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IMMUNE CELL COMPOSITION AND CYTOKINE EXPRESSION IN THE PREGNANT AND NON-PREGNANT UTERUS

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Immune cell composition and cytokine expression in the pregnant and non-pregnant uterus

Thesis for Doctoral Degree (Ph.D.)

By

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To my husband Olof, for always having my back.

Perfection is the enemy of progress.

- Winston Churchill

SVENSKSPRÅKIG SAMMANFATTNING

Livmodern är ett fascinerande organ som under en kvinnas liv genomgår dramatiska förändringar i relation till menscykeln, graviditet och efter förlossning, samt efter menopaus. Livmoderns immunförsvar inkluderar vita blodkroppar som har till uppgift att skydda mot bakterier, virus och tumörceller, men har även en avgörande roll vid etablerandet av en graviditet och fortsatt acceptans av det växande fostret som ju genetiskt inte är helt likt modern. Kunskapen om de vita blodkropparnas betydelse vid uppkomsten av sjukdomar i livmodern eller vid graviditetskomplikationer såsom missfall, havandeskapsförgiftning eller tillväxthämning av fostret är bristfällig. I denna avhandling har jag undersökt vilka vita blodkroppar som finns i livmoderns slemhinna endometriet och studerat deras egenskaper. Eftersom antalet vita blodkroppar och deras egenskaper kan variera under menscykeln, vid graviditet och efter menopaus, så har jag studerat dem i dessa olika grupper för att öka kunskapen om livmoderns immunförsvar under en kvinnas liv.

I den första studien använde vi oss av mensblod som källa till att studera vilka signalsubstanser som finns i endometriet mot slutet av menscykeln. Friska kvinnors mensblod samlades in med hjälp av en menskopp för att sedan föras över till en behållare innehållande bland annat antikoagulerande ämnen. Vi har i tidigare studier visat att mensblod insamlat på detta sätt innehåller en stor andel levande vita blodkroppar som kan isoleras och analyseras. Vita blodkroppar och endometriets celler kommunicerar med varandra via signalsubstanser, vilka kan påverka sin mottagarcell till att bland annat lämna eller söka sig till ett visst organ, genomgå mognadsförändringar, dela sig eller dö. Vi ville utröna om dessa signalsubstanser i mensblod skiljer sig åt mellan olika friska kvinnor och från menscykel till menscykel. Vi undersökte också om vi kunde upptäcka ett annorlunda uttryck av signalsubstanser efter en så kallad "endomerial scratching", dvs en mindre skrapning av livmoderns insida som tidigare användes i syftet att öka chanserna till graviditet hos kvinnor som upplevt upprepade missfall. Hypotesen bakom denna behandling var att en "skada" på endometriet skulle kunna ge upphov till en inflammatorisk reaktion som underlättar implantation av ett embryo. Våra resultat visade att sammansättningen av signalsubstanser i mensblodet var klart skild från hur den såg ut i venöst blod samt att det var mycket små skillnader mellan olika individer. När vi jämförde hur uttrycket av signalsubstanser såg ut i mensblod efter en "endometrial scratching", syntes inga förändringar jämfört med den föregående menscykeln. Vår slutsats blev att mensblod från friska kvinnor innehåller en specifik sammansättning av signalsubstanser som inte skiljer sig åt mellan individer och inte heller förändras över tid. Denna kunskap kan användas i framtida studier där man kan jämföra sammansättningen av signalsubstanser i mensblod hos kvinnor med olika reproduktiva sjukdomar såsom endometrios och upprepade missfall.

Vår andra studie undersökte förekomst av och genuttryck hos MAIT-celler (mukosa-associerade invarianta T-celler) i endometriet hos pre- och postmenopausala kvinnor samt i tidig graviditet. MAIT-cellen har till skillnad från de adaptiva T-cellerna, egenskaper som annars förknippas med det ospecifika medfödda immunsystemet och därmed förmågan att direkt gå till angrepp mot inkräktande sjukdomsalstrare. Tidigare forskningsresultat har visat att män och kvinnor har olika andelar MAIT-celler i blodet och att MAIT-celler från livmodern och blod har olika egenskaper. Mycket tyder på att MAIT-celler är mycket viktiga i moderkakan vid graviditet där de både skyddar fostret från infektioner och bidrar till immuntolerans. Vi fann att frekvensen av och genuttrycket hos MAIT-celler från endometriet inte skiljde sig åt signifikant mellan våra tre kohorter vilket sannolikt innebär att andra faktorer än könshormoner påverkar hur MAIT-celler utvecklas. Vi undersökte också populationen av MAIT-celler i mensblod och venöst blod hos enäggstvillingar och såg att mängden MAIT-celler i blodet korrelerade i hög grad mellan tvillingparet medan mängden MAIT-celler i endometriet inte gjorde det. Samma mönster kunde inte ses i mensblod och venöst blod hos 19 obesläktade kvinnor. Det fick oss att dra slutsatsen att genetiska faktorer i hög grad påverkar mängden MAIT-celler i venöst blod men att yttre påverkan såsom kontakt med vaginala mikroorganismer och seminalvätska, i större utsträckning påverkar mängden MAIT-celler i livmodern.

Vaginal blödning i tidig graviditet är ett vanligt symtom som drabbar omkring 20% av alla gravida kvinnor. Det är ett symtom som ofta ger upphov till stark oro och ängslan hos paret och rädsla för missfall. I Stockholmsregionen står denna patientgrupp för mer än 10 000 läkarbesök per år. Vid vaginal blödning där ett levande foster kan ses med ultraljud, är det idag mycket svårt att förutsäga om ett missfall kommer att ske eller inte. Detta leder ofta till upprepade läkarbesök, fördröjd tid till behandling och ytterligare oro för patienten. Specifika diagnostiska tester saknas. Det är känt sedan tidigare att mot slutet av menscykeln domineras de vita blodkropparna i endometriet av natural killer (NK)-celler. NK-celler finns i blod och alla solida organ och har där till uppgift att döda celler som visar spår av att vara infekterade, ha tumörcellsegenskaper eller inte är kroppsegna. I livmodern är NK-celler inte i första hand cell-toxiska utan reglerar implantationen av embryot. NK-celler från endometriet och moderkakan har ett annorlunda genuttryck jämfört med NK-celler från blod och andra vävnader. I vår tredje studie har vi undersökt sammansättningen av vita blodkroppar i vaginalt blod från kvinnor som sökt akut vård pga. blödning i graviditetens första trimester. Därefter har vi korrelerat de vita blodkropparnas mängd och genuttryck till utfall av graviditeten, dvs om kvinnorna fick missfall eller om graviditeten fortskred till fullgången tid. Våra resultat visade att vaginalt blod innehöll alla de stora grupperna av vita blodkroppar och att de till stor del inte skiljde sig avseende mängd och genuttryck från vita blodkroppar i venöst blod. Intressant nog innehöll vaginalt blod från de kvinnor som senare fick missfall en större andel NK-celler med genuttryck som visar att de kommer från moderkakan. Detta resultat har vi tolkat som att NK-celler blöder ut från moderkakan när en tillräckligt stor skada har uppstått och att det är dessa

graviditeter som slutar med missfall. Detta fynd har potential att kunna användas som ett diagnostiskt test i den kliniska vardagen, förkorta tiden till diagnos och behandling, och minska oron för kvinnor med blödning i tidig graviditet.

