required homeostasis and the tight regulation needed for healthy placentation, while preserving immune competence at the fetal-maternal interface. Unfortunately, the low amount of B cells that can be isolated per sample restricts experimental possibilities to assess their suppressive capacity on T cells. Even though their contribution in numbers within decidual lymphocytes is small, we propose that their effect might be amplified through close proximity and effect on T cells and possible Treg induction (Figure 4E).

In conclusion, the current study highlights the complex dynamics of the human uterine immune landscape, including B cells with the potential to contribute to the immune-regulatory environment of the uterus during pregnancy.

MATERIALS AND METHODS

Experimental model and subject details

Peripheral blood was collected from 36 healthy female volunteers (mean age 22.8 years ± 3.4 years). Exclusion criteria were smoking, chronic disease, medication, carrier of an infectious disease and pregnancy. Menstrual blood was provided by 28 healthy women (exclusion criteria: known fertility disorder, potential carrier of an infectious disease, auto-immune disease, drug abuse, or use of hormonal or intra-uterine device contraceptives) with regular menstrual cycle. First and 2nd trimester material was obtained from discarded placental and decidual tissue upon elective pregnancy termination of healthy women at a local reproductive health clinic. No additional data other than gestational age at time point of termination was acquired. Exclusion criteria were carrier of an infectious disease (active systemic infection), suspected to be a potential carrier of an infectious disease or an increased risk for infection (HIV, Hep B/C, HTLV or similar), auto-immune diseases, drug abuse, intoxication with heavy metals or pesticides. Term placental tissue (>37weeks gestation) was collected from planned cesarean section following uncomplicated pregnancy. Exclusion criteria were gestational age <37 weeks, use of immunosuppressive drugs, biological or antidepressants, HIV positivity, active infection during caesarean section, signs of infection (maternal fever or signs of intrauterine infection), use of antibiotics prior to caesarean section. All gestational samples were assessed visually and excluded for processing in case of signs of infection (discoloration) or excessive blood clots. Written informed consent was obtained for all volunteers donating blood or tissue for this study in accordance with the Dutch Medical Research Involving Human Subject Act (WMO). The study was approved by the local review board (Commissie Mensgebonden Onderzoek region Arnhem-Nijmegen, 42561.091.12, 2017-3253, 2014-232, 2009/004). An overview of all study participants is given in Supplemental Table S1. The amount of individual participants included per experiment is indicated in the according legend as the amount of cells isolated per sample limited the number of experiments carried out simultaneously.

METHOD DETAILS

Sample collection

Peripheral blood was collected using EDTA tubes. For menstrual blood, women were asked to use a menstrual cup (Femmecup Ltd, London, UK) and collect its content every 12h during the first 36h after initiation of menstruation. Contents of the cup were poured in a 30 ml tube containing 10 ml of supplemented RPMI 1640 medium (1 mM pyruvate, 2 mM glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin (all Thermo Fisher Scientific Waltham, USA) 0.3% v/v sodium citrate (Merck Darmstadt, Germany) 10% v/v human pooled serum; HPS, manufactured in-house) for storage at room temperature until processing. After collection, menstrual blood samples were transferred to the lab immediately to undergo processing within max. 24h. First

and second trimester tissue was transported to the lab at room temperature and processed immediately upon arrival.

Cell isolation

Flow cytometry staining was performed on either whole blood or, for Treg staining, on isolated mononuclear cell populations. Peripheral blood mononuclear cells were isolated from whole blood based on density gradient separation (1.077 ± 0.001 g/ml, 290 ± 15mOsm, Lymphoprep , Axis-Shield, Oslo, Norway). Menstrual blood mononuclear cells were isolated following an established protocol (58, 59). This protocol typically results in cell viability of ~96%. In brief, menstrual blood was washed with PBS (Braun, Melsungen, Germany) and filtered (70μm cell strainer Falcon®, Corning Inc., NY, USA) to remove clots or excess mucus. A granulocyte depletion cocktail RosetteSep™ (Stemcell technologies Inc, Vancouver, Canada) was used according to manufacturer's instructions. After sterile density gradient centrifugation (Lymphoprep™), menstrual blood mononuclear cells (MMC) were collected. First and 2nd trimester decidual leucocytes were isolated from maternal mucosal tissue that was carefully selected during visual assessment of the individual tissue pieces. Villous tissue, blood clots, glands and areas of blood infiltration were discarded. At these early stages of gestation, the selected pieces of membrane do not contain fused fetal membranes yet. Tissue pieces were minced mechanically using scissors and washed in PBS until the supernatant became transparent. For cells used for phenotyping, tissue was transferred to c-tubes (gentleMACS system) for additional mechanic processing using a MACS dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) prior to incubation with Accutase (Stempro, Gibco Life Technologies, Waltham, USA) in a 1:2 tissue to enzyme ratio; a gentle dissociation method to preserve marker expression (60). For B cell assays, washed tissue was digested using 0.2% collagenase (Gibco Life Technologies, Waltham, USA) and 0.04% DNAse (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Tissue digestion was performed in a shaking water bath at 37 °C for 45 minutes. After digestion, the solution was filtered through 100μm, 70μm and 40μm cell strainers consecutively. Lymphocytes were obtained through density gradient centrifugation by diluting the washed filtrate in 20 mL 1.023 g/ml Percoll (GE Healthcare, Little Chalfont, UK) to be layered on gradient of 10 mL 1.080 g/ml and 15ml 1.053 g/ml Percoll (centrifugation for 25min at 2000rpm). Large cells accumulating at the 1.053 – 1.023 g/ml interface were discarded and decidual leucocytes were isolated from the 1.080 – 1.053 g/ml interface. Leucocytes were washed twice in RPMI before further use. For term decidua, decidua parietalis was separated from the two fetal layers. The amnion was removed prior to scraping decidua parietalis from the chorionic membrane. After this, the isolation procedure was identical to that of 1st and 2nd trimester.

Flow cytometry staining and analysis

For whole blood staining, 25ml red blood cell lysis buffer [NH_4CL + $KHCO_3$/ Na_4EDTA (Merck, Darmstadt, Germany) diluted in H_2O (Versol, Lyon, France)] was added to 1ml of peripheral blood for 10 min, and washed 3times with PBS. A minimum of 200.000 cells was used for staining with

fluorochrome-conjugated monoclonal antibodies (moAbs) of interest for 20 min at RT in the dark. For Treg subset characterization and chemokine markers assessment a minimum of 500.000 mononuclear cells were used; 50.000 for intracellular cytokine assessment. For intracellular staining, fixation and permeabilization was carried out according to manufacturer's instructions (eBioscience, San Diego, USA). IL-10 detection was preceded by incubation with Fixable Viability Dye-eFluor 780/Krome Orange (eBioscience) in PBS for 30min at 4 °C in the dark. Samples were measured using a 10-color Navios™ flow cytometer (Beckman Coulter, Fullerton, CA, USA). For manual gating, data were analyzed using Kaluza V2.1 (Beckman Coulter). Gates were set based on isotype controls and a fluorescent-minus-one strategy. Locations of gates were fixed except for CD3/CD56 based selection of NK cells as shown in Supplemental Figure S1. A detailed gating strategy for the various cell subsets and tissue types is shown in Figure S1B.

CITRUS clustering

For unsupervised analysis, manually gated lymphocytes (Figure 1) or CD3 negative lymphocytes (for B cell analysis in Figure 3) were extracted (plug in provided by Beckman Coulter) before further processing using the web-based analysis platform Cytobank (http://cytobank.org/) (61). Lymphocytes and $CD19^{+}$ B cells, respectively, were chosen as input population. Files were assigned to the appropriate gestational-age group, i.e. menstrual blood, 1st trimester, 2nd trimester and term. Unsupervised clustering of lymphocyte and B cell frequencies was performed using the CITRUS tool. Clustering was performed based on abundance with a minimum cluster size of 2% of total cells and a false discovery rate of 1%. For B cell clustering, samples of less than 200 B cell events were excluded from analysis (lowest amount of events included was 246). Clustering was analyzed with prediction analysis for microarrays (PAM), a predictive model to identify features determining gestational age-dependent variation.

B cell stimulation assays

50.000 peripheral blood mononuclear cells or decidual lymphocytes were cultured in the presence or absence of CpG (5μg/ml) and CD40L (1μg/ml) stimulation, in 10% HPS culture media (RPMI 1640 medium supplemented with 1 mM pyruvate, 2 mM glutamax, 100 U/ml penicillin, and 100 μg/ml streptomycin) in a 96-well U-bottom plate for 24h at 37°C in a humidified 5% CO2 incubator. Phorbol-12-myristate-13-acetate (PMA, 50 ng/ml) and ionomycin (1 ug/ml) were added for the final 5h and brefeldin A (5μg/ml; Sigma-Aldrich, St. Louis, USA) for the final 2h of incubation. IL-10 expression of viable B cells was measured by flow cytometry as described.

Immunohistochemistry

Tissue samples were fixed in neutral buffered 4% formalin (Mallinckrodt Baker Inc, Deventer, The Netherlands) for 4h, transferred to 70% ethanol before preparation in a Tissue-Tek VIP tissue processor for embedding in paraffin. Slides were deparaffinized in xylene before rehydration, washing in tap water and boiling in Tris-EDTA buffer (pH 9, Klinipath). 6 μm sections were stained for either IHC or IF staining. For IHC staining, antibody binding was visualized by diaminobenzidine

(Thermo Scientific) or Poly-HRP-goat-anti-mouse/rabbit/rat IgG (BrightVision, Duiven, the Netherlands) with permanent red. IHC staining was assessed microscopically (AxioImager M2; Zeiss, Sliedrecht, the Netherlands) and sections were photographed using a high resolution color camera for bright field microscopy (AxioCam 105 color, Zeiss). Images were assessed using ZEN blue edition version 2.3 (Zeiss). For IF staining, visualization was performed using the Opal seven-color IHC Kit (Akoya Biosciences) on the BOND RX IHC & ISH Research Platform (Leica Biosystems). All staining cycles contained heating steps in between cycles. Tissue sections were counterstained with DAPI and mounted in Fluoromount-G (SouthernBiotech). Images of stained slides were acquired using the Vectra (Vectra 3.0.4, PerkinElmer) and exported with inForm software (Version 2.4.8, Akoya Biosciences). All staining Abs are listed in order of application in Supplemental Table S2.

4

Quantification and statistical analysis

All statistical analysis was performed using GraphPad Prism 5 (La Jolla, CA, USA). All values represent mean percentages ± SEM (error bars). Kruskal-Wallis and post-hoc Dunn's multiple comparison test (non-parametric) were performed to assess differences between mucosal isolates compared to peripheral blood as well as when assessing differences between mucosal tissues. Details on sample size and statistical outcomes can be found in the respective figure legends.

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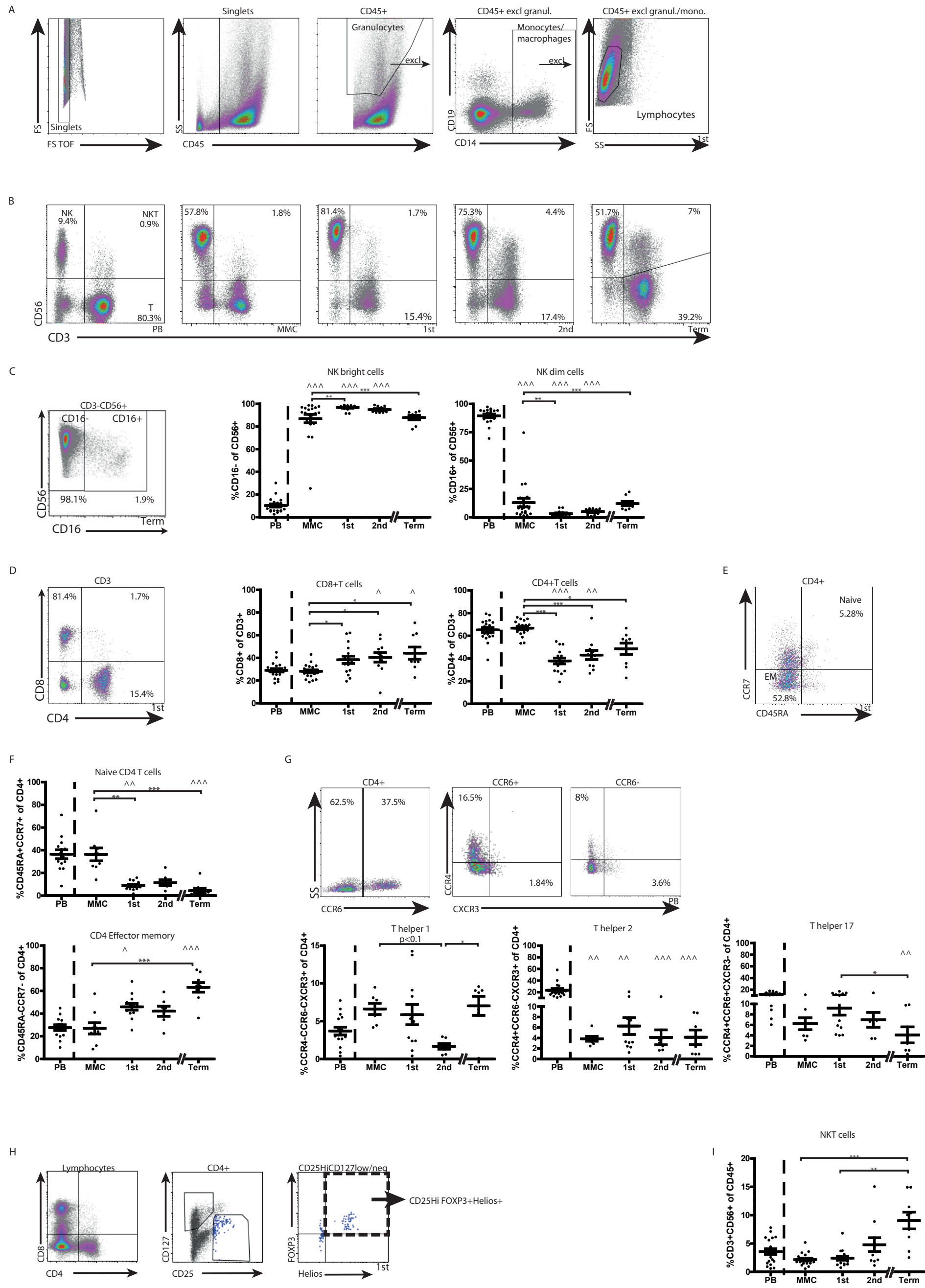
SUPPLEMENTAL INFORMATION



Supplemental Figure S1. Gating strategies and frequencies of menstrual blood and decidual tissue derived lymphocyte frequencies assessed by manual analysis. Related to Figure 1.

(A) Gating strategy to select lymphocytes as starting population of all samples used.
(B) Representative flow cytometry plots to assess general lymphocyte composition of single cell suspensions based on CD3/CD56 expression resulting in frequencies of total NK cells, T cells, and NKT cells.
(C) NK cells separated in NKbright and NKdim subsets based on CD16 expression.
(D) CD4/CD8 expression within all $CD45^+CD3^+$ lymphocytes.
(E +F) Naive and effector memory (EM) cells of all $CD4^+$ lymphocytes.
(G) T cells with $CCR4^-CCR6^-CXCR3^+$, $CCR4^+CCR6^-CXCR3^+$ and $CCR4^+CCR6^+CXCR3^-$ profile. representing respectively T helper 1, T helper 2 and T helper 17 cells
(H) Gating strategy to assess regulatory T cells.
(I) Frequencies of NKT cells.
Except for the depicted gates of CD3/CD56 expression, gate settings across tissues were identical and representative plots show cell distribution within these gates as frequency of parent population. PB, peripheral blood; MMC, menstrual blood mononuclear cells; 1st, first trimester decidual lymphocytes; ^ $p < 0.05$, ^ ^ $p < 0.01$, ^ ^ ^ $p < 0.001$ Kruskal-Wallis and post-hoc Dunn's multiple comparison test (non-parametric) to assess difference between isolated mucosal tissues and peripheral blood; * $p < 0.05$, *** $p<0.001$ Kruskal-Wallis and post-hoc Dunn's multiple comparison test (non-parametric) to compare menstrual blood mononuclear cells, 1st trimester, 2nd trimester and term decidua. Data are represented as mean±SEM.

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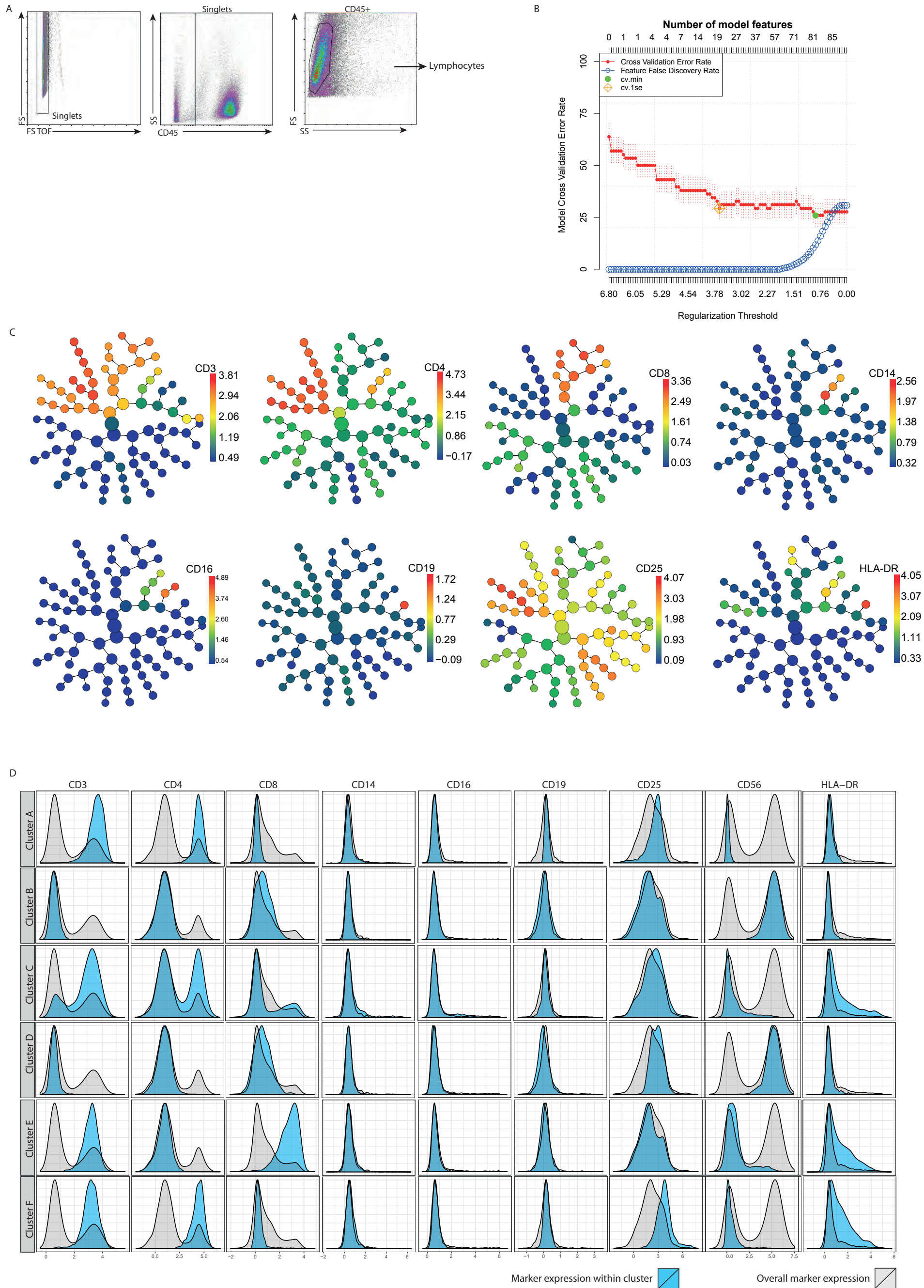
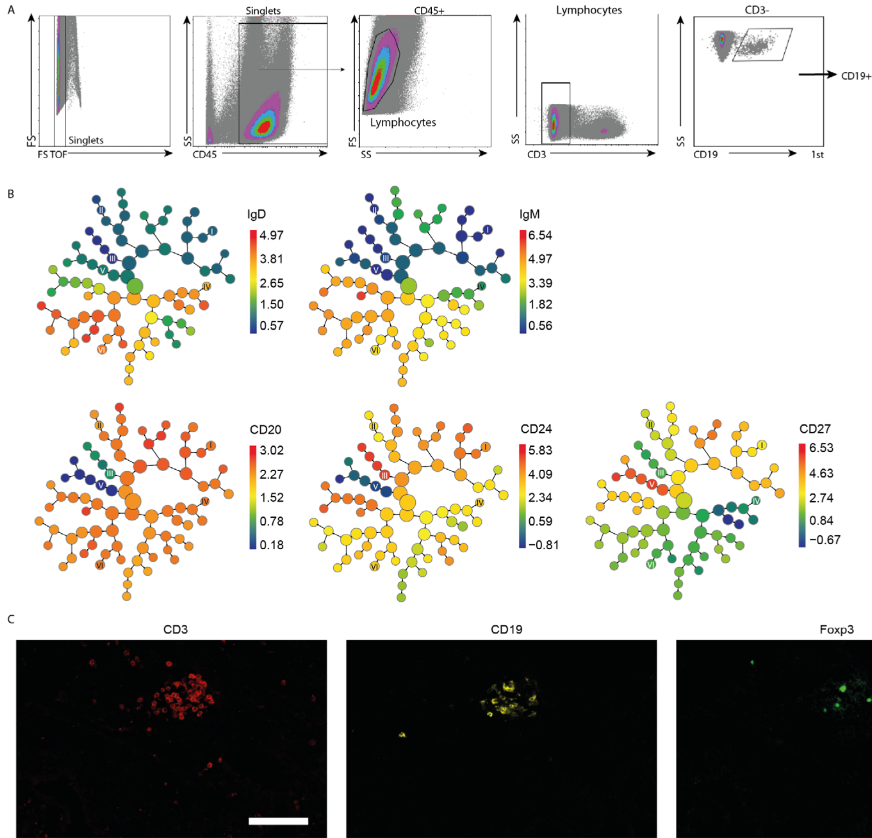


Supplemental Figure S2. CITRUS modelling of general decidual lymphocyte assessment. Related to Figure 1.

(A) Gating strategy preceding unsupervised clustering.
(B) Cross-validation error rate of the nearest shrunken centroid predictive association model. Predictive group assignment based on automatic feature generation using CD3, CD4, CD8, CD14, CD16, CD19, CD25, CD56 and HLA-DR expression data of menstrual blood mononuclear cells (n=18), first trimester (n=18), second trimester (n=10), and term (n=12) decidua. 5686 events were clustered at a minimum cluster size of 2% of all events.
(C) Clustering tree illustrating the relationship between nodes after hierarchical clustering. The color scale shows marker expression per cluster while size of each node represents event frequency.
(D) Cluster-specific expression of the individual markers.



Supplemental Figure S3. Details of decidual B cell analysis. Related to Figure 3 + 4.

(A) Flow cytometry gating strategy to select B cell population within all samples. Gate positions were not changed except for stringent selection of $CD3^-$ and $CD19^+$ events only.

(B) Unsupervised analysis of peripheral blood vs decidual B cells Marker expression of clusters identified by unsupervised analysis of IgD, IgM, CD20, CD24, CD27 and CD38. Color scales illustrates the relative marker expression per cluster while size of each node represents event frequency.

(C) Single components of multiplex assay. Representative example of individual components of CD3 (red), CD19 (yellow), and Foxp3(green). Original magnification x20, scale bar indicates 100μm.

Supplemental Table S1. Demographic characteristics of donor cohort. Related to STAR methods section.

	Peripheral blood[1]	Menstrual blood[2]	1st trimester	2nd trimester	Term[3]
Total subjects (n)[4]	36	28	69	27	12
Age at delivery (years)					
Mean (SD)	22.7 (3.4)	29.5 (6.9)	Anonym.	Anonym.	32 (3.6)
Range	19 - 32	19 - 44	Anonym.	Anonym.	28 - 39
Duration gestation (weeks)					
Mean (SD)	NA	NA	7.7 (2.0)	14.8 (1.1)	>37
Range	NA	NA	4.7 - 12.9	13.0 - 17.3	NA

[1]includes 6 anonymous donors, [2]includes 7 anonymous donors, [3]includes 4 anonymous donors, [4]samples used per experiment listed in according figure legends as not all samples could be used for multiple stainings

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Data S1. Statistics Cluster Analysis, Related to Figure 3. Can be found online at https://doi.org/10.1016/j.celrep.2020.108204.

CHAPTER 5

A combination of immune cell types identified through ensemble machine learning strategy detects altered profile in recurrent pregnancy loss

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ABSTRACT

The underlying cause of recurrent pregnancy loss (RPL) remains largely unknown. As successful implantation and placentation depends on a tightly regulated immune response to facilitate adequate interaction with trophoblast cells, dysregulation of immunity might account for idiopathic RPL. So far, studies focusing on single parameter analysis did not allow for clear classification of immunity in women with RPL versus healthy pregnancies. We here defined immune profiles in peripheral blood (PB) as well as menstrual blood (MB), a source of endometrial cells, of RPL patients and compared them to those of women with healthy pregnancies. Frequencies of 63 immune cell types defined by flow cytometry were included in the analysis, next to age and CMV status. By harnessing the combined value of 8 machine learning classifiers in an ensemble strategy and recursive feature selection, we were able to determine a combination of immune parameters that separated RPL from controls. In PB, the combination of four cell types (non-switched memory B cells, $CD8^{+}CD4^{-}$T cells, $CD56^{bright}$ $CD16^{-}$ Natural Killer (NKbright) cells, $CD4^{+}$ effector T cells) classified samples correctly to their respective cohort. The identified classifying cell types in PB differed from the results observed in MB, where a combination of 6 cell types ($Ki67^{+}CD8^{+}$ T cells, (HLA-DR^{+}) regulatory T cells, $CD27^{+}$ B cells, NKbright cells, Treg cells, $CD24^{Hi}CD38^{Hi}$ B cells) plus age allowed for assigning samples correctly to their respective cohort. This unbiased phenotyping approach focusing on immune profiles rather than single parameters might be of promising diagnostic value and deserves further large-scale validation.

INTRODUCTION

Only 30% of recognized pregnancies progress from conception to live birth (1). Spontaneous pregnancy loss poses a great medical and emotional burden. One to 2% of women suffer from recurrent pregnancy loss (RPL), defined as the consecutive loss of two or more pregnancies before 20 weeks gestation (2, 3). In absence of embryonal chromosome abnormalities, fractions of RPL can be attributed to genetic (2-5%), anatomic (10-15%) endocrine (17-20%) or autoimmune related factors (20%), but over 40% of cases remain unexplained (4, 5). These unexplained cases may be linked to an inadequate maternal immune response during key processes of implantation and placentation at the fetal-maternal interface (6-8). As the mechanisms behind this dysregulation are unknown, it is unclear whether they are contained to the uterus or manifest systemically.

Most investigations to identify dysregulation of immunity in RPL have focused on specific immune cell populations isolated from peripheral blood (PB). This sample source is readily available to study large populations and, due to its limited invasiveness, allows inclusion of large control cohorts. Altered levels of circulating T (helper) cell profiles, regulatory T cells (Treg) and ratios of Natural Killer (NK) cell subsets were observed in RPL patients but none of these cell types were observed individually at levels that allowed robust distinction from women without pregnancy complications (9-13). Large variance, due to inter-populational differences, the time point of sampling or the measurement itself, and the complexity of relationships between immunological parameters hamper detection of cohort differences by commonly applied univariate approaches (14). Furthermore, it has to be considered that endometrial and decidual immune cells are highly specialized to enable adequate interaction with trophoblast cells, through mechanisms independent from PB derived cells (15-19). Consequently, PB cannot serve to detect dysregulation of uterine immunity (20). Menstrual blood (MB) offers non-invasive access to uterine immune cells that display tissues-specific markers and NK cell profiles characteristic to decidua (21-26). This non-invasive sample source is only limitedly investigated until now, yet allows large scale studies to understand reproductive disorders.

Here, we assessed immunophenotypic profiles of peripheral and menstrual blood using an ensemble machine-learning strategy. This approach enabled unbiased multivariate detection of immunological differences in women suffering from RPL.

RESULTS

As immune responses are never limited to a single cell type, minor changes in frequency of a specific subset can affect neighboring cells through cell contact or secretion of soluble factors. While a subtle change in numbers or characteristics of a given immune cell population might fall within a physiological range, hampering its detection, machine learning can identify a change in overall patterns and the underlying cell types involved. To collect a dataset suited for multivariate analysis, we established a phenotypic flow cytometry-based overview of immune cell frequencies (Figure 1A) of PB and MB (n=15, n=18, respectively) of women who suffered from at least two consecutive unexplained miscarriages (patient characteristics, Table 1). The analysis covered total leucocyte populations, T, B, and NK cell subsets using five established staining panels (27)(Supplementary Table 1). In total, 63 immune subsets, age and CMV status were assessed, which results in 65 features that are taken into account for further analysis (Supplementary Figure S1, Supplementary Table 2). Data were compared to a control cohort, of women with uncomplicated pregnancies (PB, n=13; MB n=14). We used machine-learning based cohort classification to identify immune cell subsets that discriminate RPL from control, based on either MB or PB profiles. To achieve this, we employed an ensemble strategy as it allows for robust feature selection in a low sample size setting (28). Through combining 8 distinct classification algorithms, the ensemble overcomes any possible bias of its individual classification algorithms (Lopez-Rincon et al. 2019; Lopez-Rincon et al. 2020). The outcome of the individual algorithms were weighted and combined into a single ensemble ranking (29). The 80% top features of this list were then used to run the individual algorithms, including 10-fold cross-validation to ensure generality of the results, and the average classification accuracy is calculated. By repeatedly reducing the list of top-contributing features by 20%, the optimal number of features to achieve robust classification was determined (Figure 1B). With this approach, we identified that a combination of 4 cell types for PB (non-switched memory B cells, $CD8^{+}CD4^{-}$T cells, NKbright cells, $CD4^{+}$ effector T cells; Figure 1C left panel, Supplementary Figure 3), and 6 cell types for MB ($Ki67^{+}CD8^{+}$ T cells, HLA-DR^{+} Treg, $CD27^{+}$ B cells, NKbright cells, Treg cells, $CD24^{Hi}CD38^{Hi}$ B cells; Figure 1C right panel, Supplementary Figure 3), together with age, allowed for optimal classification as RPL versus control. After determination of the features used for classification, the individual classifying algorithms were run using the respective immunological parameters. This resulted in a maximum average area under the curve (AUC) of 0.82 ± 0.23 for PB, and 0.90 ± 0.17 for MB when analyzed by PassiveAgressive Classifier (Figure 1D), with an accuracy of 0.87 ± 0.16 and 0.84 ± 0.14, respectively (Supplementary Table 3).

Reducing the number of features included in a multivariate approach allows for more robust outcomes as features of high variance, with minor distinctive value, can be excluded. However, this approach might mask notable differences of an individual feature, as only the most contributing

node in a network of related variables is considered. Thus, we additionally assessed all studied features by classical bivariate analysis. No differences in total leucocyte populations or frequently studied T helper cell subsets were detected (Supplementary Figure S3). Whereas no altered NK cell subset frequencies were observed in PB of RPL patients, MB revealed decreased fractions of NKbright cells, and an increased percentage of $CD16^{+}CD56^{+}$ NKdim cells (Figure 2 A,B).

Table 1. Cohort characteristics.

		Control	RPL
PB	n	13	15
	Age in years, median (range)	34 (27 - 46)	34 (28 - 42) ^
	CMV positive	6/13 (46%)	4/12 (33%) ^ ^
MB	n	14	18
	Age in years, median (range)	34 (27 - 46)	33 (28 - 46)
	CMV positive	6/13 (54%) ^	6/14 (43%) ^ ^ ^

^ Information NA for one donor, ^ ^ three, or ^ ^ ^ four donors. RPL: Recurrent pregnancy loss. PB: peripheral blood, MB: Menstrual blood.

Latent cytomegalovirus (CMV) infection is known to selectively affect expansion of leucocyte subsets, especially regarding subsets of NK cells, T cells and B cells (30-33). Of all assessed features, a difference based on CMV status was only observed for CD27+ B cells in MB of RPL patients (Figure 2C). RPL patients who were CMV seropositive showed highest frequencies of CD27 expressing B cells.

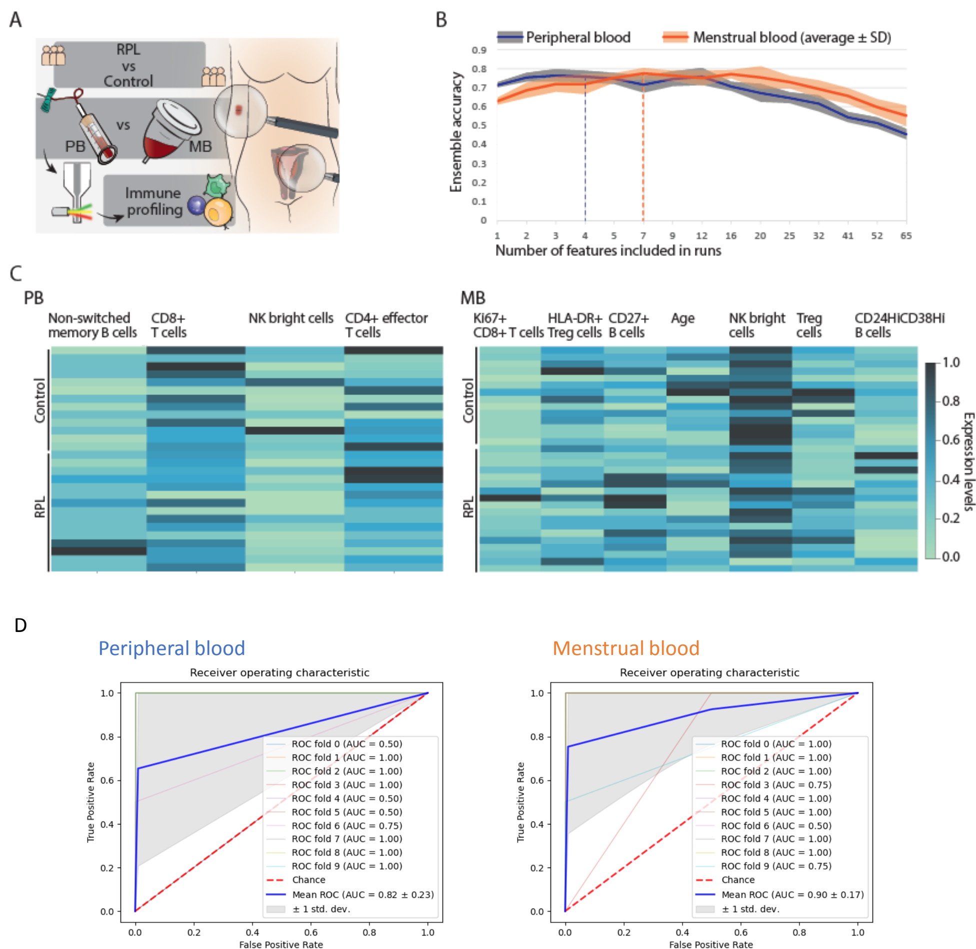

Figure 1. Machine learning approach using ensemble strategy to determine immune features that identify patients of recurrent pregnancy loss (RPL).

(A) Schematic representation of study design. A flow cytometry based dataset was established to describe the immune profile of systemic or uterine immunity, through leucocyte isolation from peripheral blood (PB) or menstrual blood (MB), respectively. **(B)** Number of feature combinations yielding optimal classification accuracy. Eight machine learning classifiers were used to established a ranked list of top features that were recursively reduced. Accuracy is depicted as average and standard deviation of 10 runs. **(C)** Heatmap of the features allowing for optimal accuracy regarding data from PB (left panel) or MB (right panel). **(D)** Receiver operating characteristics (ROC) curve showing the average area under the curve (AUC) using PassiveAgressive classifier of PB (left panel) and MB derived immune features (right panel) after stratified 10-fold cross-validation. ROC curves of the remaining classifiers are depicted in Supplementary Figure S2.

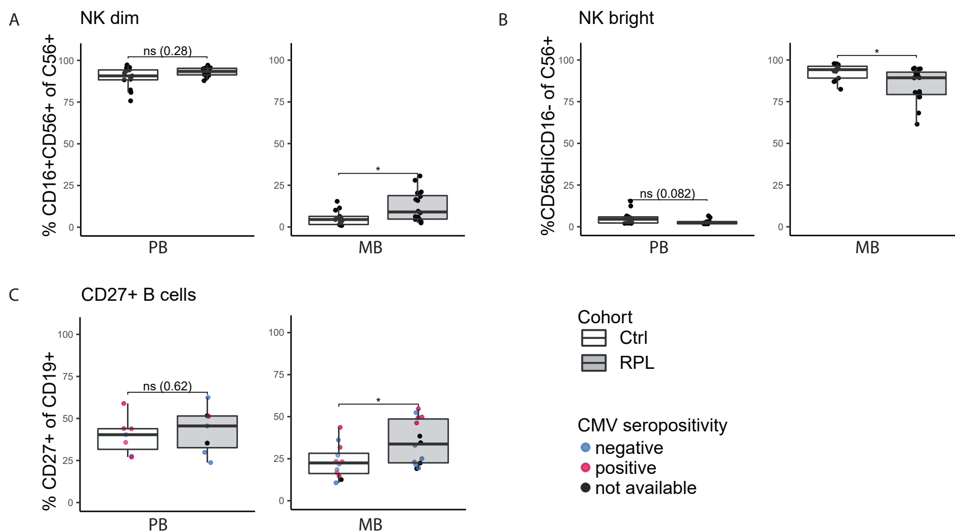

Figure 2. Univariate analysis of immune profiling shows uterine-specific deviations in RM patients.

(A+B) NK cell profiles of peripheral blood (PB) and menstrual blood derived cells (MB). **(C)** Cytomegalovirus (CMV) seropositivity of CD27+ B cells. Additional results of univariate analysis are depicted in Supplementary Figure S3. Data were compared by Mann-Whitney/Wilcoxon tests (non-parametric), * $p < 0.05$, ns= non-significant. Ctrl, control; RPL, recurrent pregnancy loss.

DISCUSSION

Adaptations of the maternal immune system during pregnancy occur not only systemically, but also locally, where immune cells and soluble factors contribute to the contact of trophoblast cells and the maternal mucosa. Subtle changes in immunological profiles of both compartments may contribute to the etiology of recurrent pregnancy loss. To create a better understanding of dysregulated immunity in RPL, we assessed in how far the joint assessment of leucocyte subsets might reveal differences in PB or MB of affected women. By using an ensemble machine learning strategy and recursive feature selection, we were able to identify 4 cell subsets of PB and 6 cell subsets and age for MB, which, when combined, robustly allowed for cohort classification for either PB or MB. Based on general definitions describing the discriminative power of a diagnostic test, both PB and MB had “excellent” distinctive value (34, 35). Thus, RPL is associated with immune adaptations that can be traced back systemically and in the uterus.

Classic univariate approaches often do not suffice when studying immunological data (14). Large overlap between frequencies in RPL and control cohort measured in PB and MB hampers the individual discriminative value of a cell type. Furthermore, differences in cell types of low

5

abundance and high variation are challenging to detect through univariate analysis but could be revealed using the presented approach. As the differences in AUC of the different individual classifiers show, algorithms of multivariate approaches also present with varying power to classify a sample to its cohort. The ensemble machine learning strategy overcomes the bias associated with choosing a single machine learning algorithm.

The power of combining classifiers, even in this low sample size setting, allowed to detect the discriminative value of low frequency cell types in MB that have a possible regulatory phenotype. $CD8^+$ T cells positive for Ki67 (indicating proliferation) were identified as most classifying feature in MB samples. In healthy pregnancy, decidual $CD8^+$ T cells are known to take longer than systemic cells to initiate proliferation upon stimulation, possibly through interaction of their coinhibitory molecules with the decidual microenvironment (36). Also involved in local regulation (19, 37-39), altered levels of Treg (overall cell population and $HLA\text{-}DR^+$), and $CD24^{Hi}CD38^{Hi}$ B cells contributed to robust RPL classification, fitting with the theory that pregnancy is negatively affected when local mechanisms of tolerance fail (40, 41). This focus on immune regulatory subsets was not observed in PB, except for NKbright cells, which contribute to cohort classification in both PB and uterine samples. Of note, age contributed as risk factor in uterine, but not systemic, immunity. The observed differences in classifying leucocyte subsets, depending on the sample source, affirm that systemic and local immunity of reproduction demand independent consideration.

Circulatory non-switched memory B cells contributed strongly to cohort classification of PB, which is in line with previously shown higher levels of circulatory non-switched memory B cells of RPL patients (13, 42). The presented data highlight that B cells deserve consideration in context of RPL. Memory B cells are altered in many autoimmune diseases such as systemic lupus erythematosus, systemic sclerosis, and antiphospholipid syndrome, known to be associated with poor reproductive outcome (43). B cell-related pregnancy complications might also result from incorrect activation through soluble factors that regulate B cell activity, as serum from women suffering from spontaneous abortion failed to induce normal levels of IL-10 production by B cells (44). Of note, we observed higher levels of $CD27^+$ B cells in CMV seropositive RPL patients. It has previously been shown that CMV affects frequencies of $CD27^+$ memory B cells in women, but not men (45). Thus, CMV status is important to include in similar approaches studying leucocytes due to its known effect of selectively expanding specific subsets (30, 33).

RPL is a multifactorial condition (46). Small sample size is a limitation of the current study, as we cannot take into account subgroups of different in disease etiologies. Nevertheless, we were able to show that investigating a immunity by multivariate approach in RPL is worthwhile. The applied flow cytometry-based profiling of MB holds the possibility to detect locally involved rare cell types (47-50). Compared to the trending, high-dimensional approaches using mass spectrometry or single-cell sequencing, flow cytometry is relatively cheap to perform and thus

allows analysis of large cohorts. More women suffering from RPL have to be enrolled to account for the heterogeneity of this cohort. Combined with inclusion of a validation cohort, this will ensure generality of results to enable translation towards diagnostic tests. This is of special interest for RPL, a condition that not only needs proper diagnosis, but would benefit from repeated sampling in order to assess the success of intervention strategies. To both ends, classification needs to be robust and accurate, and deserves further assessment.

Taken together, RPL patients present with a dysregulated immune environment, systemically and within the uterus. Cohort classification of possible diagnostic value cannot rely on individual immune cell frequencies but rather depends on a combination of immune cell subsets. The non-invasive source of menstrual blood, allowing to investigate important regulatory mechanisms, holds many opportunities for the assessment and monitoring of reproductive health.

MATERIALS AND METHODS

Sample collection

Peripheral and menstrual blood was collected from women without known disorders of reproduction, with regular menstrual cycles, and women suffering from recurrent pregnancy loss (RPL). Characteristics of women included in this study are documented in Table 1. Exclusion criteria were autoimmune diseases, smoking and current use of an intra-uterine device or hormonal contraceptives. 10 ml of PB was collected in ACD-A tubes. Women used a menstrual cup (Femmecup Ltd, London, UK) for 3 12h intervals during the first 36h of menstruation. Menstrual effluent was collected in supplemented RPMI 1640 media (pyruvate (1 mM), glutamax (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) (all Thermo Fisher Scientific, Waltham, USA), 10% human pooled serum (HPS, manufactured in-house), and 0.3% sodium citrate (Merck, Darmstadt, Germany) for storage at room temperate until processing. Samples were transferred to the lab for immediate processing, within max. 24h after the last collection. Written informed consent was obtained for all volunteers in accordance with the Dutch Medical Research Involving Human Subject Act (Commissie Mensgebonden Onderzoek Arnhem-Nijmegen, Nr 2017-3256).

Leucocyte isolation

Leucocytes from PB were isolated through density gradient centrifugation (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway). MB was processed as described previously (25, 37). Briefly, menstrual blood derived cells were obtained after granulocyte depletion using RosetteSep (Stemcell technologies Inc, Vancouver, Canada) followed by density gradient centrifugation (Lymphoprep).

Flow cytometry

For surface staining, a minimum of 250.000 PB or MB was stained using fluorochrome-conjugated monoclonal antibodies (moAbs) for 20min at room temperature in the dark. An overview of all moAbs used is shown in Supplementary Table 2. Staining of intracellular moAbs was performed with a minimum of 500.000 cells. Samples were fixed and permeabilized according to manufacturer's instructions (eBioscience, San Diego, USA). Flow cytometry data were analyzed using Kaluza (Beckman Coulter, v2.1). Gate settings (Supplementary Figure S1) were based on a fluorescence minus one strategy. Isotype controls were applied.