Genom att kartlägga olika aspekter av livmoderns immunförsvar bidrar denna avhandling till ökad kunskap om reproduktiv fysiologi hos den friska kvinnan. Kunskapen kan användas i framtida studier om reproduktiva sjukdomar. På sikt kan denna kunskap bidra till utveckling av nya diagnostiska metoder och behandling av reproduktionsstörningar.

Abstract

The success of implantation and further development of the embryo is heavily dependent on the endometrial immune cell composition and its ability to communicate with fetal semi-allogeneic trophoblast cells. Although our understanding of the immune cell population in the uterus has improved, its precise role in normal reproduction and reproductive disorders is still not fully resolved. Here, we examined immune cells and signal molecules derived from the endometrium around the time of implantation, in postmenopause, and in early pregnancy.

In study I, we analyzed cytokine and chemokine characteristics in menstrual blood from healthy nulliparous women with regular menstrual cycles, both before and after luteal phase endometrial scratching. The menstrual blood cytokine profile showed little inter-individual variation and differed distinctly from peripheral blood. Endometrial scratching did not affect the cytokine profile in menstrual blood.

Study II examined the dynamics of endometrial MAIT cells in various reproductive states, including pre- and postmenopausal endometrium and in first trimester decidua. We also evaluated the impact of genetic and environmental factors on the endometrial MAIT cell population by comparing the size of the MAIT cell compartment in menstrual and peripheral blood from monozygotic twins. Additionally, we examined the tissue-residency of endometrial MAIT cells by using transplanted uteri as a model. Finally, we assessed the ability of MAIT cells to react against *N. gonorrhoeae*, a pathogen known to infect the female genital tract and pose a growing threat of antibiotic resistance. We found that the frequency of endometrial MAIT cells remained stable throughout the different reproductive stages of the endometrium, and that they exhibited both a more activated state and a tissue-resident phenotype compared to their peripheral counterparts. However, in the transplanted uteri, only MAIT cells positive for the recipients HLA were present within the uterus, suggesting that endometrial MAIT cells are transiently tissue-resident and replenished over time from the circulation. Last, we demonstrated that MAIT cells are functional and respond to *N. gonorrhoeae*.

In study III, we investigated the immune cell characteristics in vaginal blood from women with first trimester pregnancy bleeding and associated findings with pregnancy outcome (miscarriage/ not miscarriage). Saliva and serum proteome was analyzed and correlated to vaginal immune cell phenotype and outcome of pregnancy. We found that vaginal blood contained all main immune cell lineages, and that a higher frequency of tissue-resident CD49a^{+} NK cells in vaginal blood was associated with pregnancy loss. The frequency of vaginal blood tissue-resident NK cells correlated with levels of several maternal serum proteins.

In summary, this thesis provides valuable new insights into reproductive physiology and sheds light on various aspects of the uterine immune system. The findings from this research can be used for future comparisons with reproductive pathological states that may involve altered cytokine and immune cell composition.

List of scientific papers

The following articles will be referred to by roman numerals.

I. THE CYTOKINE PROFILE OF MENSTRUAL BLOOD
   Crona Guterstam Y, Strunz B, Ivarsson MA, Zimmer C, Melin AS, Jonasson AF, Björkström NK, Gidlöf SB
   Acta Obstet Gynecol Scand. 2021 Feb;100(2):339-346

II. HUMAN ENDOMETRIAL MAIT CELLS ARE TRANSIENTLY TISSUE RESIDENT AND RESPOND TO NEISSERIA GONORRHOEAE
   Bister J, **Crona Guterstam Y**, Strunz B, Dumitrescu B, Haij Bhattarai K, Özenci V, Brännström M, Ivarsson MA, Gidlöf SB, Björkström NK
   Mucosal Immunol. 2021 Mar;14(2):357-365

III. IMMUNE CELL PROFILING OF VAGINAL BLOOD FROM PATIENTS WITH EARLY PREGNANCY BLEEDING
   Crona Guterstam Y, Acharya G, Schott K, Björkström NK, , Gidlöf SB Ivarsson MA
   Re-submission following favorable comments in progress, Am J Reprod Immunol

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
cAMP	Cyclic adenosine monophosphate
CAIX	Carbonic anhydrase IX
CCL	Chemokine C-C motif ligand
C/EBPβ	CCAAT/enhancer-binding protein-β
COX-2	Cyclooxygenase 2
CRH	Corticotrophin-releasing hormone
CTL	Cytotoxic $CD8^{+}$ T cell
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CuZn-SOD	Copper Zinc Superoxide dismutase
CXCL	Chemokine C-X-C motif ligand
DC	Dendritic cell
DH	Prostaglandin dehydrogenase
DKK1	Dickkopf-related protein 1
ECM	Extracellular matrix
EMILIN1	Elastin microfibril interface 1
ESC	Endometrial stromal cell
EVT	Extravillous trophoblast
FOXO1	Forkhead box protein O1
FSH	Follicle stimulating hormone
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GnRH	Gonadotrophin releasing hormone
hCG	Human chorionic gonadotropin
HLA	Human leukocyte antigen
HSPG	Heparan sulfate proteoglycan
IDO	Indoleamine 2,3-dioxygenas
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor 1

IGFBP-1	Insulin-like growth factor binding protein 1
IL	Interleukin
IFN-1	Interferon regulatory factor 1
KIR	Killer immunoglobulin-like receptor
LEFTY2	Left-right determination factor 2
LH	Luteal hormone
LIF	Leukemia inhibitory factor
MAIT	Mucosal-associated invariant T cell
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MNC	Mononuclear cell
mPR	Membrane-bound progesterone receptor
MR1	Major histocompatibility complex class I-related protein 1
MUC-1	Mucin 1
NK	Natural killer cell
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PGE2	Prostaglandin E2
PD-1	Programmed cell death protein 1
PDGF-AA	Platelet derived growth factor AA
PGR	Progesterone receptor
PRL	Prolactin
PTH	Parathyroid hormone
PTHLH	Parathyroid hormone-like hormone
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
TWEAK	TNF-related weak inducer of apoptosis
VEGF	Vascular endothelial growth factor
WNT-5A	WNT family member 5A

1 INTRODUCTION

The immune system of the endometrium serves as an important defense against invading microbes, defending tissue integrity to prevent disease. However, it also plays a pivotal role in facilitating embryo implantation and ensuring successful pregnancy outcomes. The immunological paradox of pregnancy, which involves the maternal immune system accepting paternal antigens expressed by fetal trophoblasts without provoking tissue rejection, has fascinated researchers for decades.

As a practicing gynecologist, I was introduced to the subject of uterine immunology, and felt like I was opening the door to a new universe. A universe that much like the Milky Way, is ever-expanding and almost impossible to grasp. While significant progress has been made in understanding the systemic alterations during pregnancy and the communication between immune cells, endometrial cells, and trophoblasts, much remains to be explored. The rationale for further research in this field remains strong, considering the health burden associated with infertility, miscarriage, and obstetrical disorders, but also to further investigate the immunological aspects of many gynecological conditions such as endometriosis, abnormal uterine bleeding, PCOS and neoplastic lesions.

Now, as a researcher and practicing gynecologist, I feel eager to further enhance the diagnostic tools for my patients and if possible, contribute to developing new therapies that are more effective than current treatments. This thesis provides new insights into endometrial and reproductive immunology, physiology, and pathophysiology, which adds a few more pieces to the puzzle.

2 THESIS AT A GLACE

Study	Aims	Materials and methods	Conclusions
I	To investigate the difference between the menstrual and peripheral blood cytokine profiles. To compare the menstrual blood cytokine profile before and after luteal phase endometrial scratching.	Menstrual and paired peripheral blood sampled for two continuous cycles (n=19). Intervention: Endometrial biopsy during "window of implantation". Cytokines analyzed with Luminex® bead-based multiplex assay.	The cytokine profile of menstrual blood is markedly distinct from that of peripheral blood and exhibit very few inter-individual differences. The menstrual blood cytokine profile demonstrates consistency across repeated menstrual cycles and after endometrial scratching.
II	To characterize eMAIT cells from pre- and postmenopausal women and in first trimester decidua. To assess MAIT cell function by stimulation of N. gonorrhoeae.	Endometrial samples from Study I, and hysterectomy specimens from postmenopausal women and two uterus transplants. Decidual samples from three first trimester abortions. Menstrual blood from eight pairs of monozygotic twins. Matched peripheral blood from all aforementioned groups. Flow cytometry PBMC and decidual MAIT cells stimulated with either *E. coli* or *N. gonorrhoeae*.	The phenotype and size of the eMAIT cell population remain largely unchanged throughout the menstrual cycle and pregnancy, as well as after menopause. eMAIT cells exhibit a phenotypically activated state and demonstrate a tissue-resident profile. eMAIT cells represent a transiently tissue-resident population. MAIT cells can respond to *N. gonorrhoeae*.
III	To assess immune cell composition and phenotype in vaginal blood from women with first trimester pregnancy bleeding. To assess serum and saliva proteome in relation to outcome (miscarriage/not miscarriage) of early pregnancy bleeding.	Vaginal blood, peripheral blood, and saliva from women with first trimester pregnancy bleeding (n=28). Flow cytometry, PEA Olink® proteomics assay, Luminex® bead-based multiplex proteomics assay.	Vaginal blood from women that subsequently miscarry contain significantly higher levels of tissue-resident decidual NK cells compared to the ongoing pregnancy group. The frequency of vaginal blood tissue-resident NK cells correlated with serum levels of granzyme A and H, CSF1, CAIX, and TWEAK.

3 BACKGROUND

3.1.1 The human endometrium and hormonal regulation of the menstrual cycle

The endometrial function is to prepare for implantation, hold a pregnancy and if no pregnancy occurs, menstruate, and rapidly repair. This dynamic process of regeneration, degradation and repair repeats itself, without loss of function, about 400 times during a woman's reproductive period of life. The menstrual cycle is orchestrated by a complex dialogue involving the endometrial stoma, epithelial cells, endovascular cells, and the endocrine and immune system. The menstrual cycle affects not only the uterus and mammary glands, but also the bone and skeletal system, osmotic balance as well as the brain and behavior.

The endometrium is composed by a simple columnar epithelium that overlies a multicellular stroma. The stroma includes endometrial stromal fibroblasts and connective tissue but also mesenchymal and epithelial stem cells important for tissue-repair and mucosal surface regeneration (1-3). Indeed, glandular epithelium may be monoclonal suggesting derivation from one single stem cell (4). Endometrial mesenchymal stem cells (MSC) can be identified by the expression of CD146 and platelet derived growth factor receptor beta (PGDF-β) (5). The endometrial structure is formed by a superficial *stratum functionalis* and a *stratum basalis* penetrated by tubular glands that reach into the underlying connective tissue. The endometrium is endowed with blood supply through the end branches of the uterine arteries, the so-called spiral arteries. In the neonate, the endometrium is thought to be inactive, consisting of a thin layer of columnar epithelium with simple glands and compact stroma (6). The neonatal endometrium can potentially undergo shedding shortly after birth, as a result of the rapid decrease in progesterone levels due to the withdrawal of the mother's sex steroid hormones. This suggests endometrial maturation. Nonetheless, neonatal uterine bleeding happens in only a small percentage of newborns, suggesting the presence of an inherent resistance to progesterone. Complete maturation of the endometrium does not take place until after menarche (7). In the postmenopausal endometrium, regressive morphological changes are seen increasingly with age and time interval from menopause due to ovarian hormone insufficiency. The change consists mainly in atrophy including loss of the functional layer, shallow glands, calcification, and fibrotic stroma (8). Mitotic activity is minimal and vessels are often obliterated by fibrosis (9). Nevertheless, immunohistochemical analysis have stated presence of both estrogen and progesterone receptors in epithelial and stromal cells of the postmenopausal endometrium. When exposed to estrogen stimulation, the postmenopausal endometrium proliferates and both glandular epithelium and stromal cells acquire progesterone receptors which if exposed to progesterone, may undergo decidualization (9, 10). This indicates that atrophy of the senescent endometrium is more

of a dormant state and the fact that transplanted uteri from postmenopausal women can result in live births further supports this notion (11).

During reproductive age, the menstrual cycle is divided into separate phases that differ in duration both between individuals and within the same individual across cycles. The normal interval is 22-36 days and average cycle length is 28.9 days (12). The hypothalamus secretes gonadotrophin releasing hormone (GnRH) into the portal bloodstream, which in turn stimulates the production of follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the gonadotropic cells in the pituitary gland. The frequency and magnitude of GnRH pulses, along with signals from the central nervous system and feedback inhibition from the gonads by estrogens, progesterone, and androgens, all play a role in controlling these processes. FSH is released in response to low-frequency GnRH pulses, while high-frequency pulses stimulate the production of LH (13).

The first phase of the menstrual cycle, the follicular phase, commences after a drop in the levels of estrogens and progesterone, which leads to the shedding of the top layers of the thickened endometrium and consequent bleeding. Concurrently, there is a slight increase in FSH levels, prompting the growth of multiple follicles within the ovary.

The follicle is surrounded by an outer layer of theca cells and an inner layer of granulosa cells. Theca cells are stimulated by LH to produce testosterone that subsequently enters the granulosa cells, where FSH stimulates the conversion of testosterone to estradiol. As a result, the progressive increase in estradiol levels during the follicular phase initiate the proliferation of the endometrium, which will persist throughout the entire menstrual cycle. Estradiol induces a rise of FSH receptors on the granulosa cells and during cycle day 5-7, the follicle most rich in FSH receptors develops into a leading follicle whereas the remaining follicles will stop growing and become atretic. The increase in estradiol results in a decrease of FSH by negative feedback on the pituitary gland. Despite the falling levels of FSH, the leading follicle will continue to grow due to its high concentration of FSH receptors.