Data analysis

Data were processed using R v.4.0.2 and the ggpubr, ggplot2, ggsignif, tidyr packages. Non-parametric Mann-Whitney test was used. Values of $p < 0.05$ were considered statistically significant. An ensemble feature selection was used to detect of features allowing for cohort classification. This strategy was previously designed and validated to overcome the bias of using a single machine-learning algorithm, thus allowing for a more robust selection of classifying features (29, 51). Eight classifiers (Bagging, Gradient Boosting, Logistic Regression, Passive-Aggressive regression, Random Forest, Ridge regression, SGD (Stochastic Gradient Descent on linear models), SVC (Support Vector Machines Classifier with a linear kernel) classifier) were run in 10-fold and used to score features on their importance for classification. Scoring of the individual algorithms was combined in an ensemble ranking: for Bagging, Gradient Boosting and Random Forest analysis that work with classification trees, features of the trees' splits were counted and ranked by frequency; for PassiveAggressive, Logistic, and Ridge regression, SGD, and SVC classifier feature importance was assigned by the coefficients' value associated with each feature. The ranking of each classifier was scored based on times it appeared within the top classifying features. A detailed description of the ranking used for the ensemble strategy has previously been presented (29). To reduce the number of features to the ones that allow for optimal classification, classifiers were run repeatedly with the top 80% features in a recursive feature selection approach. All classifiers were subjected to stratified 5-and 10-fold cross-validation. Having determined which features allow for the most robust classification, the set of parameters was used to run the individual classifying algorithms, combined with 10-fold cross-validation.

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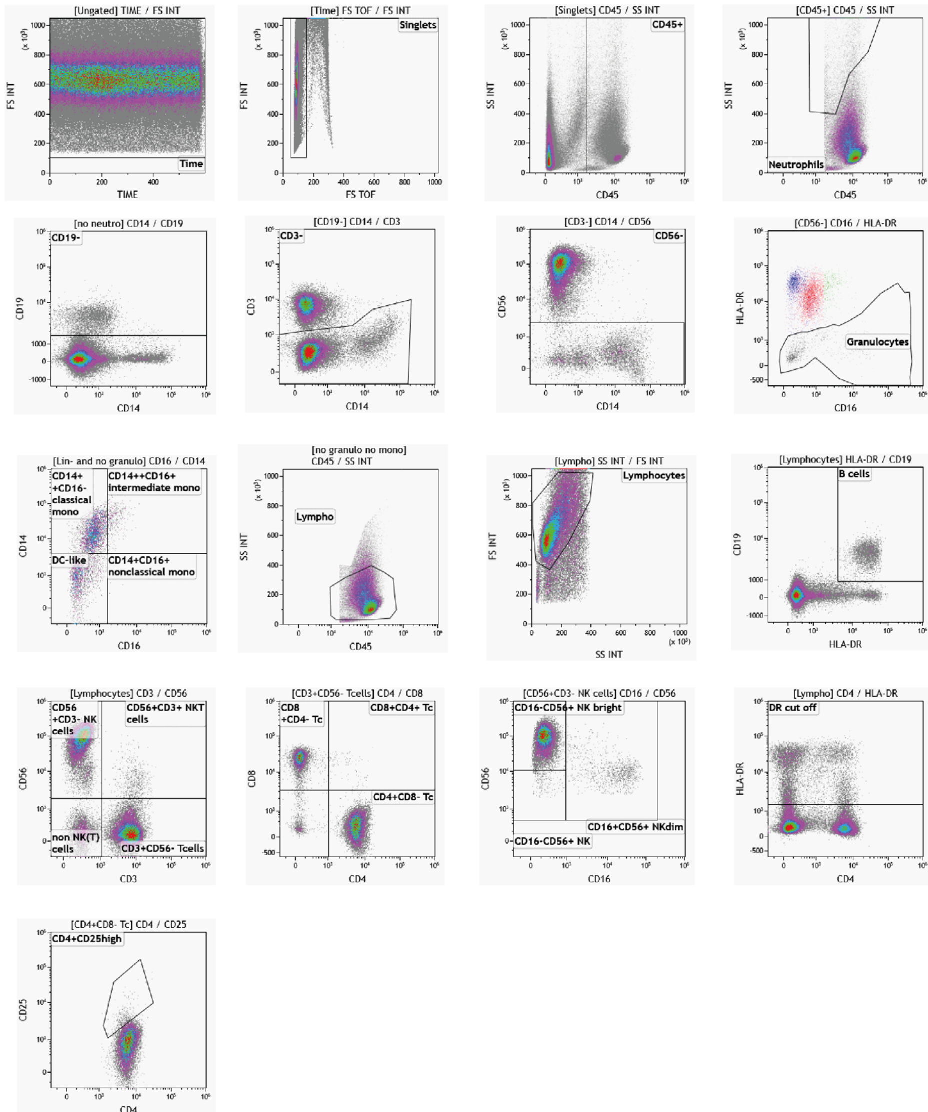
SUPPLEMENTAL INFORMATION

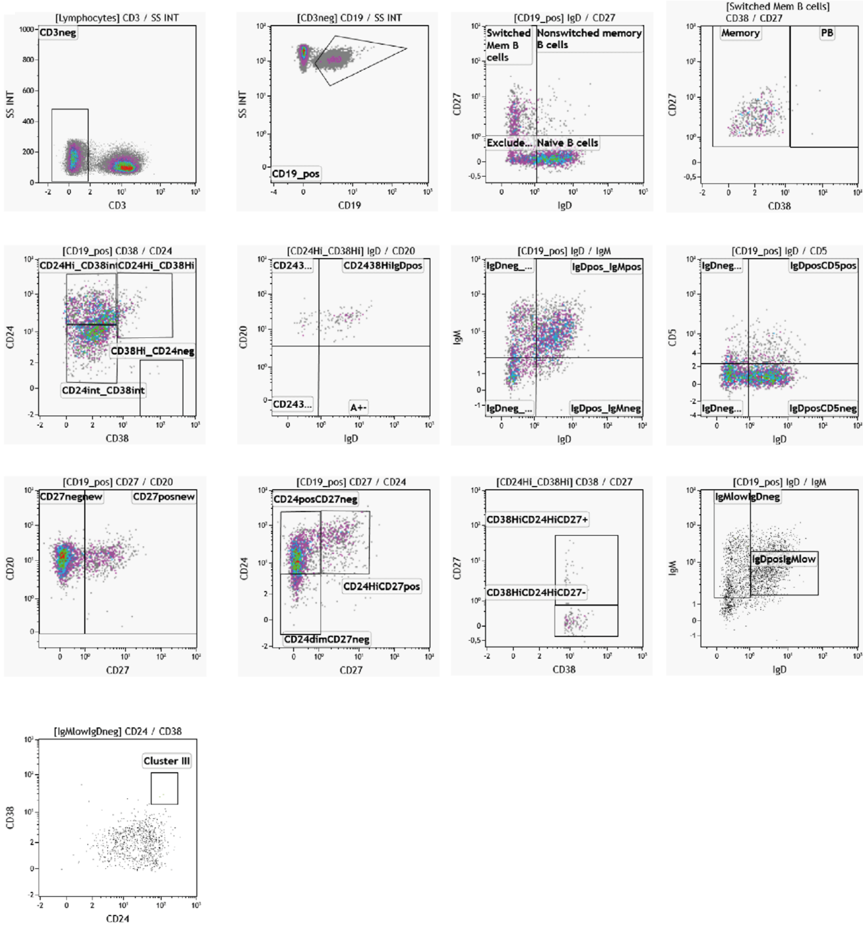
A



Supplementary Figure 1. Flow cytometry gate settings for features included in analysis.

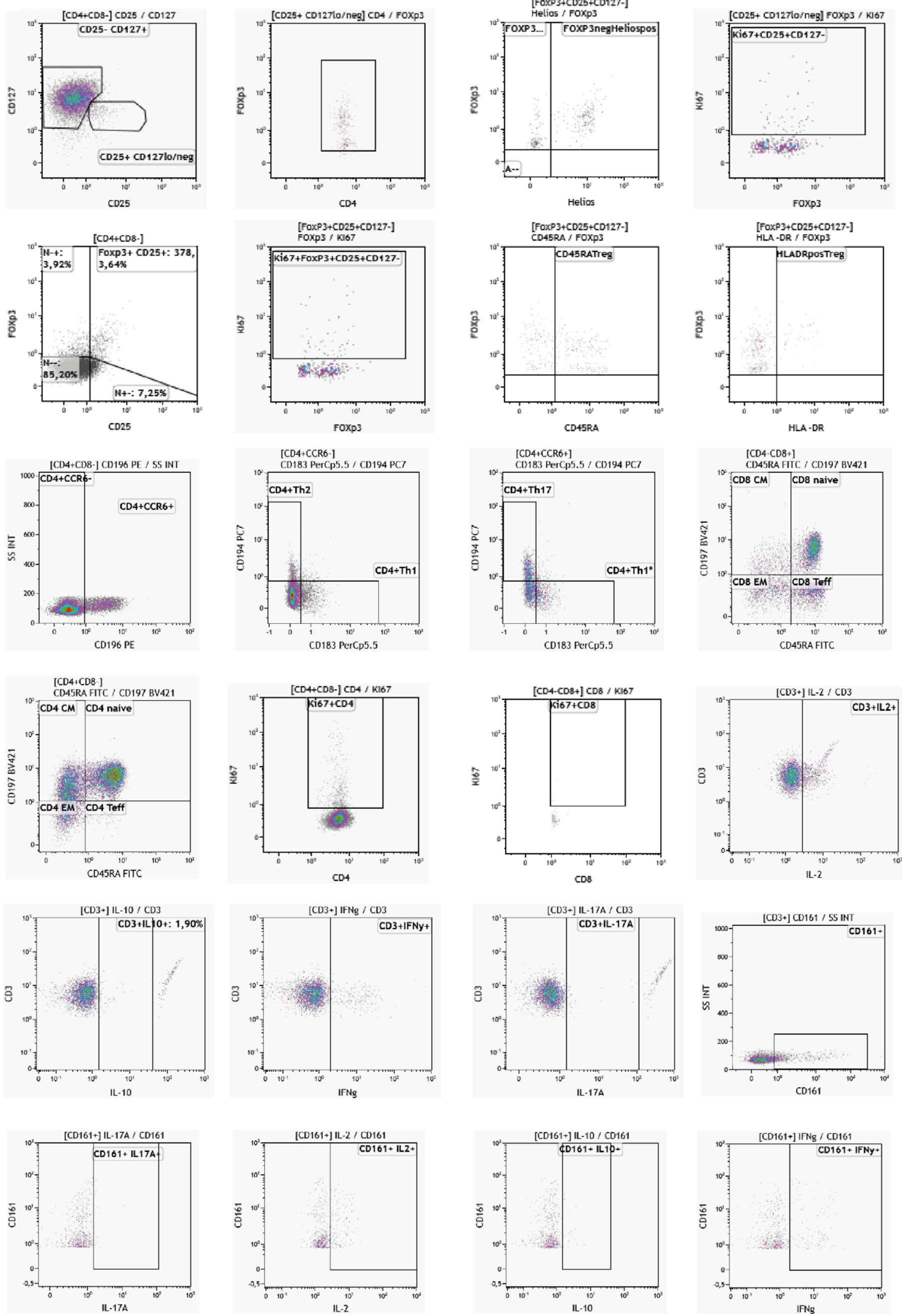
(A) Overall leucocyte subsets. **(B)** B cell subsets and **(C)** T cells subsets are shown on the following pages. An overview of all included leucocyte subsets is shown in Supplementary Table 2.

B

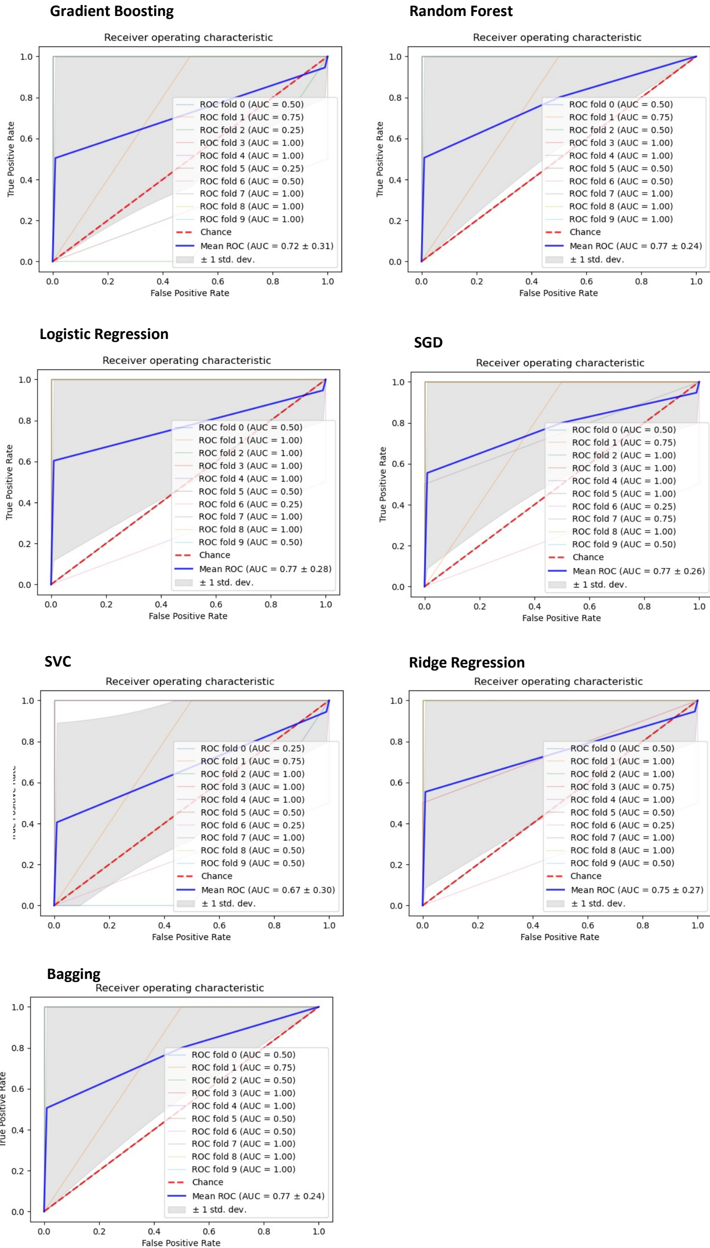


Supplementary Figure 1. Continued

C



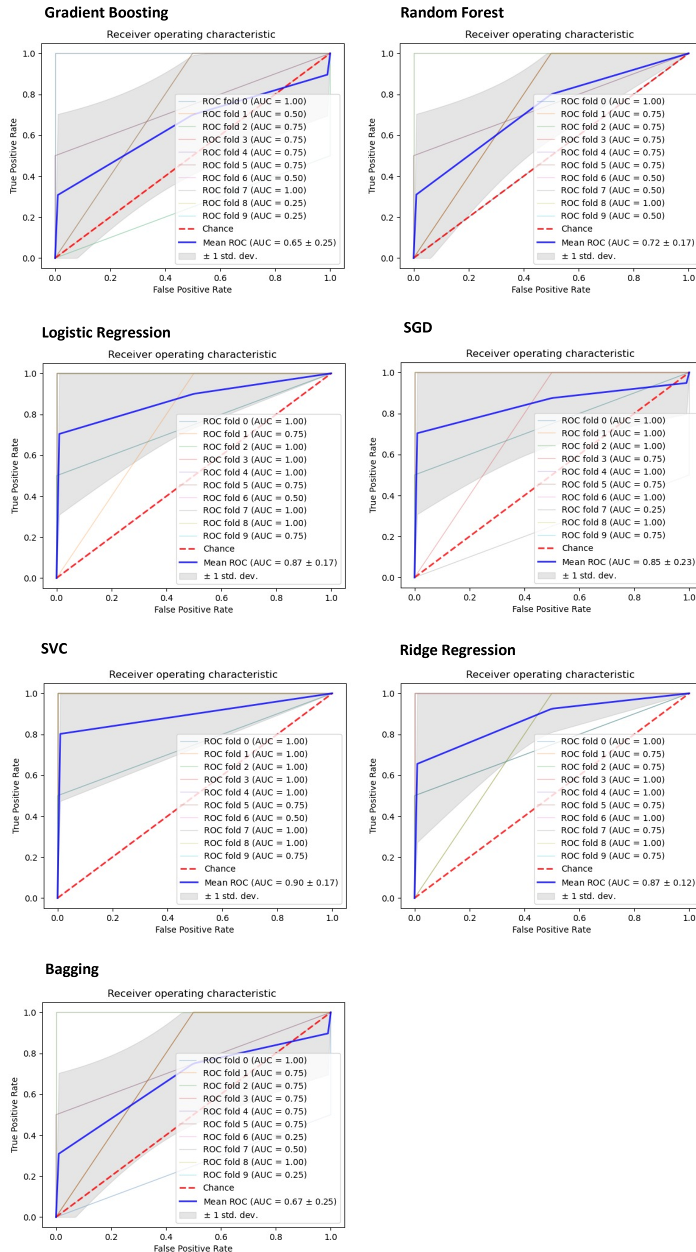
Supplementary Figure 1. Continued



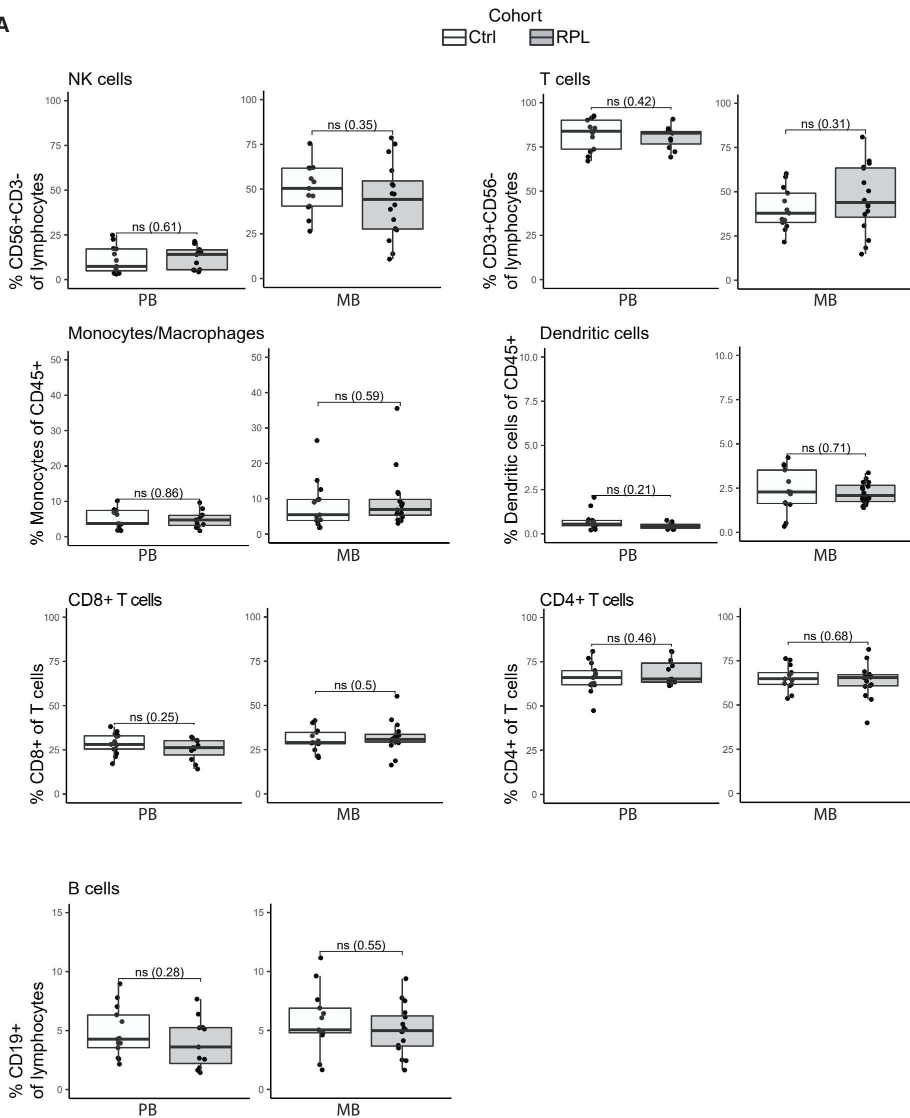
Supplementary Figure S2. Receiver operating characteristic curve of the individual classifiers based on features selected by the ensemble approach.

(A) Results for peripheral blood derived mononuclear cells (PBMC), **(B)** menstrual blood derived mononuclear cells (MBC) on next page.

B **Menstrual blood**

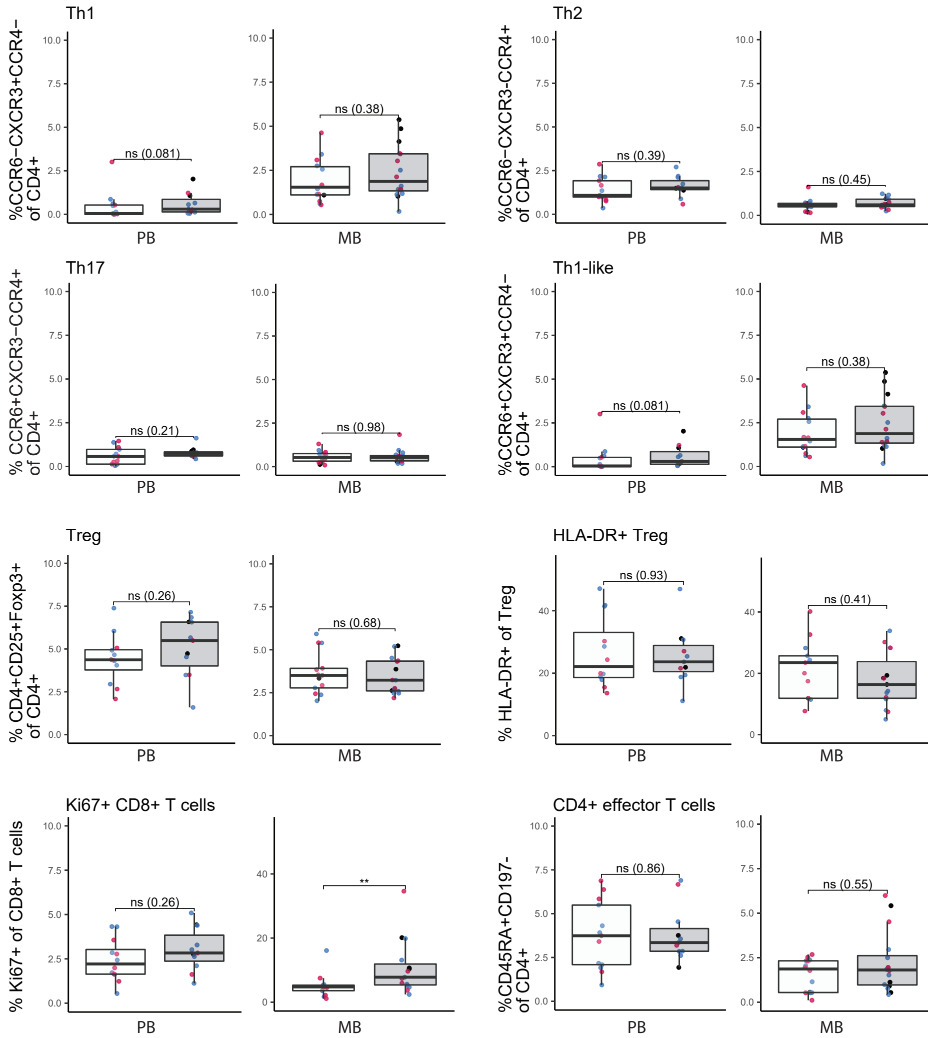


Supplementary Figure S2. Continued



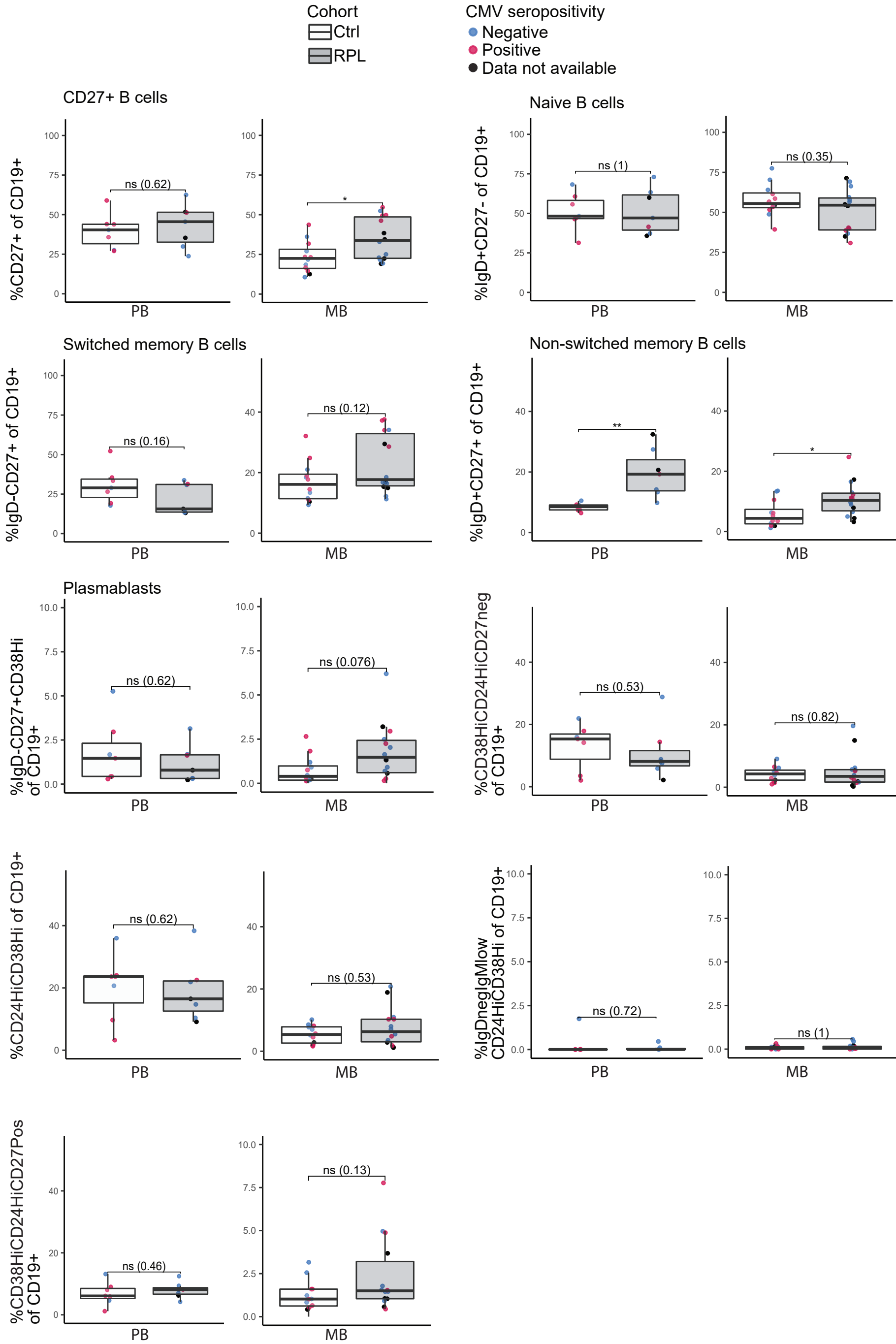
Supplementary Figure S3. Univariate analysis of immune cell subsets of leucocytes isolated from peripheral blood (PB) and menstrual blood (MB).

Continued on following pages. Data were compared by Mann-Whitney/Wilcoxon tests (non-parametric), * $p < 0.05$, ns= non-significant. Ctrl, control; RPL, recurrent pregnancy loss.



Supplementary Figure S3. Continued

5



Supplementary Figure S3. Continued

Supplementary Table 1. List of monoclonal antibodies used in 5 panels.

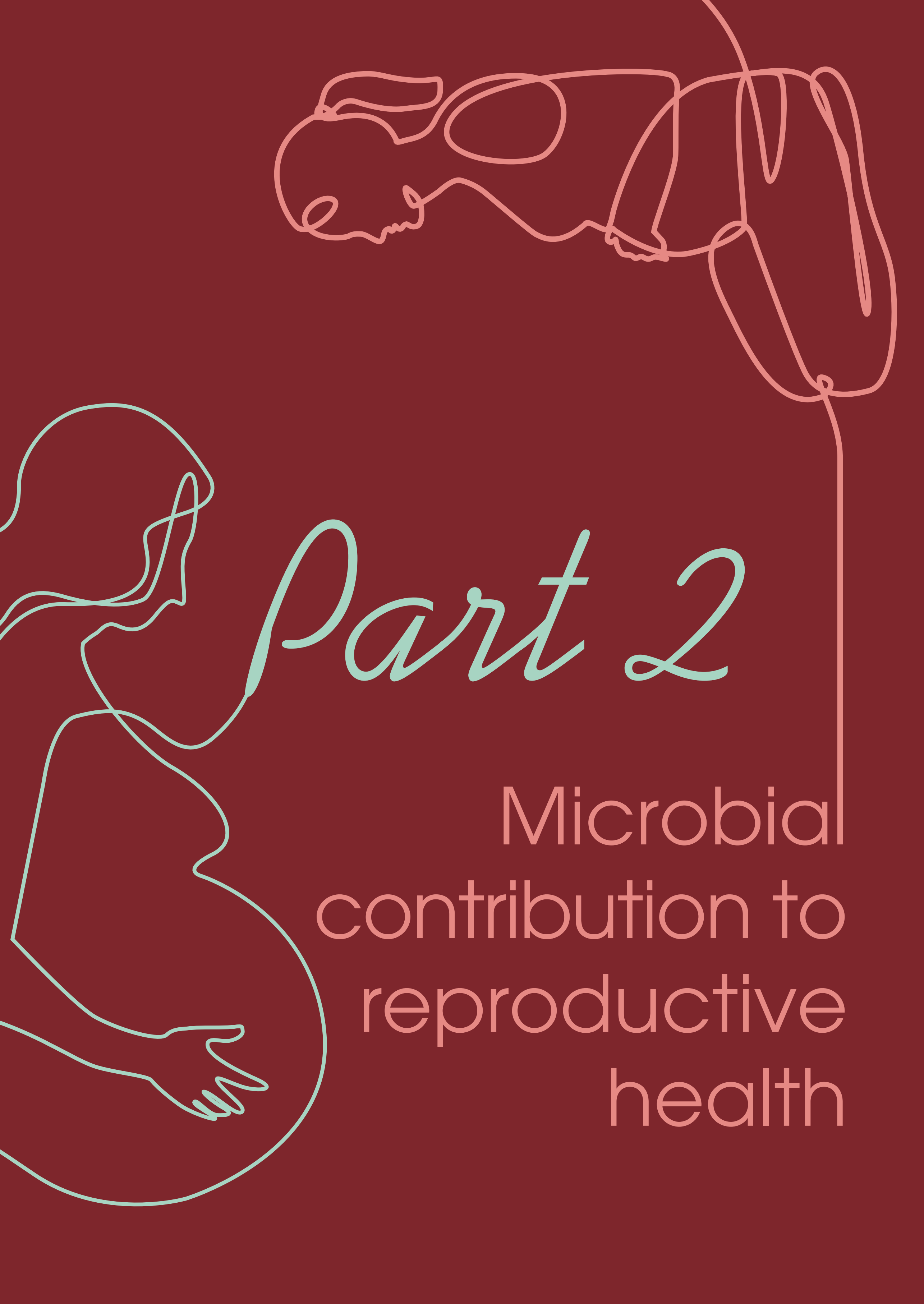
Antibody	Producer	Catalogue #
CD3-ECD	Beckman Coulter	A07748
CD3-PB	Beckman Coulter	B49204
CD45-KO	Beckman Coulter	B36294
CD45RA-ECD	Beckman Coulter	B49193
CD45RA-FITC	Beckman Coulter	A07786
CD4-AF700	eBioscience	56-0049-42
CD4-PE-Cy.5.5	Beckman Coulter	B16491
CD56-APC	Beckman Coulter	IM2474
CD5APCAF700	Beckman Coulter	A78835
CD8-APCAF700	Beckman Coulter	B49181
CD8-APCAF750	Beckman Coulter	A94683
CD8-ECD	Beckman Coulter	737659
CD14-ECD	Beckman Coulter	B92391
CD16- FITC	Beckman Coulter	B49215
CD19-APCAF750	Beckman Coulter	A94681
CD20-PB	Beckman Coulter	B49208
CD24-APC	Beckman Coulter	A87785
CD25-APC	BD	340907
CD25-PC7	BD	557741
CD25-PC7	BD	557741
CD27-PE-Cy5.5	Beckman Coulter	B21444
CD38-PC7	Beckman Coulter	B49198
CD127-APCAF700	Beckman Coulter	A71116
CD161-APC	Miltenyi	130-092-678
CD183-Percp5.5	Biolegend	353714
CD194-PC7	BD	557864
CD195-APCCy7	BD	557755
CD196-PE	BD	559562
CD197-BV421	Biolegend	353208
FoxP3-PB	eBiocience	48-4776-42
Helios-APC	Biolegend	137218
HLA-DR- PE	Beckman Coulter	IM1639
HLA-DR-PE	Beckman Coulter	IM1639
IFN-y-PC7	eBioscience	25-7319-82
IgD-FITC	Southern Biotech	2032-02
IgM-PE	Beckman Coulter	B30657
IL-10-PE	BD	559330
IL-17-APCAF780	eBioscience	47-7179-42
IL-2-FITC	BD	554565
KI67-FITC	BD	561165

Supplementary Table 2. List of leucocyte subsets included in multivariate analysis.

General leucocytes:	B cells:	T cells:		
CD45Pos	CD19pos CD3neg	CD4Pos CD8Neg	CD25Pos CD127loToneg	CD3Pos IL2Pos
TotalNrCD45	CD27pos	CD8 CM	Heliosneg	CD3Pos IL10Pos
Monocyte_all (=classical, intermediate and nonclassical monocytes)	Naive B cells	CD8 EM	Heliospos	CD3Pos IFNyPos
DC (=Number of DC-like/Number of CD45+)	Nonswitched memory B cells	CD8 naive	Ki67Pos CD25Pos CD127Neg	CD3Pos_IL17A
Lympho	Switched Mem B cells	CD8 Teff	Foxp3Pos CD25Pos	CD161Pos IL2Pos
B cells	Memory B cells	CD4 CM	Ki67Pos FoxP3Pos CD25Pos CD127Neg	CD161Pos IL10Pos
T cells	Plasmablast	CD4 EM	CD45RApos Treg	CD161Pos IFNyPos
NK cells	CD24Hi CD38Hi	CD4 naive	HLADRpos Treg	CD161Pos IL17APos
NKT cells	Cluster III (=IgMlowIgDnegCD24^{Hi}CD38^{Hi})	CD4 Teff	Ki67Pos CD4	CD4Pos IL2Pos
CD8Pos CD4Neg Tc	CD38Hi CD24Hi CD27Pos	Th1	Ki67Pos CD8	CD4Pos IFNyPos
CD4Pos CD8Neg Tc	CD38Hi CD24Hi CD27Neg	Th2		CD4Pos IL17APos
CD4Pos CD25high		Th17		CD4Pos IL10Pos
CD16Neg CD56Pos NK bright		Th1like		
CD16Pos CD56Pos NKdim		Th1Th2 ratio		
CD16Neg CD56Pos NK				
CD56pos CD16neg (=CD56 bright plus CD56dim)				

Supplementary Table 3. Average accuracy of individual classifiers, upon 10-fold cross-validation.

	Peripheral Blood		Menstrual Blood	
	Average	**SD**	**Average**	**SD**
SGDClassifier	0,9	0,15	0,84	0,11
SVC(linear)	0,83	0,22	0,9	0,08
LogisticRegression	0,83	0,22	0,85	0,09
PassiveAggressive Classifier	0,87	0,16	0,84	0,14
GradientBoosting Classifier (n_estimators=300)	0,72	0,21	0,62	0,08
RandomForest Classifier (n_estimators=300)	0,75	0,17	0,64	0,21
RidgeClassifier	0,87	0,22	0,81	0,19
BaggingClassifier (n_estimators=300)	0,78	0,18	0,63	0,2



Part 2

Microbial contribution to reproductive health

CHAPTER 6

How uterine microbiota might be responsible for a receptive, fertile endometrium

By Marilen Benner, Gerben Ferwerda, Irma Joosten,
and Renate G. van der Molen.

Human Reproduction Update, 2018

ABSTRACT

Background

Fertility depends on a receptive state of the endometrium, influenced by hormonal and anatomical adaptations, as well as the immune system. Local and systemic immunity is greatly influenced by microbiota. Recent discoveries of 16S rRNA in the endometrium and the ability to detect low-biomass microbiota fueled the notion that the uterus may be indeed a non-sterile compartment. To date, the concept of the "sterile womb" focuses on in utero effects of microbiota on offspring and neonatal immunity. However, little awareness has been raised regarding the importance of uterine microbiota for endometrial physiology in reproductive health; manifested in fertility and placentation.

Objective and rationale

Commensal colonization of the uterus has been widely discussed in the literature. The objective of this review is to outline the possible importance of this uterine colonization for a healthy, fertile uterus. We present the available evidence regarding uterine microbiota, focusing on recent findings based on 16S rRNA, and depict the possible importance of uterine colonization for a receptive endometrium. We highlight a possible role of uterine microbiota for host immunity and tissue adaptation, as well as conferring protection against pathogens. Based on knowledge of the interaction of the mucosal immune cells of the gut with the local microbiome, we want to investigate the potential implications of commensal colonization for uterine health.

Methods

PubMed and Google Scholar were searched for articles in English indexed from January 1st, 2008 to March 1st, 2018 for "16S rRNA", "uterus" and related search terms to assess available evidence on uterine microbiome analysis. A manual search of the references within the resulting articles was performed. To investigate possible functional contributions of uterine microbiota to health, studies on microbiota of other body sites were additionally assessed.

Outcomes

Challenging the view of a sterile uterus is in its infancy and, to date, no conclusions on a "core uterine microbiome" can be drawn. Nevertheless, evidence for certain microbiota and/or associated compounds in the uterus accumulates. The presence of microbiota or their constituent molecules, such as polysaccharide A of the *Bacteroides fragilis* capsule, go together with healthy physiological function. Lessons learned from the gut microbiome suggest that the microbiota of the uterus may potentially modulate immune cell subsets needed for implantation and have implications for tissue morphology. Microbiota can also be crucial in protection against uterine infections by defending their niche and competing with pathogens. Our review highlights the need for well-designed studies on a "baseline" microbial state of the uterus representing the optimal starting point for implantation and subsequent placenta formation.

Wider implications

The complex interplay of processes and cells involved in healthy pregnancy is still poorly understood. The correct receptive endometrial state, including the local immune environment, is crucial not only for fertility but also placenta formation since initiation of placentation highly depends on interaction with immune cells. Implantation failure, recurrent pregnancy loss, and other pathologies of endometrium and placenta, such as pre-eclampsia, represent an increasing societal burden. More robust studies are needed to investigate uterine colonization. Based on current data, future research needs to include the uterine microbiome as a relevant factor in order to understand the players needed for healthy pregnancy.

INTRODUCTION

Maternal microbial colonization contributes to development of the unborn child by micronutrient provision, xenobiotic metabolism and enhancing maternal energy conversion (1). Currently, the concept of the "sterile womb", the paradigm that the fetus grows up in a sterile environment, is highly debated (2). If bacteria (or their compounds) were naturally present in the uterus, their role even before pregnancy, in maintenance of the uterus deserves attention. Various recent reviews focused on correlations between commensal uterine colonization, fertility problems and pregnancy complications (3-6).

Bacteria are known to affect immunity (7, 8). If they already impact the immune environment of the uterus before pregnancy, this would greatly impact the receptive potential of the endometrium, as well as the ability to correctly initialize placenta formation. Successful embryo implantation requires both a synchronous development and an intricate interplay between the hatched blastocyst and endometrium. This depends on a receptive state of the endometrium. In the first week after fertilization, the blastocyst makes initial contact with the highly specialized endometrium. During the female cycle, if the endometrium fails to undergo the proper adaptations to acquire this receptive state, infertility and impaired placentation may be the consequence. Since bacteria can play a role in morphological changes of, for example, mucosal cells, implications of microbiota in decidualization can be envisioned. At the same time, commensal microbiota may convey protection towards pathogenic species contributing to uterine health.

Studies evaluating the endometrial microbiome and its role in fertility are limited and therefore we depend on extrapolating knowledge from other colonized body sites. A vast number of studies focusing on the gut microbiota have pointed out the contribution of microbes to immunity and development, and the necessity of commensal colonization to achieve a basal, healthy immune state (7, 9, 10). We hypothesize that, if indeed present, the endometrial microbiome or its compounds can have implications for (pre-) decidualization, and therefore fertility. The objective of this review is to outline the available evidence regarding uterine microbiota, focusing on recent findings based on 16S rRNA, and to depict the possible importance of uterine colonization for a receptive endometrium. We aim to highlight a possible role of uterine microbiota in host immunity and tissue adaptation, as well as conferring protection against pathogens.

THE NON-STERILE UTERUS?

Microbiota and mammals depend on their symbiotic relationship. While microbes receive a steady nutrient supply through the host, the host benefits from vital contributions of the microbe to physiological processes such as epithelial homeostasis, and is supplied with a natural barrier against colonization by pathogenic species (11, 12). Bacterial colonization also plays a crucial role in modulation of host immunity. Metagenomic analysis has propelled research on natural colonization of the human body forward, revealing microbiota at body sites that were previously considered to be sterile. This includes the upper reproductive tract and placenta, challenging the classic dogma of a 'sterile womb' as coined by Henry Tissier more than a century ago (13). Due to the earlier limitations in microbial characterization and challenges in sample acquisition, the significance of endometrial bacteria may have been missed or overlooked (14). Now, increasing evidence in favour of an endometrial microbiome (15-23) and a placental commensal colonization (24, 25) indicates the need for a paradigm shift.

The endometrium: An immunologically suited niche for microbiota

In the gut, the intestinal immune system needs to be highly adapted to withstand the continuous threat posed by colonization of the large mucosal surface, separated from host tissue by only a single layer of epithelial cells (26). In a symbiotic colonization, bacterial growth is safe for the host as long as it is contained within an assigned compartment. Therefore, tissue invasion must be limited, thus preventing potentially harmful inflammation or disruption of the niche for the necessary symbiotic relationship. Hooper and Macpherson (26) described three types of immunological barriers needed for intestinal microbial homeostasis: anatomically limiting exposure of resident bacteria to the systemic immune system; immune mediators restricting direct contact between epithelia and microbes; and rapid detection and killing of bacteria upon barrier breach. All three pre-requisites are met by the endometrial mucosa. In the uterus, the single layer of columnar epithelial cells that proliferate to form glandular cells in the secretory phase of the menstrual cycle forms a strong barrier through tight junctions (27). The uterine mucosal surface and the endometrial fluid (EF) contain infection-controlling molecules, known as antimicrobial peptides (AMPs), with fluctuating levels during the menstrual cycle (28). AMPs are known to contribute to the health of the female reproductive tract with implications for fertility and pregnancy (29). An example of an AMP found in the uterus is the secretory leukocyte protease inhibitor, which has antiviral and antifungal properties, and also acts as a bactericidal against gram-negative as well as gram-positive bacteria such as *Escherichia coli* and *Staphylococcus aureus* (30). Furthermore, the endometrial lymphocytes in the mucosal layer are present throughout all stages of the menstrual cycle, ready to act upon pathogen invasion (31). Therefore, according to the required properties, the uterus could offer a safe niche for symbiotic colonization.