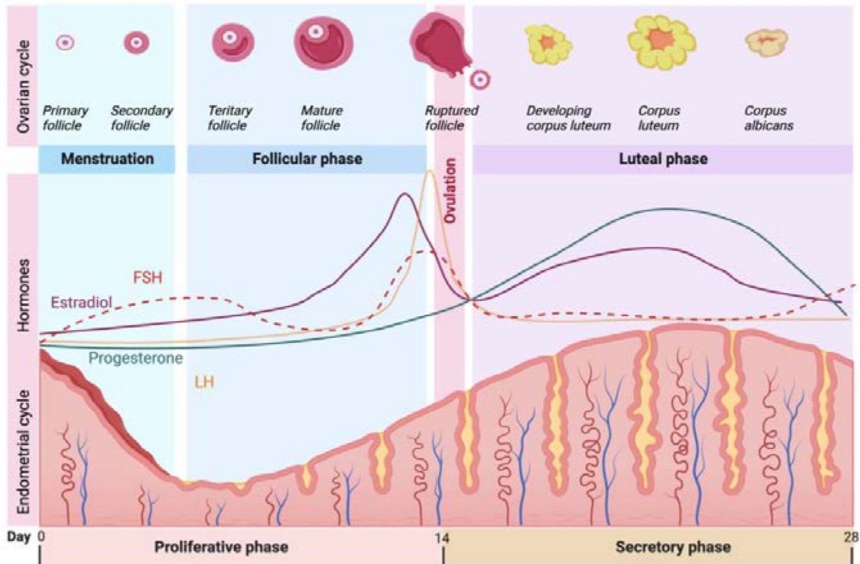


Figure 1. Schematic illustration of the endometrium and sex hormones during the menstrual cycle. Created with BioRender.com by Ylva Crona Guterstam.

Around day 13 of the menstrual cycle, the second so-called ovulatory phase begins, accompanied by a significant increase of LH. At this stage, concentrations of estradiol peak, and exert positive feedback on the pituitary gland leading to a rapid surge of LH. This LH surge, triggers the rupture of the follicle, resulting in the release of the oocyte.

The last phase of the menstrual cycle, termed the luteal phase, is characterized by the decline of both LH and FSH levels, and the formation of a corpus luteum from the ruptured follicle. In the corpus luteum, the granulosa and theca cells change, become larger and start to produce great amounts of progesterone as well as estrogens. Progesterone initiates the process of decidualization which causes the endometrium to become receptive to embryo implantation in the event of fertilization. The endometrial glands and vessels grow and become tortuous. If fertilization does not occur, the corpus luteum undergoes degeneration, resulting in decreased levels of both progesterone and estrogens, thereby triggering the onset of a new menstrual cycle. However, in the presence of an early-stage embryo called a blastocyst, hCG produced by the syncytiotrophoblast will halt the degeneration of the corpus luteum, and the secretion of progesterone will support further development of the pregnancy.

3.1.2 Decidualization of the endometrium

Decidualization is the transformation of endometrial stromal cells to specialized secretory decidual cells. In humans, together with certain mammals such as apes, old world monkeys, a few bat species, and the elephant shrew, decidualization occurs without the presence of an embryo. This contrasts with all other mammals. Other characteristics associated with spontaneous decidualization are extended mating beyond the time of ovulation, invasive hemochorial placenta, giving birth to only one or two offspring, and cyclic endometrial shedding (14). The postovulatory rise of progesterone in the circulation induces a differentiation of the endometrium. However, the morphological tissue changes, such as swelling of the cytoplasm in endometrial stromal cells (ESC) around the spiral arteries, expansion of the extra cellular matrix (ECM) and influx of innate immune cells, are seen around 9 days post ovulation suggesting that other factors are needed to trigger this process (15). Commencing the decidual process, ESC modifies the expression of numerous genes affecting cellular fate; cell cycle regulation, cytoskeleton remodeling, angiogenesis, response and signaling of hormones and cytokines or chemokines, ion, lipid and water transport, ECM changes, defense against oxidative stress, transcription and posttranslational modifications (4). PCR array experiments assessing proinflammatory cytokines and chemokines have shown an immediate rise in inflammatory mediators in decidualized cells, whereas after 8 days of decidualization only a few remained elevated and some even less abundant than before the onset of decidualization (16). This suggests that the process of decidualization is biphasic, with an initial acute inflammatory phase which is followed by an anti-inflammatory phase. It is likely that local expression of relaxin from the ovaries around ovulation and endometrial corticotrophin-releasing hormone (CRH) as well as prostaglandin E2 (PGE2), controls the start of decidualization through activation of cAMP signaling pathways in ESCs (17-19). Inhibitory signals, such as parathyroid hormone-like hormone (PTHLH) expressed in ESCs during the menstrual proliferative phase (20) and IFN-γ from clusters of immune cells in the basal layer are probably equally important in regulating the decidualization (21). Progesterone is lipophilic and can pass though cellular membranes to the nucleus, where it binds to the progesterone receptor (PGR), attaches to the DNA, and starts regulating transcription (22). A family of membrane-bound progesterone receptors (mPRs) mediate rapid non-genomic actions. During early pregnancy, these receptors promote myometrial inactivity and an intact cervix, while in term pregnancies, they have been suggested to trigger the onset of labor instead (23). Further, mPRs have been associated with oocyte maturation and regulation of apoptosis (24, 25).

The recognized practice to assess ESC differentiation and decidualization status in cell cultures is measurement of the markers prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP-1). PRL is produced by decidual stoma cells around cycle day 22 and accumulated in ECM, whereas endometrial epithelial and endothelial cells express the prolactin receptor in mid to late secretory phase (26-28). All functions of PRL on

decidualizing cells are not entirely known but PRL appears to regulate the expression of the transcription factor interferon regulatory factor 1 (IRF-1) (29) and via insulin-like growth factor 1 (IGF-1) and IGFBP-1 the angiogenesis targeting endothelial cells (30). Other highly expressed proteins in decidualizing cells are transcription factors LEFTY2, FOXO1, C/EBPβ, the signal mediator WNT-5A and its inhibitor DKK1 and prokineticin-1, which together with the morphological changes of the stroma are examples of a mesenchymal-epithelial transition (4). The reverse process, transition from epithelial to mesenchymal phenotype, is seen in the early stages of implantation (31). Several paracrine and autocrine signals are secreted by ESCs once the differentiation is initiated. One is interleukin (IL)-1β which promotes full decidualization by stimulation of αVβ3 integrin production (32). The adhesion molecule αVβ3 integrin is a receptor for various ECM ligands, such as vitronectin, laminin and osteopontin, and promotes the morphological transition of decidualizing stoma cells by EMC degradation and cytoskeleton remodeling (33). The upregulation of cyclooxygenase 2 (COX-2) induced by IL-1β, results in increased concentration of PGE2 and cAMP (34). Endometrial leukocytes, spiral arteries and endometrial gland epithelium generates local signal gradients further enhancing the stromal transformation (4).

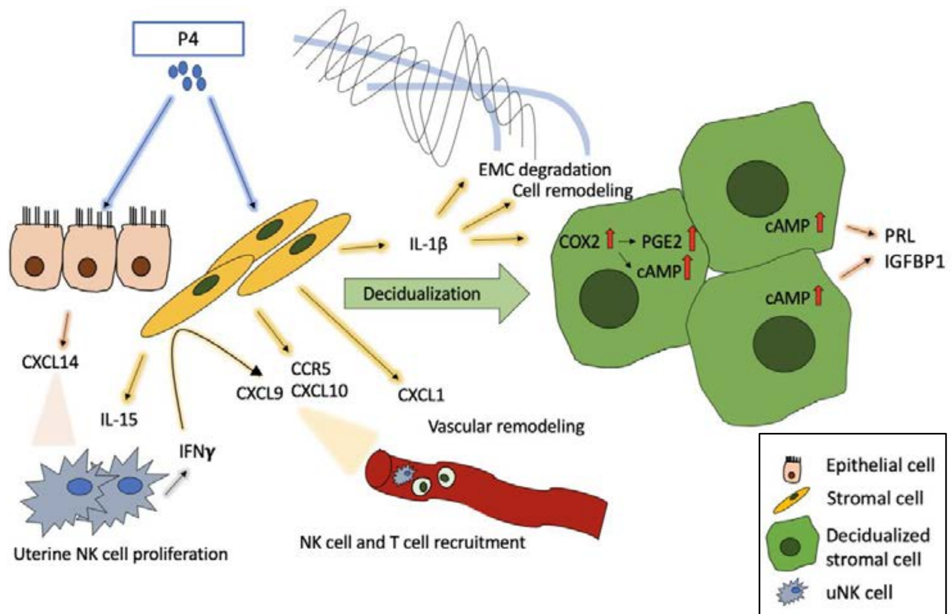


Figure 2. Schematic illustration of the decidualization process under the influence of progesterone. © Ylva Crona Guterstam

The major function of tumor suppressor protein p53 is to protect the genome from letting irreparable toxic mutations reproduce in the coming cell lineages. It is constantly expressed but continuously degraded during normal conditions. However, if DNA damage occurs, the p53 protein becomes stable and as a transcription factor initiates cell cycle arrest, DNA repair or the process of apoptosis (35). During secretory phase, ESC express p53 in detectable amounts and in decidualization stimulus experiments, withdrawal of stimulation causes the stroma cells to dedifferentiate. Further, the expression of PRL and IGFBP-1 decreases and p53 levels in the nucleus becomes undetectable (36). A potential role for p53 in decidualizing endometrium could be to protect the genome in circumstances of oxidative stress secondary to acute inflammation, and to stop proliferation in favor to differentiation.

To summarize, decidualization is the process of specialization of the endometrium to become receptive of a coming conceptus. Decidualization is accompanied by inflammation and alters the expression of various ligands, receptors, and downstream transcription factors, resulting in significant morphological and functional changes. This process is initiated by progesterone and in the absence of implantation the decidua will be shed when the concentration of progesterone decreases.

3.1.3 Implantation of the embryo and placentation

Three main factors are essential for successful implantation: the quality of the embryo, a receptive endometrial phenotype, and a well-tuned communication. The window of implantation occurs in mid-luteal phase, 6–10 days after the LH-surge, and is the period that the decidualized endometrium demonstrate a receptive phenotype. Within 24-48 hours from ovulation, the oocyte is fertilized in the fallopian tube, and the single-cell zygote forms a morula through a series of cell divisions. The morula is surrounded by a non-adhesive zona pellucida and enters the uterine cavity around four days post fertilization (37). After continued cell division and the emerge of a fluid-filled central cavity, the morula has transitioned to a blastocyst with two differentiated cell layers. The inner cell mass which will form the embryo, and the surrounding trophectoderm that will later form the extraembryonic structures including the placenta. Within three days from entering the uterine cavity, the blastocyst hatches from the zona pellucida, prepared to start the implantation. Studies of implantation of the human embryo entail inherent difficulties. Hence, much of what we know to date comes from *in vitro* and *in vivo* murine experimental models. Although human and mouse are genetically distant from one another, the mouse has proven to be an important proxy for human endometrial cell responses during early gestation (38). However, results generated in mouse studies are not applicable to the human situation without a translational follow-up in a human model (39).

The course of implantation is a step-wise process starting with positioning of the embryo near the fundus uteri. This is thought to happen due to directed peristaltic waves of contraction moving from the cervix to the fundus at the most receptive time of the decidua (40, 41). Next, the apposition is initiated by close proximity between the blastocyst and the luminal epithelium. Pinopods, several micrometers wide protrusions, are found on the surface of the epithelium only during the window of implantation. Pinopods endocytose uterine fluid leading to a reduced uterine volume taking the uterine walls closer to the blastocyst. To attach, the mature blastocyst needs to be perfectly synchronized with the receptive endometrium before the endometrium becomes refractive. It is likely that chemokine gradients direct the trophoblast to the site of implantation (42). The luminal epithelium is normally non-adhesive but in contact with the blastocyst starts to reorganize baso-lateral adhesion complexes to the apical region of the cell facing the uterine cavity. Embryonic L-selectins can interact with carbohydrate ligands on the luminal epithelium and create low avidity bonds (32, 43). Once the blastocyst has attached, the proximity of the cell surfaces enables stronger adhesions. The $\alpha V\beta 3$ integrin receptor is upregulated by IL-1β produced by the trophoblast, and sex steroid hormones from the mother, and expressed on both trophoblasts and apical endometrial epithelium. Several ECM components can act as bridging ligands but osteopontin, laminin and heparan sulfate proteoglycan (HSPG) are likely to be the most important ones (32). Other adhesion molecules such as MUC-1, E-cadherin, and multiple types of integrins, plays a role in the steps of implantation. Signals from both mother and embryo influence the expression of adhesion molecules and are necessary to create a successful interaction. Upon attachment, the blastocyst inner cell mass orients toward the endometrial epithelium and the trophoblasts differentiates into protrusions of extravillous trophoblast (EVT) invading the endometrial stroma and spiral arteries, and cytotrophoblast surrounding the inner cell mass. The EVT migrate toward the connective tissue glycoprotein EMILIN1 produced by the decidua in a process termed haptotactic directional migration, and at the same time, decidual stromal cells migrate to encapsulate the blastocyst and facilitate its expansion (43). The final steps of implantation are penetration of the epithelium and basal layer, and invasion of the stroma and spiral arteries. However, the decidual stromal cells contributes significantly to the extreme tissue remodeling by producing a variety of matrix metalloproteinases that modulates ECM and adhesions, and they have a motile and invasive capability comparable with that of trophoblast (44). Consequently, ESCs respond with chemotactic and invasive migration to trophoblast signals such as PDGF-AA (45, 46).