Assessing commensal uterine colonization

Not only theoretically does the endometrium offer an environment suited for co-existence with bacteria but also, in accordance with earlier hypotheses, such as Viniker's theory of the "bacteria endometrialis" inhabiting the uterine cavity (14), various approaches have now provided evidence for uterine colonization, as outlined below. Initial assessment of endometrial colonization was based on observations of cells in culture. It has long been thought that the few observed growing species from endometrial cultures after hysterectomy resulted from the sterile character of the uterus, and the bacteria that were found were ascribed to a pathogenic condition (32, 33). However, women with no sign of infection also showed some bacterial growth in samples maintained *in vitro*. Species such as *Lactobacillus* (34, 35), *Gardnerella vaginalis*, *Enterobacter*, and *Streptococcus agalactiae* (33), *Escherichia coli* and *Enterococcus faecalis* were typically found in patients with uterine pathologies, such as endometritis (36). Other studies used catheter tips that were used in embryo transfer for microbial assessment based on culturing. Bacterial growth was associated with outcomes of IVF treatment, with varying results. If bacterial growth was observed upon culturing the catheter tips used for embryo transfer during IVF, live birth rates were found to be either decreased (37-40), increased or not affected at all, depending on the study or bacterial species cultured (41, 42). This underlines the need to carefully assess the outcomes and limitations of such an approach, as discussed below. However, even if smears led to growing cultures, pregnancy was possible (e.g. clinical pregnancy rate 28% versus 17% in the positive culture group, n= 279) (38). Also, using isolates other than from catheter tips, endometrial colonization could be shown for asymptomatic as well as symptomatic women (43-45). Taken together, microbial presence could not be ascribed to a certain pregnancy outcome based on culture-dependent methods.

As we now know, culture-dependent characterization of microbial communities is associated with limitations (Supplementary Table I). In culture-based approaches, rapidly growing, aerobic species dominate, leaving rare species that demand specific culture conditions undetected (e.g. (46, 47). Molecular approaches allow detection of species that will not be revealed by culture-dependent techniques. Mitchell and colleagues examined uterine swabs and EF from hysterectomies by quantitative PCR (qPCR) for 12 bacterial species, including *Atopobium vaginae, Prevotella* spp.*, Lactobacillus crispatus, Lactobacillus iners, G. vaginalis,* and bacterial vaginosis-associated bacterium 1 (BVAB1) (48). All of the selected species could be detected in vaginal samples and to a varying extent in the endometrium. Clear differences could be found between vaginal and endometrial samples. While *A. vaginae* was more commonly detected in vagina, *L. iners* and BVAB1 were more likely to be detected in endometrial samples. Of note, 95% of hysterectomy samples showed the presence of bacterial DNA. Also based on selected targets, Swidsinski and colleagues used fluorescent in-situ hybridization probes for *G. vaginalis, A. vaginae, Lactobacillus, Bacteroides, Prevotella, Enterobacteriaceae,* and *Eubacteria* (49). Again, the endometrial microbiotic environment was shown to be different than that of the

vagina. However, the need of these studies to select specific probes targeting a certain species is intrinsically biased.

Rather than evaluating the presence and/or abundance of a certain species, amplicon sequencing of the hypervariable 16S region of the ribosomal RNA makes it possible to identify species present in a sample (50). The metagenomic molecular approach of high-throughput 16S rRNA sequencing allows for a more complete view, reflecting the diversity and relative abundance of microbiota. However, also this technique comes with its limitations as outlined below. While shotgun whole genome sequencing (WGS) offers advantages to amplicon 16S sequencing (51, 52), as yet this approach has not been employed to study endometrial microbiota.

Evidence of endometrial microbiota by 16S sequencing

In the Human Microbiome Project (HMP), efforts are made to understand more about the natural colonization of various body sites, its physiological importance and implications for disease (53). In the context of the HMP, data on gut and vaginal colonization increase, but the upper reproductive tract has not been characterized extensively yet (54). However, the importance of microbiota for human reproduction is increasingly acknowledged (55). Recently, several studies targeted a putative endometrial microbiome through 16S rRNA sequencing, and each of the studies documented the presence of uterine microbiota (15-23). An overview of these recent studies assessing human uterine microbial composition based on 16S rRNA sequencing is given in Table I. Tips used for embryo transfer, swabs, biopsies or aspirates were employed to obtain endometrial samples. The studies used next-generation sequencing and assigned the sequence reads to different operational taxonomic units (OTUs). Phylotypes or, depending on the fit against the database used for taxonomic annotation, genotype or species could be assigned to the 16S rRNA gene amplicon sequence. Depending on the study, varying 16S rRNA regions were targeted simultaneously, since depending on which hypervariable V1 to V9 region is targeted, more differentiation at genus or species level is possible (56). Alpha (within a subject) and beta (between different subjects) diversity were assessed using different diversity indices (Hugerth and Andersson,2017). One of the classifications often used to express alpha diversity and species richness (i.e. how different species are sequenced in total) is the Shannon Diversity Index. Another often-used index is Chao1, using rare classes of OTUs (e.g. Franasiak *et al.,*2016; Moreno *et al.*, 2016). Beta diversity is often expressed through the Bray-Curtis dissimilarity (e.g. Walther-Antonio, *et al*.,2016). Taxonomic distribution profiles of identified genera or species are markedly different between the studies.

Table 1. Studies presenting uterine microbiome assessment based on 16S rRNA

		Cohort			Sampling			Results			
Ref.	**Aim**	**Subjects (nr of women)**	**Cohort specification (Inclusion criteria)**	**Age (years)**	**Technique**	**Number of endometrial samples taken**	**Controls**	**Sequencing platform**	**Variable regions**	**Consistently found species**	**Additional findings**
Franasiak et al., 2016	Characterization endometrial microbiome at the time of embryo transfer by reproductive outcome	• 33	Undergoing ART	Average 35.9 (range 22.5- 3,0).	Transcervical; distal portion of transfer catheter used for embryo transfer	1	2 Escherichia coli controls, negative controls from reagents.	Ion PGM™ system sequencing (Thermo Fisher)	V2,3,4,6, 7,8,9	*Lactobacillus* and *Flavobacterium*	No association in Lactobacillus content and pregnancy outcome
Verstraelen et al., 2016	Investigation of the presence of a uterine microbiome	• 11	Recurrent implantation failure	Median 32 (range 25-39)	Transcervical; Tao Brush™ Endometrial Sampler	1	Not defined	MiSeq® (Illumina)	V1 - V2	Bacteroidetes phylum, making up one third of overall population, second most abundant: Proteobacteria (incl Pelomonas, Beta-and Gammaproteobacteria related to *Escherichia/ Shigella*)	High similarity in 90% of women of 75% (25% Bray-Curtis dissimilarity). Additional high abundance of *Lactobacillus iners*, *Prevotellaamnii* or *L.crispatus* in 5 women.
		• 7	Recurrent pregnancy loss								
Kahn et al., 2016	Investigation of endometrial microbial colonization related to endometriosis	• 32	Endometriosis, undergoing laparoscopy (16 of which undergoing GnRH treatment)	Range 21 - 47	Transcervical swabs	1	Not defined	MiSeq® (Illumina)	Custom primers, see Mori et al., 2014	*Lactobacillacae, Streptococcaceae, Staphylococaceae, Enterobacteriaceae,* and *Moraxellaceae* as predominant families of 58 bacterial candidates.	Increase in microbial colonization during menstrual phase. GnRH reatment impacts bacterial proportions. Microbial accumulation in endometriosis patients compared to control.
		• 32	Fertile (16 of which undergoing GnRH treatment, uterine myoma)	Range 21 - 52							

Study	Aim	n	Population	Age	Sampling method	Samples	Control	Sequencing	Region	Findings	Conclusion
Fang et al., 2016	Investigation of endometrial microbial colonization related to endometrial polyps	• 10	Fertile	Average 30.9 (±1.56)	Transcervical swabs	1	Vaginal swabs	MiSeq® (Illumina)	V4	Proteobacteria (73%), Firmicutes (14%) and Actinobacteria (5%) on phylum level; *Enterobacter* (33%), *Pseudomonas* (24%) and *Lactobacillus* (6%) on genus level in healthy cohort	Differences in detected phyla/ genera in patient versus control cohort less Enterobacter and Pseudomonas whereas more Lactobacillus than in diseased. Higher Shannon diversity in patient cohort.
		• 10	Endometrial polyps	34.4 ± 2.44							
		• 10	Endometrial polyps and chronic endometritis	Average 35.2 ± 1.3							
Moreno et al., 2016	Investigation of the presence of a uterine microbiome	• 13	Fertile	Range 18-35	Transcervical; Endometrial fluid aspirated through a catheter	2 samples endometrial fluid per woman, obtained in prereceptive and receptive phase within the same menstrual cycle (n=26)	Vaginal aspirates	454 pyro-sequencing on 454 Life Sciences GS FLX+ instrument (Roche)	V3-V5	71.7% *Lactobacillus*, 12.6% *Gardnerella*, 3.7% *Bifidobacterium*, 3.2% *Streptococcus*, 0.9% *Prevotella;* if samples *non-Lactobacillus* dominated *Atopodium, Clostridium, Gardnerella, Megasphaera, Parvimonas, Prevotella, Sphingomonas or Sneathia genera abundant*	Stratification into Lactobacillus versus non-Lactobacillus dominated group (containing high proportion of Atopodium, Clostridium, Gardnerella, Megasphaera, Parvimonas, Prevotella, Sphingomonas or Sneathia genera).
	Hormonal regulation of microbiome	• 22	Fertile	Range 18-35		1 endometrial sample taken in menstrual cycle before embryo transfer					No variation in bacterial community composition in prereceptive versus receptive phase in most subjects (n=18 of 22)
	Impact on reproductive outcome	• 35	Infertile, undergoing IVF, receptive endometrium	Range 25-40							Negative association of non-Lactobacillus dominated subjects with pregnancy outcome (decreased implantation of 23.1% versus 60.7%; pregnancy rates 33.3% versus 70.6%; ongoing pregnancy rates 13.3% versus 58.8%, and live birth rates 6.7% versus 58.8%).

Table 1. Continued

Study	Aim	n	Population	Age	Sampling		Controls	Platform	Region	Taxa	Findings
Walther-Antonio et al., 2016	Composition of the uterine microbiome, its role in endometrial cancer	• 10 • 4 • 17	Benign uterine conditions (pelvic pain, abnormal bleeding, fibroids, prolapse) Endometrial hyperplasia (cancer precursor) Endometrial cancer	Median 44.5 (range 43.5- 2.5) Median 54 (range 50.75- 2.5) Median 64 (range 58-71)	Hysterectomy; Uterus, Fallopian tubes and ovaries excised, biopsy and scrapes taken.	1	Lysogeny broth kept open during sample acquisition swabbed, controls of DNA extraction/ microbiome enrichment process. Pre-operative vaginal/ cervical swabs	MiSeq (Illumina)	V3-V5	*Shigella, Barnesiella, Staphylococcus, Blautia, Parabacteroides*	*Bacteroides* and *Faecalibacterium* dominant in cancer group, enrichment of Firmicutes (*Anaerostipes, ph2, Dialister, Peptoniphilus, 1-68, Ruminococcus, Anaerotruncus*), Spirochaetes (*Treponema*), Actinobacteria (*Atopobium vaginae*), Bacteroidetes (*Bacteroides and Porphyromonas*), and Proteobacteria (*Arthrospira*). Higher diversity in cancer cohort, especially within the uterus. Presence of *Atopobium vaginae* and spp. 99% matching *P.somerae* as predictor of disease status.
Miles et al., 2017	Investigation of microbiota composition within female reproductive tract of women undergoing hysterectomy/ bilateral salpingo-oopherectomy (pilot)	• 10	Undergoing hysterectomy and bilateral salpingo-oopherectomy	Median 49 (range 41 - 59)	Varying route of hysterectomy, transvaginal (1/10), abdominal (1/10), robot-assisted (1/10), and laparoscopic (7/10)	1	Vaginal swabs (preceding surgery)	454 pyro-sequencing on Life Sciences instrument (Roche)	V1 - V3	*Lactobacillus, Acinetobacter, Blautia, Corynebacterium Staphylococcus*	Various attempts to sequence endometrial samples of patient with atrophic endometrium were negative.
Tao et al., 2017	Investigation of endometrial microbial colonization at the time of embryo transfer during IVF using ulta-low bacteria counts	• 70	Undergoing IVF	Average 36.2 (range 22.3 - 46)	Transcervical; distal portion of transfer catheter used for embryo transfer	1		MiSeq (Illumina)	V4	*Lactobacillus* spp 33 samples contained over 90% of *Lactobacillus* abundance, 50 samples over 70%, *Corynebacterium*(40 patients), *Staphylococcus* (38 patients) *Streptococcus* (38 patients), *Bifidobacterium* (15 patients)	16S analysis tested on diluted single- and poly-microbial samples. Sample processing allows reliable results on species classification and abundance with low biomass sampling of 60 bacterial cells.

Chen et al., 2017	Investigation of microbiota composition within female reproductive tract	• 80	Surgery for conditions not known to involve infection (hysteromyoma, adenomyosis, endometriosis, salpingemphraxis)	Median 31 (range 22-48)	Laparoscopy or laparotomy swabs, transcervical swabs	2 (1 obtained transcervically, 1 surgically)	Dry swabs, pre-operative skin area, swabs of gloves used by surgeons. Swabs taken through cervical os to test influence of contamination compared to operative sampling, PBS/ physiological saline as diluent negative controls controls for sample processing, DNA extraction, and real-time qPCR (the latter included additional ultrapure water control)	Ion PGM™ system sequencing (Thermo Fisher)	V4 - V5	*Lactobacillus (30.6%), Pseudomonas (9.1%), Acinetobacter (9.1%), Vagococcus (7.3%)* and *Sphingobium (5%)*	Higher diversity in uterus compared to vaginal and cervical samples. High similarity between samples taken by entrance through cervical os versus surgical access through abdomen. Microbial profiles different than controls. High reproducibility. Determination of signature OTUs increasing in abundance from vagina to peritoneal fluid. High intra-individual correlation versus clear distinction between upper and lower RT also in inter-individual analysis
	Estimation of bacterial biomass of female reproductive tract				Copy number obtained by qPCR species-specific for 4 major vaginal *Lactobacillus* species, divided by corresponding relative abundance based on 16S rRNA sequencing	1					Lowest biomass compared to other sites of reproductive tract (decreasing from 10^10 - 10^20 copies/sample at lower third of vagina towards posterior fornix and cervical canal and endometrium with 10^2 - 10^3 copies/sample). Much lower CT value of endometrial samples compared to negative controls. Higher diversity corresponds to lower biomass.
	Validation if sequencing live bacteria or debris	• 15		Median 33 (range 24 - 41)	Culturing of live bacteria (additional to 16S rRNA sequencing)	1					Positive cultures for 5 out of 15 samples; 8 different isolates belonging to 7 genera such as *Lactobacillus, Staphylococcus* and *Actinomyces*

CT, cycle threshold; GnRH, gonadotrophic releasing hormone; GnRHa, gonadotrophic releasing hormone agonist; OTU, operational taxonomic unit; PGM, personal genome maschine; qPCR, quantitative PCR; RT, reproductive tract

6

Franasiak and colleagues characterized the endometrial microbiome of 33 women by collecting material from the tip of the catheter used for embryo transfer (ET) during ART (57). Both in the group of non-ongoing and ongoing pregnancies, *Lactobacillus* and *Flavobacterium* were identified as the most abundant genera. However, in this initial study only women undergoing ART for unspecified reasons were included. Therefore, it is unclear to what extent these findings represent the colonization of a healthy, fertile endometrium. The same holds true for the study by Tao and colleagues (22) assessing the microbiota obtained from catheter tips during ET of 70 women. *Lactobacillus* was abundantly detected (>90% of OTUs in 33 women, >70% in 50 women). Samples also frequently contained *Corynebacterium* (40 women), *Staphylococcus* (38 women), *Streptococcus* (38 women) and *Bifidobacterium* (15 women). To achieve reliable sequencing results, particular attention was given to sample preparation, as sampling using remaining tissue from catheter tips only yields ultra-low bacterial cell counts. The authors were able to reliably determine bacterial abundance when more than 60 bacterial cells were present per sample, although no estimation was given of how many cells the catheter tips likely contain.

Verstraelen and colleagues identified *Bacteroides* as the dominant genus, present in >90% of the women that were included. All women underwent hysteroscopy in the absence of uterine anomalies (16). Again, the cohort of women employed sought medical help due to recurrent implantation failure (RIF) or recurrent pregnancy loss. Twelve of the 19 women included showed 75% similarity in bacterial composition (Bray-Curtis dissimilarity of 24.6%, range 13.2 – 34.3%) due to general high abundance of *Bacteroidetes* and *Proteobacteria* taxa. Next to this core of *Bacteroidetes* and *Proteobacteria,* five women also presented with *L. iners* (n=2) , *P. amnii (n=1) or L. crispatus* (n=1) as the most abundant species. Large dissimilarities were observed in two women (79.0 and 90.7% Bray-Curtis dissimilarity) showing *Prevotella* spp. or *L. crispatus* dominance, which the authors ascribe to possible contamination from the vagina. Control swabs from the vagina could have given an indication if the distinctive microbial profile of these women indeed reflected an artifact from the transcervical sampling approach but no such data are available.

In a study focusing on the relation of endometrial polyps to local microbiota, Fang et al. included 10 fertile women as a control population in their 16S analysis of transcervical uterine swabs. Proteobacteria, Firmicutes, and Actinobacteria were consistently found at phylum level; *Enterobacter, Pseudomonas,* and *Lactobacillus* at genus level. Moreno and colleagues (2016) were also able to include fertile women (n=13 for comparison of vaginal aspirates and EF, n=22 for compositional differences between pre-receptive and receptive phase) of reproductive age (19). *Lactobacillus* (71.1%), *Gardnerella* (12.6%), *Bifidobacterium* (3.7%), *Streptococcus* (3.2%), and *Prevotella* (0.9%) were the most identified genera. Endometrial microbial composition was categorized into either *Lactobacillus*-dominated (>90% *Lactobacillus* spp.) versus non-*Lactobacillus* dominated (>10% bacteria other than *Lactobacillus,* such as *A. vaginae, G. vaginalis,*

species of the genera *Clostridium, Megasphaera, Parvimonas, Prevotella, Sphingomonas* or *Sneathia*). Eighteen of the 22 women presented with stable microbiota profiles when comparing pre-receptive and receptive phase, of which 12 women were continuously categorized in the *Lactobacillus*-dominated group, and six women categorized in the non-*Lactobacillus* group independent from sampling time point. This indicates that bacterial community composition was relatively stable in most women. Contradictory to the results by Franasiak et al. (2006), Moreno et al. (2016) found an association between *Lactobacillus* abundance and pregnancy outcome. Implantation was decreased (23.1% versus 60.7%), and pregnancy rates declined (13.3% versus 58.8%) when the women showed a non-*Lactobacillus* dominated endometrial phenotype at the time of ET. An especially negative impact on reproductive outcome was observed when *G. vaginalis* and *Streptococcus* species were present in abundance. These results were independent of pH of the sample, known to be affected by *Lactobacillus* species (58).

Unlike the other transcervical sampling approaches, Walther-Antonio et al. (2016) studied samples taken from the uterus, Fallopian tubes, and ovaries removed during hysterectomy (n=31), in addition to pre-operative vaginal and cervical swabs and scrapes. Their results showed *Shigella* and *Barnesiella* as dominant species of the endometrial microbiome in accordance with earlier culture-based observations (36). High abundance of *Staphylococcus, Blautia* and *Parabacteroides* was found in benign uterine conditions (n=10), and *Bacteroides*, and *Faecalibacterium* were associated with women who presented with cancer as reason for hysterectomy (n=17). Miles and colleagues (2017) also took samples from various tissue types obtained during hysterectomy. Their data on 10 women, comprising a wide range of uterine pathologies, shows high variability. Species such as *Lactobacillus, Acinetobacter, Blautia, Corynebacterium and Staphylococcus* were abundantly found in some of the women. The authors also note that even after various attempts, no sequencing data could be obtained for endometrium from a patient with atrophic endometrium. Similarly, Chen and colleagues investigated the microbiota of different sites of the female reproductive tract accessed through surgery (23). Tissue was obtained during laparoscopy or laparotomy, therefore not reflecting healthy, fertile women of reproductive age but the patients enrolled had conditions known not to involve infection. In these conditions, *Lactobacillus, Pseudomonas, Acinetobacter, Vagococcus,* and *Sphingobium* were frequently detected.

When evaluating the results of the recent 16S endometrial studies, certain species were found in more than one study (Table I). Some of the described findings are addressed in two recent reviews (4, 59), neither including a concluding summary of the species found in the different studies, nor a meta-analysis.

MICROBIOTA IN THE UTERUS - REAL OR ARTIFACT?

Until now, available 16S data on uterine microbiota do not allow conclusions on a “core uterine microbiome” due to a number of limitations. When investigating the questioned low-biomass microbiome, studies need to be designed with a special focus on possible contamination. In the following paragraph, we outline the pitfalls of the presented studies.

Contamination acquired during sample acquisition and processing

It is currently highly debated whether microbiota found to be involved in reproduction, such as in the placenta, are in fact merely the result of contamination and an artifact of the study design (60). Contamination always has to be a concern when studying 16S data. Especially when investigating a suspected low-biomass microbiome, as found within the uterus, the impact of various (contaminating) handling steps makes it hard to detect low-abundance microbes originating only from the sampling site (61-63). To overcome the hurdles associated with the need for highly sensitive detection, the 16S studies on endometrial colonization employed different controls (Table I). To examine the possible contribution of contamination when studying the placenta, Lauder and colleagues examined the results from “air swabs” waved in the laboratory space, as well as unused sterile swabs, in comparison to placental tissue samples (64). They could not distinguish between contamination controls and placental samples. Compositions of microbiota in negative controls are known to be associated with the DNA extraction kits (65). Misrepresentation of data needs to be minimized by using special DNA isolation kits for low microbial biomass samples and committing to one kit type for all samples (65).

Low-biomass microbial communities are sensitive to contamination and misinterpretation

Lauder et al. suggest the presence of a placental microbiome below their level of detection as a reason for not being able to distinguish between air swabs and sampled material (64). Detection of a low-biomass microbiome (where for 1g of placental tissue only 20 – 2000 ng of bacterial DNA is estimated to be extracted) is a major issue when assessing endometrial microbiota (66). Chen et al. included an assessment of the bacterial biomass in their approach. Copy numbers, as calculated from qPCR on vaginal typical *Lactobacillus* species, were related to the relative abundance of the species as found in 16S rRNA sequencing, showing that microbial numbers decrease from vagina (10^{10} – 10^{20}copies/sample) through the cervix (10^{8} – 10^{10}copies/sample) to the uterus (10^{2} – 10^{3}copies/sample) (23). As the authors point out, this still represents higher numbers than those that can potentially be detected in background noise (62) or in their controls of sterile PBS, sterile saline, and ultrapure water. Accordingly, even though the detected biomass is small, the detected species are not an artifact or contamination.

Another source of misinterpretation that is gaining attention is the approach in which species distribution is related to the obtained sequencing library. Disease-associated changes in *Prevotella* and *Bacteroides* abundances found in Crohn's disease were found to be rooted in proportional profiling and had to be ascribed to a general decrease in microbiota richness in Crohn's disease (67). If abundance is interpreted as a relative proportion, a change in the presence of a certain species presents as increase or decrease of a possibly unrelated species. Low-biomass microbiota, as detected in the uterus, are highly sensitive to this misrepresentation of data. A quantitative assessment of genus abundance proportional to cell counts and microbial load of the sample would allow a more precise representation of data (67).

Vaginal microbiota as a source of contamination

Due to its high biomass relative to the uterine microbiome and the high abundance of *Lactobacillus* in the vagina, misrepresentation of species distribution cannot be excluded. Especially when using a transcervical approach, contamination by vaginal bacteria needs to be controlled for. Moreno and colleagues compared endometrial and vaginal samples, and only two of the 26 sample pairs (two pairs per woman) of EF and vaginal aspirate showed the same microbial profile (19). While in some samples only minor differences were observed, six paired samples exhibited totally different profiles when women belonged to the group of non-*Lactobacilli* dominated subjects. In nine of the 13 women, *Lactobacillus* consistently reflected the dominant genus with more than 90% colonization in the pre-receptive and receptive phase of endometrium and vagina. Notably, some common species, such as *A. vaginae, G. vaginalis, Prevotella, and Sneathia,* of both endometrium and vagina were identified. Also, the study that circumvented this risk, by sampling from uteri obtained during hysterectomy, showed a correlation between the vaginal and uterine microbiomes (20). However, it is unclear how the uterine microenvironment is contaminated by transfer of cervicovaginal bacteria into the excised uterus. As manipulation of the uterus during surgery might enable passage of bacteria from the lower reproductive tract, it cannot be excluded that the correlation of vaginal and uterine microbiota is just an artifact. This correlation was also seen by Verstraelen et al. (2016), who detected endometrial phylotypes known to be associated with the vagina. *L. crispatus* was present in 12 of the 19 women, and most abundant in five of 19 individuals (range from 17.1% to 79.1% of the total number of sequence reads). Also, species associated with vaginal microbiota imbalance, such as *Prevotella* (*P. amnii, Prevotella* sp.*, P. timonensis, P. disiens*), were abundantly expressed in two subjects (18.4% and 55.4% of total sequence reads) (47, 68, 69). Likewise, *G. vaginalis* was present in six women, but at an abundance of less than 1%. While trying to avoid vaginal contamination through the use of a sheath-protected sampling surface and trying to prevent cervicovaginal contact of the sampling tool, it cannot be excluded that insertion or extraction of the device facilitates bacterial carry-over. Verstraelen et al. pointed out that, even though a certain degree of species overlap exists, it is not to be assumed that endometrial samples reflect vaginal colonization, since the diversity found in endometrial samples is higher than in the vagina (16).

While we believe that it is unlikely that the distribution profiles from endometrial swabs are merely a representation of vaginal colonization, the possibility of carrying over traces from vaginal microbiota onto the sample cannot be neglected. Likewise, insertion of a sampling device or speculum could carry over bacteria from the lower reproductive tract into the uterus. It is also unclear how surgical access of the uterus coincided with disruption of the cervical barrier allowing passage of bacteria that would normally not reside in the uterus as a result of the pre-operative preparation.

Live bacteria?

It cannot be ignored that, without further measures, sequencing of 16S rRNA does not differentiate between living bacteria or (dead) bacterial fragments. Chen et al. studied cultures of freshly collected peritoneal fluid samples from the Pouch of Douglas (the lowest part of the abdominal cavity, between uterus and rectum), a site shown to be similar in microbial diversity, and even lower in terms of microbial abundance, as compared to the endometrium (23). Eight different isolates were cultured belonging to seven genera, such as *Lactobacillus, Staphylococcus,* and *Actinomyces,* in five out of 15 samples, whereas various negative controls did not lead to bacterial growth when cultured. These results indicate that the detected 16S reads do not solely reflect dead bacterial fragments.

Conclusions on the validity of the current evidence

Based on the current study designs, no information on bacterial detection and distributions of a “core uterine microbiome” can be extracted. Contamination during sampling by surrounding microbiota and reagent kits have not been addressed systematically on a large cohort of healthy, fertile women yet, in order to allow any conclusions on the continuous baseline colonization of the uterus independent of pathologies. Future studies also need to address the question of whether detected 16S results from bacterial fragments rather than live organisms by using a more robust approach than culture *in vitro* since (even though proving that culture is not impossible) often no growth can be established in cases of these ultra-low biomass samples. The variability in culturing success probably also results from variation between women in species composition and biomass of live bacteria. Aside from this, even 16S rRNA data, reflecting a proportion of the overall community that is dead or fragmented, represent ligands for the host cells to recognize and act upon. Thereby, these inactive bacterial fragments can still contribute to a physiologic interaction with host cells.

We conclude that well-setup large cohort studies are needed to define a healthy uterine microbiome. We want to stress that even though no uterus-typical bacterial profile can be established yet, existing data are not to be underrated as evidence towards a natural microbial presence within the uterus. A number of the presented studies do employ negative controls and/or additional vaginal swabs. Since species abundance of these controls was not identical

to the results from endometrial material, we should consider a physiological importance of local microbiota.

ENDOMETRIUM – STARTING POINT FOR DECIDUALIZATION, IMPLANTATION AND PLACENTATION

To understand how microbiota could play a role in the uterine interplay of cells involved in the per-implantation period, we first need to understand how the endometrium forms the basis for successful implantation and placentation. Despite the growing interest from both clinicians and scientists in the process of implantation, the mechanisms underlying human implantation remain poorly understood. Each month, before the presence of a developing embryo, the stage for formation of the placenta is set. During the mid-to-late luteal phase spontaneous decidualization occurs; i.e. the transformation of the endometrium into a receptive state, independent of the presence or absence of a conceptus. Six to 10 days after ovulation (during day 19-24 of the menstrual cycle) the receptive state of the endometrium is shaped. This is referred to as 'the window of implantation' (WOI), lasting for 2-4 days (70, 71). The substantial physiological adaptations needed for correct cyclical changes of the endometrium are orchestrated by fluctuations of progesterone and oestrogen (72, 73), as well as by immune cells and their products (74, 75). Endometrial stromal fibroblasts differentiate to become larger, rounded fibroblast-like stromal cells. To achieve this, the cytoskeleton and plasma membrane undergo modifications (76-78). The luminal epithelium must be able to interact with the blastocyst. Interactions also involve adhesion molecules, such as integrins, L-selectin ligands (e.g. L-selectin ligands expressed by the luminal epithelium and L-selectin receptors of the blastocyst), and oligosaccharides (79-82). We summarized key endometrial adaptations in the peri-implantation period (Figure 1) to show the importance of enabling the correct interaction of endometrium and blastocyst.

Already at this stage, irreversible shortcomings in placental vascularization pose an origin for diseases such as pre-eclampsia or intra-uterine growth restriction (83). On the maternal side, the underlying mechanisms regulating correct migration of the trophoblast and vascular remodeling may be rooted in the endometrium. Due to its importance for correct placentation, the endometrium is crucial for developing a healthy placenta, and therefore a healthy pregnancy (84). If microbial compounds constitute another physiologic player in the complex uterine environment, its natural impact will likely also contribute to implantation and placentation.

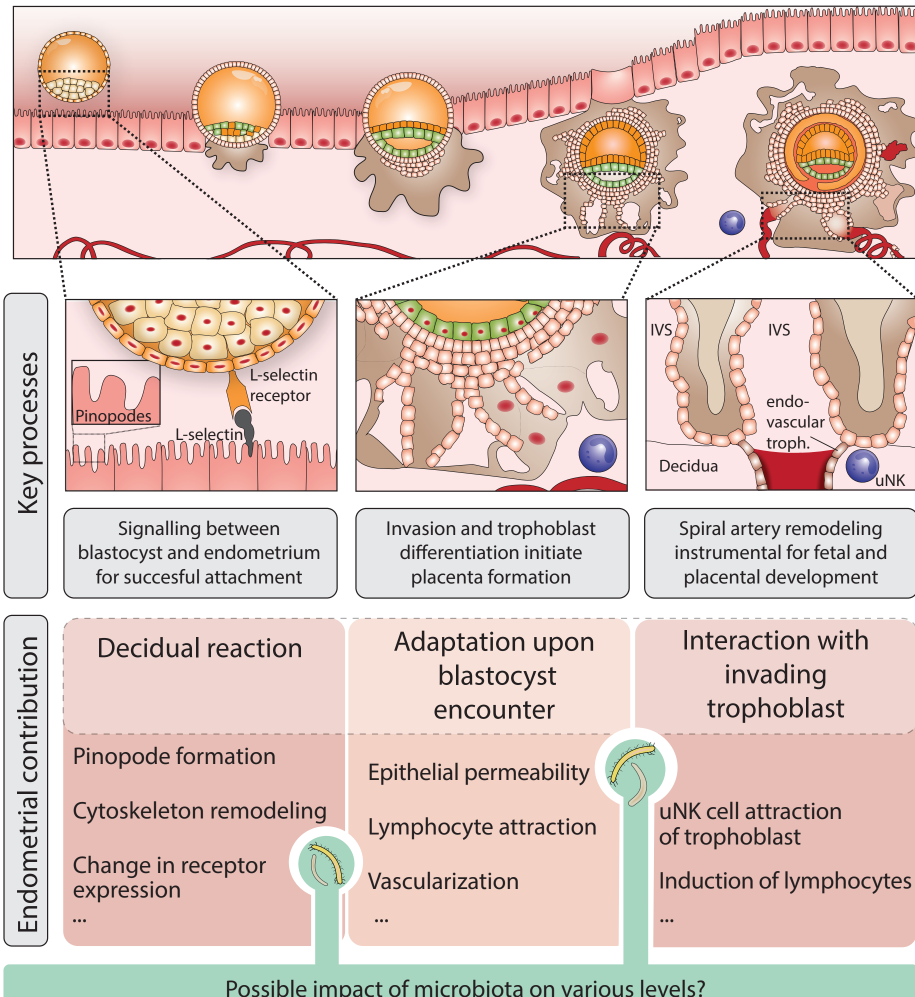


Figure 1. Illustration of key elements in blastocyst-endometrium interaction needed for a receptive endometrium.

Due to its important contributions during early placentation, the endometrium is key to healthy pregnancy. During the window of opportunity, day 19-24 of the menstrual cycle, the endometrium resides in a receptive state that allows selection, apposition and attachment of a healthy blastocyst. Implantation is marked by invasive growth and differentiation of trophoblast cells. Proper interaction of invading trophoblast and local immunity is needed to achieve correct villi development and a healthy placenta. All these highly regulated properties of the endometrium needed in the initial phase of pregnancy are possibly affected by uterine microbiota. *IVS , intervillous space; uNK, uterine natural killer cell.*

UTERINE IMMUNITY DURING IMPLANTATION AND PLACENTATION AS A POSSIBLE INTERACTION PARTNER OF LOCAL MICROBIOTA

Local immune cells belong to the factors known to influence extravillous trophoblast migration, and are therefore instrumental to maternal spiral artery remodeling (85). The immune system is intimately involved in all aspects of the reproductive process, particularly around the time of conception, and in the peri-implantation period (86). Different stages during the initiation of pregnancy hold specific immunologic challenges: to enable a healthy pregnancy, the maternal immune system must support the introduction of semen to allow fertilization, the initial contact of blastocyst and endometrium, and correct formation of the placenta (87). All of these processes are subjects of extensive study, and a comprehensive overview is beyond the scope of a single review. Natural killer (NK) cells, T cells and antigen presenting cells (APCs) are a common focus when investigating the immunology of pregnancy (88-95).

We wish to highlight the importance of a tightly-regulated uterine immune environment that could potentially be affected by microbiota-induced factors. Recent discoveries on the interplay of the intestinal microbiome, their metabolites, and host immunity, give an impression of the vast impact that the endometrial microbiome could have (96-98). We outline here the implications of gut microbiota affecting cell types known to be important within the uterus.

Lymphocytes involved in the peri-implantation period

NK cells are the most abundant of immune cells in the endometrium representing ~ 70% of all hematopoietic cells present (99, 100). The term endometrial NK cells (eNK; or uterine NK cells; uNK cells) refers to NK cells in the non-pregnant endometrium, and decidual NK (dNK) cells residing in the placental membrane are phenotypically and functionally different from eNK cells or peripheral blood NK (pbNK) cells (100, 101). In the endometrium, the non-cytolytic and potent cytokine secretors, $CD56^{bright}CD16^{-}$ NK cells, dominate. Only a small fraction of eNK cells belongs to the $CD56^{+}CD16^{+}$NK population specialized in killing infected cells (94, 102), contrary to the NK cell division in peripheral blood where the majority are $CD56^{+}CD16^{+}$. Throughout the female cycle, eNK cells continue to accumulate until the first half of gestation. Abnormal levels of NK cells, measured 20 days after ET, were shown to be associated with RIF (103). Direct involvement of eNK cells in implantation remains unclear, as successful implantation is possible in NK cell deficient mice (104). More evidence points towards an involvement of dNK cells in placenta-formation processes after initial implantation. However, an indirect role of the human $CD56^{bright}$CD16- eNK cells already during implantation cannot be neglected due to their high abundance and ability to secrete large amounts of cytokines, such as IFN-γ, tumor necrosis factor alpha (TNFα), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 10 (IL-10) (102).

Just as eNK and dNK cells are distinct from pbNK cells, the mucosal NK cells of the gut that are continuously exposed to the gut microbiome are distinct from pbNK cells. This distinction is characterized by limited IFN-γ production and an absence of cytotoxic effector perforin, granzymes, FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in mucosal NK cells of the gut (105). Of note, germ-free mice lacked these IL-22^{+}NKp46^{+} cells (106). Hence, the specialized intestinal NK cells might be the result of an adaptation to the microbiota-rich environment. It needs to be evaluated whether the reduced cytotoxic capacity of uNK cells (107) has any relation to the uterine microbiota.

APCs, such as macrophages (Mϕ) and dendritic cells (DCs), constitute 10-20% of uterine leukocytes, expressing major histocompatibility complex class II molecules (108, 109). Through integrating microbial, environmental, and self-derived stimuli, APCs are key to the successful initiation of an appropriate immune response (110, 111). Throughout the menstrual cycle, mature CD83^{+} DCs and CD68^{+} Mϕ increase, peaking in the late secretory phase (112). Mϕ are, together with NK cells, the main cytokine producers in the human endometrium (113). Through secreting leukemia inhibitory factor (LIF) and IL1B, Mϕ play a known role in the establishment of endometrial receptivity by increasing cell surface fucosylated structures, allowing trophectoderm attachment (87).

Even more pronounced than seen for NK cells, monocytes of the heavily colonized gut mucosa are markedly different in phenotype and function compared to blood-derived monocytes. Even though they reside in close proximity to bacteria colonizing the intestinal lumen, the ability of the local macrophages to mount a pro-inflammatory response seems to be tuned down. Their expression of, for example, lipopolysaccharide (LPS)-binding protein CD14 receptor (114), CD89, implicated in IgA-enhanced phagocytosis is decreased (115). Also, various FCγ receptors (CD16, CD32, CD64), integrins (CD11a, CD11b, CD11c, CD18) and the IL-2 receptor CD25 are, amongst others, downregulated on intestinal macrophages. Their ability to produce cytokines (IL-1, IL-6, TNFα) is diminished (116). It needs to be investigated whether this Smad-induced IκBα expression and NF-κB inactivation-mediated phenotype, termed “inflammation anergy”, is paralleled at the fetal-maternal interface (117).

T cells represent the third largest fraction of immune cells found in human endometrium (118-120). Since, in the absence of pregnancy, T cells mostly reside in the deeper layers of the endometrium, their relation to fertility disorders more likely reflects a role in early placenta formation after implantation, rather than a contribution to the pre-implantation period. Experimental evidence on the functional importance of T cells in the WOI is scarce. It has been suggested that a switch in the ratio of Th1 and Th2 cells (Th1/Th2 paradigm) within the endometrium is needed to prepare for implantation, as a pronounced decrease in Th1 was accompanied by an increase in Th2 cells

seen from the secretory phase towards early pregnancy (121). More recently, regulatory T (Treg) cells and Th17 cells have been included in the discussion (122, 123).

Increasing evidence on the shaping of T cell subsets by microbiota based on studies of the gut is emerging (124). Like in the endometrium, mucosal immunity of the intestines depends on an interplay of IFN-γ producing Th1 cells, Th17 cells that secrete IL-17 and IL-22, and innate lymphoid cells with Th2-cytokine secreting functions, and suppressive Foxp3^{+} Treg cells. Differentiation of various T cell subsets is impaired in mice with disrupted gut microbiota (125, 126). Commensal segmented filamentous bacteria were shown to be important for Th17-mediated mucosal protection (127). A detailed understanding of the mechanisms underlying bacteria-mediated T cell induction is not yet available. One of the few examples in which the link between microbe and host is known, is polysaccharide A (PSA) originating from the capsule of *Bacteroides fragilis* (127). Bacteroides, found in a number of the endometrial studies analyzing 16S rRNA, tunes the Th17 response of intestinal T cells, and causes a systemic increase of circulating CD4+ T and Th1 cells (128-130).

Other microbiota are known to induce and cause accumulation of Treg cells, essential in maintaining tolerance and known to be important for pregnancy (131). In the gut, Treg mediated IL-10 production is thought to be critical to maintain intestinal microbial homeostasis by limiting continuous activation of Th1 and Th17 cells. Commensal microbiota promote *de novo* generation and activation of mucosal Treg cells (132) as shown by mice colonized with altered Schaedler flora species (*Lactobaillus, Bacteroides,* species of *Flexistipes* phylum and gram-positive bacteria of the *Firmicutes, Bacillus-Clostridium* group) (132, 133). Focusing on specific strains, colonization of mice with *Clostridia* strains was shown to induce expansion of Treg cells locally in the lamina propria and systemically, resulting in increased resistance to colitis and IgE responses in adult mice (134). These intestinally induced Treg were shown to express a T-cell receptor repertoire specific for bacteria of the luminal content of colonized mice, allowing tolerance towards individual species (135). Similar generation and activation of immune cell types by endometrial microbiota could be essential for correct homeostasis regarding maintenance of a microbiome and programming the local immunity within the uterus. The majority of work on microbiota and immunity focuses on T cell homeostasis, but especially cells of the innate arm of the immune system are involved in the peri-implantation period.

Cytokines and chemokines belong to the soluble factors known to be associated with reproductive health. Induced by either endometrial cells or immune cells recruited upon blastocyst encounter, the implantation period is characterized by an increase in pro-inflammatory Th1 cytokines, such as IL-6, IL-8, LIF, and TNF-α, with implications for immune cell recruitment and activation of the endometrium, as reviewed previously (136, 137). Throughout the female cycle, healthy endometrial stromal cells constitutively secrete the chemokine CCL2, also known as monocyte

chemoattractant protein 1 involved in directing monocytes, T cells and DCs to inflammatory or tumor tissue (138) (139, 140). Also, decidual stromal cells obtained during first trimester (~8 weeks in both studies) secrete CCL2, which was shown to attract Th2 and Th17 cells, thereby influencing the local T cell balance (141, 142). In addition, macrophage recruitment and polarization (143), and plasmacytoid DCs were affected by CCL2. It was recently shown that microbiota are important in establishing baseline CCL2 secretion controlling homeostatic trafficking of plasmacytoid DCs (144). As no functional contribution of endometrial microbiota has been shown yet, the physiologically closest possible microbiome, as indicated by Chen et al. (2017), is probably the cervicovaginal microbiome. Cytokine concentrations were shown to be affected depending on the microbial profiles of 16S rRNA analysis of cervicovaginal lavage. This was reflected in lower IL-1beta, IL-8 and IL-10 levels in bacterial vaginosis (BV) (145).

Also other cytokines, such as LIF, GM-CSF, colony-stimulating factor-1, heparin binding EGF like growth factor, insulin-like growth factor Iand II, contribute to fertility through their role in supporting blastocyst development in the pre-implantation phase (87). Decreased LIF expression of endometrial epithelial cells was shown to be associated with infertility (146). Cervicovaginal levels of cytokines were affected by the bacterial flora found (147) and a probiotic supplement was shown to affect vaginal interleukin changes (148). It remains to be seen if different uterine microbial profiles could be associated with altered chemokine/cytokine profiles and effects on reproductive success.

LESSONS LEARNED FROM THE GUT MICROBIOME: POSSIBLE INTERACTIONS BETWEEN LOCAL MICROBIOME AND ENDOMETRIUM

Besides influence on systemic and tissue resident lymphocytes, the microbiota and host interact on various levels. The relation between microbes and host is not a black and white phenomenon; and thus surpasses the simplified view of commensal versus pathogen. The immune system shapes a homeostatic co-existence, i.e. by eliminating species that might not only be harmful but also disturb the balance (12). Two different types of battles are continuously fought when hosting a microbial community: one that is responsible for facing the daily challenge posed by containment of symbiotic microbes, and one for defense against pathogenic microbes that breach containment (12, 149). Although our understanding of the microbiome – host interaction is still rudimentary, its functional importance, especially on various arms of innate and adaptive immunity, is evident (7, 150). Below, we give an overview of the different mechanisms of how endometrial colonization might have implications for the uterine immune-environment, and thus for fertility.

Pattern recognition receptors

To detect viral, fungal and bacterial pathogens, the innate immune system senses pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), RIG-I-like receptors, NOD-like receptors (NLRs) and C-type lectin receptors, such as collectins, selectins, endocytic receptors and proteoglycans (151-153). PAMPs include, for example, cell wall mannans (yeast), formylated or lipo-peptides, peptidoglycans, teichoic acids and bacterial cell-wall components such as LPS (154). Activation of TLR induces Nuclear factor-kappa B (NFκB), which initiates an inflammatory cascade including upregulation of markers to mount an adaptive immune response (155). PRRs form the first line of defense against sexually transmitted disease or any other pathogens that can access the female reproductive tract through the vagina (156). For example, decidual and uterine stromal and epithelial cells express various TLRs and other PRRs, and the cells mount a potent inflammatory response upon receptor recognition (157-162). An overview of TLRs of the endometrium is given in Table II. The expression of TLR mRNA and protein changes throughout the menstrual cycle, as shown by different groups (163-167). Receptor expression on endometrial cells was found to be low in the proliferative phase and increased in the secretory phase for TLRs 1 – 10. Together with intracellular receptors, there is a constitutively high expression of pathogenicity sensors lining the female reproductive tract (160). All of these receptors can sense their specific PAMP and react by inducing signaling cascades.

Evidence accumulates that PRRs are one way for microbiota and host to communicate. Round and colleagues examined how *B. fragilis* induces symbiosis via TLR2 of CD4^{+} T cells. PSA of *B. fragilis* induces Foxp3^{+} regulatory cells that are needed to counteract the otherwise Th17-mediated killing of *Bacteroides* (168). *Nod2*-deficient mice were more susceptible to pathogenic infection (169). Nod2 was shown to control dysregulated commensal colonization, and commensal resident bacteria are in turn needed to induce *Nod2* expression. TLRs and NLRs are suspected to play a role in the periconceptual regulation as they are major players in the cascade of cytokine induction (86, 170).

Protection against pathogens

Maintaining colonization of commensal bacteria protects the host against pathogens. Residential microbiota successfully compete for the niche by exhibiting better adaptation than an occasionally invading pathogenic species, termed the “colonization resistance” concept (171, 172). Commensal microbiota are specialized in nutrient usage of their habitat, and deplete the environment of nutrients needed for pathogenic species. Consumption of a limited resource can starve the pathogenic invader (173). *E. coli,* for example, competes in consumption of sugars, amino acids and other nutrients with the pathogenic enterohaemorrhagic *E. coli* (EHEC) (174, 175). Symbionts also defend their niche (176). An example is the commensal *Clostridium scindens* whose colonization protects against *C. difficile* infection by production of secondary

Table 2. Pattern recognition receptor expression on endometrial cells

Pattern recognition receptor	Expressed on	EEC	ESC	Cyclical changes	Pathogen-associated molecular pattern	Pathogen	Reference
Toll-like receptor							
TLR1	Plasma membrane	x	(x)	-	Lipid-containing PAMPs e.g. LTA (together with TLR2/6)	Gram pos. bacteria, fungi,	Aflatoonian et al., 2007, Young et al., 2004
TLR2	Plasma membrane	x	x	x	Lipid-containing PAMPs e.g. LTA (together with TLR2/6), zymosan	Gram pos. bacteria, fungi	Aflatoonian et al., 2007, Hirata et al., 2007, Young et al., 2004
TLR6	Plasma membrane	x	(x)	x	See TLR1/2		Aflatoonian et al., 2007
TLR4	Plasma membrane	x	x	x	LPS, LipidA	Gram neg. bacteria	Aflatoonian et al., 2007, Hirata et al., 2007, Young et al., 2004
TLR5	Plasma membrane	x	(x)	x	Flagellin	Flagellated bacteria	Aflatoonian et al., 2007, Young et al., 2004
TLR3	Endosome	x	x	x	dsRNA	Virus	Aflatoonian et al., 2007, Hirata et al., 2007, Jorgensen et al., 2005, Schaefer et al., 2005 Young et al., 2004,
TLR7	Endosome	x	x	-	ssRNA	Virus, bacteria	Aflatoonian et al., 2007
TLR8	Endosome	x	x	-	ssRNA	Virus, bacteria	Aflatoonian et al., 2007
TLR9	Endosome	x	x	x	CpG-rich unmethylated ssDNA	Virus, bacteria	Aflatoonian et al., 2007, Hirata et al., 2007, Young et al., 2004
TLR10	Endosome	x	x	x	Unknown		Aflatoonian et al., 2007
Nod-like receptor							
Nod1	Cytoplasm	x	low	-	Peptidoglycan (gram pos. bacteria cell wall component)	Gram pos./neg. Bacteria	*Ghosh et al., 2013, King et al., 2009*
Nod2	Cytoplasm	x	x	x	Muramyl dipeptide	Gram pos./neg. Bacteria	Ghosh et al., 2013, King et al., 2009
Inflammasome	Cytoplasm	x	low	nd	Muramyl dipeptide	Gram pos./neg. Bacteria	D'Ippolito et al., 2016

RIG-1-like receptor							
RIG-1	Cytoplasm	x	low	nd	Short / 5'triphosphate dsRNA	Virus	Ghosh et al., 2013,
MDA-5	Cytoplasm	x	low	nd	dsRNA, preference for long segments	Virus	Ghosh et al., 2013,
C-type lectin							
E.g. Dectins, mannose-binding lectin	Plasma membrane/ endosome/ secreted	(x)	(x)	nd	-Glucan, zymosan (yeast cell wall), carbohydrates	Virus, Fungi	Oger et al., 2009

dsRNA/DNA, double-stranded RNA/DNA; EEC, endometrial epithelial cells; ESC, endometrial stromal cells; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MDA-5, Melanoma Differentiation-Associated protein 5; nd, not defined; (x), PAMPs, pathogen-associated molecular pattern; presence shown for whole endometrial sample; RIG-1,retinoic acid-inducible gene 1; ssRNA, single-stranded RNA

bile acids (177). Another contribution by commensals is continuous receptor stimulation leading to TLR upregulation; needed to sense potentially dangerous bacteria (178). Mice and humans treated with antibiotics, diminishing the intestinal microbiota, are highly susceptible to antibiotic-resistant strains because of a reduced expression of antimicrobial defense mechanisms (179). In the female reproductive tract, inhibition of gonococci (*Neisseria gonorrhoeae*) adherence is observed in the presence of *Lactobacilli* when using an *in vitro* model of endometrial epithelial cells (180). In an endocervical epithelial cell model, TLR agonists, as a surrogate for microbial products, were shown to stimulate antimicrobial products and mucins (181). Maintaining commensal colonization within the endometrium may likely offer a similar means of effective protection against uterine infections.

Tissue adaptation

In addition to lymphocytes, epithelial cells play a crucial role in co-existence with commensal colonization. An intact epithelium allows for safe colonization without extensive (pathogenic) barrier breach, through a physical barrier and reaction of the epithelial cells towards bacterial ligands. On the other hand, bacteria also contribute to a healthy barrier development. Increasing evidence highlights the importance of microbiota for gut development and morphogenesis. Known adaptations of microbiota-implicated tissue adaptations include intestinal epithelial cell differentiation (182), support of epithelial cell regeneration (183), modulation of epithelial cell permeability (184), and vascularization (185). In the uterus, commensal bacteria might contribute to the remodeling needed for a receptive state of the endometrium. The genus *Bacteroides*, constituting 30% of the endometrial community in 90% of women, is known to be tightly involved in intestinal tissue development by influencing epithelial cell maturation and maintenance (186, 187). Hooper and colleagues showed that *B. thetaiotaomicron* induced gene expression of proteins involved in nutrient absorption, angiogenesis, intestinal maturation, and mucosal barrier reinforcement (188). *Bacteroides* was shown to be essential for intestinal vascularization through signaling mediated by Paneth cells, the main type of epithelial cell in the intestine secreting anti-microbial compounds (189). Further studies using endometrial epithelial cells are warranted to ascribe similar physiological functions to the uterine microbiome during endometrial remodeling. The continuous reparative processes following the secretory phase might benefit from microbial support mechanisms. Dysbiosis in this critical period of preparing for blastocyst invasion might be one of the unrevealed causes leading to recurrent implantation failure due to failed remodeling.

Another interesting tissue adaptation involved in the peri-implantation period is the change in barrier function of the endometrial epithelium. Features of tight junctions, such as area of whole junction and the area enclosed by junctional strands, decrease from day 13 to day 22 of the menstrual cycle, which could be beneficial for implantation (190). Redistribution of adherens junctions and desmosomes during the WOI may also prepare for trophoblast invasion (191). The tight junctional barrier function is regulated by nutrients and cytokines (192). As a side effect

of increased barrier permeability, more barrier breach of the uterine microbiota can occur. This in turn can lead to a more pro-inflammatory immune environment with mucosal immune cells stimulated to secrete the cytokines that are beneficial for the peri-implantation period.

DISCUSSION

Conclusions on the possible presence of uterine colonization

Even before the era of 16S RNA analysis, it was put forward that "it is difficult to envision that a mucosa that is continuously exposed to microorganisms present in the lower genital tract and that is regularly invaded by sperm that can carry microorganisms into the endometrial cavity, may be free of bacteria and its products such as endotoxin" (193). We also agree that, based on the various lines of evidence for the existence of an endometrial microbiota, the era of the sterile uterus, free of any microbial compounds, has to come to an end.

Taken together, this variety of immunological adaptations due to microbial presence contributes to establishing the right balance of tolerance versus reactivity towards bacterial ligands, and likely has a physiological impact on pregnancy (Figure 2). Following the suggestion by Espinoza and colleagues, it can be envisioned that as long as no pathologic pro-inflammatory response against the microorganisms of the endometrial mucosa is started, the presence of a certain microbial coexistence does not harm the conceptus (194), and might well contribute to vital processes in the peri-implantation period and pregnancy.

Additional studies required to define the core microbiome

Sample cohort

Even though several studies have evaluated the microbiome of the endometrium, a "baseline" or "core" microbiome of this healthy endometrium has not been defined. The studies presented here on 16S rRNA evaluation of endometrial microbiota vary to a large extent in experimental approach. Differences in sample population, sample acquisition method, 16S region, controls, and bacterial genotyping make it impossible to combine the data in a more powerful meta-analysis. Of note, all studies enrolled small and highly selected cohorts. Except for the studies by Fang and colleagues (2017) assessing 10 healthy women, and Moreno and colleagues (2016) including 22 healthy women, all studies enrolled subjects seeking treatment because of reproductive complications or uterine abnormalities (18, 19). Therefore, the majority of data does not reflect a putative steady-state healthy endometrial microbiome. Only recruitment of healthy, fertile women would provide insight into the microbiome of a fertile, receptive endometrium. However, without underlying medical reasons, sampling in healthy women is unethical and therefore a major limitation to investigating uterine microbiota.

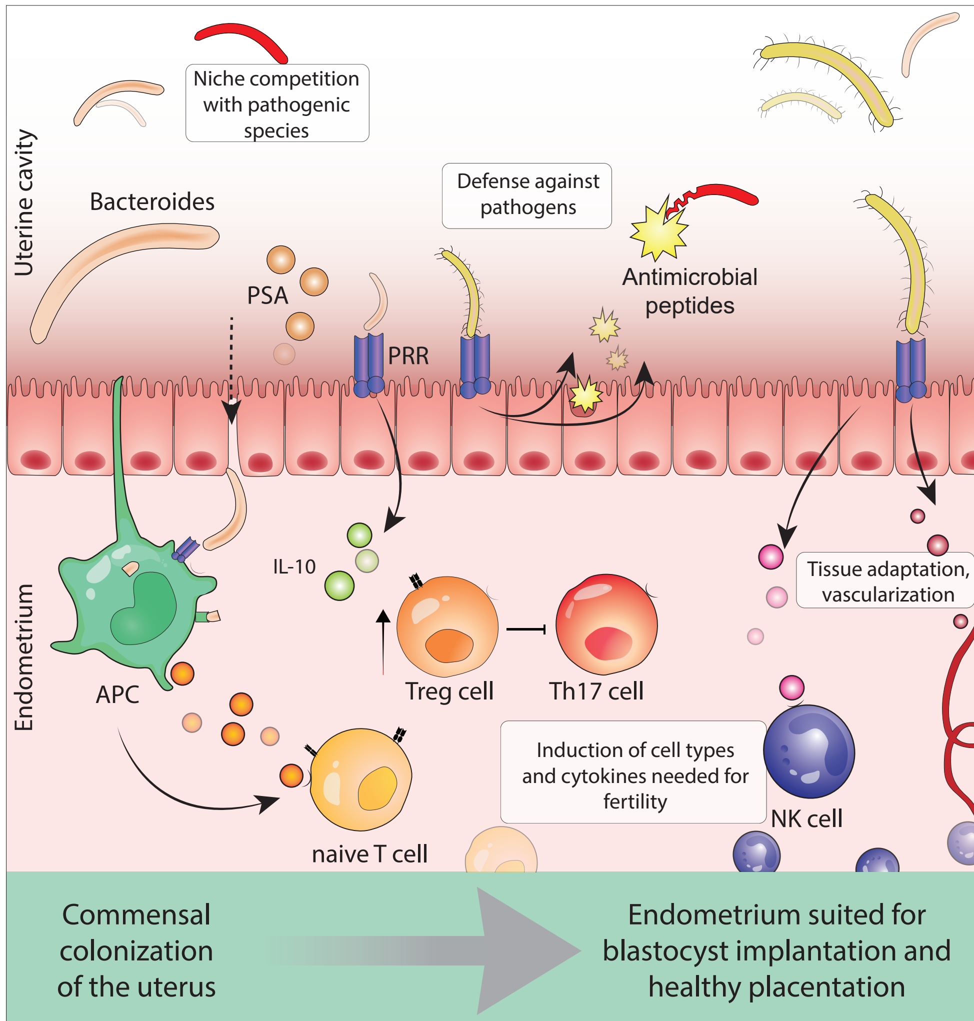


Figure 2. Uterine microbiota may contribute to healthy endometrium physiology.

Upon pathogen recognition receptor (PRR) stimulation, epithelial cells release soluble factors such as cytokines affecting local lymphocyte populations. If bacteria are naturally present in the uterus, similar interaction with host cells as seen in mucosa of the gut can be envisioned. Local lymphocytes e.g. antigen presenting cells (APCs) could sense microbes by probing through the epithelia or upon barrier breach initiating a signaling cascade. Presence of commensals might alter the mucosal T cell balance and the involved cytokines can have an effect on the local immune environment. Cells important for healthy implantation and placentation such as uterine natural killer (NK) cells are potentially affected. Therefore colonization might have an impact on important initial steps of endometrial physiology. *APC, antigen presenting cell; IL-10, interleukin 10; NK cell, natural killer cell; PSA, Polysaccharide A; PRR, pattern recognition receptor; Th17, T helper 17 cells; Treg, regulatory T cell.*

Controlling for vaginal and environmental contamination

A transvaginal approach carries the risk of contamination by the cervicovaginal microbiome. Due to the high biomass of the vaginal relative to the uterine microbiome and the high abundance of *Lactobacillus,* misrepresentation of species distribution cannot be excluded. To achieve more conclusive results in the near future, transvaginal studies on endometrial microbiota need to strictly incorporate control samples from vagina and cervix.

Additionally, contamination by reagents needs to be controlled for by including controls of all extraction kits used, a common source for misleading data (195). Also, air swabs and swabs of other environmental sources of microbial exposure need to be taken into account when trying to establish conclusive results on the low-biomass microbiome of the uterus.

Standardized sample processing

In the presented studies, major differences in the processing of material, data collection and analyses are all sources of variation (196). The choice of hypervariable region (V1-V9) of the 16S rRNA, containing species-specific sequences used for assessing microbial diversity, influences the detection results. V1 for example was found to be well suited to distinguish between *S. aureus* species, whereas V2 was more successful in *Mycobacterium* species differentiation (56). Depending on the variable region of the 16S rRNA, different species are preferentially detected. A standardized approach in which study design, sampling method, DNA extraction with generation of 16S amplicons and sequencing, assigning of OTUs to genus/species, and reporting of the findings is needed (197) to achieve conclusive results for endometrial and placental microbiome studies. Without these standardized, well-controlled study designs the uterine microbiome will still not come into its own in the future.

Points of interest

Timing of intervention

Owing to the dynamic fluctuations of microbiota in the uterus, the timing of microbial influence(s) on a healthy pregnancy needs attention. Depending on when the pregnant (or soon-to-be pregnant) woman undergoes any external microbial (pro-/pre or antibiotic) intervention, this might alter the physiologic processes of implantation, placenta formation or placenta maintenance if microbiota are involved. There is increasing evidence that BV is associated with infertility and pregnancy complications (198). Interestingly, the risk of delivering preterm because of BV is not reduced after clearance of infection by antibiotics (199). This might be explained by the fact that the effects of BV have already taken place and the initiated cascade of changes induced by the bacterial imbalance cannot be reversed. Accordingly, treatment of BV before 20 weeks of gestation might decrease the risk of BV-associated preterm birth, suggesting that the early phase of pregnancy in particular is sensitive to microbial impact (200). Systematic reviews on other conditions of bacterial imbalance associated with pregnancy and their treatment might

narrow down the window in which a certain microbe-associated effect on pregnancy takes place. In the future, robust evidence for a uterine microbiome might also fuel novel options for intervention concerning fertility and prevention of preterm birth risk in women with a history of BV.

Chicken or egg

It is possible that the state of inflammation is a sign of dysregulated immunity, rather than only a result of the presence of pathogenic, bacterial colonization. Chronic endometritis, the persistent inflammation of the endometrial lining, is associated with RIF (201) and successful antibiotic treatment of endometritis could clear the condition (as characterized by accumulation of plasma cells in biopsy samples), but clearance did not improve the chance of implantation. In this case, a certain microbial profile might not represent the cause but, instead, the effect of an immune state that is unfavorable for pregnancy. Interestingly, bacteria found in the endometrium causing the inflammation were not found in the vagina, suggesting that they are commensals in a dysbiotic state, rather than pathogenic intruders (36).

However, at present, we cannot exclude an active effect of microbiota on the physiological changes needed for a receptive endometrium. Functional studies are needed to understand the host-microbe interaction in the uterus. More robust information on the species involved in uterine colonization will allow further functional assessment using *in vitro* endometrial models (202). Next to this, the association of bacterial metabolites as a cause or consequence of certain microbial colonization and the state of fertility needs to be examined. Yeoman et al. (2013) evaluated metabolomic profiling as a fast and cost-effective diagnostic tool for BV (203). It remains to be elucidated whether uterine metabolites can be a proxy for the infertility related to dysbiosis. Also, since naturally occurring microbes continuously breach the endothelial barrier and are a part of homeostasis, microbes will continuously shape the immune environment. How this impacts the tightly regulated immunological processes involved from endometrium preparation towards a healthy pregnancy is yet to be discovered.

Dynamic bugs

Recently, the importance of the microbiome for regulation of rhythmic biological changes has been stated (204). Fluctuating microbial community structures might direct hormonal changes (205). It is tempting to speculate that similar dynamics are involved in the female cycle. Moreno and colleagues observed differences in the microbiota detected in the pre-receptive versus receptive phase in the endometrium (19). The extent of this effect varied. However, also for the vaginal microbiota it was shown that while in some women low constancy and high species turnover is observed, the extent of these changes can be very small for others (206). Until now, sample size prohibits conclusions on putative characteristic changes of the endometrial microbiota associated with the induction of the WOI. More in-depth studies on changes in the endometrial microbiome are needed to assess whether microbiota play a role in orchestrating

the cyclic endometrial changes and establishing a receptive endometrium. Studies examining 16S rRNA from endometrium alongside first trimester and term placental samples are needed to show if, and how far, the microbes of the fetal-maternal interface relate to the pre-pregnancy state.

Origin

The origin of the endometrial, and especially the placental, microbiome is highly discussed. Different routes of colonization have been suggested, including the gut, vagina, or oral cavity. We believe that with placental development originating from decidualized endometrium, it would be highly unlikely that the endometrial and placental microbiome are completely independent. In the intimate relationship of endometrium/decidua and placenta, the trophoblast cells invading the uterine mucosal tissue have to be in contact with the residential microbiota. Systematically comparing the healthy, pre-pregnancy endometrium to placental samples will help to elucidate whether the placental colonization results from the bacteria already present in the uterus. A vaginal origin is one of the suggested routes of uterine colonization, as a number of species seem to be residents of both body sites. Transfer of labeled macrospheres by a "uterine peristaltic pump" activity from vagina to uterus has been shown (207, 208). Evidence from mouse studies points out that a change in TLRs and antimicrobial peptide expression and function allows the passage of vaginal bacteria through the cervix into the uterus upon cervical infection. Remarkably, in nonpregnant mice, bacteria could travel from the lower reproductive tract into the uterus, but this was prohibited during pregnancy. These findings suggest that a healthy, uninfected cervix, regulates the ascents of vaginal bacteria (209). This theory is supported by earlier findings of differences in biochemical and chemical properties of the cervix depending on the pregnancy state (210, 211).

6

In line with theories on oral transmission of bacteria into the uterus, an association of periodontal disease and pregnancy complications is highly discussed (212). Indeed, bacteria such as *Lactobacillus* or *A.vaginae* that were found in the 16S assessment of the endometrial microbiome, are associated with caries, but not healthy plaques (213, 214). Interestingly, the placental microbiome resembles that of the oral cavity more than that of gut or even vagina (24). Several studies also show an association of cytokines and bacteria from amniotic fluid with oral microbiota (215, 216). It remains to be seen whether bacteria of the oral cavity can indeed travel via the circulatory system to selectively colonize the uterus before or during pregnancy (217).

Jimenez and colleagues elegantly showed that *in utero* bacterial colonization of the fetus takes place, which can be influenced by maternal oral uptake of microbiota (218). Pregnant mice were fed with a genetically labeled *E. fecium* strain isolated from human breast milk, and the bacteria could be detected in pups delivered by C-section. The authors point out that next to the possible oral route, APCs of the gut might sense and actively harvest intestinal bacteria, facilitating their

spread to other body sites. This sampling of luminal bacteria by DCs has been shown for the gut (219). A similar way of harvesting might already take place before pregnancy to prepare the uterus for placentation. If and how microbiota of gut and uterus might interact remains to be established. The study by Jimenez et al. (2008) fits with the "in utero colonization hypothesis", which in itself is heavily debated. Recently, Perez-Muños and colleagues have pointed out the shortcomings of published data surrounding the theory that newborns acquire first microbial colonization *in utero* (2). Studies on *in utero* colonization and microbial colonization of the non-pregnant uterus share the same methodological and technical limitations that have not been addressed sufficiently to allow any conclusions to be drawn about a core uterine microbiome. We wish to point that even though possibly not determining colonization of the pup *in utero,* as highlighted by the authors, maternal fertility or uterine health might have still have been affected by the microbial status. Importantly, a role in establishing protection against pathogenic invasion can be envisioned, a threat that mice in protected breeding facilities are a lot less exposed to than humans. Therefore, the fact that germ-free mice are fertile does not rule out a possible impact of uterine microbiota on normal physiology of the human endometrium.

CONCLUSION

Microbiota might be another piece in the complex mechanism contributing to the cogwheels of hormones, immune cells and physiological adaptations that are needed for successful pregnancy. As described in this review, a vast number of possible physiological contributions of the uterine microbiota can be inferred from other body sites. We highlight that the assessment of uterine microbiota still suffers from many limitations. Nevertheless, the available evidence shows that the presence of uterine 16S rRNA is not solely the result of sampling or analysis errors and deserves to be acknowledged. We conclude that the concept of the sterile endometrium, and the uterine compartment in general, is outworn, although the true core uterine microbiome still needs to be assessed. Functional studies are needed to elucidate the physiological importance of the microbiome in fertility. The challenge of studying reproductive immunology and the microbiota involved is that research on all of the different aspects is still in its infancy; microbiome, immunity, endocrinology in pregnancy, and placental and fetal development need to be studied together to obtain a more comprehensive overview. Thus, experts in various fields, such as microbiology and immunology, need to co-operate. Without this, such a basal process as pregnancy, needed for healthy offspring, will continue to be a mystery.

METHODS

PubMed and Google Scholar were searched for articles in English indexed from January 1st, 2008 to March 1st, 2018 for "16S rRNA", "uterus" and related search terms (Supplementary Figure I) to assess available evidence on uterine microbiome analysis. Studies on the upper female genital tract were investigated regarding data on specifically the uterine compartment. A manual search of the references within the resulting articles was performed. Studies on microbiota of other body sites were additionally assessed.

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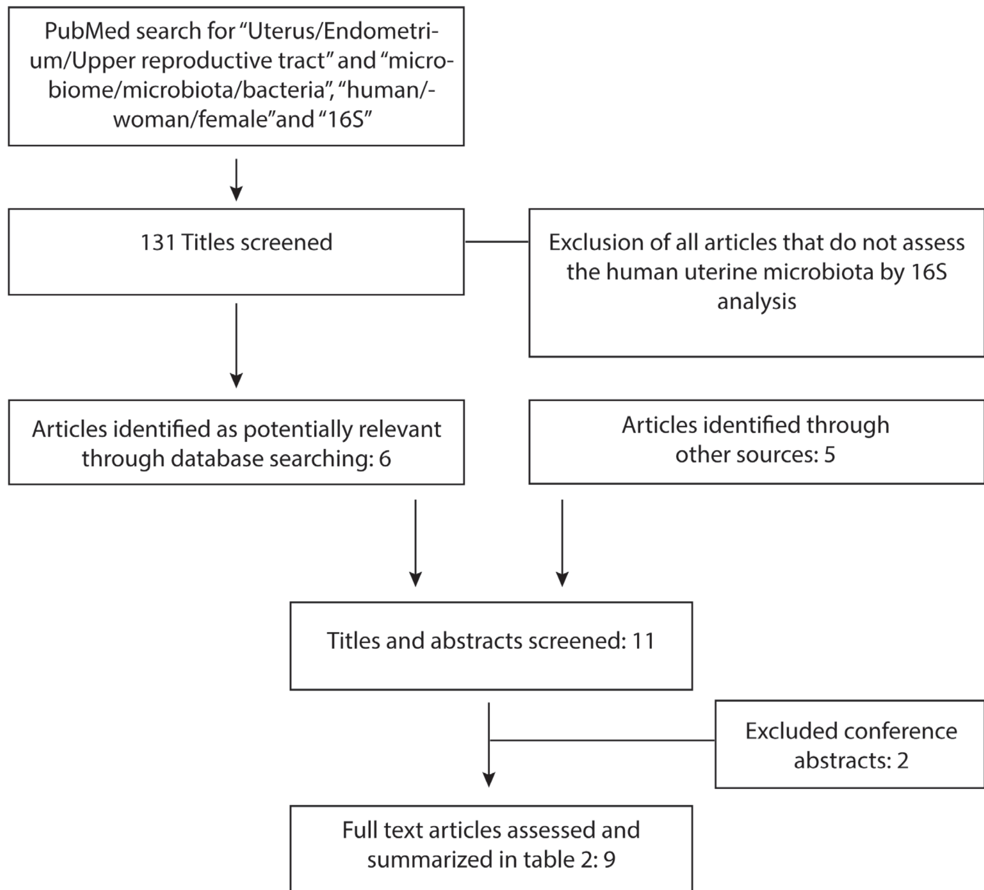
SUPPLEMENTAL INFORMATION



Supplementary Figure 1. Flow chart of the performed literature search to assess the available uterine 16S rRNA data

Supplementary Table 1. Commonly used microbial detection methods

	Strengths	Limitations
Culture-based techniques	Allows detection of live bacteria	Hard to detect slow-growing or fastidious species; some species are non-culturable
	No costly apparatus for read-out needed	No indication of relative species representation within a sample
		Labor and time-consuming
		Safety concern for involved staff
qPCR	Less labor intensive and time-consuming compared to culture-based methods	Biased approach as species-specific primers are needed
	High throughput possible	
	Sensitive	
16S amplicon sequencing	Possible to study fastidious or cultivation resistant organisms	Sensitive to contamination, from reagents and environment of the sample acquisition/processing. Thorough controls needed.
	Phyla / genus (to lesser extent) level distinction also possible	Little accuracy on species level
	Allows assessment of community diversity	Relative abundance of species detected can be affected by biomass of sample
	Unclassified microorganisms detectable	Detection of bacterial DNA does not necessarily mean presence of live bacteria; additional assay needed for live/dead discrimination
		Complex data analysis
Whole genome shotgun sequencing	Enhanced species detection (debated, see Tessler et al., 2017)	Probability of lower taxon count, likely resulting from limitations in mapping reads to databases
	Increased information on diversity	Expensive
	Details on species' genome	
	Information on viruses, fungi and protozoa additional to bacteria	

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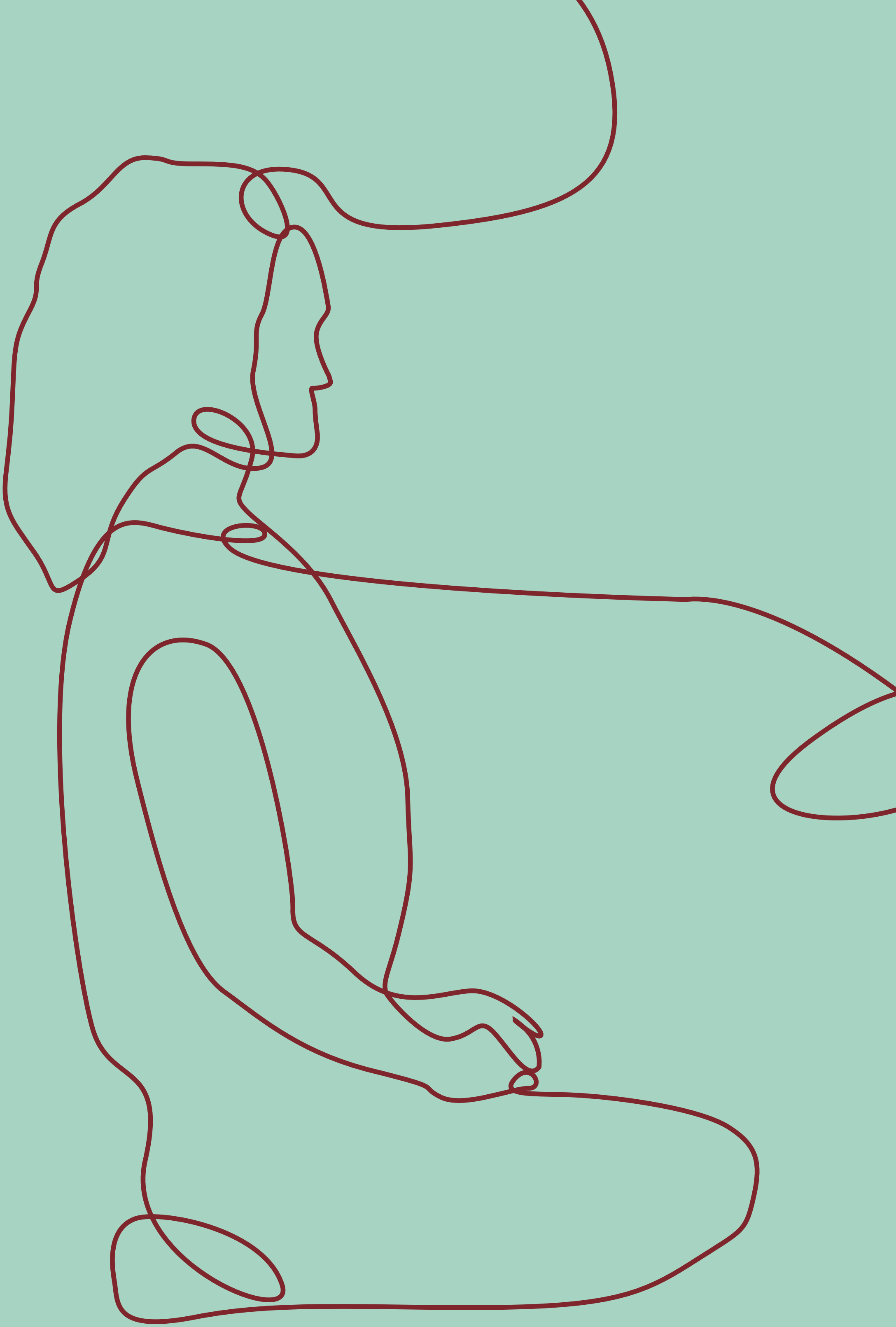

CHAPTER 7

Antibiotic intervention affects maternal immunity during gestation in mice

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Manuscript in preparation

ABSTRACT

Background

Pregnancy is a portentous stage in life, during which countless events are precisely orchestrated to ensure a healthy offspring. Maternal microbial communities are thought to have a profound impact on development. Although antibiotic drugs may interfere in these processes, they constitute the most frequently prescribed medication during pregnancy to prohibit detrimental consequences of infections. Gestational antibiotic intervention is linked to preeclampsia and negative effects on neonatal immunity. Even though perturbations in the immune system of the mother can affect reproductive health, the impact of microbial manipulation on maternal immunity is still unknown.

Aim

To assess whether antibiotic treatment influences maternal immunity during pregnancy.

Methods

Pregnant mice were treated with broad-spectrum antibiotics. The maternal gut microbiome was assessed. Numerous immune parameters throughout the maternal body, including placenta and amniotic fluid were investigated and a novel machine-learning ensemble strategy was used to identify immunological parameters that allow distinction between the control and antibiotics-treated cohort.

Results

Antibiotic treatment reduced diversity of maternal microbiota, but litter sizes remained unaffected. Effects of antibiotic treatment on immunity reached as far as the placenta. Four immunological features were identified by recursive feature selection to contribute to the most robust cohort classification (splenic T helper 17 cells and $CD5^+$ B cells, $CD4^+$ T cells in mesenteric lymph nodes and RORγT mRNA expression in placenta).

Conclusion

In the present study, antibiotic treatment was able to affect the carefully coordinated immunity during pregnancy. These findings highlight the importance of inclusion of immunological parameters when studying the effects of medication used during gestation.

INTRODUCTION

Any medication during pregnancy demands careful consideration, however treatment is essential when infections need to be controlled to ensure safe progression of the pregnancy. In western society, antibiotics for systemic use are amongst the most frequently prescribed drugs during gestation (1). Most commonly, gestational respiratory tract infections or urinary tract infections (UTI) require systemic anti-infectious medical intervention (2-4). If left untreated, 20-40% of asymptomatic UTI advance to acute UTI, which can lead to premature labor in up to half of the women affected (5). Antibiotic intervention has been shown to reduce complications of UTI such as preterm birth and/or low birth weight (6). Approximately 1 in 5 pregnancies is exposed to antimicrobial treatment nowadays (1, 7). While this can safeguard pregnancy, microbial intervention during pregnancy is known to have long term effects on the offspring as well. Gestational use of antibiotics is associated with an increased risk for the offspring to develop non communicable diseases like asthma, obesity, and even increased susceptibility to infections (8-10).

In general, antibiotic prescriptions require rational and critical use, not only to limit the selection towards drug-resistant pathogens (11). Awareness of how the natural microbiomes' contribution to physiology increases, and a diverse microbiome is key to healthy immunity (12-14). Systemic antibiotics drastically reduce the diversity of the gut microbiome and, depending on the compound and its target, impact on bacterial taxa can last for years (15, 16). This interference through broad-spectrum microbial modulation resulted, among others, in colonic infiltration of innate inflammatory cells in mice (17). In addition, antibiotics treatment skewed T cells towards an activated T helper (Th) 1 profile, together with a reduced proportion of FOXP3^{+}CD4^{+} regulatory T cells (Treg) (17). After antibiotic treatment, a general increase in pro-inflammatory transcriptional and cellular responses was observed, such as an activation of dendritic cells and upregulation of genes of pro-inflammatory cytokines interleukin 6 (IL-6), IL-8 and CXCL2 (18). The adverse effects of this inflammatory state might be limited in young adults (>65 years age) (18) but it is unsure if this holds true for pregnant women.

Pregnancy relies on tightly regulated immunity to allow trophoblast invasion and prevent infection, while excessive inflammation of the prenatal environment has to be avoided as well (19-27). Especially at the direct fetal environment, dysregulated immunity can cause pregnancy complications, such as preeclampsia (PE) and preterm birth, with long-term consequences on the offspring's development (28-30). As such, antibiotic-mediated changes of immunity can endanger prenatal development. Based on this, we questioned in this study whether gestational antibiotic use translates to maternal immune adaptations with possible impact on reproductive health. We here evaluated immunity of different maternal immunological compartments, including the placenta, using a murine model of gestational microbial modification.

RESULTS

Microbial disruption does not result in pregnancy complications in mice

Gestational antibiotic intervention of metronidazole, neomycin and polymyxin was previously shown to influence immunity of the offspring without observed pregnancy complications (31). Following this established treatment regime (Figure 1A), we assessed effective maternal microbial modification. 16S rRNA analysis of the gut showed a distinct microbiota composition in antibiotic treated mice compared to the control cohort (Figure 1B-C). In total 39 different genera showed a significant change in relative abundance as well when both groups were compared using ANCOM pipeline (Supplementary Table 1). In addition, a significant decrease of total alpha diversity represented by Shannon index diversity was observed in the antibiotic treated group (p-value = 0.0001616, Figure 1D). As major microbiota metabolites, SCFAs are involved in regulating intestinal integrity and intestinal immunity (32). Antibiotic treatment did not affect SCFA levels in maternal cecum, as no significant differences were observed regarding levels of acetic acid, propionic acid and butyric acid (Supplementary Figure S1A). Iso-butyric acid, valeric acid, and iso-valeric acid were not quantifiable in the cecal content of all sampled mice. To investigate a possible direct microbial effect of treatment on the prenatal environment, the 16S rRNA profiles of placental tissue were analyzed as well. Although some samples had reads as revealed by Qiime2 and DADA2 analysis, these represented mostly unspecific contamination, probably due to the high concentration of eukaryotic DNA in the samples. Therefore, based on our methods, we could not identify the presence of a specific bacterial community in the placentas analyzed. Pregnancy outcome was assessed as the number of pregnancies, intact and resorbed fetuses. No statistically significant differences were observed between groups. Eleven pregnant mice of the control group, and 8 of the antibiotic-treated group had a mean litter size of 7.8 (range 3-11, mean resorption rate 1) and 9.4 (range 3-11, mean resorption rate 0.8), respectively (Supplementary Figure S1B+C). No clinical symptoms in the antibiotic-treated group were observed that would suggest adverse effects on maternal health and as a consequence, pregnancy. Building on this intervention model, we then proceeded to analyze the consequences of antibiotic treatment on the maternal immune system.

Antibiotic treatment associated with shift in immune parameters differentiating from control cohort

Maternal immunity was assessed by flow cytometry of placenta, spleen, ILN, MLN and peritoneal cavity lavage fluid (PCLF), mRNA of intestines and placenta, and cytokine levels of maternal serum, amniotic fluid and splenocytes. A total of 129 different parameters were analyzed (Supplementary Table 2). To detect the classifying immune alterations occurring upon antibiotic treatment, a previously validated machine learning ensemble classification strategy was used (33, 34) suited for robust feature selection in the given low sample size setting. This method combines 8 classification algorithms, which compensates for possible biases inherent to the

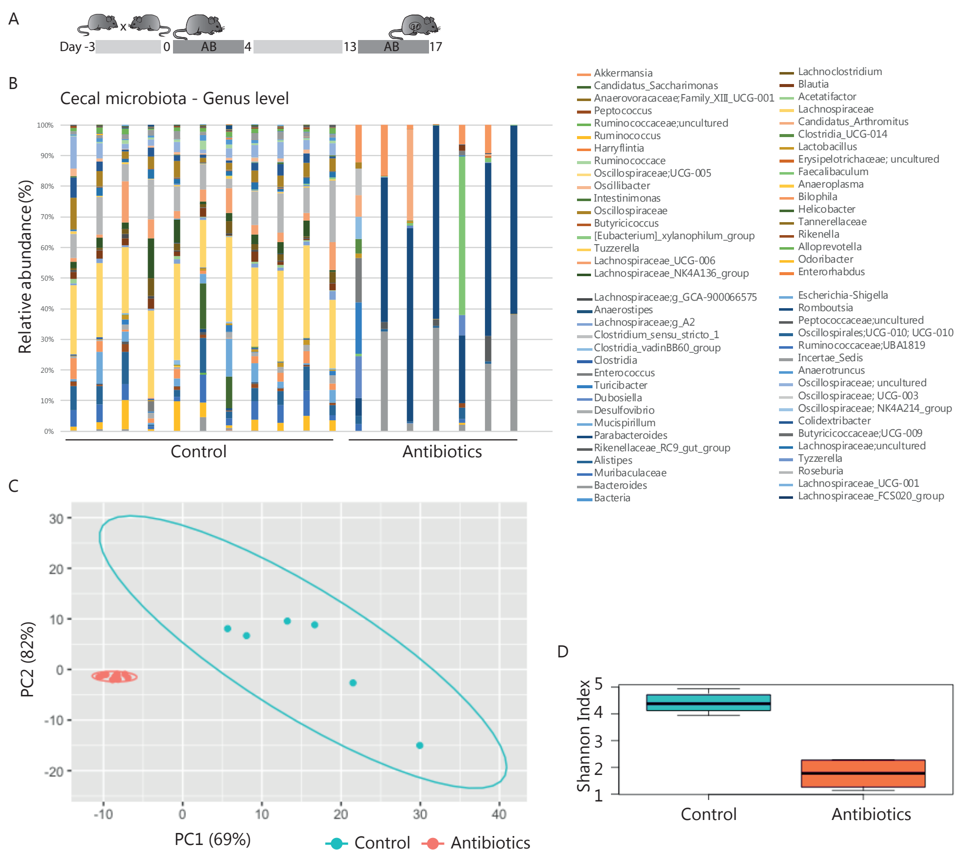

Figure 1. Antibiotic intervention in pregnant Balb/c mice.

(A) Schematic representation of the experimental model. After randomly assigning mated mice to the treatment groups, 11 mice of the control and 8 mice of the treatment cohort were pregnant and included for further analysis. **(B)** 16S rRNA composition of the cecal samples per individual animal (1 sample of the antibiotic treatment cohort excluded due to read count <500). **(C)** Principal component analysis of 16S rRNA sequencing data on genus level. **(D)** Shannon Index presenting microbial diversity.

individual algorithm. The output of the 8 classification algorithms each yielded a ranked list of features, representing the contribution of the 129 immune features to cohort classification. Each list was weighted based on coefficients and frequency of an individual feature contributing to classification (34) to aggregate classifiers to a single ranking. Classification algorithms were repeatedly run using the top 80% of the ensemble ranking (recursive feature selection). Each classification run was carried out 10 times, each run being subjected to 10-fold cross validation. At a global average accuracy of 90% as cutoff to ensure robust classification (Figure 2A), ensemble accuracy for each iteration of feature combinations showed optimal classification

when combining 4 features: frequency of Th17 cells and CD5^{+} B cells of the spleen, fold-change of RORγT assessed in placental tissue, and frequencies of CD4^{+} T cells of MLN (normalized expression values, Figure 2B). Principal component analysis illustrated separation of maternal immunity after antimicrobial intervention from healthy control (Figure 2C). The area under the curve (AUC) of 0.99 of the receiver operating characteristic (ROC) analysis confirmed robust classification based on the 4 identified features (Figure 2D).

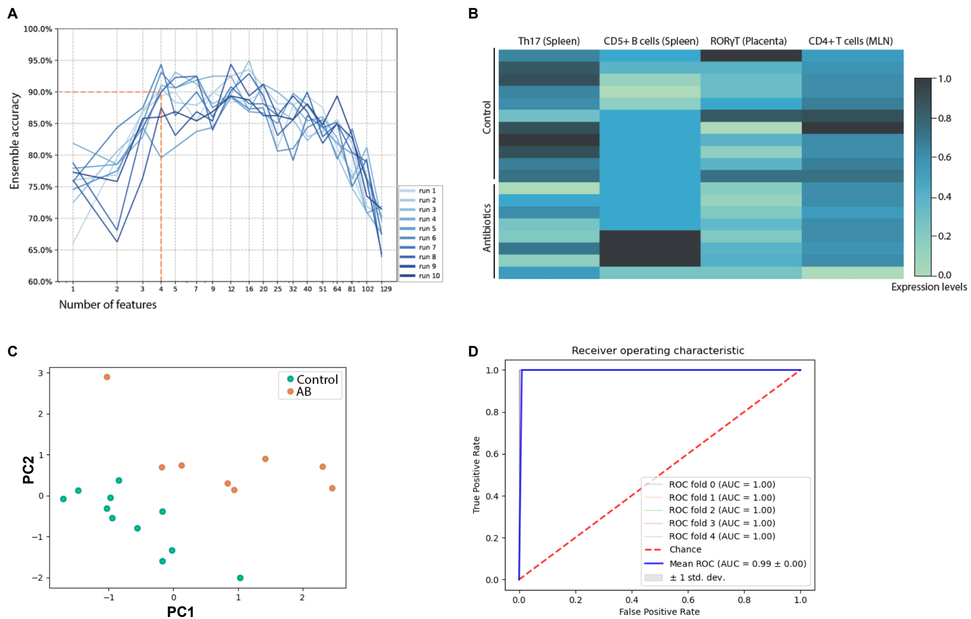

Figure 2. Multivariate analysis of immunologic assessment across samples of spleen, mesenteric lymph node, inguinal lymph node, peritoneal cavity lavage fluid, amniotic fluid, and placenta.