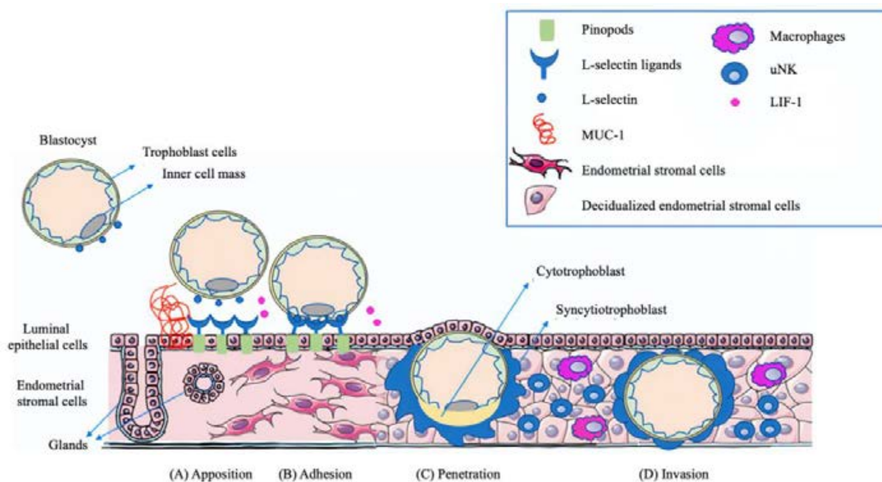


Figure 3. Schematic illustration of the implantation process. Ochoa-Bernal et al. Physiologic Events of Embryo Implantation and Decidualization in Human and Non-Human Primates. 2020. Reproduced with permission from the International Journal of Molecular Science.

During implantation, the decidua does not show degenerative signs or apoptosis which suggests that the invasion process is organized to prevent impairment to the fetomaternal interface (47). Normal decidua discriminates between high- and low-quality embryos and limits excessive trophoblast invasion (44). The invasion of EVT is expected to cease at the inner third of the myometrium anchoring the placenta to the uterus. However, in pregnancies with abnormal invasive placenta, the invasion continues and may extend outside the uterus, resulting in conditions such as placenta accreta, placenta percreta, or placenta increta. EVT invade the uterine glands and replace the glandular epithelium to open the lumen of the glands to the intervillous space (37). Hereby glandular secretion products are released into the intervillous space, supplying the growing embryo with histiotrophic nutrition. Perfusion of the placenta with blood and nutrients from the mother is not fully established until the end of the first trimester of pregnancy. In humans, the hemochorial placentation entails trophoblast in direct contact with maternal blood. EVT invade the spiral arteries through the vessel muscle media and replace the endothelium. However, the vascular remodeling starts prior to trophoblast invasion with uterine NK (uNK) cells causing medial loosening transforming narrow muscular vessels to expanded low-pressure tubes (48). In the lumen of arteries, trophoblast accumulate and create plugs which block the blood flow while the placental structure is finished. Maternal blood plasma leak through the trophoblast plugs, contributing to the histiotrophic nutrients filling the intervillous space. The endovascular plugs contribute to a low oxygen tension in the early placenta since plasma only contains physically dissolved oxygen, making the total oxygen content much lower than whole blood. Increased oxygen prior to

ten weeks of gestation is shown to induce massive placental stress and risk of spontaneous abortion (49).

3.1.4 Immune system of the uterus

The immune system plays a pivotal role in several uterine processes. Besides providing a defense against invading pathogens, the uterine immune cells help orchestrate decidualization and implantation of the embryo.

3.1.4.1 Natural killer cells

In the peripheral blood NK cells make up 2-20% of lymphocytes and most NK cells differentiate to $CD56^{dim}CD16^{+}$ cells, obtain killer immunoglobulin-like receptors (KIRs) and CD57, and function as cytotoxic killers against MHC-null tumor cells (50). However, 10% remain $CD56^{bright}$ and are thought to act as regulators of the adaptive immune response by cytokine production (51). In the endometrium, NK cells are the most abundant lymphocyte during mid-to late secretory phase and during first trimester decidua of pregnancy (52). Uterine NK cells differ in phenotype from peripheral blood NK cells being $CD56^{superbright}$ with high expression of CD94 and KIRs but little expression of CD16 and CD57. uNK cells are large, with prominent cytoplasmic granules containing granzymes and other cytolytic proteins but are normally not cytotoxic against standard cancer cell lines (e.g., K562) (51). This suggests that other regulatory functions are important in this anatomical niche. uNK cells are found mainly around spiral arteries and endometrial glands but relatively sparse in the stroma underlying the luminal epithelium. It is still unclear whether cyclic regeneration of the uNK cell population is derived from local precursors residing in the endometrial basal layer or through active recruitment from the bone marrow via the circulation. There is data supporting both hypotheses as uNK cell precursors have been found in the endometrium and peripheral blood NK cells can obtain uNK cell phenotype when exposed to transforming growth factor β (TGF-β) (53). Strunz et al. recently showed that, by using a human uterus transplantation as a model, uNK cells can be replenished from bone-marrow derived cells in the recipient (54). They concluded that uNK cells are transiently tissue resident, being replenished from the circulation and acquire tissue-residency markers in the uterus. In response to endometrial regeneration, uNK cells differentiate and in the absence of pregnancy sheds with menstruation (55). One important function of uNK cells is to contribute to correct spiral artery remodeling and thereby the blood supply to the growing fetus (56). This is done by allorecognition of trophoblast cells by uNK cells and studies indicate this is decisive for sufficient placentation and healthy pregnancy outcome. uNK cells can respond to a wide range of signals, differing between adhesion molecules, cytokines, MHC class I ligands and more (4). Chemokines such as CXCL9, CXCL10, CXCL14 and trophoblast-derived CXCL12 induces chemotactic trafficking of uNK cells (57). The uNK cells lack progesterone

receptors but proliferates and mature primarily in response to IL-15, as well as to IL-11 and IL-33 produced by decidualizing stromal cells under the influence of progesterone (58-61) . The NK cell receptor family KIR, recognize HLA molecules and are the most variable of the NK receptors being either inhibitory or activating. KIRs exhibit high polymorphism with numerous genes and alleles. They are expressed in a variegated manner on NK cells, which means that they can be present or absent depending on an individual's genetic makeup, and that their expression can be turned on and off in a particular cell. During NK cell development, KIRs interact with self HLA molecules, thus educating the cells to acquire tolerance to self but also to respond in the absence of self. The semi-allogeneic fetus exposes the maternal immune system to a different set of HLA molecules, and whilst villous cytotrophoblast and covering syncytiotrophoblast that are in contact with maternal blood are HLA null (62), EVT express both non-classical HLA-E and HLA-G which are oligomorphic, as well as the polymorphic HLA-C molecules which are the primary ligand for KIRs (63). Genetic studies have demonstrated a higher frequency of certain KIR haplotypes (KIR AA) in combination with a group of paternal allotype HLA-C2, in cases of preeclampsia and other obstetrical disorders such as intrauterine growth restriction and stillbirth (64-67). The KIR A haplotype gene KIR2DL1, which strongly inhibits uNK cells, binds to HLA-C2 on EVT resulting in low levels of factors that are needed for optimal placentation. On the other hand, presence of the activating KIR2DS1 which also binds HLA-C2, is associated with a lower risk of developing pregnancy disorders such as preeclampsia. Consequently, the levels of regulatory cytokines and chemokines will differ depending on the specific KIR–HLA-C interaction of a given fetomaternal interface and modify the EVT invasion accordingly.