(A) Recursive feature reduction used in an ensemble machine-learning strategy to determine the number of top features needed to achieve robust (>90% accuracy) cohort classification. **(B)** Top 4 immune features that allow for distinction between cohorts. Normalized expression levels are depicted. **(C)** Principal component analysis based on the top 4 features that allowed for optimal cohort classification. **(D)** Individual classification algorithms were run with the top 4 features of the ensemble ranking. The receiver operating curve of Ridge regression is shown. The same results were observed using Passive-Aggressive or Logistic regression. Additional receiver operating curves are depicted in Supplementary Figure 2. AB: Cohort treated with antibiotics, MLN: mesenteric lymph nodes.

Systemic and placental T cell adaptations mediated by gestational antibiotics

Immune features of adaptive immunity contributed to cohort stratification as shown by machine-learning. Additionally, by univariate analysis, extra attention was payed to the different T cell subsets, whose differentiation is known to be influenced by symbiotic microbiota (35-37). While frequencies of Th1 (CD4^{+} CXCR3^{+}) and Th2 (CD4^{+} T1/ST2^{+}) in spleen, ILN, MLN and PCLF were not affected by treatment (Supplementary Figure S3A and B), a significant increase in placental Th2 cell frequencies was observed (29.0% ± 2.8% compared to 20.8% ± 4.4% in the control

group, Figure 3A). Percentages of CD4^{+}CCR6^{+}RorγT^{+} Th17 cells were lower in the spleens of mice treated with antibiotics (1.4% ± 0.1% compared to 1.1% ± 0.1% in the control group, Figure 3B) but no such differences were observed in other tissues. A significantly lower percentage of splenic CD4^{+}CD25^{+}FOXP3^{+} regulatory T cells (Treg) was observed in the antibiotic-treated group (5.3% ± 0.4% compared to 6.7% ± 0.3% in the control group, Figure 3C), but not in other compartments (Supplementary Figure S3C). Overall, T cell activation as observed through CD25 and CD69 expression was not affected in any of the tissues (Supplementary Figure S3D). Functional assessment of maternal lymphocytes through splenocytes stimulated ex vivo with LPS revealed only limited alterations of detectable cytokine levels in the supernatant. IL-6 production was significantly affected by the antibiotic treatment (Figure 3D) and a trend towards a higher IL-22 production was observed (p 0.061, Figure 3E). No significant differences were observed for any of the other cytokines analyzed (IFNγ, TNFα, IL-1β, IL-10, IL-2).

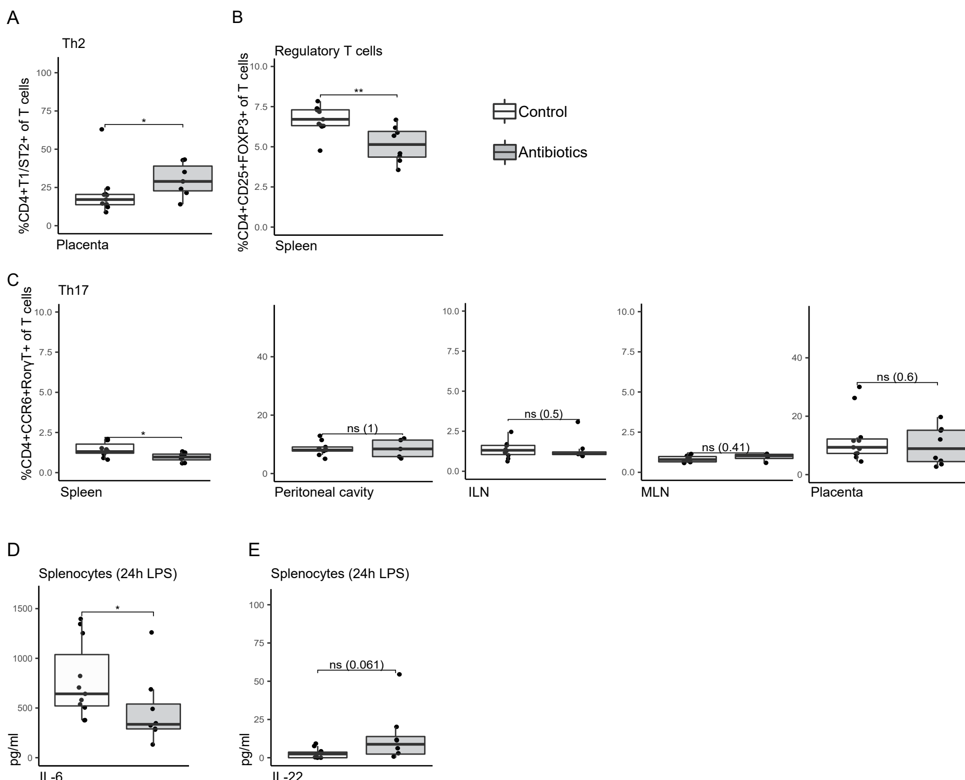

Figure 3. Univariate analysis comparing T cell subsets of antibiotic-treated and control mice.

(A) Frequencies of T helper 2 cells within T cells isolated from placental tissue, staining CD4+T1ST2+. **(B)** Splenic CD4+CD25+FOXP3+ regulatory T cells as frequency of total T cells. **(C)** CD4+CCR6+RORγ T+ T helper 17 cells isolated from spleen, peritoneal cavity lavage fluid, inguinal lymph nodes, mesenteric lymph nodes, and placenta. Data were compared by Mann-Whitney/Wilcoxon tests (non-parametric), * $p < 0.05$ ** $p < 0.01$. ILN: Inguinal lymph nodes, MLN: mesenteric lymph nodes, ns: non-significant.

DISCUSSION

In utero exposure to antibiotics could impact neonatal immunity through two different routes: by disrupting microbial colonization of the child, needed for healthy immune development, or by the antibiotics' impact on maternal immunity. The latter is associated with pregnancy complications known to alter offspring development (38-41). In the current study, we examined the effect of antibiotic treatment on maternal immunity using a murine gestational model. The chosen microbial intervention strategy was shown to affect offspring immunity (31). In line with this previous investigation of this combination of antibiotics, antibiotic treatment significantly decreased microbial diversity in the ceca of pregnant mice, but none of the mice were observed to have any clinical symptoms or reduced reproductive success. It was however possible to detect a maternal shift in immunological profile by using a machine learning ensemble classification strategy to assess >100 immune parameters of different sampling sides simultaneously (33, 34). Recursive feature elimination reduced the assessed parameters to a selection of 4 immunological features that distinguished control from antibiotic treated mother animals with an accuracy of >90%. Of note, these immune adaptations were observed throughout the maternal body, reaching as far as the placenta.

Antibiotics are known to affect intestinal immunity due to microbial manipulation of this immunological compartment (17, 42). Little is known on how this translates to other immune sites, especially regarding gestational tissues. In this exploratory approach, samples of placenta and amniotic fluid were included, and immune features were obtained through multiple methods, i.e. a combination of flow cytometry, mRNA expression, and analysis of soluble factors, to cover a wide range of possible effects. Using machine learning and recursive feature selection allowed for an open assessment of studied parameters. This dimensionality reduction enabled an unbiased focus on the strongest induced changes. Additionally, the applied ensemble strategy offered stable feature selection in this low sample size setting. After mating, of the 40 females that were randomly allocated to the control and treatment cohort, 11 and 8 mice respectively were pregnant and available for a final readout. Small sample size and large variation of the input data could weaken reproducibility when single selection algorithms are used, a limitation that could be overcome by the applied ensemble approach (34, 43). The presented high accuracy of the classification underlines how computational methods may help to reduce the number of animals needed for in vivo studies.

While earlier findings showed that antibiotic treatment resulted in a macrophage-dependent increase in inflammatory colonic Th1 responses mice (17), we did not observe any differences in Th lymphocyte populations in the MLN, the gut-draining lymph nodes, of the antibiotic treated animals. Of note, the study by Scott and colleagues investigated immunity in male mice (17) and thus could not take into account the highly specialized immune dynamics of

pregnancy. We additionally investigated placental and amniotic fluid samples, showing that systemic immune features do not represent immunity of gestational tissues. For example, placental $CD4^{+}CD25^{+}FOXP3^{+}$ Treg populations remained unaffected, whereas the percentage of splenic Treg in antibiotic treated mice was reduced. Especially Treg of the fetal-maternal interface are considered critical to maintaining the anti-inflammatory environment necessary during the implantation period and throughout gestation (44). The increase in decidual Treg upon conception is hypothesized to be facilitated locally; through seminal fluid (45), human chorionic gonadrotropin secreted by the blastocyst (46), extravillous trophoblast cells (47), or local immune cells such as decidual macrophages (47). This local induction, independent from systemic immunity, might be connected to the observed lack of antibiotic effect on placental Treg. On the other hand, we detected an increase in the Th2 cell populations in the placentas after antibiotic treatment. Based on the premise that gestational immunity depends on a tightly regulated Th1/Th2 mediated cytokine balance (48), it is tempting to consider this a protective counteraction to prevent from a possible proinflammatory load upon external modifications during gestation. Thus, while the underlying mechanisms of how gestational antibiotics may perturb maternal immunity and fetal development are not yet clear, our results emphasize the need to study parameters of the fetal environment. The observed altered immunological profile upon antibiotics may be linked to side effects that were previously exclusively ascribed to its antimicrobial effect.

During pregnancy, any dysregulation of immunity might affect placentation and thus fetal development (21, 49-51). Gestational immune adaptations are highly specialized to enable selective tolerance towards invading fetal cells, immune-competence to overcome pathogenic invasion, and immune-mediated support of establishing vascularization during placenta formation (52-55). Imbalance of immunity is thought to hamper correct placentation and thus contribute to the etiology of preeclampsia (PE) (56, 57). Indeed, prescription of antibiotics during pregnancy is associated with an increased risk of PE, which is also concerning considering that a fairly large proportion of pregnant women are prescribed antibiotics without an indication (58, 59). Still, it is unclear whether infections like UTI themselves, or their treatment is associated with an increased risk of developing PE (60). In case of an infection that requires antibiotic intervention, the involved inflammatory cascade may elicit systemic maternal inflammation and endothelial injury, which could also increase the risk of PE (61-65). Nevertheless, in vitro assays have shown that an alteration of immune responses can also occur independent of altered microbiota as phagocytosis by macrophages was inhibited directly through addition of antibiotics (66). Moreover, the current study shows that also in absence of infection, antibiotics affect the immune balance during pregnancy.

In conclusion, either mediated by manipulation of the microbial profile or by direct effects of antibiotics, treatment affects the tightly regulated immunity of pregnancy. The associated

poorly understood - but possibly far-reaching - consequences underscore the need for careful assessment and restraint use of antibiotics during pregnancy. Pregnant women are generally excluded from clinical trials, and, other than what can be deduced from retrospective studies, few approaches take into account the unique adaptations occurring to maintain a healthy pregnancy. These present results highlight the importance of in vivo studies on medication used during gestation to employ pregnancy models, taking into account the unique immunological properties, and possible tissue-specific effects, of this period.

MATERIALS AND METHODS

Animals

8-week-old, specific pathogen-free, male C57Bl/6 mice and 8-week-old nulliparous female Balb/c, purchased from Envigo (Horst, The Netherlands) were housed at the animal facility of the Utrecht University (Utrecht, The Netherlands) on a reversed 12 h light/dark cycle with unlimited access to water and semi-purified AIN-93G soy protein-based rodent diet (Ssniff Spezialdiäten GmbH, Soest, Germany). Upon arrival, mice were habituated to the laboratory conditions for two weeks prior to the start of the study. The male mice were mated with a separate set of Balb/c females (Envigo, Horst, The Netherlands) prior to the experiment, and males with proven fertility were selected to mate with the experimental females. Males were housed individually before and after mating, and female mice were housed 2 per cage. Animal procedures were approved by the Ethical Committee for Animal Research of the Utrecht University and conducted according to the European Directive 2010/63/EU on the protection of animals used for scientific purposes (AVD108002016597).

Experimental design

After 14 days of acclimatization, bedding from the cages of assigned breeder males was added to the cages of experimental female mice to facilitate synchronization of the females' cycle. After three days, males and females were housed together for 72 hours in a 1:2 ratio. Vaginal plugs were scored to assess the time of mating. After mating, the females were randomly assigned into the control group or the antibiotic treatment group. Antibiotic treatment was carried out by adding a mix of 2.5 mg/ml neomycin (Sigma Aldrich, Zwijndrecht, The Netherlands), 0.5 mg/ml metronidazole (Sigma Aldrich), and 0.09 mg/ml polymyxin (Sigma Aldrich) in the drinking water. The treatment consisted of two courses, starting with 4 days antibiotic treatment with followed by 10 days without antibiotics and another 4 days with antibiotics (Figure 1A). The animals were weighed before mating and at the end of experiment to evaluate the weight gain. The pregnant mice were killed by cervical dislocation on day 17 after mating, after which the number of fetuses and resorptions were assessed and tissue samples were collected for further analysis. During the sectioning, which was carried out in a laminar flowhood, sterile surgical instruments were

used. Pregnant mice were killed in a separate part of the flowhood to avoid contamination of the location where murine tissues were collected. The skins of the mice were carefully swabbed with ethanol, after which an intraperitoneal lavage was carried out by flushing the peritoneal cavity with 2 ml of PBS to collect intraperitoneal leukocytes. Hereafter, the abdominal cavity was opened using one set of surgical instruments, carefully avoiding contact of the skin with the abdominal cavity by pinning the skin of the animals back. Another set of sterilized surgical instruments was used to isolate placental and fetal tissues, and amniotic fluid was collected from individual amniotic cavities of the fetuses. Hereafter, other tissues (e.g. spleen, intestinal tissue and lymph nodes) were isolated, for which purpose the carcass of the mouse was moved to another section of the laboratory, to avoid contamination of the laminar flowhood. Samples isolated for analysis of mRNA-expression were immediately snap-frozen using dry ice and stored at -80°C. Samples used for flow cytometry or cell culturing were kept on ice until further processing.

Microbiota-analysis placenta & cecum

Total DNA was isolated from 50-225 microgram of cecal content feces and 125-200 microgram of placental tissue using the QIAamp Stool DNA mini kit (Qiagen). DNA was quantified by NanoDrop assay. The 16S rRNA gene profiling was analyzed as described by Paganelli et al 2019, by the Exposome HUB Utrecht. Briefly, 16S rRNA regions V3 and V4 were sequenced with an Illumina MiSeq reagent Kit v3 (600-cycle) on an Illumina MiSeq instrument (Illumina)(67). Samples were analyzed with the QIIME™ 2 microbial community analysis pipeline (68). For the cecum samples, significant differences between treatment and control groups at genus level were detected using the statistical framework analysis of composition of microbiomes (ANCOM)(69). p-values were adjusted for multiple comparisons using false discovery rates. RStudio 1.4.1103 (RStudio Team) was used to calculate alpha diversity using the Shannon index, and significance was calculated by the Wilcoxon test. The global difference in microbiota composition was assessed using principal component analysis (PCA), employing zCompositions, centered log-ratio (CLR) transformation, and ggplot R packages.

SCFA analysis cecal content

The cecal SCFA levels of acetic, propionic, butyric, isobutyric and valeric acids were quantitatively determined as well as levels of lactic acids as described previously (70, 71). The SCFA were captured using a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionisation detector. SCFA concentrations were determined using 2-ethylbutyric acid as an internal standard. Lactic acids were determined enzymatically using a d/l-lactic acid detection kit with d- and l-lactate dehydrogenase (EnzyPlus, BioControl Systems, Inc., Bellevue, WA, USA).

Lymphocyte subset analysis

For flow cytometric analysis of lymphocytes, single cells suspensions were prepared from isolated placental tissue, spleens, mesenteric lymph nodes (MLN) and inguinal lymph nodes (ILN). ILN and MLN single cell suspensions were obtained by crushing the tissue through 70 μm cell strainers on ice. The strainers were washed with RPMI 1640 medium, after which the cells were counted, resuspended in PBS and kept on ice until further processing. Splenocytes were similarly isolated, but red blood cells were lysed prior to counting the cells using lysis buffer (8.3 g NH4Cl, 1 g KHC3O, and 37.2 mg EDTA dissolved in 1 L demi water and filter sterilized). Placental tissues were cut into small pieces and incubated with Accutase (Stempro, GIBCO Life Technologies, Waltham, USA) for 35 min at 37°C under slight agitation. Hereafter, red blood cells were lysed as described for splenocytes, and placental cells were washed, counted, resuspended in PBS and kept on ice until further processing. Prior to staining cells for flow cytometric analysis, they were washed in PBS and 50μl of cell suspension (4.10^6 cells/ml) was incubated with a fixable viability dye eFluor® 780 (eBiosciences, Thermo Fisher Scientific, San Diego, CA, USA) for 30 min at 4°C. After washing, cells were incubated with anti-mouse CD16/CD32 (1:100 dilution in PBS/1% BSA; Mouse BD Fc Block, BD Pharmingen, San Jose, CA, USA) to block non-specific binding sites. For flow cytometric analysis of surface marker expression, cells were incubated at room temperature for 1 h in the dark with corresponding antibody-cocktails, washed with PBS/1% BSA and fixed in 1 % paraformaldehyde-solution until flow cytometric analysis. For the analysis of intracellular markers, cells were first stained for extracellular markers, washed with PBS/1% BSA and incubated overnight in Fix/Perm buffer (eBiosciences). The following day, cells were washed with permeabilization buffer (eBioscience), and incubated with anti-mouse CD16/CD32 for 15 min at 4°C in the dark. Next, the cells were stained for intracellular markers for 30 min at 4°C in the dark, washed in PBS/1% BSA and immediately used for flow cytometric analysis. The following fluorochrome-conjugated monoclonal antibodies were used: CD4-PerCP-Cy5.5 (eBioscience), CD69-APC (eBioscience), CXCR3-PE (eBioscience), T1ST2-FITC (MD Biosciences, St. Paul, MN, USA); CD11b- PerCP-Cy5.5 (eBioscience), NK1.1-APC (eBioscience), CD49b-FITC (eBioscience), CD94-PE (eBioscience); CD4- Brilliant Violet 510, CCR6-PE (BioLegend, San Diego, CA, United States), CD25-PerCP-Cy5.5, (eBiosciences), CD196 (CCR6)-PE (BioLegend), CD127-PE-Vio770 REA (Miltenyi Biotec, Bergisch Gladbach, Germany), Neuropilin-eFluor450 (eBioscience), RorγT-Alexafluor 647 (BD Pharmingen, San Jose, CA, USA), CD1d-PerCP-Cy5.5 (BioLegend), CD5-Alexa Fluow 647 (BioLegend), CD19-PE-Cy7 (BD), CD21/CD35-FITC (BD), CD23-PE (BD), CD24-Brilliant Violet 510 (BD), Tim-1-Brilliant Violet 421 (BD), Viability-APC-Cy7 (eBioscience). Results were collected with BD FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed with FlowLogic software (Inivai Technologies, Mentone, VIC, Australia) and Kaluza software (v2.1, Beckman Coulter, Fullerton, CA, USA).

Placental and intestinal mRNA-expression analysis

Total RNA was isolated from maternal intestinal tissues and placenta using the RNeasy mini kit (Qiagen, Germantown, USA) and cDNA was prepared using the iScript cDNA synthesis kit (Bio Rad, Veenendaal, the Netherlands), according to the manufacturer's instructions. For quantitative real-time PCR, the reaction mixture was prepared by adding specific forward and reverse primers and iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) to the cDNA samples, and amplifications were performed according to the manufacturer's instructions using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Validated qPCR primers for FOXP3, T-bet, GATA3, ROR-γT, β-Actin and IL-10 were obtained from SABiosciences (Qiagen, Germantown, USA). mRNA expression levels were calculated relative to the expression of β-actin reference gene with CFX Manager software (version 1.6).

Determination of cytokine profiles (in amniotic fluid, and after ex vivo stimulation of splenocytes)

Splenocytes collected from pregnant mice were cultured at a concentration of 4.10^6 cells/ml RPMI 1640 culture medium in 96-well U-bottom culture plates at 37°C in a humidified environment containing 5% CO_2, in the presence or absence of 10 μg/ml lipopolysaccharide (LPS) (Sigma). Cell culture supernatants were collected after 24 hours and stored at −20°C until further analysis. A ProcartaPlex multiplex protein assay kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used to assess the concentrations of interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-22, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ in amniotic fluid and cell culture supernatants, according to manufacturer's instructions. To calculate expression levels of cytokines by maternal splenocytes, cytokine concentrations in supernatants of LPS-stimulated cells were corrected for those measured in supernatants of unstimulated cells.

Recursive automatic ensemble feature selection

To discover the selection of immunological parameters that allow classification as control or antibiotics-treated cohort, a previously established ensemble feature selection was used (33, 34). This strategy allows for a more general selection of stratifying features than a single classifier, overcoming the bias of each individual algorithm. In brief, 8 classifiers (Bagging, Gradient Boosting, Logistic regression, Passive-Aggressive regression, Random Forest, Ridge Regression, SGD (Stochastic Gradient Descent on linear models), and SVC (Support Vector Machines Classifier with a linear kernel) classifier) generated a list of relative feature importance that is scored for a combined summary of top most relevant features. To ensure generality of the results, each classifier was run 10 times together with a 10-fold cross validation. This was repeated in a stepwise reduction of the 129 initial features by 20%, while determining the accuracy for each classifier.

Univariate analysis

Data were analyzed using R v.4.0.2 and the ggpubr, ggplot2, ggsignif, tidyr packages. Non-parametric Mann-Whitney test was performed. Values of $p < 0.05$ were considered statistically significant.

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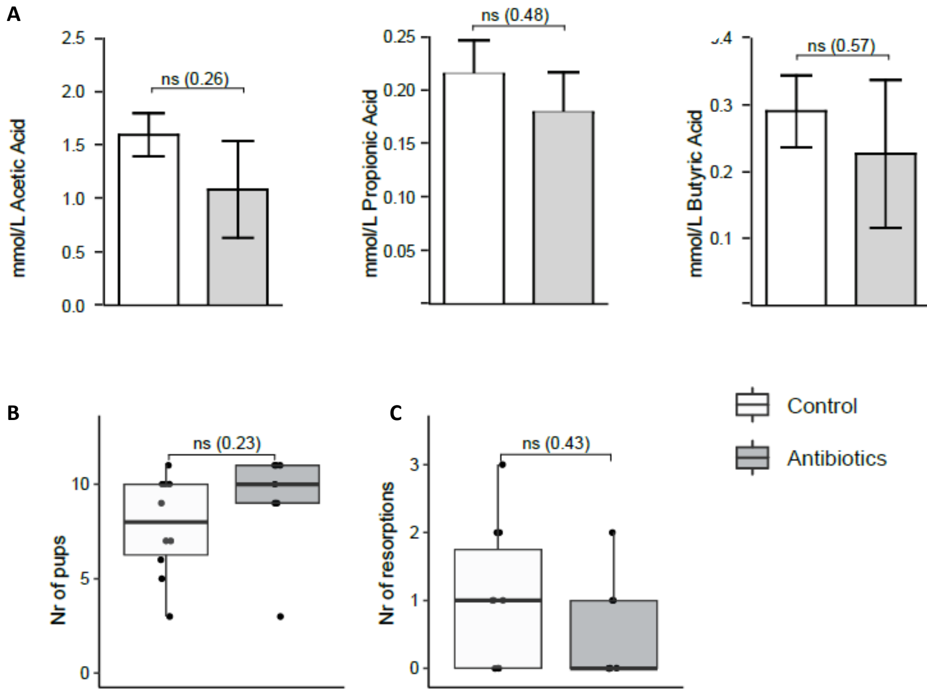
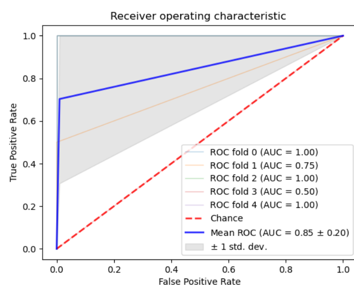
SUPPLEMENTAL INFORMATION



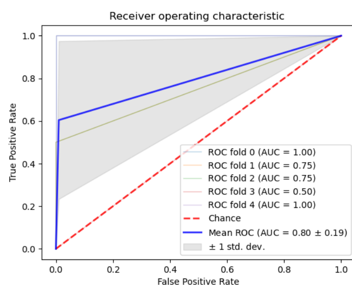
Supplementary Figure S1.

(A) Short chain fatty acids of maternal cecum. **(B)** Reproductive outcome assessed as litter size, and **(C)** numer of resorptions per animal. Data were compared by Mann-Whitney/Wilcoxon tests (non-parametric), ns: non-significant

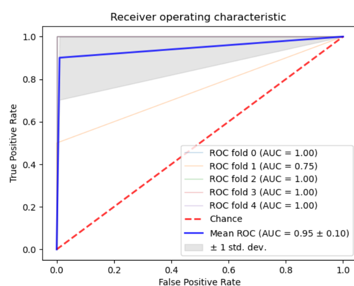
A **Gradient Boosting**



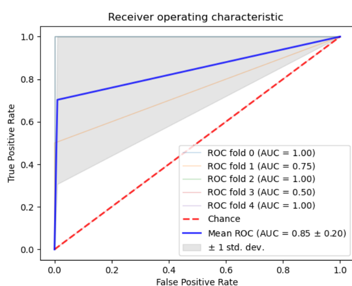
B **Random Forest**



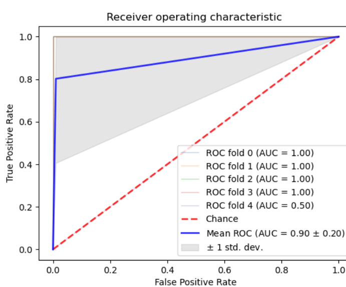
D **SVC**



E **Bagging**



C **SGD**



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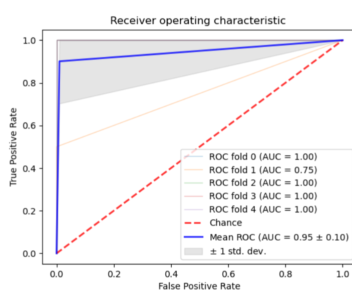

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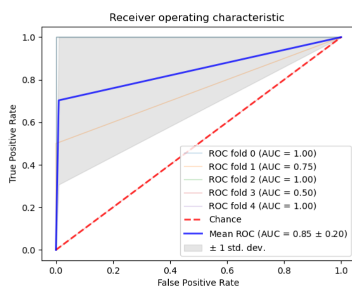
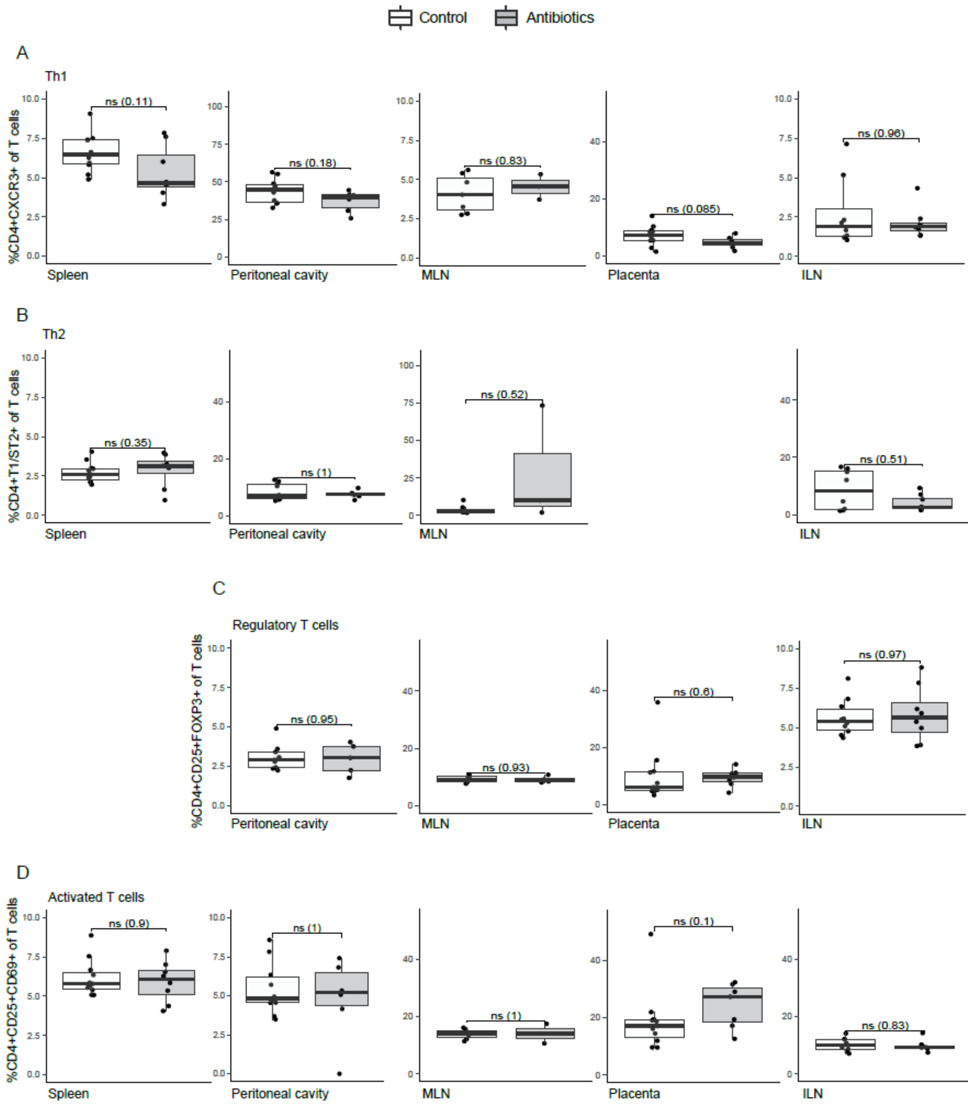
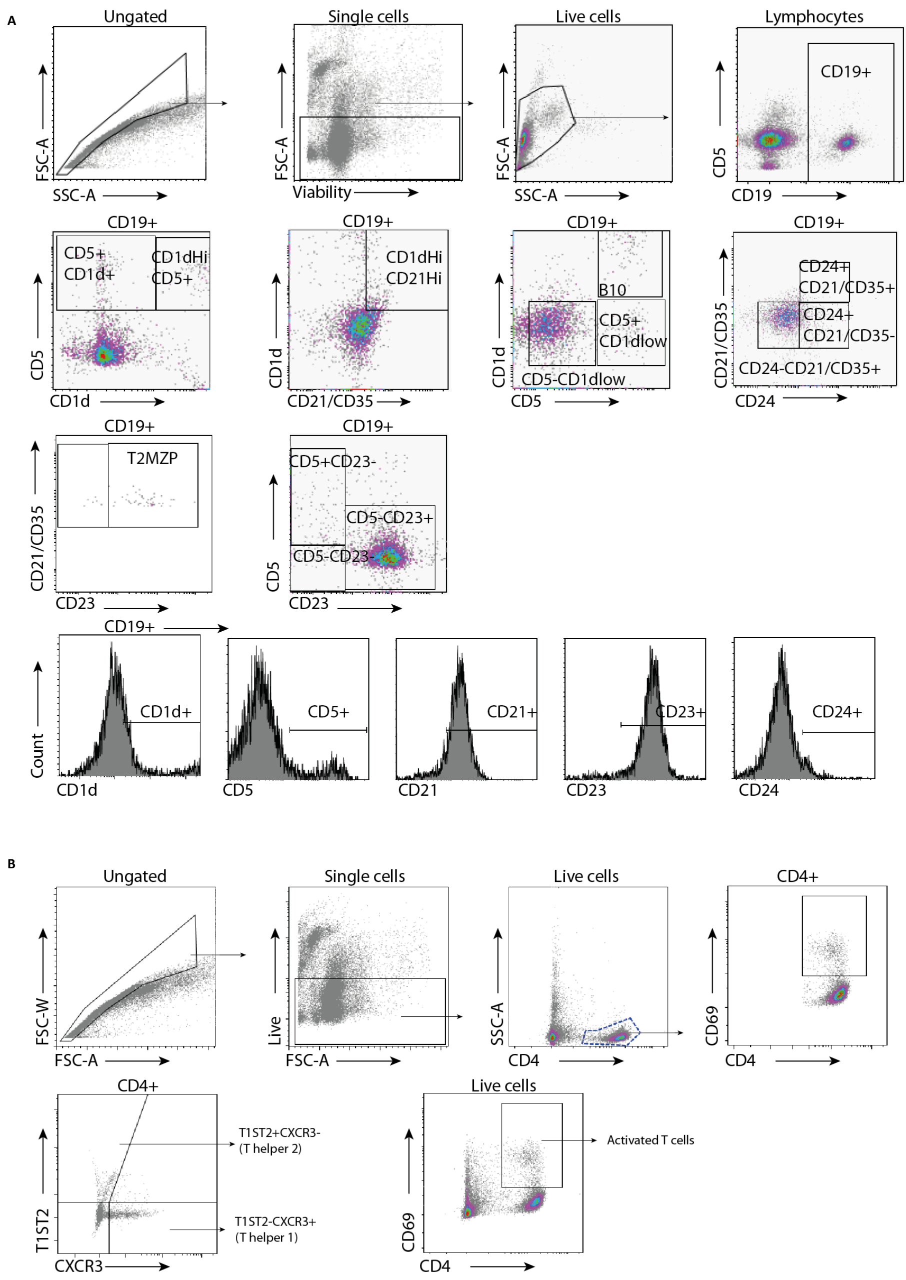


Supplementary Figure S2. Receiver operating characteristic curves of the individual classifiers used in the ensemble strategy after feature selection.



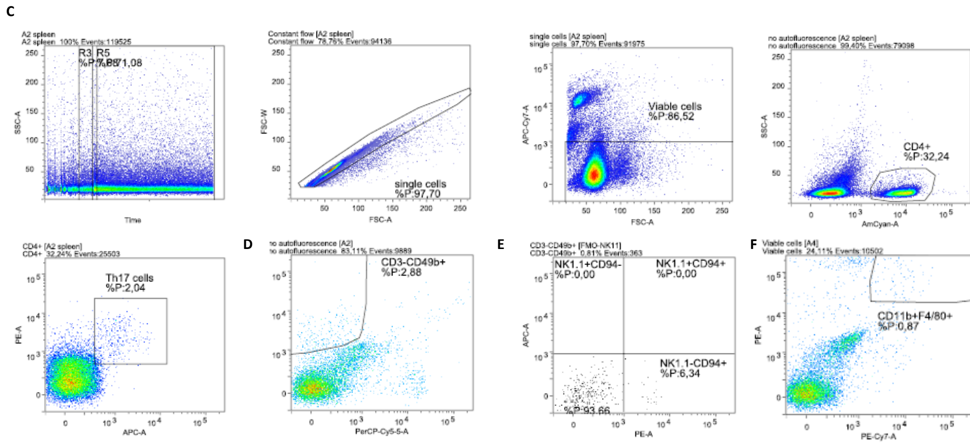
Supplementary Figure S3. Univariate analysis of T cells.

(A) T helper 1 cells, **(B)** T helper 2 cells, **(C)** Regulatory T cells, and **(D)** activated T cells. ILN: inguinal lymph nodes, MLN: mesenteric lymph nodes, ns: non-significant, Th: T helper cells.



Supplemental Figure S4. Gating strategy of features included in machine learning approach.

(see Supplemental Table T2 for list of features).



Supplementary Figure S4 continued.

(A) Flow cytometry plots of B cells and **(B-C)** T cells from spleen, **(D)** – **(F)** flow cytometry plots of macrophages and NK cell features from placenta. Gates were set based on fluorescence minus one stainings and isotype controls to adjust for autofluorescence. Single stainings were performed to calculate compensation.

Supplementary Table T1. Genera affected by antibiotic treatment as observed through 16S rRNA analysis of cecal microbiota.

Sample_ID	Average	SD	Average	SD
Bacteroidaceae_Bacteroides	0,006759	0,01322	0,187006	0,16813
Marinifilaceae, g.Odoribacter	0,039366	0,03153	0,000874	0,00231
Muribaculaceae, g.Muribaculaceae	0,047996	0,02772	0,005422	0,00919
Rikenellaceae, g.Rikenella	0,009005	0,00705	0,002075	0,00549
Tannerellaceae, g.Parabacteroides	0,002205	0,00303	0,456937	0,22845
Deferribacteraceae, g.Mucispirillum	0,041651	0,03989	0	0
Desulfovibrionaceae, g.Bilophila	0,025431	0,01803	0	0
Desulfovibrionaceae, g.Desulfovibrio	0,013822	0,00826	0	0
Erysipelotrichaceae, g.Dubosiella	1,88E-05	6,23E-05	0,0303	0,05198
Erysipelotrichaceae, g.Faecalibaculum	3,75E-05	0,00013	0,079191	0,19371
Erysipelotrichaceae, g.uncultured	0	0	0,0011	0,00206
Lactobacillaceae, g.Lactobacillus	0,007371	0,00608	0,002768	0,00512
Lachnospiraceae	0,25393	0,04748	0	0
Lachnospiraceae, g.A2	0,01104	0,00653	0	0
Lachnospiraceae, g.Acetatifactor	0,004405	0,00451	0	0
Lachnospiraceae, g.Blautia	0,020167	0,01053	0,00273	0,00722
Lachnospiraceae, g.GCA900066575	0,005693	0,00407	0	0
Lachnospiraceae, g.Lachnoclostridium	0,021349	0,01669	0	0
Lachnospiraceae, g.Lachnospiraceae_NK4A136_group	0,040543	0,03569	0,000135	0,00036
Lachnospiraceae, g.Lachnospiraceae_UCG006	0,048969	0,03913	0	0
Lachnospiraceae, g.Roseburia	0,100611	0,04559	0	0
Lachnospiraceae, g.Tuzzerella	0,004984	0,00231	0	0
Lachnospiraceae, g.uncultured	0,015858	0,00927	0	0
Butyricicoccaceae, g.Butyricicoccus	0,000863	0,00052	0	0
Butyricicoccaceae, g.UCG009	0,001958	0,00148	0	0
Oscillospiraceae	0,042516	0,02367	0,002801	0,00741
Oscillospiraceae, g.Colidextribacter	0,024567	0,01768	0	0
Oscillospiraceae, g.Oscillibacter	0,00919	0,00701	0	0
Oscillospiraceae, g.uncultured	0,039401	0,02487	0	0
Ruminococcaceae	0,009283	0,00657	0	0
Ruminococcaceae, g.Anaerotruncus	0,007639	0,00593	0	0
Ruminococcaceae, g.Harryflintia	0,000823	0,00125	0	0
Ruminococcaceae, g.Incertae_Sedis	0,014978	0,00666	0	0
Ruminococcaceae, g.UBA1819	0,000903	0,00081	0	0
Ruminococcaceae, g.uncultured	0,012825	0,00504	0	0
Peptococcaceae, g.Peptococcus	0,002752	0,00223	0	0
Peptococcaceae, g.uncultured	0,004379	0,00163	0	0
Anaerovoracaceae, g.Family_XIII_UCG001	0,000717	0,00066	0	0
Akkermansiaceae, g.Akkermansia	1,88E-05	6,23E-05	0,06528	0,06483

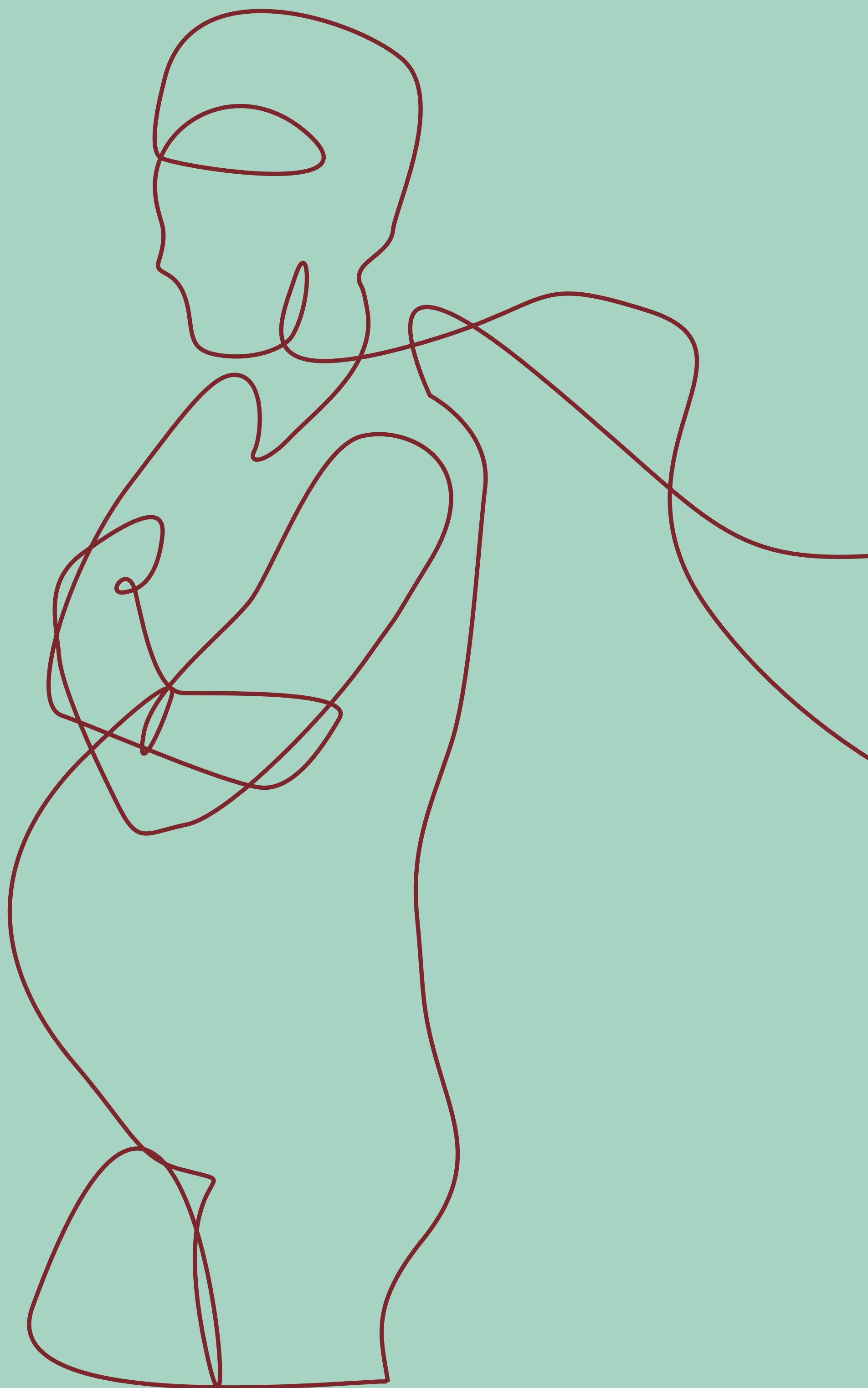
Supplementary Table T2. Immune features assessed after antibiotic treatment in mice

Technique	Tissue	Assessed parameters						
Flow cytometry	Spleen	T cells ^						
	Placenta	T cells						
	MLN	T cells						
	ILN	T cells						
	Peritoneal cavity	T cells						
	Spleen	B cells ^ ^						
	Placenta	B cells						
	Peritoneal cavity	B cells						
	Placenta	NK_CD3-	CD11b+	CD301+				
		CD49b+	F4/80+					
Luminex	Splenocytes after 24h stimulation	IL-2	IL-6	IL-10	TNFa	IL-1b	IFNg	IL-22
	Amniotic fluid	IL-2	IL-6	IL-10	TNFa	IL-1b	IL-4	IL-5
qPCR	Colon	FOXP3	Gata3	Rory	T-Bet			
	Placenta	FOXP3	Gata3	Rory	T-Bet	IL-10		

^ T cell panel: CD4pos, activated CD4pos, Th1 cells, Th2 cells, Th1Th2 ratio, Th17 cells, Treg cells, CD4pos, Neuropilin-1pos percent of Treg cells ^ ^ B cell panel: CD19+, CD1dHi CD5+, CD5+ CD1d+, CD1dHi CD21Hi, B10, CD5-, CD1dlow, CD5+, CD1dlow, T2MZP, CD24- CD21+ CD35+, CD24+ CD21- CD35-, CD24+ CD21+ CD35+, CD5- CD23-, CD5+ CD23-, CD5+ CD23+, CD1d histogram, CD5 histogram, CD21 histogram, CD23 histogram, CD24 histogram

Part 3

Connecting the pieces



CHAPTER 8

Summary and discussion

Human reproduction depends on complex adaptations of the uterus. Every month, hormones, tissue, and local immunity orchestrate the optimal conditions for implantation of a fertilized egg. Successful initiation of pregnancy depends on a balanced reciprocal interaction of maternal and fetal cells. A key challenge in this interaction is the natural antigenic mismatch between mother and child. How does the maternal immune system allow semi-allogeneic fetal cells to connect to the uterine circulation enabling formation of the placenta? In this thesis, we investigate this fetal-maternal interface to unravel a number of involved immunological adaptations. In **Part I** of this thesis, we investigate the role of uterine immune cells for healthy pregnancy. We illustrate how mucosal immunity of the pregnant uterus gain memory throughout gestation. Previously unassessed Treg and B cells, unique to the uterus, are part of the complex interaction of local immune cells. We show that a combination of immune cell subsets, isolated from the uterus and systemically, is altered in women suffering from recurrent pregnancy loss (RPL). In **Part II**, we examine if, and to what extent, microbiota could contribute to the modulation of the uterine immune environment. We conclude that microbiota and their compounds have a potential influence on receptivity. Maternal immunity is influenced through antibiotics, with possible impact on the developing child. This thesis highlights how an open approach is needed to discover new pieces in the puzzle that reproductive immunology still poses.

LOCAL IMMUNITY EVOLVES THROUGHOUT PREGNANCY

The endometrium, the mucosal lining of the uterus, forms the basis for implantation. Endometrial stromal fibroblasts differentiate in the mid-to-late luteal phase of the menstrual cycle to form the decidua. Formation of this specialized tissue allows for engulfment of villous cells in species such as humans, where trophoblast cells invade beyond the luminal epithelium (hemochorial placentation) (1). This transformation, termed decidualization, forms the basis for successful placentation. Existing data on uterine immunity mostly focus on specific cell types in either endometrium or decidua. We first investigated the immune signature of the non-pregnant uterine mucosa in parallel with term decidua to assess pregnancy-induced adaptations **(Chapter 2)**. Monthly, in absence of conception, decreasing progesterone levels initiate shedding of the superficial layer of the decidualized uterine mucosa to allow for renewal of the receptive surface (2). Discharge of the endometrial lining involves an efflux of immune cells that reside in the tissue. We asked women to collect menstrual blood using a menstrual cup, to allow for a non-invasive access to endometrial lymphocytes. We previously established that the subsequent isolation of immune cells yields cells similar to lymphocytes obtained through endometrial biopsies (3). We also made use of this now broadly accepted strategy in **Chapter 4 and 5**. At term, placentae can be easily obtained, and the maternal side of the placental membranes (decidua parietalis) can be separated from the fetal layers, which allows for the retrieval of maternal uterine cells post pregnancy. Analyses of material sampled at either time point, non-pregnant and post-pregnancy,

confirmed that we successfully isolated immune cells of the uterine mucosa; the observed NK cell profile consisting of almost exclusively NKbright cells displays the phenotype associated with uterine cells (4). Additionally, the presence of $CD103^+$, an integrin subunit mostly observed on tissue resident cells in combination with the absence of CD62L, a marker associated with circulating leucocytes, further confirmed that our observed results reflect uterine immunity rather than peripheral blood contamination.

Our results again reveal the general consensus of NK and T cell fluctuations throughout gestation: NK cells are abundant in non-pregnant endometrium but decrease in term decidua parietalis (5-7). The opposite is seen for T cells, accumulating throughout gestation (8-10). Of note, all samples of uterine mucosa contained, albeit small, a $CD19^+$ B cell fraction.

Using high dimensional flowcytometry, we showed a gain in immune cell memory from pre-pregnancy mucosal immunology up to term decidua. Less naïve, and more effector and central memory $CD4^+$ and $CD8^+$ T cells were observed in decidua parietalis **(Chapter 2)**. Likewise, an increase in B cell memory was observed in the study described in **Chapter 4**. Especially in contrast to lymphocytes of the non-pregnant mucosa, this suggests that cells become activated and differentiate over the course of pregnancy. Indeed, *in vitro*, endometrial cells were able to differentiate and display a similar switch in phenotype upon T cell activation. The T cell signature we observed in term decidua is in accordance with earlier data by Tilburgs and colleagues that highlights the distinct functional profiles of these highly differentiated decidual T cells, compared to their counterparts in peripheral blood (11). Building up on this, it was later shown that decidual T cells are able to recognize fetal antigen as previously hypothesized (12). Frequencies of HY-specific T cells, in response to male fetuses, were 10-fold higher in decidua (0,6% of $CD8^+$ T cells) compared to peripheral blood highlighting the local character of the occurring recognition. In an in vitro mixed lymphocyte reaction, decidual $CD4^+$ and $CD8^+$ T cells were shown to proliferate more potently than peripheral blood T cells in response to irradiated fetal cord blood cells. Powell and colleagues additionally observed how Treg are essential to control this proliferation (12). These and other studies highlight the importance of local Treg to contain inflammation of the decidua (8).

To establish an overview of the evolving immune environment throughout pregnancy, we also investigated Th subsets in **Chapter 2**. The paradigm that a Th2-type immunity, marked by IL-4, IL-5, and IL-13 secretion, dominates a Th1-type, characterized by an IL-2, and IFNγ secretion profile, dominated the field in the 1980s to 1990s (13). This Th2 profile was thought to dampen a harmful Th1-type response of the maternal immune system towards fetal antigens. It has now been established that there are more aspects to consider, such as Treg and IL-17 secreting Th17 cells (13, 14). Likewise, asides from Treg, we investigated a number of Th subsets. In the flow cytometric approach used in this study, we assessed Th frequencies using

a chemokine receptor-based characterization (15). A limitation of this approach may be that as this phenotypical assignment has been established for peripheral blood lymphocytes, marker expression might not always refer to cells of equal functional characteristics when examining mucosal tissue. We observed relatively more Th1 compared to Th2 cells in uterine samples, which is in accordance with previous data (16). Comparing non-pregnant endometrium and decidua at term, we observed no significant change in levels of Th1, Th1-like, Th2, and Th17 cells. However, in this study, we only examined the pregnant mucosa at the very end of gestation. Pregnancy complications such as PE and RPL, manifest in the first trimester, during spiral artery remodeling. Thus, if fetal/paternal antigens or other possible triggers induce changes in Th profiles during the peri-implantation period, this cannot be conclusively investigated with this approach that lacks observations of early pregnancy.

The discovery of immune subsets and immune-modulatory molecules continues. Measuring a broad range of cytokine profiles can directly point out pro- or anti-inflammatory trends. Indeed, recurrent implantation failure after ART has been associated with dysregulated uterine profiles of soluble factors (17). Analyzing endometrial cells obtained from menstrual blood of healthy, fertile women offers a way to research profiles of healthy, fertile women in future approaches.

In the work presented in this thesis, we sought to exemplify that healthy pregnancy, much like the immune response in general, depends on the interaction of cells and soluble factors, rather than a particular type of cell that, on its own, determines the faith of gestation. The initial, exploratory study described in **Chapter 2** led us to question what other possible key immune-modulators are involved in pregnancy that fulfill roles beyond our current understanding.

THE DECIDUA CONTAINS SPECIALIZED REGULATORY CELLS: REGULATORY T CELLS

A major focus in studying the maintenance of tolerance at the fetal-maternal interface has been put on Treg cells. We observed that compared to non-pregnant endometrium, frequencies of $CD4^{+}CD25^{Hi}$ were increased in term decidua **(Chapter 2)**. This is in line with previously reported high levels of functional $CD4^{+}CD25^{Hi}$ T cells with suppressive capacities and *in vitro* experiments confirming an induction of $CD4^{+}Foxp3^{+}$ Treg upon co-culture with EVT (18-20). However, additional to a phenotypic overview in **Chapter 2**, functional assays are needed to confirm if the observed T cell subset is truly regulatory, i.e. suppressive. The number of studies assessing functional capacities of decidual T cells with a regulatory phenotype is limited (18, 21, 22).

In **Chapter 3**, we advance knowledge on Treg at the fetal-maternal interface. We describe, next to CD25Hi Treg, two additional functionally and phenotypically distinct Treg subsets of the decidua with regulatory capacities; PD1^{Hi} and TIGIT^{+} T cells. All three Treg subsets express distinct transcriptional profiles compared to their phenotypic counterparts of peripheral blood. Both PD1 and TIGIT^{+} Treg selectively inhibit CD4^{+} and not CD8^{+} Teff cells. The ability of decidual PD1^{Hi} to induce IL-10 expression in effector T cells suggests a positive feedback loop. This emphasizes the heightened capacity of decidual cells to produce IL-10, of which reduced presence is associated with pregnancy complications (23-26). Based on their lack of FOXP3, high PD1, IFNγ, and granzyme expression, and capacity to suppress CD4^{+} T cell proliferation through IL-10, PD1^{Hi} resemble Tr1 cells. However, they do not express CD49b and LAG3, supporting the notion that decidual Treg are a unique result of pregnancy-related antigen encounter. Accordingly, direct cell contact with extra villous trophoblast cells induced PD1^{Hi} cells. Also decidual macrophages were able to induce CD25^{Hi} and PD1^{Hi} Treg, upregulating FOXP3 and HELIOS expression. This fits with the timing of the observed differential expression, as Helios expression is associated with potent suppressive capacities. This was displayed by first trimester-derived decidual Treg, but only to a small extent by Treg isolated from term. In-depth mass cytometry-based phenotyping of decidual lymphocytes supports the presented findings of decidual Treg as heterogenous population with differential FOXP3 and HELIOS expression (27). Bright CD25 and Helios expression is associated with natural Treg (nTreg), which arise from the thymus to prevent anti-self-responses, in contrast to induced Treg (iTreg), that can dampen allo-recognition. It is tempting to speculate, based on our observed marker profiles, that the balance between nTreg to iTreg shifts towards more iTreg during gestation. However, the lack of clearly defining surface markers to specifically identify nTreg or iTreg leaves us questioning where (and to what purpose exactly) decidual Treg are induced. The functional and transcriptional differences between the selected Treg subsets of first trimester and term decidua underline that both timepoints deserve independent assessment.

THE DECIDUA CONTAINS SPECIALIZED REGULATORY CELLS: IL-10 PRODUCING B CELLS

The individual comparison of cells isolated from non-pregnant endometrium to decidua parietalis, and first trimester decidua to decidua parietalis, point out how focus on one time point will not suffice to understand the role of a particular cell at the fetal-maternal interface. We investigated lymphocytes isolated from menstrual blood alongside first trimester and term decidua parietalis by high-dimensional phenotyping **(Chapter 4)**. All studied time points contained uterine B cells, albeit at a low abundance (<2% of total lymphocytes). Systemically, B cells, together with T cells, are known as a major mediator of adaptive immunity. Next to their classical antibody-secreting function, B cells can act as APC, and modulate the local immune environment through cytokine

production (28-30). Especially their capacity to secrete IL-10 has gained attention, starting with the observation that the inability of B cell-deficient mice to recover from experimental autoimmune encephalomyelitis stems from the lack of IL-10 producing B cells (31, 32). The idea that B cells are not the cause, but a suppressor of autoimmune disease contrasted all other approaches focusing on auto-antibody production. The intriguing observation that a major part of decidual B cell samples, especially of the second trimester, expressed CD24 and CD38 to a high extent **(Chapter 4)**, often connected to the ability to produce IL-10, sparked a new question: could B cells of the fetal-maternal interface contribute to local maintenance of tolerance? Recently, few studies have started to acknowledge a possible role for B cells in healthy pregnancy. Rolle et al. initially demonstrated that systemic B cells of pregnant women were able to suppress TNFα production of $CD4^{+}$ T cells, which could not be mimicked in the same setting by non-pregnant donors (33). An increase in the pool of $CD19^{+}CD24^{Hi}CD27^{+}$ B cells due to pregnancy was not shared by women who had suffered from a spontaneous abortion. Serum of women during pregnancy, but not after pregnancy loss, was able to induce IL-10 production by B cells of peripheral blood (33). This was attributed to pregnancy-induced high levels of human chorionic gonadotropin (hCG) as 95% of $CD19^{+}CD24^{Hi}CD27^{+}$ expressed the hCG receptor and $CD19^{+}$ cells produced IL-10 upon hCG stimulation (33). HCG is secreted by the blastocyst even before its implantation and before serum levels are measurable (34, 35). In this period, it likely serves a juxtacrine function by modulating levels of e.g. metalloproteinases, insulin-like growth factor-binding protein -1 (IGFBP-1), Leukemia Inhibitory Factor (LIF), M-CSF, vascular endothelial growth factor (VEGF) (36). Its local action in stimulating IL-10 production by B cells was then shown in co-cultures of trophoblast cell lines, and murine placental explants, with B cells. The interaction of these fetal cells and B cells induced IL-10, but this was abrogated when hCG was blocked (37). Supporting the notion that induction of IL-10 production of B cells is a local phenomenon upon trophoblast encounter, we observed that decidual B cells are potent producers of IL-10 upon CpG/CD40L stimulation, an accepted strategy to detect IL-10 secretion by B cells (38-40), and do so to a higher extent than systemic B cells. This fits the results of Fettke et al., who pointed out that co-culture with fetal cells had an additive effect on IL-10 levels compared to CD40L/CpG stimulation alone (37). Likewise, we observed that, in a number of samples, decidual B cells produced IL-10 without the need for CpG/CD40L stimulation supporting the idea that B cells have already been primed *in utero*, through hCG, trophoblast cells, or other trophoblast related receptor-ligand interactions, to secrete IL-10.

Our observation that B cells are located in clusters, together with T cells, points out that even though their numbers are limited, decidual B cells might facilitate IL-10 mediated inhibition of neighboring cells. Of note, lymphocyte clusters could not be found for all decidual samples that we investigated by immunohistochemistry, but in approximately half of all cases. This might be sampling issue, clusters might not be located all throughout the decidua and only a small proportion of tissue is embedded for imaging. Another possible explanation might be time

dynamics; induction of clusters might occur at specific time points during gestation, which may vary between individuals. Although the presence of uterine B cells as being a part of healthy pregnancy has been disputed in the past, several studies now confirm their presence and they clearly warrant additional investigation (41-44).

Where do these B cells come from? Circulatory B cells are decreased from mid-gestation to delivery, which might result from a gestational reduction in lymphopoiesis, as shown in mice (45-50). This might not be the only explanation for reduced systemic B cells; evidence supporting a relocation of B cells to the uterus increases. Placental explants are able to attract B cells through CCL20 (placenta) - CCR6 (B cell) interaction (51). Also, data on decidua support the notion that B cells are retained within the uterus during gestation. Matching our observation that naïve B cells can be isolated from all decidual samples, an increased proportions of mature/naïve B cells were found within the intervillous space (51). However, probably not all of the observed decidual B cells are the result of pregnancy-induced homing to the uterus as menstrual blood samples also contain B cells. This hints towards a tissue-resident population independent of implantation. Furthermore, we observed that B cells of the decidua contain subsets with phenotypes distinct from peripheral blood. Either the adaptations to the uterine environment itself, or pregnancy induced interaction with B cells causes differential marker expression of local B cells. As such, primary human samples of the fetal-maternal interface cannot be replaced by investigation of systemic cells.

A QUESTION OF TIMING

Origin of immune cells, as well as gestational duration, strongly impacts immune phenotype and function. In our efforts to understand the source of pregnancy complications, we need to consider the characteristics of the immune cells at the respective time point involved in the physiological adaptation needed for a healthy continuation of pregnancy. Generating an overview of the immunological fingerprints associated with the different gestational time points, we assessed general lymphocyte composition from menstrual blood, to first and second trimester decidua, and up to decidua parietalis. Unsupervised-clustering offers analysis of flow cytometry data without the need for manual gating which depends on arbitrary, biased selection of cell populations to assign a classification. This confirmed that $CD4^{+}$ T cells and NK cells are the most stratifying features of uterine lymphocytes allowing for assignment of an immune-profile to its respective gestational time point **(Chapter 4)**. Both NK and T cells frequencies strongly change upon onset of pregnancy. Of note, in MMC, a relatively large variation in NK and T cells frequencies was observed, and some donors presented with similar lymphocyte levels as observed in first trimester decidua samples. We can only speculate whether this lack of difference compared to the gestational profile is due to natural variation, or to an underlying

and undetected pathologic cause. For example, we do not have data on whether the samples were collected at the end of a cycle during which unprotected intercourse took place. Exposure to seminal fluid or even failed implantation could affect the lymphocyte composition observed in menstrual blood-derived lymphocytes (52-54). Moreover, fertility status was confirmed for about half of the participating women, as they gave birth in the past. Thus, while we made the assumption that the majority of women was able to conceive, it might be possible that for some this was not the case and consequently women with aberrant immunological profile were included. We investigated in how far menstrual blood can identify an unfavorable immunological environment connected to RPL in **Chapter 5**.

The decidualized endometrium forms the "fertile ground" for a healthy placenta; successful crosstalk of the blastocyst and the decidua determines if correct placenta formation can be initiated (55). Immune alterations can impact the trophoblast invasion, and endometrial biopsies showed a reduction in NK^{bright} and $CD8^{+}$ T cell frequencies compared to healthy controls (56). However, the number and extent of these data is limited, and establishing an immunological profile of the endometrium of healthy women is challenging when relying on tissue from biopsies. Not only the invasive nature of the procedure, and thus ethical concerns, hamper sample collection, but the number of lymphocytes isolated from a single tissue sample limits the information that can be gathered from each volunteer. We were able to analyze more than 10 times more cells through non-invasive menstrual blood collection and cell isolation. This way, in **Chapter 5**, we investigated the immunological landscape of the non-pregnant uterus in women suffering from recurrent pregnancy loss. The general immune profiling strategy as described in Chapter 2 was expanded with an additional investigation of B cell subsets based on our findings of Chapter 4. Using machine learning, we were able to define key immune subsets of either peripheral or menstrual blood that allow for correct classification. Thus, RPL is connected to altered immunity. Of note, B cell subsets contributed to cohort distinction for both sample types, emphasizing the possible value of considering B cells in future approaches.

We included cytomegalovirus (CMV) seropositivity of our donors in the analysis. CMV causes lifelong latent infection and with, a prevalence of estimated 40 to 100 percent globally, affects a majority of women, especially with increasing age (57). While it rarely elicits symptoms in immunocompetent individuals, it persistently affects immunity (58). Immune evasion mechanisms of CMV are known to especially alter NK and T cell responses, as both are major components of the defense against the virus (59). As such, whether or not a woman has encountered CMV has to be considered a possible confounder. Primary CMV infection during pregnancy is the most frequent cause for congenital disease in developed countries, but it is unclear whether latent CMV infection also deserves a negative connotation with regard to healthy pregnancy. CMV has coevolved with human development for millions of years. Hampering reproduction of its host would negatively impact persistence of the virus. The data presented in **Chapter 5** show that

CMV seropositivity did not contribute to classification as healthy control or RPL patient. Likewise, in **Chapter 3**, CMV infection did not affect the EVT's ability to induce Treg. The human immune system is not just exploited by the virus to allow persistence, but relies on being shaped by early infection. The neonatal immune system is vulnerable to viral infections, but while not suffering from disease, the young child gets equipped with an alert immune system enabling efficient elimination of harmful pathogens (60). Lifelong CMV infection was shown to improve pathogen recognition, by allowing for recruitment of a broad cross-reactive antigen repertoire in mice (61). Pregnancy-induced training of uterine NK cells, hypothesized to be connected with improved placentation (62), was shown to be dependent on CMV-seropositivity (63).

CMV seropositivity caused a segregation in frequencies of B cell subtypes of the RPL cohort. Without taking CMV status into account, switched memory B cells and naïve B cells are present at similar frequencies in control vs RPL patients. Higher frequencies of CD27 positivity of B cells, a marker for activation, were observed in the CMV^{pos} group of the RPL cohort. Increased abundance of CD27 on B cells is a known association with CMV seropositivity (64). Unfortunately, information on CMV status was limited for donors of peripheral blood in this study. Frasca et al. compared vaccine responses based on CMV status, showing that a pre-activation of B cells, also reflected in increased intracellular TNF-α levels, negatively impacted their ability to undergo class switch recombination, and generate proper antibody and memory response (65). The authors propose that a CMV-mediated proinflammatory profile can be kept under control in young individuals, while elderly present with higher inflammatory levels. It remains to be elucidated whether CMV seropositivity poses a risk factor for RPL in case of additional deregulation of tolerance. In that case, failure to suppress a CMV-related proinflammatory profile might negatively impact implantation. An earlier study by Radcliffe et al. pointed out the difficulty to assess an association of CMV and RPL based on measuring anti-CMV antibodies as performed in **Chapter 5**: the authors observed an inability of RPL patients to respond to CMV through proliferation or antibody secretion (66). The difficulty of these individuals to adequately mount a humoral response to specifically CMV infection, not to control stimulation, underlines the importance to study CMV and B cells with regard to RPL in future approaches.

HUMAN REPRODUCTION AND MICROBIOTA ARE INSEPARABLE

The concept that human immunity is naturally shaped through foreign influence is manifested in immunology. The term *microbiome*, describes all genomes from microorganisms inside the body is often also referred to as *microbiota*, which describes the specific microorganisms. The microbiome does not only refer to natural colonization by bacteria, but also covers viruses, fungi, protozoa, and archaea (67, 68). The importance of microbial colonization of the human body has been extensively reviewed, focusing especially on the contribution of the gut microbiome

to systemic immunity (69-74). In **Chapter 6**, we studied the available evidence regarding the possible impact of microbiota on endometrial immunology and fertility. With the chance to analyze microbial composition through measuring 16S rRNA rather than culturing methods, even rare species, and low biomass colonization, can be assessed. The discussion on the contribution of a local microbiome for healthy pregnancy lifted off with the exposure of a possible placental microbiome in 2014 (75). At the same time, bacteria were also observed within cells of the placenta, without signs of inflammation such as accumulation of immune cells in the proximity (76, 77). As outlined throughout this thesis, the placenta evolves from, and depends on, a healthy endometrium. Therefore, we assume that in theory, the placental microbiome stems from microbiota of the endometrium, but both may differ to a large extent in composition. The microbiome is known to adapt due to gestation (78, 79). For example, when pregnant, 70% of women present with increased fractions of gut microbiota that are associated with inflammation (80). It has been suggested, that this compositional shift is dictated by pregnancy hormones (81). The same hormones also reach the uterine cavity, and can thus impact the local microbiome. In **Chapter 6**, we focus on the endometrial microbiome as a contributor to setting the stage for conception**.** We extrapolate known contributions of gut microbiota to possible mechanisms at play within the uterus. Even though only limited endometrial profiles of healthy women were available at the time point of the study, we conclude that, if bacteria are present within the uterus, a physiological role for maintenance of uterine adaptation and immunity is inevitable.

THE STERILE WOMB PARADIGM

Based on the hypothesis that newborns acquire their initial bacterial colonization through birth, rather than in the uterus, the dogma of the "sterile womb" was manifested by French pediatrician Henry Tissier (82). This concept has proven persistent; whether the uterus harbors a microbiome, or whether this is merely an artefact of sampling or detection, is still under heavy debate; research has not reached consensus regarding when the baby is initially colonized by bacteria (83-85). An overview of arguments supporting and refuting antenatal microbial exposure exceeds the scope of this thesis. It remains to be established how the neonatal microbiome relates to a gestational uterine microbiome, and might consequently disrupted upon microbial modulation during pregnancy. In **Chapter 6**, we stress how controlling for contamination, and a standardized approach to detect 16S rRNA can support a worthwhile search to find consensus on uterine microbiota. In the years following publication of this chapter, a rapid increase in studies, that support a shift in perspective on the microbiome and the uterus, could be observed (86-89).

Even now, species composition of the healthy endometrium is yet to be determined. Contrary to the well-studied, and well-defined, mucosal microbiome of the gut or the vagina, the uterine cavity contains only a very low biomass of microbiota (90). Low biomass does not equal sterility;

combining copy numbers from qPCR and 16S rRNA gene amplicon sequencing showed detectable reads above potential background noise and negative controls (90, 91). The example of Lactobacilli (e.g. *L.iners* or *L.crispatus*), identified as dominant by some (92-94), or as rare to absent by others (91) underlines how a joint, standardized effort might be needed to unite the side of researchers who support the notion that uterine microbiota deserve to be studied. Multicentered studies with strict guidelines regarding all steps from cohort selection to sampling, sample processing to analysis, are needed to credibly replace the concept of the sterile womb by the definition of a healthy endometrial microbiome.

Recent investigations showing an altered microbial profile prior to RPL, or in case of IUGR, support the notion that, whether as cause or consequence, varying uterine microbiota profiles are associated with different reproductive outcomes (94, 95). Increasing knowledge on a healthy endometrial microbiome, prior to pregnancy, might lead to treatment options for women struggling to conceive. Diet and medication can steer a microbial profile (96-100). The beneficial effect of interfering with microbiota to improve reproductive outcome can be illustrated by bacterial vaginosis, an imbalance of the vaginal flora correlating with poor reproductive outcome (101, 102). Its association with PTB can be counteracted with antibiotic intervention, if occurring in the first half of gestation (103). The importance of timing reflects that healthy pregnancy is determined in its early stages. The altered immunological profile observed in menstrual blood of RPL patients **(Chapter 5)** might (for some individuals) be connected to an unfavorable microbial profile. In that case, proper diagnosis might offer therapeutic options. 16S rRNA sequencing becomes increasingly accessible, and currently, self-testing of vaginal microbiota is already commercially available to predict ART success (104-106). As there is only limited overlap between uterine microbiota and the vaginal microbiome (90, 92), including the immune profile of menstrual blood, or sampling endometrial microbiome directly (although warranting a professional approach rather than at-home swabs) might offer improved prediction of reproductive outcome or pinpoint treatment targets.

MANIPULATING MICROBIOTA: CHANCES AND THREATS

As shown by the example of successful reduction of PTB risk in case of bacterial vaginosis, treating antibiotic disbalance to improve reproductive outcome is tempting to consider. Indeed, antibiotic treatment can alter endometrial microbiota (107). However, antibiotic intervention around the time of, or after, conception needs to be treated extremely carefully. A central theme of this thesis is how reproductive immunology relies on well-balanced, connected mechanisms, and, as such, any interference can be detrimental. However, in many cases, gestational use of antibiotics cannot be avoided as progression of an infection would be detrimental to mother and child. Urinary tract infections (UTI) are one of the most common pregnancy complications (108).

UTI itself is a risk factor for PE, which is hypothesized to originate from an increase in the systemic inflammatory burden (109-111). In **Chapter 7**, we investigate in how far antibiotic intervention in pregnant mice causes changes in systemic, as well as local (placenta) immunity. We observed that while the medication did not affect reproductive success, microbial manipulation translated to altered immunology of the mother, reaching as far as the placenta. We based our microbial intervention on the combination of antibiotics published by Tormo-Badia et al., who showed lasting immunological effects in offspring of non-obese diabetic mice treated with antibiotics (112). The authors observed a reduced diversity of intestinal microbiota and limited activation of T cells in the pups. Altered immunity of the offspring might not only result from their own altered microbial profile, but from treatment effects on the fetal prenatal environment such as demonstrated by our results. The presented data illustrate that the effect of gestational antibiotics on the offspring need to be studied in light of the treatment's effect on maternal immunity.

Unfortunately, low bacterial yield upon sequencing of placental microbiota prohibited us to draw conclusions on exact species composition, or a treatment-induced switch in frequencies or abundance. However, few samples in which detection was possible, indicate a shift in placental species distribution. It is tempting to speculate that this indicates altered prenatal microbial exposure. Could microbial priming of the developing child occur in utero, with possible lifelong effect on the offspring? Various studies have linked the gestational use of antibiotics to an increased risk for the child to develop asthma, atopic dermatitis, and allergic disease and increased childhood hospitalization of the offspring (113-116). While the possible confounding effect of other underlying causes of disease, linking maternal and fetal health, needs to be kept in mind (117), murine studies could also confirm the link in antibiotic intervention studies (118-120). Still, the causative relationship and etiology of disease needs to be elucidated. Systemic immune adaptations of the mother, immune modulation reaching the baby, direct effects of (metabolites of) the microbial intervention, effects passed on during breastfeeding, a shift in microbial prenatal exposure; the list of possible medication-related impact on the offspring is long. Neither route probably impacts childhood health exclusively. The need to treat pregnant women with antibiotics will persist in order to save mother and child of an acute threat. It remains to be revealed how gestational antibiotics are linked to long-term effects on immunity. Our study points out that effects due to an altered uterine environment have to be considered as a part of this investigation.

GENERAL CONCLUSION AND FUTURE PERSPECTIVES

Healthy pregnancy depends on a broad variety anatomic, endocrine, metabolic, and immunologic adaptations, which orchestrate local immunity (Figure 1). The findings discussed in this thesis point out that uterine immunity is highly dynamic, and is affected by even more factors than

meets the eye. The endometrium forms the basis for all immune adaptations occurring upon initiation of pregnancy. If dysregulated, complications like pregnancy loss, PE, and IUGR can arise. Pregnancy is accompanied by a general increase in a memory phenotype of T cells and B cells, and shifts in their respective subsets. Both key players of adaptive immunity influence the local immune environment, through direct suppressive effects, or cytokine secretion. Ultimately, reproductive immunology demands complex regulatory mechanisms allowing for trophoblast invasion, whilst maintaining reactivity towards infectious threats. We showed that Treg and B cells contribute to this intricate balance. Local differentiation or modification equips uterine immune cells for their pregnancy-specific regulatory tasks. Natural colonization by a uterine microbiome can influence these delicate interactions needed for implantation, progression of pregnancy, and development of a healthy child.

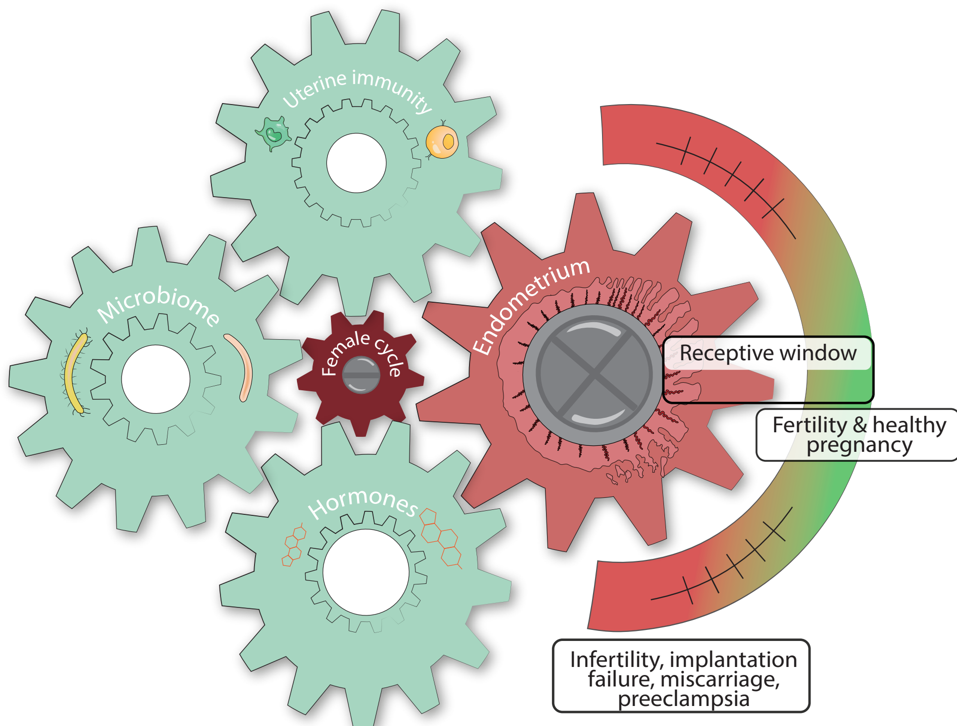


Figure 1. Interacting mechanisms direct uterine health. Only correctly orchestrated conditions allow for conception and healthy pregnancy.

The presented data underline the extraordinary characteristics of uterine immune cells, in absence of, or during, pregnancy. These investigations are challenging to model *in vitro* when using readily accessible sources of lymphocytes. No cell lines are available that are identical to

uterine lymphocytes. Even co-culture of decidual lymphocytes and commercially available target cell lines hold their limitations. Decidual NK cells spare first trimester trophoblast from cytotoxicity, but not the widely-used target cell lines (121). Peripheral blood offers a source to study systemic immunity of pregnant women, as the act of drawing blood is no threat to the baby, and can be done as part of routine checkups. However, mechanisms at play at the fetal-maternal interface, in preparation for, or during, implantation or placenta formation are to a large extent unique to local immunity. Future investigations to increase our understanding of principles underlying all human life thus strongly rely on primary samples. As outlined in this thesis, patient-derived tissue can be obtained without the need for invasive biopsies. We isolated lymphocytes from menstrual blood, first and second trimester abortions, and placentae obtained upon delivery. As no additional procedures or change in behavior are required from donors, approval for use of this material by the local ethical committee can be relatively straightforward in the Netherlands (considered "*niet-wmo-plichtig*"). The use of menstrual cups as female hygiene product becomes increasingly popular, and, consequently, collection of the effluent is of limited additional effort. Increased use of the presented primary cells can also help to reduce animal studies effectively, especially since "only humans have human placentas" and other species might not present with identical mechanisms at play (122). Rather than considered waste material, menstrual blood cannot only be vital to research, but it also holds many opportunities for large-scale screenings. Prior to planned conception, especially during ART, women want to ensure that all conditions are optimal to support a healthy pregnancy. Not knowing the underlying cause of an unfulfilled child-wish comes with a large medical and emotional burden. Harnessing the diagnostic value of menstrual blood holds many opportunities. The method can only gain strength if the taboos around female health are lifted. Funding bodies might contribute to support that menstrual blood becomes just as *attractive* as the seemingly easier act of drawing blood for in-depth analysis.

"The biologist of today […] is in much the same position as an Egyptian astronomer exploring the secrets of the universe with the naked eye." (Roberts and Lowe, 1975, The Lancet, "Where have all the conceptions gone")

This thesis explored how understanding of reproductive immunology demands a change in perspective. A number of presented dogmas were established before holistic detection options were available. Systems biology approaches and computational analysis allow for an open approach, moving away from a biased target selection or a selective readout. Next to the presented microbiome analysis, examples of such comprehensive approaches are transcriptomics (123, 124), use of mass cytometry (27), enabling highly multiplexed flow cytometry, or mass imaging, expanding our abilities for structural analysis. Increased availability of these in-depth omics approaches will allow for a better understanding of phenotype and function of uterine immune cells. Healthy pregnancy depends on the integration of all these

different pieces of the puzzle (Figure 2). Bioinformatic approaches will aid to make connections between the different omics strategies that we cannot fathom. Reproductive immunology not only affects fertility but sets the start for all prenatal development, which, on its turn, sets the stage for a healthy future (125). Development of effective screening methods, throughout, or even prior to, pregnancy will allow detection, and hopefully treatment of, diseases that manifest too late to alter their perinatal outcome. Pregnancy still seems mysterious; there are many pieces to put together, and discover, in order to understand this fascinating puzzle. However, the final picture for research to work on is clearly defined. The aim will always be to offer the best possible conditions for a future generation.

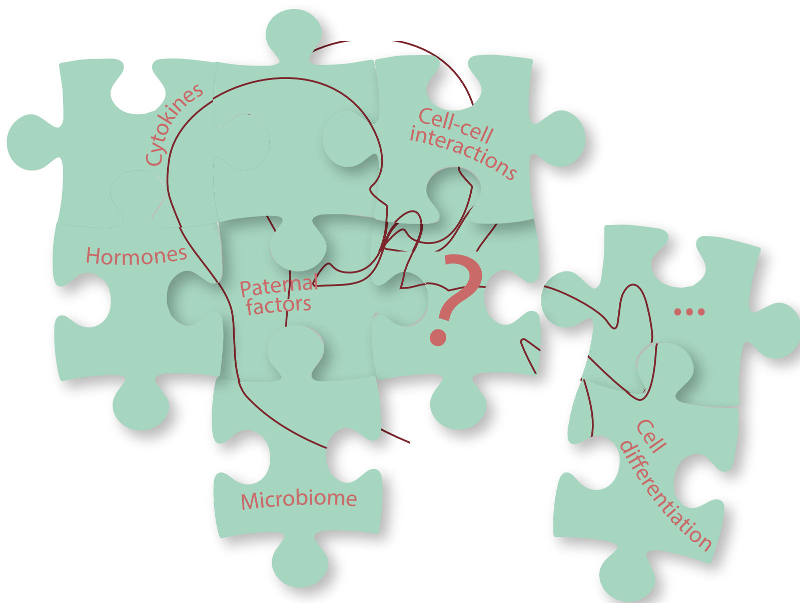


Figure 2. Reproductive immunology demands integration of multiple disciplines.

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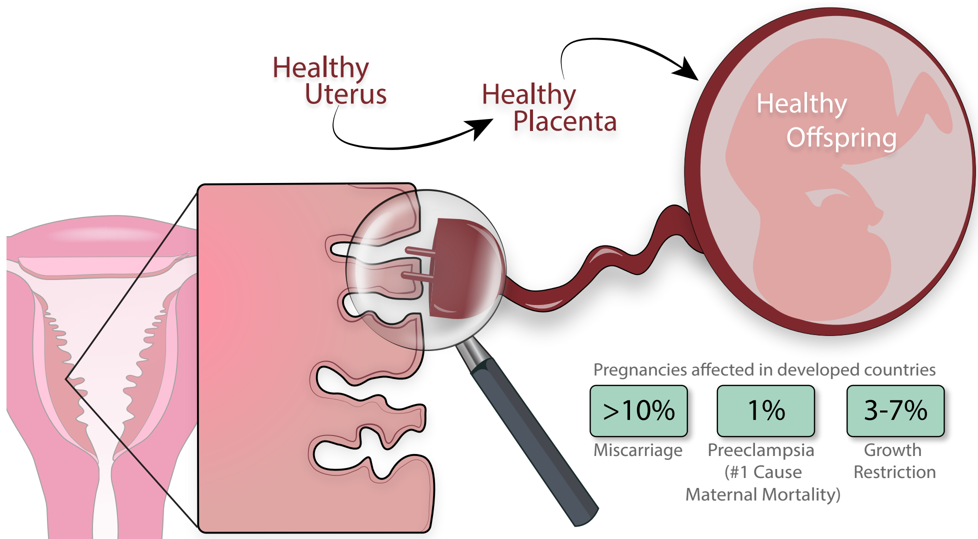
CHAPTER 9

Graphical summary

Science can be fuzzy, dark, and obtuse. There comes a point where words fail and it's easier to just draw a picture.

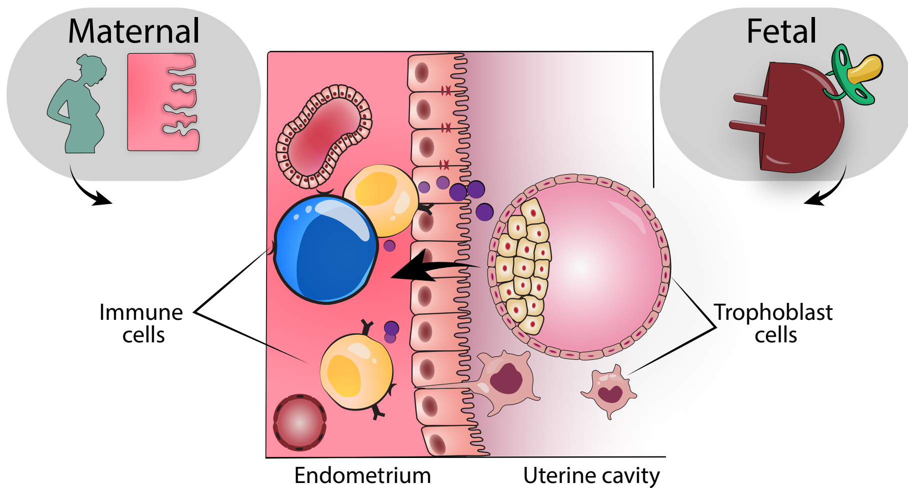
- Maki Naro

CHAPTER 1 | INTRODUCTION

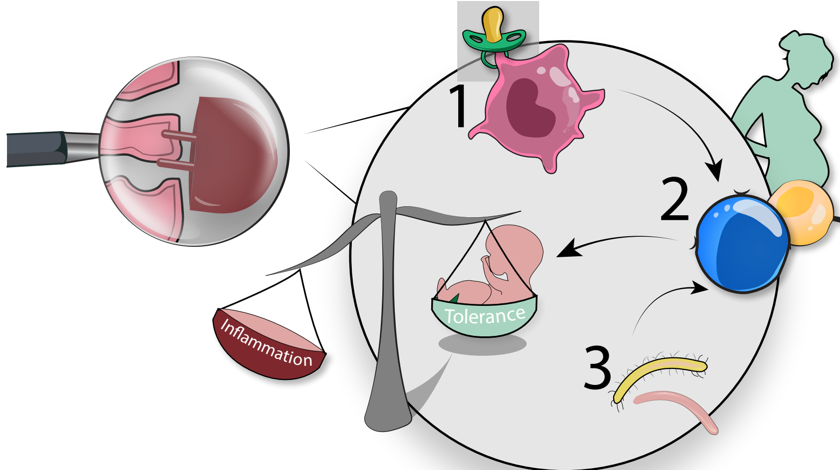


- Uterine health is closely linked to placentation and thus development of the unborn child.
- Pregnancy complications affect a large part of all pregnancies.
- Understanding how formation of placental connection is regulated is paramount to improving diagnostic methods and developing treatments.

The role of the immune system in establishing the placental connection



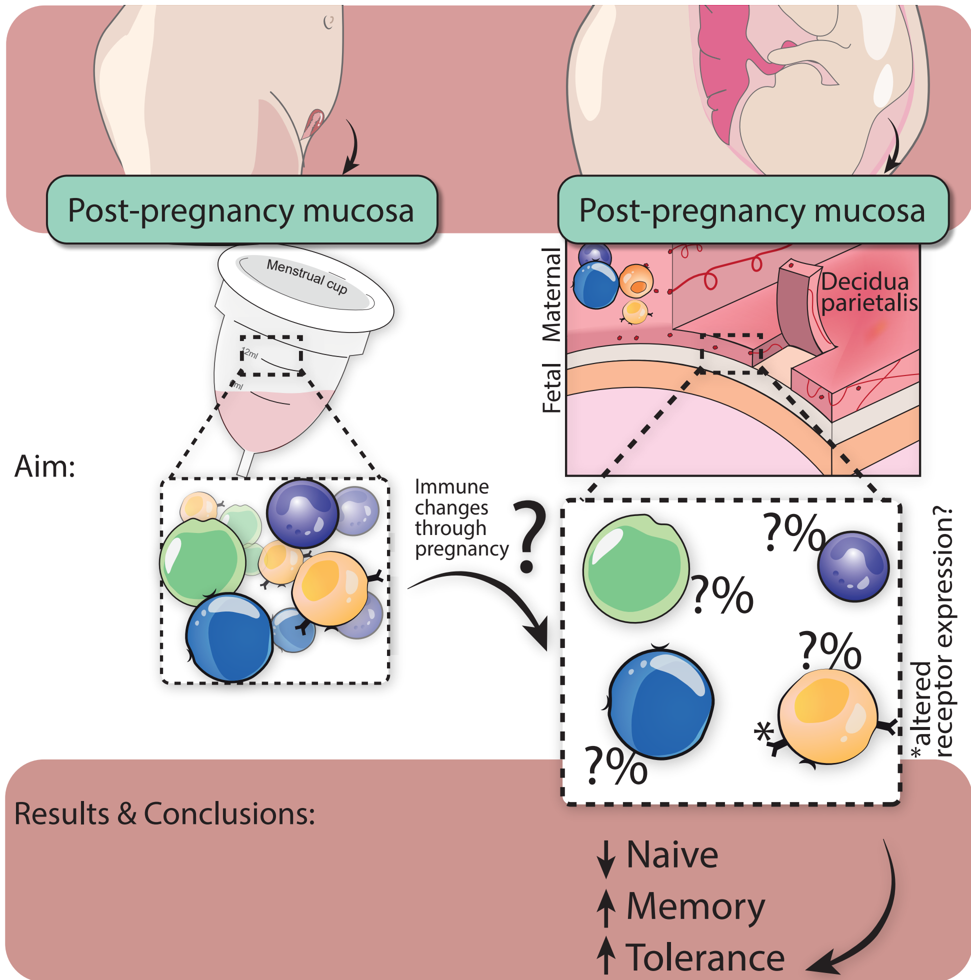
Successful trophoblast invasion depends on the correct interaction with maternal immune cells.



How is the right balance between tolerance and inflammation maintained at the fetal-maternal interface ?

1. Trophoblasts possess mechanisms to facilitate immune evasion.
2. Cells of the uterine tissue influence the local immune environment.
3. External factors, such as bacteria contribute to a tolerance-favoring milieu.