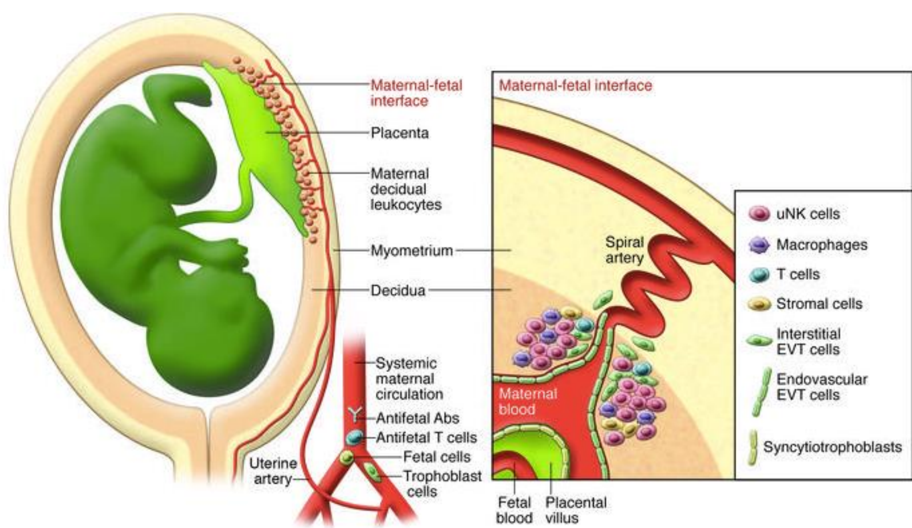


Figure 4. Maternal immune response to fetus and placenta. Moffett et al. Uterine NK cells: active regulators at the maternal-fetal interface. 2014. Reproduced with permission from the Journal of Clinical Investigation.

3.1.4.2 T cells

Different subsets of T cells are present in decidua and fetomaternal interface and many researchers have tried to determine their behavior and immunotolerance in this unique setting. However, the exact role of T cells in reproduction is still not clear and as with uNK cell physiology, much of what we know to date is derived from mouse studies.

T cells are educated in the thymus where they acquire their T cell receptor (TCR). The TCR is generated by TCR α and TCR β gene arrangements, as well as to a lesser extent arrangements of the γ and δ gene, resulting in a unique antigen specificity for each T cell. A T cell is considered naïve until it encounters its first cognate antigen in the spleen or lymph node. The peptide antigen will be presented via the MHC molecule on the surface of an antigen presenting cell (APC), usually a dendritic cell (DC). $CD4^+$ T cells recognize MHC class II and $CD8^+$ T cells MHC class I. The DC must provide sufficient co-stimulation via surface molecules CD80 and CD86 for the responding T cell to proliferate and differentiate to a specific T cell subset. The common way to define the T cell subsets are helper $CD4^+$ T_H1, $CD4^+$ T_H2, $CD4^+$ T_H17 effector T cells, regulatory $CD4^+CD25^+FOXP3^+$ T_{reg} cells, and cytotoxic effector $CD8^+$ T cells (CTLs). Once activated, the T cell exits the lymphatic organ and travels through the blood to the peripheral tissue of interest, homing via chemotactic signals. Each subset of T cells expresses its own set of transcription factors to maintain its differentiated state, and its own set of cytokines to execute its effect. Additionally, T cells have their own set of chemokine receptors that to a large extent determines what kind of T cell subset that can gain access to a certain inflammatory site. The function of T_H1 cells in peripheral tissue is mainly as promotors of inflammation and eradication of virus-infected cells. CTLs are like T_H1 cells, recruited to sites of virus- and intracellular pathogen infected cells and have the capacity of directly killing target cells upon activation. It kills through expression of cytolytic molecules such as perforin and granzymes.

Since T_H1 cells and CTLs are the major drivers of allograft rejection, these subsets have been considered to pose a threat to fetal survival and increase the risk of obstetrical disorders. T_H2 cells function primarily in allergic reactions and parasites. T_H17 cells mediate acute inflammatory responses against bacterial and fungi infections and their function in pregnancy is unclear. Human T cell proportion of total leucocytes in the decidua is estimated to 10-20% (68), of which 30-40% are $CD4^+$ and 45-75% are $CD8^+$ (69), and the greater part has already been exposed to antigens ($CD45RA^-$ or $CD45RO^+$) (70). Hereafter I will focus on T_{reg} cells since the role of this subtype in decidua and placenta is one of the most studied. T_{reg} cells function as suppressors of immune cell activity, primarily through secretion of the cytokines IL-10 and TGF-β. They aid in contracting the immune response after elimination of a pathogen or to prevent autoimmunity, and further act as a decoy for IL-2. IL-2 is the ligand for CD25 and initiates T cell proliferation. Because of expression of a wide variety of chemokine receptors, T_{reg} cells have the capacity to reach into multiple

organs. T_{reg} cells can be generated in two ways: either as naturally occurring T_{reg} (nT_{reg}) cells generated from T cell precursors in the thymus, which can react against self, or they can be induced (iT_{reg}) in the presence of TGF-β after encounters with APCs in tissues. The T_{reg} cell subset increases in numbers systemically and in decidua towards the late luteal phase, through early placentation and peaks during the second semester of pregnancy (71-73), but whether this is a clonal expansion of $CD4^+$ T cells converting to iT_{reg} cells after contact with placental antigen or if this is a real hormone dependent expansion, is not fully understood. However, both human and murine T_{reg} cells express the LH/CG receptor. Different T_{reg} cell subtypes have been evaluated within the total T_{reg} cell population in normal and pathologic pregnancies. Altered homeostatic composition and decreased suppressive activity of T_{reg} cell subsets have been shown in patients with preeclampsia and premature labor (74).

If a T cell is to react against foreign paternal antigen, it must be exposed to it first. Where this exposure occurs is still unclear. T cells from mice respond to semen antigen soon after coitus and $FOXP3^+$ T_{reg} cells can be found in regional lymph nodes and at the fetomaternal interface in early pregnancy (75). Seminal fluid contains several components, besides the cellular fraction, a non-cellular fraction including prostaglandins and TGF-β. One hypothesis is that T_{reg} cells, after exposure to human paternal antigen and the T_{reg} cell proliferating factor TGF-β, home back to the uterus where they prepare for implantation and recognize fetal HLA-C (76). Another hypothesis is that decidual APCs present placental antigens within the placenta where the extrathymic subset of T_{reg} cells proliferate after expression of FOXP3 enhancer promotor region (77). Indeed, previous work has shown that $CD83^+$ DCs form clusters with decidual T cells (78). However, naïve T cells are to a large extent excluded from peripheral tissue and the proportion of naïve T cells in placenta has been determined to be low. Further, uterine DCs in mice are unable to migrate from tissue to lymph nodes upon decidualization. This entrapment minimizes the potential of immune surveillance of the genetically fetal placental tissue and influx of CTLs (79). Activated T_H1 cells and CTLs are also actively excluded from mouse decidua because of epigenetic silencing of decidual genes encoding for the key T_H1- and CTL-attracting chemokines CXCL9, CXCL10, CXCL11 and CCL5. Whether this is true for humans as well is still not settled but the initial proinflammatory surge in decidualization followed by a downregulation of inflammatory chemokines and mediators may point in that direction. Production of IL-10, leukemia inhibitory factor (LIF), and TGF-β is thought to contribute to fetal immunotolerance, as well as indoleamine 2,3-dioxygenas (IDO) reducing levels of tryptophan necessary for T cell proliferation (80, 81). Decidual signals, such as galectin-1, which inhibit T cell proliferation and decreases T cell expression of proinflammatory cytokines, may also play a role in immune tolerance to the fetus. Taken together, T_{reg} cells are key players in immune regulation at the fetomaternal interface and dampen the proliferation and cytokine production of other $CD4^+$ and $CD8^+$ T cells, as well as inhibiting the cytotoxicity of uNK cells. Further they suppress B cells, DCs and

macrophages (82). IL-10, TGF-β and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) via IDO and IFN-γ, seems to potentiate their immune regulatory mechanisms. However, the precise pathway of T_{reg} contribution to fetal tolerance is still not known.