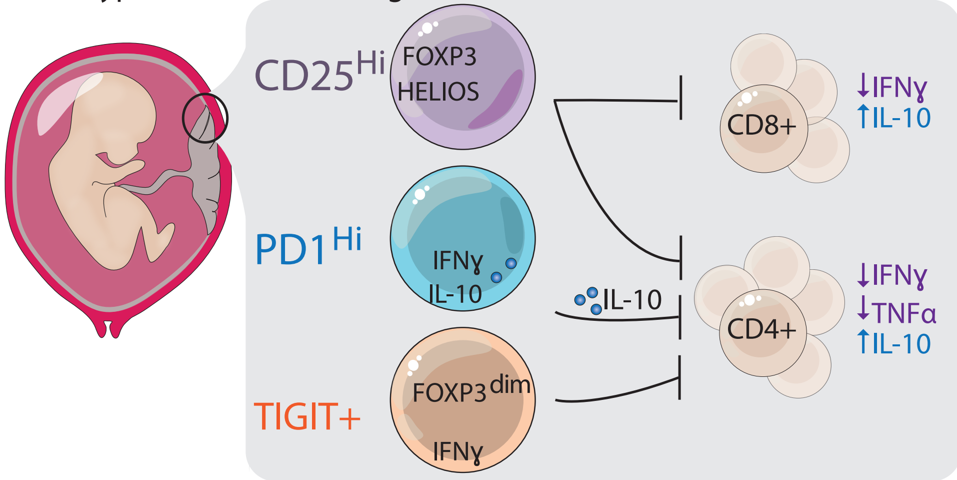
In this thesis, we explore the interaction of 1&2 and 2&3 to better understand the regulation of uterine immunity.



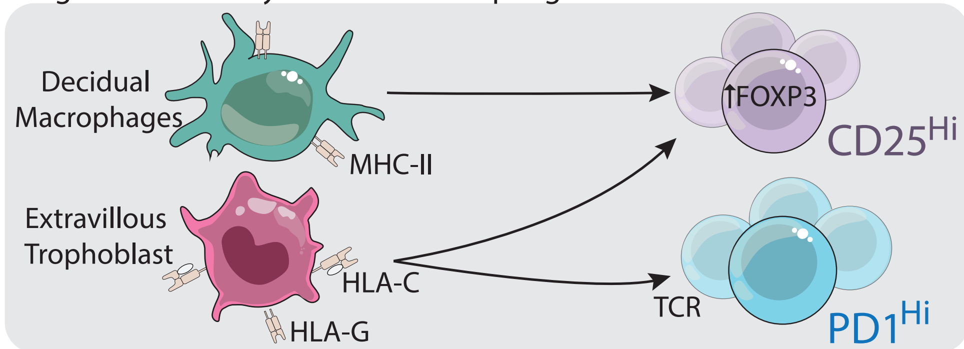
How does the uterine immune environment change during pregnancy?

- Decreased abundance of naïve T cells in decidua parietalis.
- Increase in central/effector memory T cells.
- More regulatory T cells at the end of pregnancy.
- Mucosal cells differ in phenotype from peripheral blood.

Three types of functional Treg are found at the maternal-fetal interface

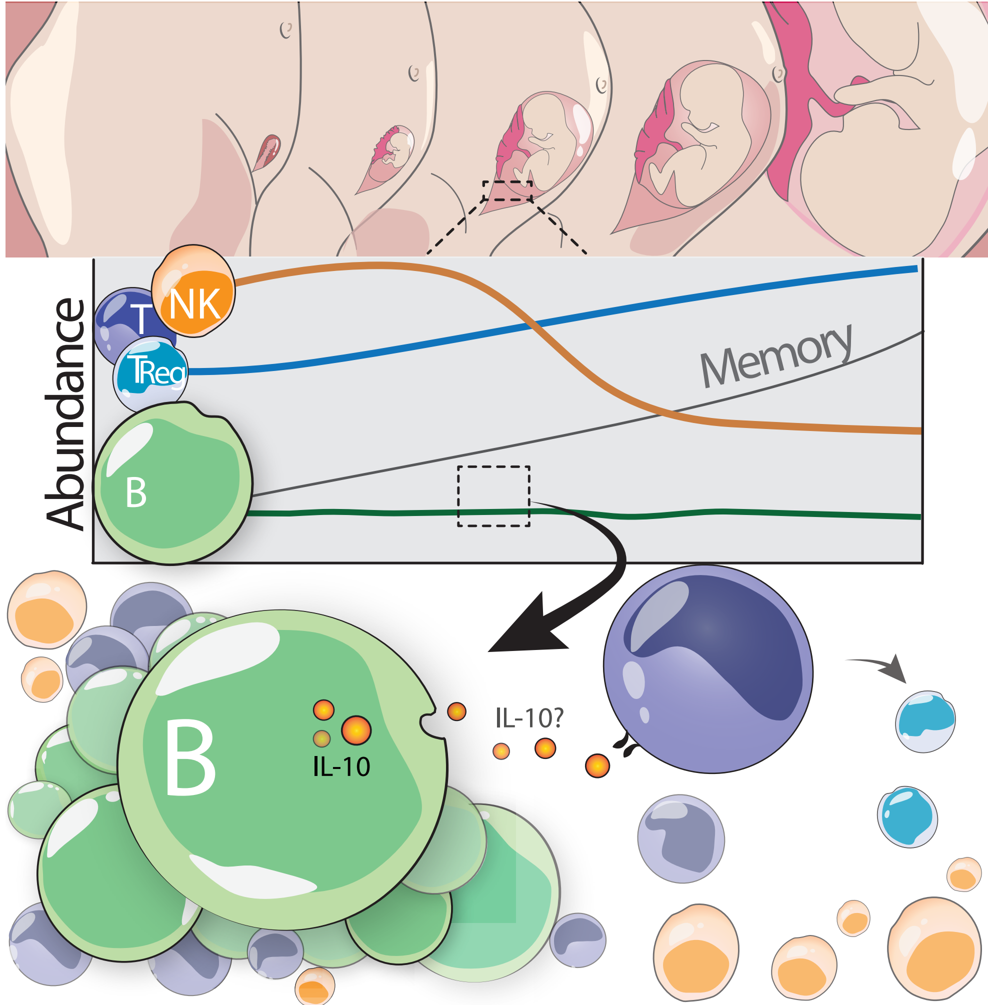


Treg are induced by decidual macrophages and HLA-G+ EVT



- Three decidual Treg types suppress Teff responses in human pregnancy.
- Decidual PD1^{HI} Tregs suppress T cell proliferation in an IL-10- dependent manner.
- EVTs and decidual macrophages directly increase FOXP3+ Tregs, while EVTs also increase PD1^{HI} Tregs.
- Induction of PD1^{HI} Tregs by EVTs may involve antigen-specificity for HLA-C.

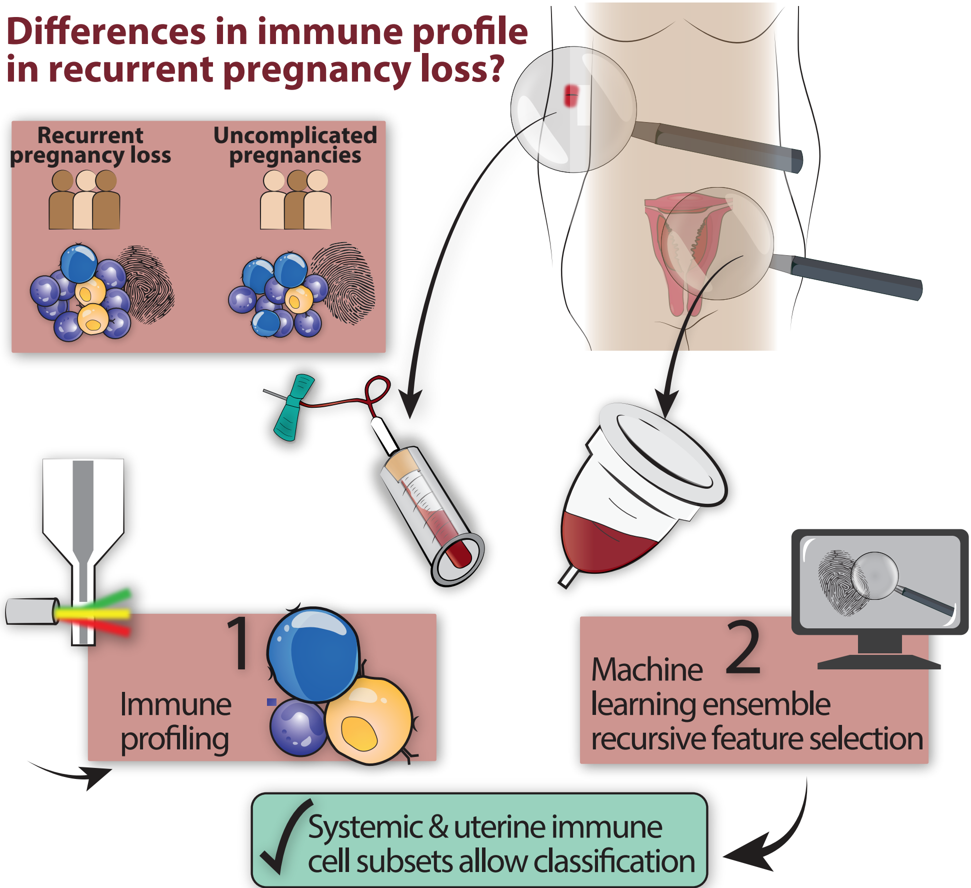
CHAPTER 4| CLUSTERS OF TOLEROGENIC B CELLS FEATURE IN THE DYNAMIC IMMUNOLOGICAL LANDSCAPE OF THE PREGNANT UTERUS



- B cells obtained from the fetal-maternal interface gain memory during gestation.
- Unsupervised analysis shows B cell subsets with marker expression unique to the uterus.
- Decidual B cells are able to secrete IL-10.
- Clusters of decidual B cells also contain T cells, including Foxp3^{+} T cells.

9

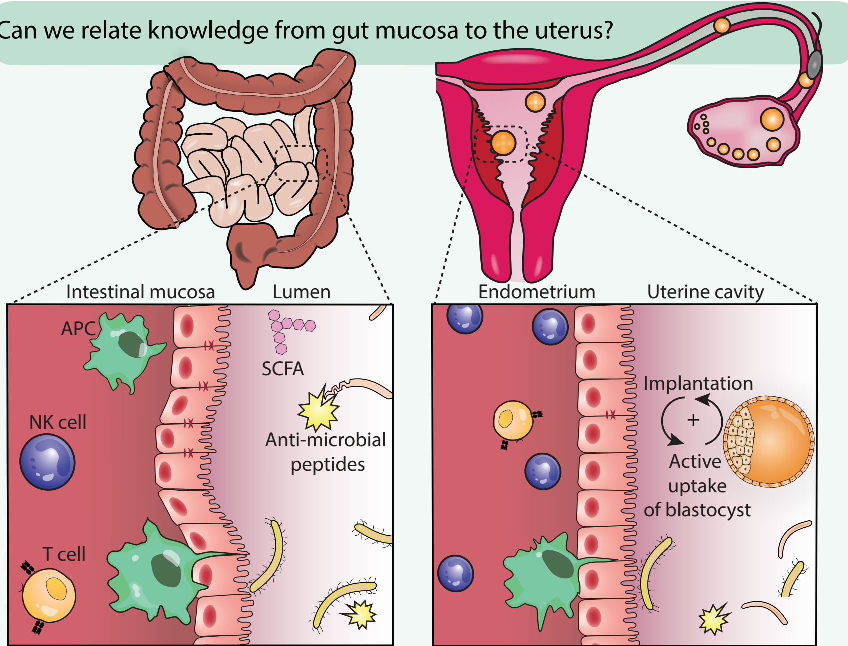
CHAPTER 5 | A COMBINATION OF IMMUNE CELL TYPES, IDENTIFIED THROUGH AN ENSEMBLE MACHINE LEARNING STRATEGY, DETECTS ALTERED PROFILE IN RECURRENT PREGNANCY LOSS

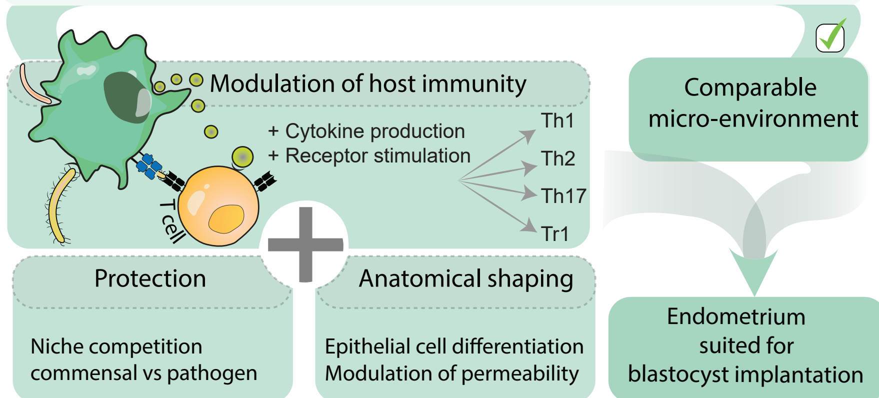


- Altered immune profiles in women who experienced recurrent pregnancy loss.
- Systemic and uterine immunity are both affected but different cell types contribute to classification.
- High accuracy → possible diagnostic value upon further validation.

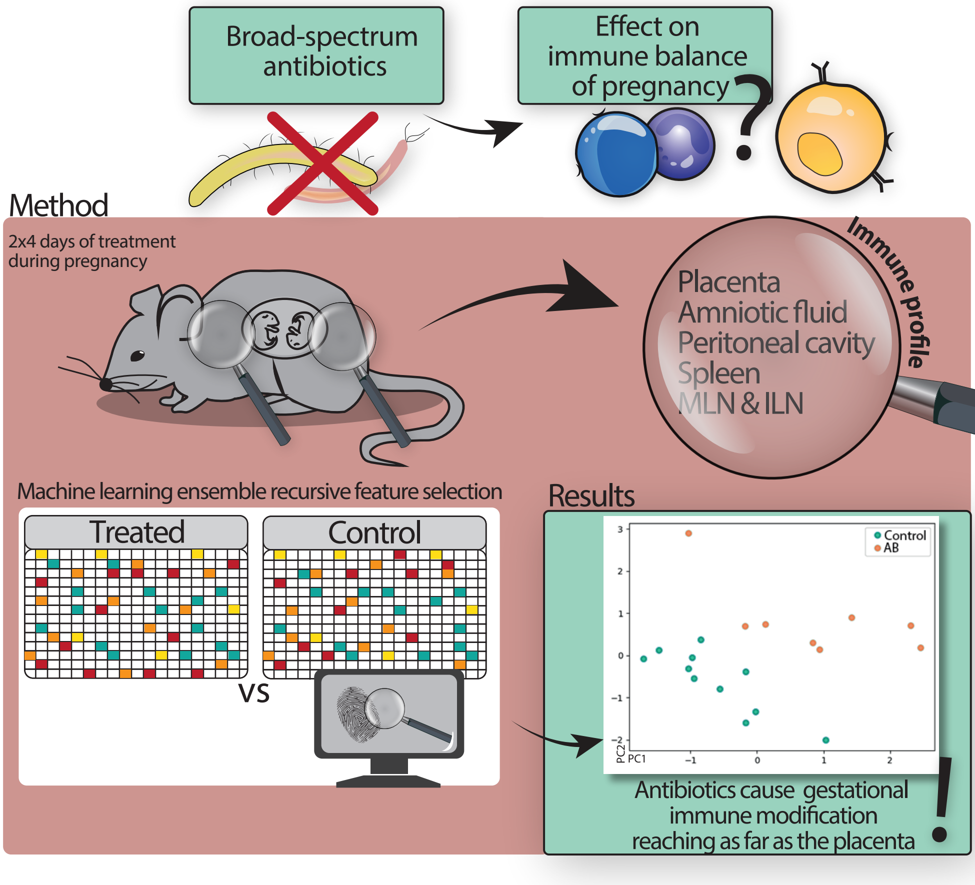
Essential properties for beneficial microbial colonisation

Needed:		Given in endometrium?
Minimizing systemic threat anatomically	✓	Strong tight junctions endometrial epithelia
Limited contact between bacteria and epithelia	✓	Secretion of anti-microbial molecules
Rapid detection and killing upon barrier breach	✓	High abundance of gatekeeping lymphocytes



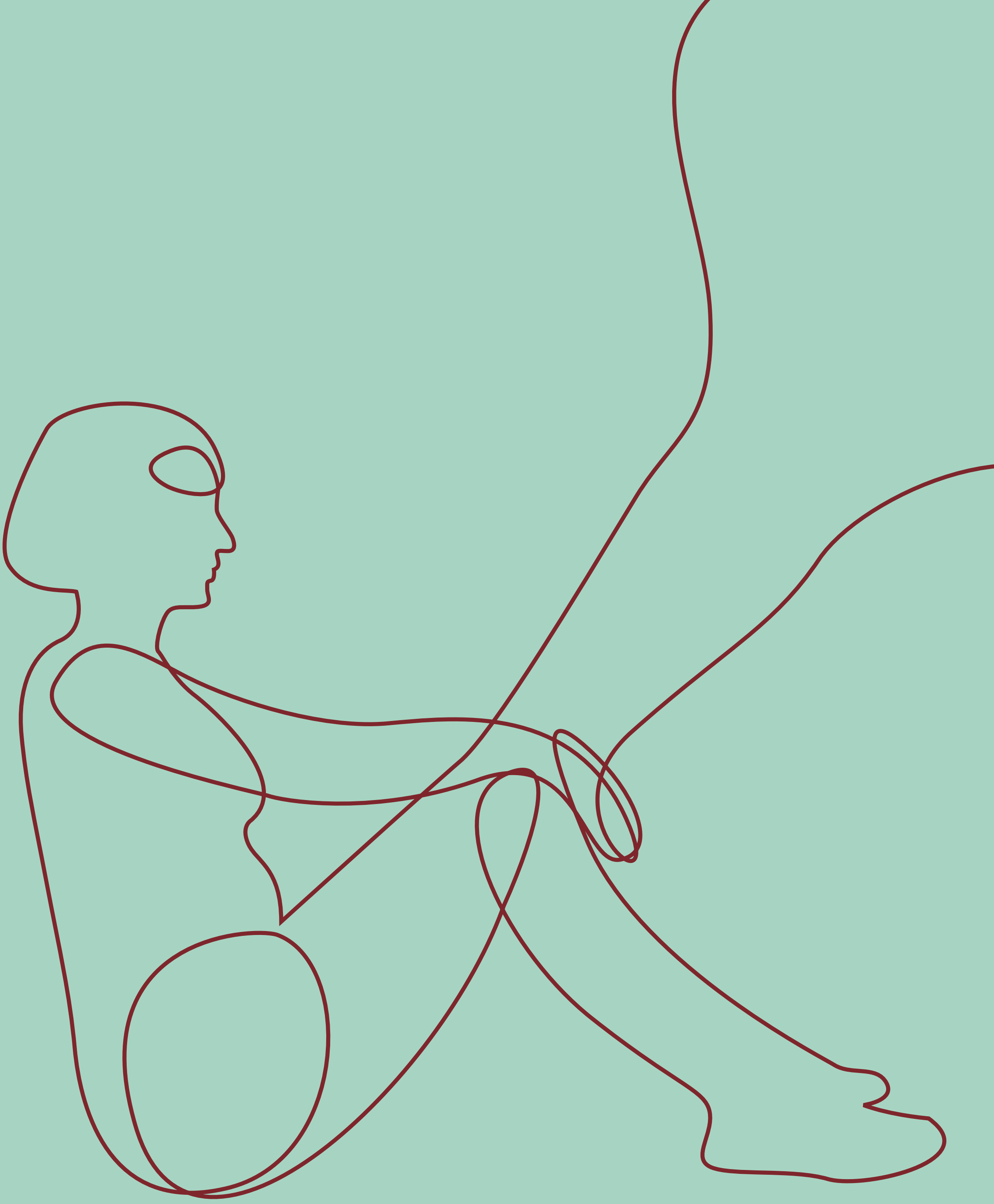


CHAPTER 7| ANTIBIOTIC INTERVENTION AFFECTS MATERNAL IMMUNITY DURING GESTATION IN MICE



- Antibiotic treatment reduced microbial diversity maternal gut but litter size remained unaffected.
- Immune features altered due to treatment reached as far as placenta (splenic T helper 17 cells and CD5+ B cells, CD4+ T cells in mesenteric lymph nodes and RORγT mRNA expression in placenta).
- Unique immunological properties of pregnancy have to be considered in safety studies.

- Uterine immune regulation depends on a complex interplay of cells.
- Even cells types or microbes that are not abundantly present can make a difference.
- The unique local processes depend on studies looking at the involved tissues. Commercially available cell lines or peripheral blood cannot reveal uterine processes.



CHAPTER 10

EEN KWETSBARE FASE

Blastocyst
Bevruchte eicel na 5-6 dagen celdeling. Een celmassa binnenin het cluster zal de embryo vormen, cellen aan de buitenkant vormen het foetale deel van de placenta (zie trofoblast).

Endometrium
Slijmvliezen (med.:mucosa) van de baarmoeder.

Pre-eclampsie
Zwangerschapscomplicatie gekenmerkt door aanwezigheid van eiwit in de urine (proteïnurie) en hoge bloeddruk. Kan overgaan in HELLP-syndroom (hemolyse, elevated liver enzymes, and low platelets). Wordt vaak ook zwangerschapsvergiftiging genoemd.

Trofoblast
Cel type van foetale oorsprong die invasief in de baarmoederwand groeien.

Zwangerschap verandert het leven van de ouders op veel niveaus. Lichamelijke aanpassingen van de moeder, lokaal in de baarmoeder en ook systemisch, zijn cruciaal om de optimale ontwikkeling van het kind te faciliteren. Maternale steun begint als het ware bij de interactie met de bevruchte eicel: de slijmvliezen van de baarmoeder moeten een geschikte basis voor innesteling vormen. Alleen als de blastocyst en het endometrium samen op de juiste manier een verbinding aangaan, kan een zwangerschap succesvol verlopen. Een gezonde zwangerschap is dus verre van vanzelfsprekend: 30% van de zwangerschappen gaat verloren voordat de moeder wist zwanger te zijn. Evenveel zwangerschappen eindigen in een miskraam, een heftige ervaring die bijna een kwart van alle vrouwen een keer in haar leven meemaakt. Daarnaast worden veel zwangerschappen gecompliceerd door pre-eclampsie (PE) of HELLP-syndroom. Wereldwijd krijgt zo'n 6-8% (1-3% in Nederland) van alle zwangerschappen de diagnose PE. Deze zwangerschapshypertensie kan gepaard gaan met langetermijngevolgen voor moeder en kind, bijvoorbeeld als het kind te vroeg moet worden geboren om ernstige cardiologische of nefrologische gevolgen voor de moeder te voorkomen. Ook al weten we niet wat de oorzaak van PE is, een hoofdrol wordt toegeschreven aan de foutieve aanleg van de placenta.

Vraagstelling van dit proefschrift

Het afweersysteem speelt een belangrijke rol bij de ontwikkeling van de placenta. Maternaal weefsel en trofoblastcellen moeten nauw op elkaar afgestemd zijn. Alleen als dit goed verloopt kan een gezond kind geboren worden. Het doel van dit proefschrift was om vanuit mogelijke nieuwe invalshoeken de betrokken immuun-regulatie te onderzoeken. Pas als meer bekend is over de cruciale mechanismen en wat er mogelijk misgaat als zwangerschapscomplicaties optreden, zijn ontwikkeling van vroege herkenningsmethodes en interventies mogelijk.

Waarom is het immuunsysteem belangrijk bij de aanleg van de placenta?

Het immuunsysteem herkent elke niet-lichaamseigen cel. Er volgt een afweer-, oftewel ontstekingsreactie, om verder binnendringen en

mogelijke schade te voorkomen. Cellen van foetale oorsprong zijn maar voor 50% lichaamseigen, omdat de helft van de genetische opmaak van de bevruchte eicel door de vader wordt bijgedragen. Het maternale immuunsysteem herkent de trofoblastcellen die de spiraalarteriën van de baarmoeder binnendringen om de placentale connectie tussen moeder en kind te vormen. Dit wordt beschouwd als een immunologische paradox: in tegenstelling tot de afweerreactie die optreedt bij herkenning van een donororgaan bij een transplantatie, vormt de reactie op de deels vreemde cellen géén bedreiging voor zwangerschap. Deze herkenning en enige mate van ontstekingsreactie binnen de decidua is zelfs nodig om de juiste mate van innesteling te faciliteren. Hierbij staat de immunologische balans centraal: elke reactie van het afweersysteem wordt gedempt door regulerende cellen, die via celcontact of afgifte van oplosbare factoren reacties van omliggende cellen onderdrukken. Voor een gezonde zwangerschap is het essentieel dat een activatie van het immuunsysteem niet uit de hand loopt. Hoge ontstekingswaardes ontregelen de aanleg van de placenta, kunnen een vroegtijdige geboorte inleiden en negatieve invloed hebben op de ontwikkeling van het kind. Het onderzoeksveld van de reproductie-immunologie bestudeert de bijzondere controle- en regulatie mechanismen die voor elke zwangerschap van belang zijn. De immuunregulatie die is betrokken bij het vormen van de placenta is samen te vatten in 3 hoofdlijnen:

1. Mechanismes van de trofoblast om een sterke afweerreactie te voorkomen.
2. Bijzondere eigenschappen van de maternale immuuncellen tijdens zwangerschap/in de baarmoeder.
3. Externe factoren die mogelijk tolerantie bevorderen, zoals componenten van zaadvloeistof, of natuurlijk-voorkomende bacteriën.

Deze elementen beïnvloeden elkaar, maar zijn te divers om als geheel te worden onderzocht. De verschillende puzzelstukjes moeten dus apart worden bestudeerd. In dit proefschrift werd zo veel mogelijk gekeken naar de raakvlakken om de bevindingen in context te kunnen plaatsen.

Spiraalarteriën
Bloedvaten van baarmoederwand, en -slijmvlies die de bloedtoevoer naar de placenta leveren.

Decidua
Gedifferentieerd endometrium, vanaf het einde van elke menstruele cyclus. Functioneel gedeelte van het slijmvlies wat betrokken is bij de innesteling en aanleg van de placenta.

DIT PROEFSCHRIFT

Zwangerschap induceert een toename in geheugen van afweercellen in de baarmoeder

In hoofdstuk 2 gaven we een overzicht van de verschillende cellen van het afweersysteem die in de baarmoeder betrokken zijn bij zwangerschap. We bestudeerden hiervoor endometriale cellen van niet-zwangere vrouwen. In eerdere studies werd dit vooral gedaan door een biopt van het endometrium te nemen, een invasieve methode die niet zomaar wordt toegepast als er geen medische noodzakelijkheid voor de ingreep is. Dit maakt het moeilijk om een controle-cohort te rekruteren die een beeld van de immuun-omgeving bij gezonde vrouwen kan geven. Dit probleem kan worden verholpen door de betrokken cellen uit menstruatiebloed (MB) te isoleren. Elke maand wordt de functionele laag van het endometrium samen met de hierin voorkomende cellen van het afweersysteem afgestoten. De vloeistof kan met een menstruatiecup, een milieuvriendelijk alternatief voor tampons, worden opgevangen. Op deze manier kunnen er zonder invasieve behandeling vele malen meer cellen worden verkregen en onderzocht. Deze techniek is ook gebruikt voor het onderzoek dat is beschreven in de hoofdstukken 4 en 5. In hoofdstuk 2 lieten we door flowcytometrie zien, dat de leukocyten uit MB inderdaad de typische kenmerken van uteriene cellen vertonen. De onderzochte MB-cellen hadden een ander fenotype dan cellen uit het bloed, wat bewijst dat ze van mucosale oorsprong zijn. We vergeleken de MB-cellen met leukocyten die we uit de decidua parietalis van à terme zwangerschappen isoleerden. Zij representeren het afweersysteem aan het einde van een gezonde zwangerschap en geven dus informatie hoe het lokale immuunsysteem verandert als gevolg van zwangerschap. Na 9 maanden zwangerschap waren de lokale immuuncellen minder naïef, dat wil zeggen dat de T-cellen geactiveerd zijn geraakt en geheugen hebben opgebouwd. Verder zijn in de d.parietalis meer cellen aanwezig die met tolerantie in verbinding worden gebracht, zoals regulatoire T-cellen (Tregs). Inmiddels is bekend dat differentiatie van deciduale T-cellen optreedt na contact met foetale cellen, een lokale reactie die maar in geringe mate in de circulatie terug te zien is. Een inductie van Tregs helpt om te voorkomen dat deze herkenning oploopt tot een (te) sterke ontstekingsreactie.

Flowcytometrie
Techniek om d.m.v. gelabelde antistoffen de uiterlijke kenmerken van een cel in kaart te brengen. Aan/afwezigheid van receptoren geeft uitkomst over het exacte cel type.

Leukocyten
Witte bloedcellen, mediëren afweerrespons. Omschrijft granulocyten, monocyten/macrofagen, lymfocyten (T-, B-, NK cellen) en dendritische cellen.

Fenotype
Uiterlijke kenmerken/opmaak van een cel

Decidua parietalis
Maternale laag van de placentale vliezen. Gevormd als foetale lagen (amnion, chorion) met gedecidualiseerd endometrium een verbinding aangaan.

À terme periode
Tijd rond de uitgerekende datum van bevalling (zwangerschapsduur tussen 37 en 42 weken).

Drie verschillende regulatoire T-cellen reguleren de T-cel respons waar maternale en foetale cellen bij elkaar komen

De betrokken Tregs, gekenmerkt door hun vermogen om T-cellen te onderdrukken, werden in meer detail onderzocht in **hoofdstuk 3**. Deze cellen zijn dus vooral betrokken bij de initiële fase van zwangerschap, wanneer er continu moet worden gewaarborgd dat er niet te veel immuunactivatie optreedt. Om deze cellen te onderzoeken zijn er dus cellen van het eerste zwangerschapstrimester nodig. Dit is mogelijk door immuuncellen uit de stukjes decidua te isoleren die bij een abortus vrijkomen. Naast het meest bekende soort Treg lieten we twee additionele Treg- types zien, gekenmerkt door unieke feno- en genotypes. Trofoblastcellen en deciduale macrofagen zijn in staat om deze Tregs te induceren.

Genotype
Genetische opmaak

Clusters van B-cellen als onderdeel van de immuunomgeving tijdens zwangerschap in de baarmoeder

Naast Tregs komt er steeds meer aandacht voor een ander cel type dat mogelijk een belangrijke bijdrage levert aan het in stand houden van tolerantie: interleukine-10 (IL-10) producerende B-cellen. In **hoofdstuk 4** lieten we zien dat deciduale B-cellen in sterkere mate IL-10 kunnen produceren dan B-cellen uit het bloed. We hebben het fenotype van leukocyten uit MB, eerste trimester decidua, tweede trimester decidua en à terme decidua in kaart gebracht en vergeleken met B-cellen uit het bloed. Door een geautomatiseerde analyse waarbij hun kenmerken objectief worden geclassificeerd werd duidelijk dat uteriene B-cellen los staan van circulerende B-cellen. Door hun positie op knooppunten, waar ook T-cellen aanwezig zijn, zouden ze ondanks hun lage aantallen cruciaal kunnen zijn voor de lokale immuunbalans. B-cellen verdienen dus aandacht in vervolgonderzoek van het uterine immuunsysteem.

IL-10
Immuunmodulerend molecuul (cytokine), uitgescheiden door cellen om een afweer-reactie te dempen.

Veranderd patroon afweercellen bij herhaalde-miskraampatiënten

In **hoofdstuk 5** hebben we onderzocht of vrouwen die herhaaldelijk een miskraam hebben gehad, op basis van afwijkingen in hun afweersysteem gediagnostiseerd kunnen worden. Naast de veel bestudeerde NK en T-cel subtypes waren ook B-cellen en hun subpopulaties onderdeel van de immunologische profielen die van MB en perifeer bloed werden gemaakt. Door middel van een machine-learning ensemble strategie hebben we laten zien dat een combinatie

Ensemble machine-learning
In plaats van één specifiek machine-learning algoritme worden er meerdere gebruikt voor classificatie. Het resultaat is een betrouwbare ranking van parameters die nodig zijn om onderscheid tussen cohorten te maken.

van immunologische parameters betrouwbaar vrouwen met herhaalde miskramen kan identificeren. Zowel het systemische afweersysteem als ook de lokale immuun-cellen zijn hiervoor geschikt. De cel types die samen een diagnostische waarde hebben zijn echter niet dezelfde voor de twee compartimenten, op NKbright cellen na. Het viel op dat in MB vooral cellen die met regulatie in verbinding worden gebracht afwijken (zoals Tregs of bepaalde B-cellen).

NKbright
Type NK cellen dat met immuun-regulatie geassocieerd wordt. Hiertegenover staan NKdim cellen die voornamelijk cytotoxisch zijn.

Een mogelijke bijdrage van het uteriene microbioom aan vruchtbaarheid

De hoge mate aan regulatie die nodig is voor een gezonde zwangerschap zou ook door micro-organismen ondersteund kunnen worden. Het is bekend dat bacteriën op veel plaatsen in ons lichaam een belangrijke rol hebben bij het aansturen van het afweersysteem. In **hoofdstuk 6** geven we een overzicht van de kennis op het gebied van endometriale bacteriën en hun mogelijke rol. Alhoewel er nog geen duidelijk profiel van een uterien microbioom bekend is, zullen de aanwezige soorten een bijdrage leveren aan de lokale immunologische balans en mogelijk ook aan de aanpassingen van het weefsel die nodig zijn voor het vormen van de placenta. Veel studies laten zien dat we er niet meer van uit kunnen gaan dat de baarmoeder steriel is, dus compleet vrij van bacteriën. Er is dus een kans dat problemen rond vruchtbaarheid, of complicatie in de aanleg van de placenta, voortkomen uit een ongunstige microbiële balans.

Microbioom
Alle micro-organismen (virussen, fungi, protozoa en archaea) die op natuurlijke manier aanwezig zijn op een bepaalde plek in het lichaam.

Behandeling met antibiotica heeft effect of afweercellen van de placenta

Behandelingen met antibiotica verstoren de natuurlijke bacteriële flora en hebben mogelijk invloed op processen die mede door bacteriën worden bepaald. Dit zou ook het verloop van een zwangerschap kunnen beïnvloeden. Tijdens één op de vijf zwangerschappen is echter wel een behandeling met antibiotica nodig, bijvoorbeeld als een urine- of luchtweginfectie gepaard gaat met een hoge systemische ontsteking. Gebruik van antibiotica is gekoppeld aan een verhoogd risico op PE, maar de causale link is niet bekend. Tijdens zwangerschap worden er alleen antibiotica gebruikt die veilig geacht worden voor de ontwikkeling van het ongeboren kind. Toch zijn er steeds meer bewijzen voor schadelijke gevolgen op de lange termijn. In **hoofdstuk 7** laten we zien dat antibioticabehandeling bij muizen tijdens zwangerschap consequenties heeft voor het maternale immuunsysteem. Ook bij

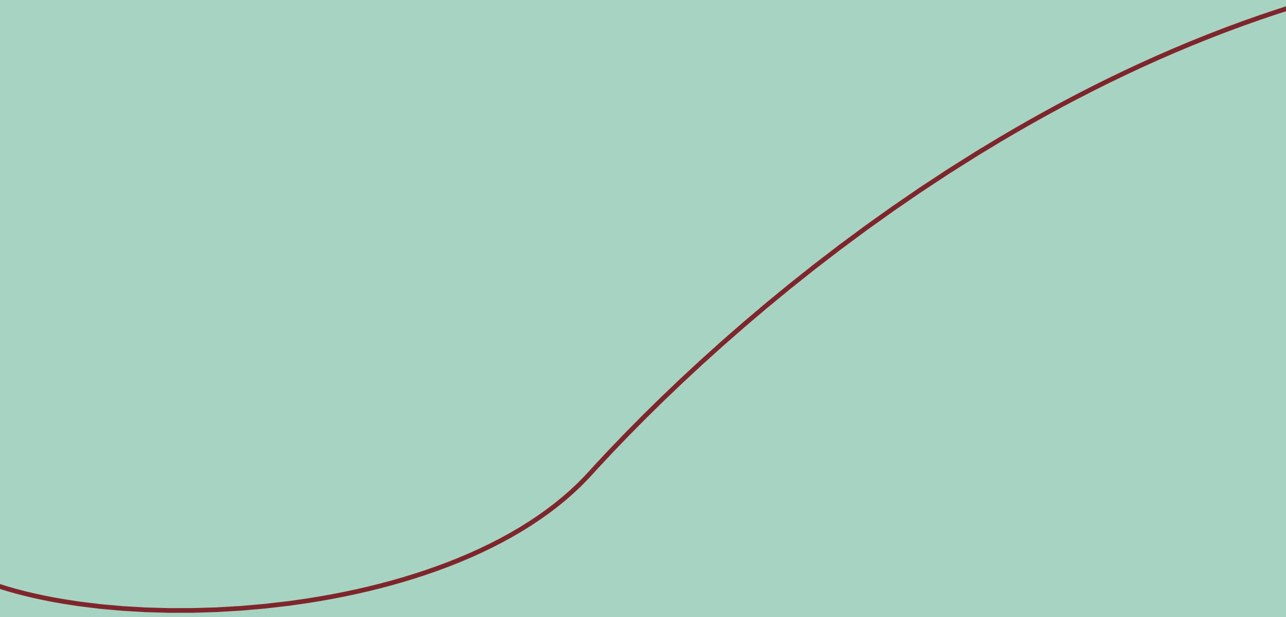
deze studie werd gebruik gemaakt van ensemble machine-learning om de verschillen in het immunologische samenspel van cel types te bestuderen. De immunologische veranderingen zijn ook in de placenta terug te vinden. Hieruit volgt dat er bij de beoordeling van medicatie op veilig gebruik tijdens zwangerschap een mogelijke impact op de directe prenatale immuun-omgeving moet worden overwogen.

Noodzaak voor multidisciplinair onderzoek

Dit proefschrift laat zien dat ook cel types of microbiota die in lage hoeveelheden aanwezig zijn belangrijk kunnen zijn voor een gezonde zwangerschap. Als er complicaties tijdens zwangerschap optreden is dit waarschijnlijk niet het gevolg van één enkel mechanisme wat ontspoort. Een complex samenspel van processen waarborgt de balans die nodig is voor het vormen van de placenta: het immuunsysteem, lokaal en systemisch, microbiota en aanpassingen van het endometrium hangen allemaal met elkaar samen. We lieten zien dat een open blik op elke factor de moeite waard kan zijn om beter te begrijpen wat er nodig is voor een goede ontwikkeling van het ongeboren kind. Om dit verder te kunnen onderzoeken is samenwerking van verschillende disciplines cruciaal. Alleen als we openstaan voor de inbreng van experts en voor nieuwe perspectieven kunnen we zwangerschapscomplicaties beter detecteren en behandelen.

CHAPTER 11

Zusammenfassung (German summary)



EMPFINDLICHE PHASE

Eine Schwangerschaft verändert das Leben der Eltern grundlegend. Diese Veränderungen sind für die Mutter schon früh spürbar, da die körperlichen Anpassungen für die optimale Entwicklung des Kindes essenziell sind. Diese Adaptionen erfolgen sowohl lokalisiert (in der Gebärmutter), als auch systemisch (in der Blutzirkulation). Mütterliche Unterstützung beginnt bereits bei der Interaktion mit der befruchteten Eizelle, da die Gebärmutterschleimhaut eine geeignete Basis formen muss, um die Einnistung zu ermöglichen. Nur, wenn Blastozyste und Endometrium korrekt in Verbindung kommen kann eine Schwangerschaft erfolgreich verlaufen. Demnach ist eine Schwangerschaft lange nicht selbstverständlich und dreißig Prozent aller Schwangerschaften enden bevor sie überhaupt bemerkt wurden. Der gleiche Anteil an Schwangerschaften führt zu einer Fehlgeburt, eine besonders belastende Erfahrung, die fast ein Viertel aller Frauen einmal in ihrem Leben macht. Mit einer weltweiten Prävalenz von 6-8% (2-3% in Deutschland) sind viele Schwangerschaften außerdem von Komplikationen wie Präeklampsie (PE) oder dem HELLP-Syndrom, dem Hauptgrund aller mütterlichen Todesfälle, betroffen. Neben möglicher akuter Unterversorgung des Kindes drohen auch Langzeitschäden, z.B. wenn dauerhafte kardiologische und nephrologische Schäden der Mutter eine frühzeitige Geburt unvermeidbar machen. Die genaue Ursache der PE ist bislang unbekannt, jedoch wird allgemein angenommen, dass eine fehlerhafte Entwicklung der Plazenta eine zentrale Rolle spielt.

Blastozyst
Befruchtete Eizelle nach 5-6 Tagen Zellteilung. Aus der Zellmasse im Inneren wird sich der Embryo entwickeln. Zellen des äußeren Randes formen den fötalen Teil der Plazenta (siehe auch Trophoblast).

Endometrium
Gebärmutterschleimhaut (med. Mukosa)

Präeklampsie (PE)
Früher auch EPH-Gestose genannt, die typischen Symptome umschreibend: Edema (engl Ödem, Wasseransammlung), Protein (Eiweißausscheidung im Urin) und Hypertension (Bluthochdruck). Kann in lebensbedrohliches HELLP-Syndrom übergehen, wobei zusätzlich zu den PE Symptomen Blutgerinnungsstörung (Hämolyse), erhöhte Leberwerte (elevated liver enzymes) und Reduzierung der Blutplättchen (low platelet count) diagnostiziert werden.

Trophoblast
Zellen fötalen Ursprungs die invasiv in die Gebärmutterwand wachsen.

Fragestellung der Dissertation

Beim Entwicklungsprozess der Plazenta spielt das Abwehrsystem eine entscheidende Rolle. Maternales Gewebe und Trophoblastzellen müssen exakt auf einander abgestimmt sein um die Geburt eines gesunden Kindes zu ermöglichen. Das Ziel dieser Dissertation war es, um aus einem neuen Blickwinkel die hierfür nötige Immunregulation zu erforschen. Ein genaues mechanistisches Verständnis der Ursachen, die der Entstehung von Schwangerschaftskomplikationen zugrunde liegen, ist ausschlaggebend um die Entwicklung von frühzeitigen Diagnose- und Behandlungsmethoden zu ermöglichen.

Die Bedeutung des Immunsystems bei der Plazentabildung

Unser Immunsystem erkennt jede körperfremde Zelle und reagiert auf diese zum Schutz vor Infektionen mit einer Abwehr-, beziehungsweise Entzündungsantwort. Auch fötale Zellen werden vom maternalen Immunsystem entdeckt, da die genetische Information dieser Zellen zur Hälfte vom Vater stammt und sie damit nur zu 50% körpereigen sind. Dennoch werden sie nicht aktiv bekämpft; die Trophoblastzellen dürfen trotz ihrer Fremdheit in die Spiralarterien eindringen um die plazentale Verbindung zwischen Mutter und Kind zu initiieren. Dies wird allgemein als ein „immunologisches Paradox" betrachtet. Anders als bei einer Abstoßungsreaktion, die bei einer Organtransplantation nach der Erkennung des gespendeten, fremden Gewebes auftritt, stellt die immunologische Reaktion auf die fötalen Zellen keine Bedrohung für die Schwangerschaft dar. Stärker noch, ein gewisser Grad an Entzündungsreaktion innerhalb der Dezidua ist sogar förderlich um die notwendige Verbindung zwischen Mutter und Embryo herzustellen. Das korrekte Funktionieren des Immunsystems ist von einer kontinuierlichen Balance zwischen Immunaktivierung und -toleranz abhängig. Jede Abwehrreaktion wird durch regulierende Zellen gedämpft um eine optimale Kontrolle des Entzündungsniveaus zu gewährleisten. Für eine gesunde Schwangerschaft ist es von äußerster Bedeutung, dass die Aktivierung des Immunsystems niedrig gehalten wird und nicht aus dem Gleichgewicht gerät. Erhöhte Spiegel von Entzündungsfaktoren können die plazentale Entwicklung stören oder gar zur Frühgeburt führen, was mit negativen Folgen für das heranwachsende Kind einhergeht. Das Forschungsgebiet der Reproduktionsimmunologie beschäftigt sich mit den besonderen Kontrollmechanismen, die beim Heranwachsen der Plazenta und dem Verlauf jeder Schwangerschaft eine wichtige Rolle spielen. Diese Prozesse zur Immunregulierung lassen sich in 3 Hauptthemen zusammenfassen:

Dezidua
Differenziertes Endometrium, ab dem Ende des Menstruationszykluses. Funktioneller Teil der Schleimhaut, der bei der Einnistung der Eizelle und der Verankerung der Plazenta mitwirkt.

1. Anpassungen der Trophoblasten um eine starke Abwehrreaktion zu unterdrücken.
2. Besondere Eigenschaften der systemischen (Blut) und lokalen (Gebärmutter) mütterlichen Abwehrzellen während der Schwangerschaft.

3. Externe Faktoren, z.B. Komponenten der Samenflüssigkeit oder natürlich vorkommende Bakterien, die möglicherweise zur Immuntoleranz beitragen.

All diese Elemente beeinflussen einander und die Thematik ist insgesamt zu komplex um im Ganzen erforscht werden zu können. Wir können also lediglich jedes Puzzleteil gesondert betrachten. In dieser Dissertation wurde besonders auf die Verbindung der einzelnen Teile geachtet, um diese wichtigen Erkenntnisse in Zusammenhang miteinander zu bringen.

INHALT DIESER ARBEIT

Schwangerschaft induziert immunologisches Gedächtnis der Gebärmutter

In **Kapitel 2** geben wir eine Übersicht der verschiedenen Zellen der Gebärmutter, die bei einer Schwangerschaft mitwirken. Hierfür wurden Zellen des Endometriums von nicht-schwangeren Frauen analysiert. In früheren Studien wurden zur Erforschung des uterinen Immunsystems hauptsächlich Gewebeproben begutachtet. Dabei wurde Material verwendet, das bei medizinischer Indikation als Biopsie invasiv entnommen werden konnte und dann für Studien zur Verfügung gestellt wurde. Dies erschwert Aussagen bezüglich eines gesunden Immunsystems. Wir konnten dieses Problem durch die Aufarbeitung von Menstruationsblut (MB) umgehen. Monatlich wird am Ende des weiblichen Zyklus die funktionale Schicht des Endometriums, einschließlich der darin vorkommenden Zellen, abgestoßen. Menstruationstassen, umweltfreundliche Alternativen zu Tampons, fangen diese Flüssigkeit auf und ermöglichen die Isolierung und Analyse von einer großen Anzahl von uterinen Immunzellen; auch von Frauen ohne Fertilitätsproblematik. Diese Methode wurde auch in **Kapitel 4** und **5** angewendet. In **Kapitel 2** zeigten wir mithilfe von Durchflusszytometrie, dass Leukozyten aus MB in der Tat die typischen Kennzeichen von Abwehrzellen der Gebärmutter aufweisen. Die analysierten MB-Zellen unterschieden sich im Phänotyp deutlich von Zellen der Zirkulation. Wir verglichen MB-Zellen des Weiteren mit Leukozyten der *decidua parietalis* von vollendeten, gesunden Schwangerschaften (> 37. Schwangerschaftswoche, SSW), um

Durchflusszytometrie
Zell-Vermessungsverfahren, bei dem mit Hilfe von fluoreszent-markierten Antikörpen Zelltyp-spezifische Merkmale analysiert werden.

Leukozyten
Weiße Blutzellen, zuständig für Abwehrreaktion. Umschreibt u.a. Granulozyten, Monozyten/Makrophagen, Lymphozyten (T-,B- NK Zellen) und dendritische Zellen.

Phänotyp
Merkmale einer Zelle. Ermöglicht z.B. Rückschlüsse bezüglich des Zelltyps, Herkunft der Zelle oder deren Aktivierung.

Decidua parietalis
Maternale Lage der Eihaut (ugs. Fruchtblase).

zu untersuchen inwieweit sich das lokale Abwehrsystem durch eine Schwangerschaft verändert. Nach neunmonatiger Schwangerschaft war das Immunsystem der Gebärmutter weniger naiv, bzw. unerfahren. Das bedeutet, dass T-Zellen aktiviert wurden und ein Immungedächtnis aufbauen konnten. Des Weiteren fanden wir in der *d.parietalis* mehr Zellen, die mit Immuntoleranz in Verbindung gebracht werden, wie z.B. regulatorische T-Zellen (TReg). Mittlerweile ist auch bekannt, dass diese Differenzierung durch den Kontakt der T-Zellen mit fötalen Zellen vor Ort induziert wird und nur ein geringer Teil dieser spezifischen Reaktion in der Zirkulation zurückzufinden ist. Das erhöhte Vorkommen der TReg Zellen wirkt einer möglichen (zu) starken Reaktion der T-Gedächtniszellen entgegen.

Drei unterschiedliche regulatorische T-Zellen steuern die Begegnung von fötalen und maternalen Zellen

In **Kapitel 3** wurden TRegs, bekannt wegen ihrer Fähigkeit Immuntoleranz zu fördern, näher untersucht. Diese Zellen sind vor allem in der ersten Phase einer Schwangerschaft essentiell, da die partielle Unterdrückung der Immunantwort in diesem Zeitraum entscheidend ist. Um die speziellen Anpassungen der involvierten TRegs zu analysieren, wurden daher Abwehrzellen aus der Gebärmutterschleimhaut isoliert, die beim Schwangerschaftsabbruch des ersten Trimesters teils entnommen wurde. Neben der meist untersuchten Art der TRegs konnten wir außerdem zwei weitere TReg-Populationen isolieren. Alle drei Zelltypen unterscheiden sich sowohl im Pheno-, als auch im Genotypen voneinander. Trophoblastzellen und Makrophagen der Dezidua konnten die Differenzierung von naiven T-Zellen zu diesen TReg-Zellen induzieren.

Genotyp
Genetische Zusammenstellung einer Zelle/ eines Organismus

B-Zell-Cluster gehören während der Schwangerschaft zum Immunsystem der Gebärmutter

Neben TReg Zellen scheinen auch weitere Zelltypen einen wichtigen Beitrag zur Induktion der Toleranz leisten, nämlich die Interleukin-10 (IL-10)-produzierenden B-Zellen. In **Kapitel 4** konnten wir zeigen, dass B-Zellen der Dezidua verglichen mit B-Zellen der Zirkulation erhöhte Kapazitäten zur IL-10-Synthese haben. Wir verglichen die Phenotypen von B-Zellen der Gebärmutterschleimhaut zu verschiedenen Zeitpunkten vor (MB-Zellen), während (erstes und zweites Trimester) und nach der Schwangerschaft (*d.parietalis* nach der 37.SSW)

IL-10
Toleranzinduzierendes Molekül, welches pro-inflammatorischen Zytokinen entgegenwirkt.

miteinander und mit Zellen der Blutzirkulation. Eine automatische Computeranalyse dieser Zellen ermöglichte eine objektive Beurteilung und zeigte, dass uterine B-Zellen sich nicht nur in ihrer Funktion, sondern auch im Phenotyp, von B-Zellen der Zirkulation unterscheiden.

Abweichende Abwehrzellmuster bei Frauen, die wiederholt Fehlgeburten erleiden

In **Kapitel 5** untersuchten wir mögliche Veränderungen im Immunsystem bei Frauen mit wiederholtem Spontanabort (WSA). Hierbei wurde die Frage gestellt, ob WSA-Patientinnen auf Basis ihres Immunsystems als solche identifiziert werden können. Neben den meist untersuchten NK- und T-Zellen, waren auch B-Zellen Teil der Immunzellübersicht, die wir sowohl für MB, als auch Blut der Zirkulation, erstellt haben. Mithilfe einer Machine-Learning-Ensemble-Strategie konnten wir zeigen, dass Frauen ohne Schwangerschaftsproblematik von Frauen mit WSA auf Basis von fünf (Zirkulation) bzw. sieben (MB) Immunparametern unterschieden werden können. Demnach sind sowohl das Abwehrsystem der Zirkulation, als auch das der Gebärmutter, bei WSA-Patientinnen verändert. Die hierbei ausschlaggebenden Zelltypen sind jedoch in den beiden Kompartimenten unterschiedlich, bis auf NKbright-Zellen, die in beiden Datensets wichtig sind. Auffallend war auch, dass in MB vor allem Zelltypen mit regulatorischer Kapazität (TReg oder bestimme B-Zelltypen), von der Kontrollgruppe abweichen.

Wiederholter Spontanabort (WSA)
Definiert als ≥3 Fehlgeburten vor der 20. SSW. Betrifft etwa 1-3% aller Paare im reproduktionsfähigen Alter.

Machine-Learning-Ensemble
Kombination von mehreren Machine-Learning Algorithmen. Hieraus folgt ein valides Ranking von Parametern, auf deren Basis zwei getestete Gruppen unterschieden werden können.

NKbright Zellen
Unterart der NK-Zellen, die vor allem mit einer regulierenden Rolle in Verbindung gebracht wird. Dem gegenüber stehen NKdim-Zellen die weitestgehend zytotoxische Eigenschaften haben.

Möglicher Beitrag des Mikrobioms zur Fruchtbarkeit

Das hohe Maß an Mechanismen zur Immunregulierung, ohne das eine gesunde Schwangerschaft nicht zu Stande kommen kann, könnte auch durch Mikroorganismen unterstützt werden. Es ist bereits bewiesen, dass Bakterien an verschiedensten Stellen des Körpers natürliche Prozesse beeinflussen und sogar steuern. **Kapitel 6** fasst unser Wissen bezüglich eines möglichen Beitrags von uterinen Bakterien zur Fruchtbarkeit zusammen. Obwohl noch nicht konkret gezeigt werden konnte aus welcher Spezieszusammenstellung ein gesundes Mikrobiom der Gebärmutter besteht, ist deutlich, dass man den Uterus nicht als steril betrachten kann und die Anwesenheit von Bakterien damit eine unabkömmliche Rolle für das Gewebe spielen muss. Die nachgewiesenen Mikroorganismen können sowohl das lokale Immunsystem, als auch die Entwicklung der Mukosa, und

Mikrobiom
Natürliche vorkommende Mikroorganismen (Bakterien, Pilzen, Viren und Archaeen), die zusammen eine Stelle des Körpers kolonisieren.

somit die der Plazenta, beeinflussen. Demnach wäre es möglich, dass Pathologien der Fruchtbarkeit oder der plazentalen Entwicklung mit einem ungünstigen Mikrobiom im Zusammenhang stehen.

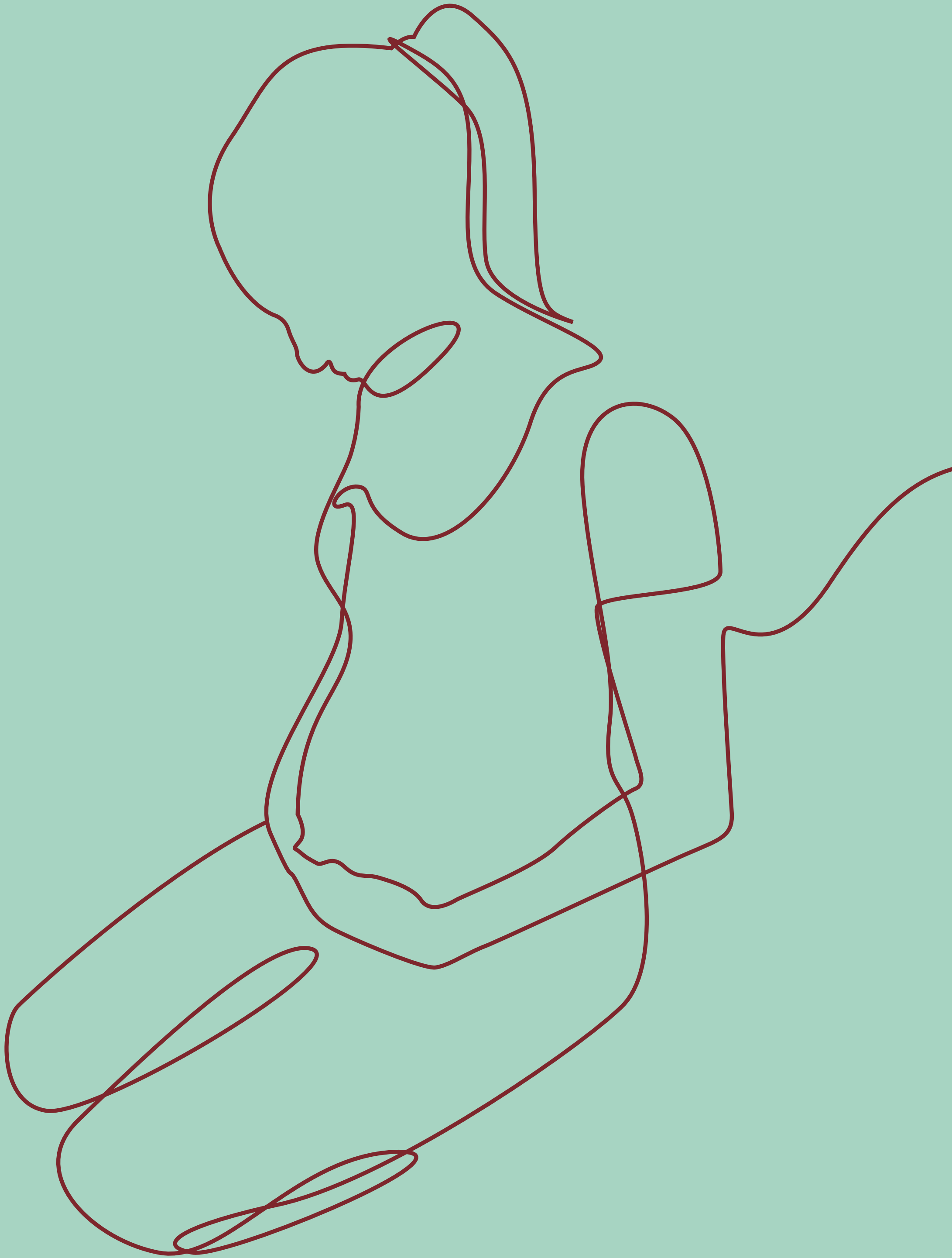
Antibiotika beeinflussen Abwehrzellen der Plazenta

Die natürliche bakterielle Flora kann nicht nur bei einer Infektion aus dem Gleichgewicht geraten. Auch Antibiotika beeinflussen das Mikrobiom und haben dementsprechend Effekt auf Prozesse, die diesem unterliegen. Somit könnte eine Antibiotikabehandlung den natürlichen Verlauf einer Schwangerschaft verändern. Ein derartiges Eingreifen ist in ungefähr einer von fünf Schwangerschaften unabdingbar, z.B. wenn es eine Harn- oder Luftweginfektion zu kontrollieren gilt, die womöglich auch in eine systemische Entzündung überzugehen droht. Allgemein sind nur ausgewählte Medikamente, die als sicher für die Kindesentwicklung ausgewiesen sind, für den Einsatz während einer Schwangerschaft zugelassen. Dennoch sammeln sich die Beweise, dass auch diese Mittel möglicherweise Langzeitfolgen mit sich bringen. Eine Antibiose bei Schwangeren erhöht das Risiko auf PE, allerdings ist die Ursache für diesen Zusammenhang noch nicht bekannt. In **Kapitel 7** zeigten wir, dass eine Antibiotikabehandlung Auswirkungen auf das Immunsystem von trächtigen Mäusen hat. Auch in dieser Studie konnte eine Machine-Learning-Ensemble-Strategie die wichtigsten Unterschiede zwischen den Testgruppen aufzeigen. Die immunologischen Veränderungen konnten bis in die Plazenta nachgewiesen werden. Diese Ergebnisse heben hervor, dass bei der Beurteilung von Medikamenten zur Anwendung während der Schwangerschaft Rücksicht auf die besonderen immunologischen Umstände dieser Phase genommen werden muss. Hierbei darf die mögliche Manipulation der pränatalen Immunumgebung nicht vergessen werden.

Multidisziplinäre Forschung gefragt

Die hier vorgelegte Arbeit zeigt, dass sogar Zelltypen oder Mikroorganismen, die wegen ihrer geringen Anzahl häufig nicht im Fokus stehen, einen wichtigen Beitrag zur Schwangerschaft leisten können. Komplikationen während der Schwangerschaft sind wahrscheinlich nicht auf einen einzigen, isolierten, fehlerhaften Prozess zurück zu führen. Die komplexe Interaktion verschiedener Mechanismen muss in Balance sein, um die korrekte Entstehung der Plazenta zu

gewährleisten. Das Immunsystem der Gebärmutter und der Zirkulation, sowie Mikroorganismen des Endometriums stehen miteinander in Verbindung um die besonderen Anforderungen einer Schwangerschaft zu gewährleisten. Der unbefangene Blick der vorliegenden Arbeit ermöglichte damit neue Einsichten bezüglich dieser diversen Faktoren und ihrem Einfluss auf die gesunde Kindesentwicklung. Um dies weiter zu untersuchen ist es essenziell, dass die einzelnen Forschungsfelder verknüpft werden. Nur, wenn wir den verschiedenen Experten offen begegnen und zusammen neue Blickwinkel schaffen, können wir zukünftig Schwangerschaftskomplikationen besser aufdecken und behandeln.



CHAPTER 12

A plea for being open to a new approach
– Changing perspectives based on evidence

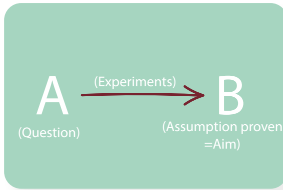
Reproductive immunology comprises a field of many unknowns. The related conferences have an active debate culture; many meals are accompanied by a lively back and forth on opposing hypotheses. The wish to provide solutions for fertility and pregnancy complications creates an urgency in developments. In many countries, clinicians will provide diagnostic tools and treatment before the needed proven efficacy, which adds to the pool of uncertainty regarding the topic. Still, the scientific discussion depends on evidence rather than opposing thoughts. Which concepts are we going to build on when we try to fill a knowledge gap?

We thought that this thesis started with a proposal based on ***commonly accepted*** positions of the field. As a starting PhD candidate, this goes along with the expectation that with a solid foundation and the right assumptions, the project is going to follow a straight path. In the end, the projects turned out rather different than we planned in 2015. Apparently, this is the classic, unpredictable path that most PhD projects follow. Likewise, the frustrations going along with failed experiments are not hugely surprising. Looking back, a lot of learning happened especially in that time of what seemed like failure. Professor Uri Alon termed this phase of frustrations ***The Cloud***[1] , as summarized on the following page, with his kind approval.

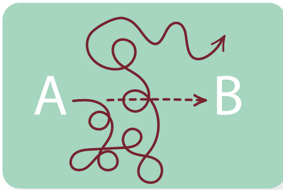
I am writing this Behind the Scenes chapter for all those, who might have started a PhD project, but who struggle because nothing seems to go according to plan. Without The Cloud, most of this thesis' chapters would not have been written. Just like young researchers and their supervisors, reproductive immunology will thrive, when we are open to a change of plans and concepts.

[1] Uri Alon, "Why truly innovative science demands a leap into the unknown", TED: Ideas Worth Spreading, June 2013, https://www.ted.com/talks/uri_alon_why_science_demands_a_leap_into_the_unknown

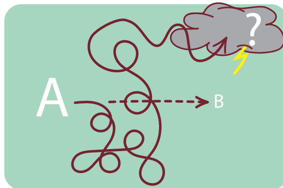
The Cloud



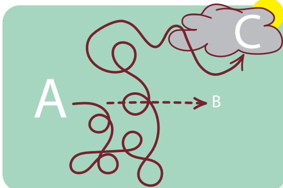
In science, we learn about the results, not the process. Likewise, this thesis presents questions and answers based on the gathered evidence. If all is well (written), the process sounds very straightforward: We identify a knowledge gap and then venture out to prove an assumption through a logical sequence of experiments.



In reality, we start at **A** but despite all plans, there is no direct path to B. Experiments do not work, other projects demand immediate attention, or personal ones come up. Sometimes we might think that we get closer to B, until we discover that we cannot even see **B** anymore. We may also start doubting **A** (and many other things…).



The diverting path has led us to *The Cloud*. On top of the stress that we still do not have results of **B** to show, frustration builds up about the fact that **A** → **B** seemingly works for everyone else.



Continuing to work in the cloud, a new outcome that is worth pursuing often comes into view. The truth is, we likely read someone's story of how they got from **A** → **C** (like the content of this thesis). C might be based on new assumptions based on all the "failed" experiments. While B was useful to get the project going, C is much more innovative.

Thus, the period of uncertainty is the part that enabled innovation. While C has never been the initial plan, C is more worth pursuing than B. The more this period of uncertainty is just related to science, and not insecurities about funding or other additional sources of pressure, the more opportunities for creative problem solving are created. By creating a supportive environment that allows letting go of the initial plan, we will enable scientific advances.

Part 4

Appendix

CHAPTER 13

FAIR principles

Acknowledgements — Dankwoord - Danksagung

Curriculum Vitae

RIMLS PhD portfolio

FAIR PRINCIPLES

This thesis is based on the results of human studies which were performed in accordance with the principles of the Declaration of Helsinki. All samples were acquired with the approval of the local medical and ethical review board. Study codes, as registered with the Committee on Research Involving Human Subjects Region Arnhem Nijmegen, Nijmegen, the Netherlands, are stated in the Materials and Methods section of the respective chapter. The murine data described in Chapter 7 were acquired in collaboration with the University of Utrecht. Animal procedures were approved by the Ethical Committee for Animal Research of the Utrecht University and conducted according to the European Directive 2010/63/EU on the protection of animals used for scientific purposes (AVD108002016597).

Experimental notes are stored on Labguru, a digital lab book platform, and accessible by the associated staff members. Raw data and analyses are stored on the Radboudumc department server (labgk_lmi-research, umcsanfsclp01) in separate folders assigned to each project (Z:\Sectie HCI_kweek\z049173_Marilen Benner\Projects). These data are backed-up on local servers of the department on a weekly basis. Samples were labeled with an ID to ensure anonymity. All databases which contain or link patient details to sample IDs are password protected. Published data associated with this thesis are accessible on the respective journal website or can be provided by the corresponding author upon request.

'What is the bravest thing you've ever said? asked the boy.
'Help,' said the horse.
'Asking for help isn't giving up,' said the horse. 'It's refusing to give up.'

— Charlie Mackesy, The Boy, the Mole, the Fox and the Horse

ACKNOWLEDGEMENTS – DANKWOORD - DANKSAGUNG

This is it, the end of my thesis! There have been a lot of people involved to get to this achievement. I am grateful for everyone who supported me through the years of blood, sweat, tears and placentas. This chapter cannot really do justice to thank everyone who contributed scientifically or who somehow took my mind off the science.

First of all, I owe this thesis to all **donors** whose (menstrual) blood and placental tissue was used for the presented work. Many of you have shared personal experiences on menstruation, fertility, or pregnancy. Thank you for your trust and for fueling my motivation.

Mijn **promotieteam** heeft mij de kans gegeven om aan het mooiste onderwerp dat er is te werken. Dit project hebben we echt samen geschreven en jullie hebben er alles voor gegeven dat ik eraan kon beginnen. Ik ben jullie ontzettend dankbaar. **Renate**, bedankt voor je enthousiasme en steun sinds dag 1 bij de zwangerschapsimmunologie. Ik ben benieuwd hoeveel PhDs ervoor nodig zijn om al je projectideeën aan te pakken. **Gerben**, ook jij bent vol ideeën en je staat altijd open voor een rondje sparren over the bigger picture. Op die manier heb je heel wat projecten helpen ontstaan. Soms was je er even een tijdje (of wat langer) niet, maar je wist me te helpen toen ik zelf nog niet om hulp durfde te vragen. **Irma**, zonder jouw gerichte vragen was ik waarschijnlijk nog een aantal jaren bezig geweest. Ik waardeer de manier waarop jij me sturing en vrijheid gaf enorm. Uiteindelijk kon ik bij jou met veel meer terecht dan ik dacht en veel van je advies zal me voor altijd bij blijven.

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Het team van **MildredClinics**, zonder jullie inzet was een belangrijk deel van dit proefschrift niet mogelijk geweest. Jullie creëren altijd een warme, veilige sfeer, zonder oordeel. Naast jullie belangrijke taken hadden jullie zelfs nog aandacht voor het onderzoek. Dat waardeer ik enorm. Bedankt dat jullie me altijd als collega hebben laten meewerken.

Alejandro, uncountable hours were spent to help me understand my data and you never lost your patience. This thesis would have looked a lot different without your input. Thanks a lot!

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A special thanks goes to my office roommates and PhD colleagues who showed me how to enjoy and survive the journey. **Dorien**, we hebben heel wat projecten en ervaringen gedeeld. Congressen waren veel leuker met jou als travel buddy (en mede-gebakliefhebber). **Yessica**, thanks for being there for all PhD joys and frustrations. Life at LMI, and many parties, would have been a lot less fun without you. Polydactylous, wise and silly **Paulo**, your continuous reminders that everything will be fi(iiiii)ne got us through the years. Maybe someday we can get together with **Josh**, **Jorge**, **Rosanne**, **Omar and Cristiane** for a Christmas market reunion. Thank you for the good times! **Sija**, wat is het lang geleden dat we samen op het lab (!) hebben gestaan. Bedankt voor de gezelligheid sinds dag 1 van mijn stage. **Xuehui**, I often think about our trip to Beerse. I am grateful for everything I learnt from you. **Ezgi**, I am happy to have seen you embark on the reproductive immunology journey. I am sure you will carry on our office mantra. All the best! **Elena**, **Patrick**, **Pieter** en onze extended lab family **Lucille**, **Rienke**, **Janeri**, bedankt voor alle gezelligheid en discussies.

Thanks to all members of the **Strominger lab**. **Tamara**, thanks for the opportunity to dive deeper into reproductive immunology. And for your long list of restaurant recommendations. **Jack**, thank you for all the tricky questions, our lab dinners and for trusting me to arrange a trip to your farm. Thanks to **Angela**, **Andrew**, **Bill**, **Ester**, **Hannah**, **Leo**, **Lloyd**, **Mathias**, **Mei-Ling**, and **Torsten** for the amazing time in and outside of the lab.

Ooit begon het op een lab en jaren later zijn jullie er nog. **Liz**, altijd klaar voor een feest, borrel, concert, dans. Dank je wel voor alle support door de jaren heen. Pep-talks en klusverhalen delen lukt gelukkig ook op afstand. Wat een gave uitdaging ga je samen met **Erwin** aan. Ik kijk uit naar

jullie verhalen. **Janin**, wer sonst fährt 5 Stunden Zug um mit mir zu Stricken. **Nathalie**, bedankt voor onze wijn- en spelletjesavonden. Gelukkig zijn we allebei even slecht met de regels. **Anita**, mijn decidua-dinsdag buddy. Mijn tijd bij het Strominger lab was niet hetzelfde geweest zonder jou. Ik kijk uit naar meer korte trips met jou en **Hussain** als het weer kan.

Marit, where to begin? Tijdens de studie? Of eigenlijk Boston? Sinds je met je 3 koffers voor mijn deur stond is er heel wat veranderd. Maar ik zal me inhouden, anders wordt dit echt een hoofdstuk op zich (en janken). Je hebt een aantal enorm knappe keuzes gemaakt. Ik ben blij dat jij straks als paranimf naast me staat. Dat is zeker ook jouw prestatie.

De leden van de **RIMLS PhD council**, dank jullie wel voor alle leuke gesprekken, de gezelligheid, gemeenschappelijk geregel en de fijne samenwerking. **Anouk**, **Bas**, **Chet**, **Clasien**, **Dov**, **Estel**, **Francesca**, **Iris**, **Judith**, **Lucas**, **Mariya**, **Michelle**, **Minke**, **Paola**, **Patrick**, **Pepijn**, **Pieter**, **Romy**, **Sónia**, **Sophie**, **Vince**, **Xander**, ik heb een hele leuke en leerzame tijd gehad met jullie! De councilprojecten dreigden soms wel belangrijker te lijken dan het onderzoek en ik ben heel trots op wat we samen hebben bereikt. **Bert** en **René**, bedankt voor al jullie inzet voor de council en voor de kans om voorzitter te mogen zijn. Ook al moeten jullie met veel verschillende stakeholders rekening houden, er was altijd aandacht voor de promovendi. **Daan**, van Chief Retreat Advisor naar gezelligheid buiten de council, bedankt! **Anique**, bedankt voor al je steun, overlevens- ,design- ,planten-, en kledingadvies. Ook ik hoop dat we nog veel voor taartjes (of wijn) bij elkaar zullen komen.

One of the best things of my PhD was the opportunity to supervise (and to learn from) an amazing and diverse group of students. **Celia**, **Nurcan**, **Sergi**, **Julia** thank you for everything. You have done so much work, you put up with my endless contemplations, and you all mastered quite some challenges (and I refer to the ones that are more profound than coming to work after an Erasmus night). Lab life would have been a lot less fun without you! It has been great to see you learn and become more confident.

MMD cohort of 2014, we learnt, survived, traveled, almost missed planes, laughed, and cried together. It's been fün! Bedankt ook aan **Helma** en **Roland**, door jullie kon MMD zijn wat het voor ons was. Het team van de MMD programme committee, **Alessandra**, **Kim**, **Loes**, **Malou**, bedankt voor de fijne samenwerking achter de schermen van de opleiding. Jullie verliezen het welzijn van de student niet uit het oog. Mooi om de afgelopen jaren te mogen meemaken en meedenken hoe zich dit in veranderingen van het programma kan vertalen.

Eigenlijk kennen we elkaar sinds dag 1 van de studie. **Lisanne**, bedankt voor alle thee-, whiskey-, spa-, en dinnerdates de afgelopen jaren! Ik bewonder wat jij allemaal voor elkaar krijgt. Ik kijk uit naar nog meer gezellige avonden samen met **Huub** en naar jullie bruiloft! **Iris**,

allebei zijn we inmiddels ver gevorderd in een promotietraject. Dat hadden wij 10 jaar geleden zeker niet voorspeld. Het valt dan ook echt niet samen te vatten wat wij allemaal samen hebben meegemaakt. Ik hou je verantwoordelijk voor mijn introductie aan de Witte Gijt en alle gevolgen van dien. Soms op afstand en soms met minder regelmaat, maar als we elkaar zien ben ik altijd verbaasd hoe goed je mij kent en begrijpt. Heel gaaf, wat je nu samen met **Arjen** hebt opgebouwd. Ik kijk uit naar **Bennie**! **Dennis**, van dans- naar wandelpartner. Bedankt voor 10 jaar met spontane koffiemomenten en feest-jives. **Niklas**, mein Thailand-Roommate, Fitti-Berater, Kummerkasten und R-Experte! Ich freue mich auf noch viele gemeinsame Dinner (-rezensionen) bzw. Kuchen mit **Neele** und **Patric**.

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To the friends gained through ASML, **Hogard** and **Lenka**, **Marta** and **Jacek**, **Greg** and **Rachel, Chris** and **Dashiell**. We had some amazing trips thanks to you. What a welcoming, crazy bunch. Thank you so much! **Joost** en **Manon**, bedankt voor alle gezelligheid!

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Die **Wild**en, obwohl unser Treffpunkt in der Mitte immer schwerer zu erreichen ist, kriegen wir hoffentlich bald wieder eine Reunion organisiert. **Anika**, **Dalina**, **Laura**, **Miriam**, **Tamara**, mit euch war die Galgenveldküche wie nach Hause kommen. Meine liebe **Birte**, mittlerweile auch trotz Lockdown, du warst immer da für Rückendeckung, Roadtrips, Zoom-Zumba, Spazieren und mittlere bis größere Panik. Ich bewundere so, was du alles wuppst. Ziehe bloß nicht weg, no pressure.

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Gabi en **Erlinde**, mijn Vlaamse familie, ook al zeggen ze dat je familie niet kunt kiezen. Jullie waren en zijn er altijd, ook voor Mama (of hoor ik Moeke te zeggen?) als ik door alle werkdrukte nauwelijks in beeld was. Bedankt voor alles! **Geke**, **Jan**, **Willemijn**, **Pim**, **Gijs**, **Wessel**, **Ilona**, **Arend** en **Alyona**, bedankt dat ik me altijd thuis mag voelen in de familie. Jullie waren altijd geïnteresseerd in het onderzoek maar bij alle BBQ, gourmet, nachtelijke borrelhapjes, spelletjes en gemeenschappelijke reizen kon ik het juist allemaal loslaten. Ik kijk ernaar uit om **Renske** te zien opgroeien. **Johan** en **Letty**, bedankt voor alle warmte en steun de afgelopen jaren. Dank ook aan **Anneke**. Waarschijnlijk zal ik nog allemaal typfouten in mijn boekje vinden, maar zeker niet in de Nederlandse samenvatting. **Nine**, ganz vielen Dank für deinen Beitrag zu dieser Arbeit. Das hat mich sehr gefreut!

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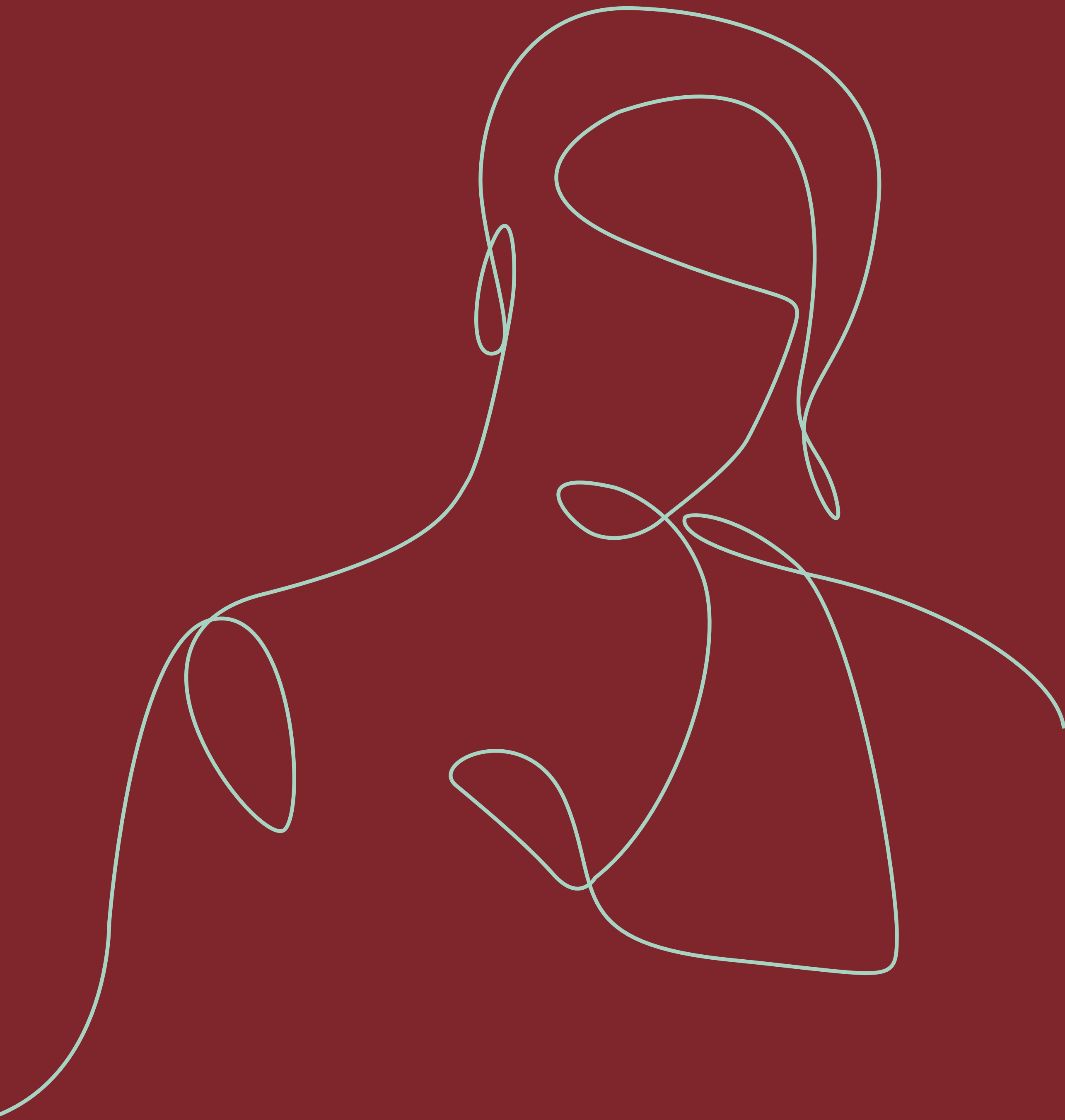
Het is even werk, maar dan heb je ook wat. Daarvoor hebben we inmiddels genoeg bewijs. Bedankt, **Rindert**, voor alles wat door jou de afgelopen jaren mooier is geworden.

13



CURRICULUM VITAE

Marilen grew up in Mettmann, Germany, where she attended the Konrad-Heresbach-Gymnasium. In 2008, she spent a semester at the Sunshine Beach State High school in Noosa, Australia. After graduating from secondary education in 2011, she moved to Nijmegen, The Netherlands, to study (Medical) Biology at the Radboud University. In her third year, she performed a research internship at the Laboratory of Experimental Nephrology under supervision of Prof. Dr. Johan van der Vlag in the context of the Radboud Honors Academy. She visited the Hadassah Medical Center in Jerusalem, Israel, for parts of this project. Finishing her bachelor's program cum laude, she was selected to enroll in the research master's program Molecular Mechanisms of Disease (MMD) of the Radboud university medical center (Radboudumc) and the Radboud Institute for Molecular Life Sciences (RIMLS). She joined the field of reproductive immunology during her first master's internship, under supervision of Dr. Renate van der Molen at the lab of Prof. Dr. Irma Joosten. She continued studying the unique immunological characteristics of the placenta at the Harvard Department of Stem Cell and Regenerative Biology in Cambridge, USA, at the lab of Prof. Dr. Jack Strominger, under supervision of Dr. Tamara Tilburgs. Both her master's theses obtained awards, as Best Thesis of the Radboudumc and the University Study Award 2016, respectively. Marilen finished her master's program cum laude in 2016. Her PhD project proposal, written together with Prof. Dr. Joosten, Dr. Van der Molen, and Dr. Gerben Ferwerda, was awarded with a Junior Researcher grant, funding the work presented in this thesis. She started her PhD at the group of Dr. Van der Molen in November 2016. Marilen has presented her work at various international conferences, which was sponsored by several awarded travel grants. Marilen supervised bachelor's and master's students during their internships. Next to her research, she was an active member of the RIMLS PhD council, organizing events, and creating opportunities for PhD candidates to exchange their experiences. Marilen chaired the council for 1.5 years, representing RIMLS PhD candidates to improve the graduate school for fellow young researchers.

Marilen lives together with Rindert in their house in Nijmegen Brakkenstein.

Institute for Molecular Life Sciences
Radboudumc

PHD PORTFOLIO

Name PhD student:	*Marilen Benner*	PhD period:	*01-01-2017 until 30-06-2021*
Department:	*Laboratory Medicine, Laboratory Medical Immunology*	Promotor(s):	Prof. dr. Irma Joosten
Graduate school:	*Radboud Institute for Molecular Life Sciences*	Co-promotor(s):	*dr Renate G. van der Molen, dr. Gerben Ferwerda*

Training activities	Year(s)	ECTS
a) Courses & Workshops		
- Graduate School introductory course	2017	1.5
- Placenta Summer School Centre for Trophoblast Research, Cambridge, UK	2017	1.75
- Mindfulness based stress reduction for PhD	2017	2.5
- Career management for PhD	2018	1
- Within sight of your PhD	2018	1
- Project management for PhD	2019	2
- Scientific Integrity	2019	1
- Introduction to R	2019	1
- Design and Illustration	2020	1
- Udemy course R Programming A-Z™: R for Data Science	2020	1
- The Art of Finishing Up	2021	1
b) Seminars & Lectures		
- Introduction day Radboudumc	2017	0.5
- Radboud Research Rounds	2017-2020	0.1
Radboud Research Round Inflammatory Diseases, organizing committee	2019	0.5
- Next Generation Metabolomic Screening Lecture	2017	0.1
- BD Flow Day	2017	0.1
- Webinar Open Science	2020	0.1
- Webinar Meet the Expert LinkedIn	2020	0.1
- RiNet meeting	2017	0.2
- Joined meeting LMI, AIG, LKI	2017-2020	1.5
c) (Inter)national Symposia & Congresses		
Conferences		
- Herrenhausen Conference “The Neonatal Window of Opportunity, Early Priming for Life”	2016	1.5
- Radboud Frontiers #	2017	1.5
Co- organizing Public event	2017	0.75
- PhD retreat *#	2017-2020	4
Organization retreat	2019	1
- Fetal Maternal Cross-Talk Keystone Symposium, Washington DC, US *#	2017	2.25
- Centre for Trophoblast Research meeting, Cambridge, UK	2017	0.5
- European Society for Reproductive Immunology, Aalborg, Denmark *	2018	1.3
- National Congress of Infertility and Reproductive Health, Bulgaria *	2019	1.25
- ENABLE conference	2020	0.8

Symposia		
- Symposium “No guts, no glory” accompanying the inaugural lecture of Dr. Aletta Kraneveld	2017	0.2
- Radboud Science Days	2017	0.5
- RIMLS Science day	2018	0.1
Organizing committee	2018	0.5
- Symposium of the Reproductive Immunology Netherlands		
Utrecht *	2018	0.25
Groningen	2020	0.25
Leiden	2020	0.25
- EPPW meeting	2020	0.5
- Janssen Interactive Career Day *	2020	0.8
d) Other		
- Radboud Talks Pitch Competition *	2018	1
- Review of scientific publications	2018-2021	0.1
- MMD program committee member (alumni)	2017-2019	1.5
- PhD Council committee member	2017-2020	2
PhD Council chair	2019 - 2020	1.5
- Journal Club	2017-2020	3
- Organization monthly social event RIMLS PhD council (10x per year)	2018-2019	2
Teaching activities	**Year(s)**	**ECTS**
e) Lecturing		
- BMW 2nd year students Infectious Diseases Introduction	2017	0.7
- Innovation project Medicine students	2017	0.1
- Jury MMD symposium	2017-2018	0.5
- Scientific skills for MMD students, class on Adobe Illustrator	2018-2020	1.5
- Presentation Labroots Webinar Beckman Coulter	2021	1
f) Other		
Supervision of internships		
- BSc, Celia Cartagena, University Barcelona, 10 months	2017	3.1
- BSc, Nurcan Inci, Istanbul University, 6 months	2018	2
- BSc, Sergi Cedó López, University Barcelona, 6 months	2019	2
- MSc, Julia Meerman, RIMLS, 6 months	2020	2
- Literature Thesis (MSc) Miriam Meijerhof, RIMLS, 2 months	2020	0.5
Total		**60.65**

Oral and poster presentations are indicated with a * and # after the name of the activity, respectively.

LIST OF PUBLICATIONS

In this thesis

Marilen Benner, Dorien Feyaerts, Celia Cartagena García, Nurcan Inci, Sergi Cedó López, Esther Fasse, Wijs Shadmanfar, Olivier W. H. van der Heijden, Mark A. J. Gorris, Irma Joosten, Gerben Ferwerda, & Renate G. van der Molen.
"Clusters of Tolerogenic B Cells Feature in the Dynamic Immunological Landscape of the Pregnant Uterus"
Cell Reports, 2020; 32(13), DOI: 10.1016/j.celrep.2020.108204

Maria Salvany-Celades*, Anita van der Zwan*, Marilen Benner*, Vita Setrajcic-Dragos, Hannah Ananda Bougleux Gomes, Vidya Iyer, Errol R. Norwitz, Jack L. Strominger, & Tamara Tilburgs (*contributed equally).
"Three Types of Functional Regulatory T Cells Control T Cell Responses at the Human Maternal-Fetal Interface"
Cell Reports, 2019; 27(9); 2537-2547.e2535, DOI: 10.1016/j.celrep.2019.04.109

Marilen Benner, Gerben Ferwerda, Irma Joosten, & Renate G. van der Molen.
"How uterine microbiota might be responsible for a receptive, fertile endometrium."
Human Reproduction Update, 2018; 24(4), DOI: 10.1093/humupd/dmy012

Dorien Feyaerts, Marilen Benner, Bram van Cranenbroek, Olivier W. H. van der Heijden, Irma Joosten, & Renate G. van der Molen.
"Human uterine lymphocytes acquire a more experienced and tolerogenic phenotype during pregnancy."
Scientific Reports, 2017; 7(1), 2884, Doi:10.1038/s41598-017-03191-0

Other

Marjolein Garsen, Marilen Benner, Henry B. Dijkman, Ton H. van Kuppevelt, Jing P. Li, Ton J. Rabelink, Israel Vlodavsky, Jo H. Berden, Angelique L. Rops, Michael Elkin, & Johan van der Vlag.
"Heparanase Is Essential for the Development of Acute Experimental Glomerulonephritis."
The American journal of pathology, 2016; 186(4), 805-815, DOI: 10.1016/j.ajpath.2015.12.008

13

Notes

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