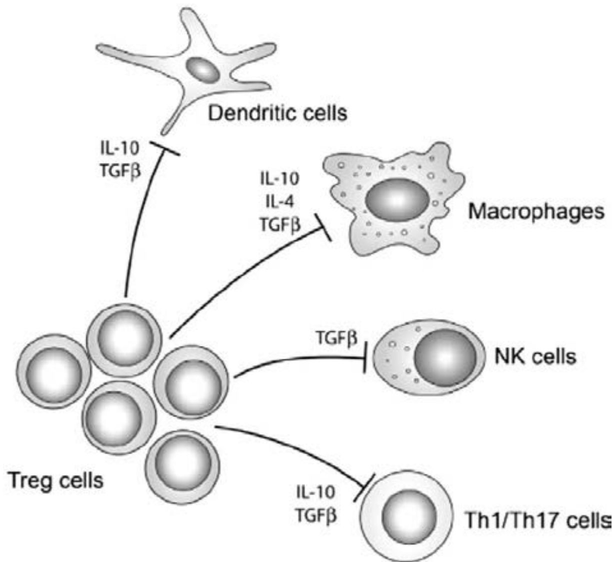


Figure 5. Suppressive pathways of T_{reg} cells. Immunology of pregnancy, Knobil and Neill's Physiology of Reproduction. Reproduced with permission from Academic Press Elsevier Science.

The maternal immune system needs to balance the act of attack against invading pathogens and at the same time tolerate the semi-allogeneic fetus. Aggressive infection often ends in fetal rejection or preterm delivery. In murine models, a combination of augmented T_H1 and T_H17 effector response and T_{reg} cells promoting persistence of infection, conjointly contribute to worsen pregnancy outcome (74, 83).

Mucosal-associated invariant T cells (MAIT) are an evolutionary conserved subset of innate T cells important for host defense against pathogens. MAIT leaves the thymus with a semi-invariant TCR (Vα7.2 coupled with a restricted Jα segment) which detects bacterial-derived riboflavin metabolites presented in the major histocompatibility complex class I-related protein 1 (MR1) by an APC. Thus, with a memory phenotype prepared with capacity to secrete cytokines and effect cytolysis. A second activating pathway in a non-TCR-dependent manner, is through the inflammatory cytokines IL-12 and IL-18. These cytokines are expressed during viral and inflammatory responses (84). MAIT cells are abundant in mucosal tissues, blood, and liver, and are also found in the upper and lower female genital tract and at the fetomaternal interface of the pregnant uterus (85, 86). In the endometrium, the MAIT cells are found predominantly near the epithelial glands in the lamina propria (85). Previous studies have investigated the MAIT

cell contribution to uterine mucosal defense against microbes and concluded that they recognize several gram^{+} and gram^{-} bacteria and yeast (87). Women of reproductive age have more MAIT cells in peripheral blood then age-matched men, and declining amounts of circulating MAIT cells are seen in women with increasing age (88). However, we recently observed no differences in MAIT cell phenotype or frequency between pre-, and postmenopausal endometrium, first trimester decidua, and in the circulation, which indicate that the hormonal changes during the menstrual cycle and after reproductive age, or during pregnancy has little effect on both peripheral and endometrial MAIT cell composition (89). In the same study, we concluded that MAIT cells can respond to the site relevant pathogen *Neisseria gonorrhoeae*, but whether the endometrium harbors a microbiota or is a sterile environment, has been a matter of debate. Chen et al. found Pseudomonas, Acinetobacter, Vagococcus and Shingobium in human endometrial samples (without signs of infection) taken during surgery both intraabdominally and via the cervical os (90), and others report low-biomass microbiota in endometrium including Bacteroidetes and Firmicutes (Lactobacillus spp.) (91). However, compared with the vaginal microbiome, the uterine cavity microbiome is significantly lower in quantity which points to the assumption that the cervix, or the endometrial environment serves as a barrier against pathogens (92). How the microbiota affects the maternal immune system during implantation and throughout pregnancy is not clearly understood.

MAIT cells are present in the intervillous space and with their quick response to microbes and inability to respond to foreign HLA antigens, they could be perfect as patrolling effector cells at the fetomaternal interface. Indeed, data show that MAIT cells from decidua parietalis express increased levels of activating markers such as programmed cell death protein 1 (PD-1), CD38 and CD25 compared to MAIT cells from decidua basalis (93). Although decidual MAIT cells express granzyme B and IFN-γ to the same extent as peripheral MAIT cells, it is not yet known if they possess the ability to secrete IL-17 and IL-22, which are important for the mucosal barrier function (85, 93, 94). However, recently a tissue repair function of MAIT cells has been described (95). This pathway is dependent on TCR-MR1 activation and hence, riboflavin-producing bacteria needs to be present. Since bacterial ligands can cross epithelial barriers, it is possible that intervillous MAIT cells act to protect the placental barrier devoid of infection (96).

3.1.4.3 Macrophages and dendritic cells

Macrophages constitutes 20-30% of all decidual leukocytes (97) and remain in the decidua throughout pregnancy. In simple terms, it could be said that decidual macrophages express a M2-spectrum regulatory phenotype with anti-inflammatory functions. They are a major source of anti-inflammatory IL-10 and TGF-β which implicates a role as suppressors of immune response to the fetus. However, they are also active

scavengers expressing the pattern recognition receptor DC-SIGN, which allows recognition of pathogen-associated molecular patterns (PAMP). Upon PAMP activation, the decidual macrophage secrete cytokines that promotes immune cell recruitment, but several protecting factors diminish their impact. Epigenetic silencing of transcription of proinflammatory molecules and the shortage of co-stimulation by CD86 on decidual macrophages, to mention a few (98). Decidual macrophages are found near the spiral arteries and invading EVT suggesting a supportive role in implantation. Several previous studies have shown that decidual macrophages clear the tissue of cell debris from apoptotic remodeling decidual cells and trophoblast, thereby protecting the fetus from immune cell activation (99). MR1 has been identified on decidual macrophages making them a potential APC for decidual MAIT cells (100).

Another APC present in decidua is the dendritic cell. Decidual DCs have a regulatory rather than activating phenotype and like decidual macrophages produce the anti-inflammatory cytokines IL-10 and TGF-β. Several other synergetic processes in the decidua contributes to the tolerant properties of decidual DCs. Estrogen makes DCs unable to stimulate T cell proliferation and cytokine production by upregulation of IDO (101) and progesterone interacts with decidual stimulus such as IL-18, TNF, IFN-γ and GM-CSF (102, 103). Decidual DCs appear in the vicinity of uNK cells and are found to contribute to the crosstalk between immune cells and promote uNK cell proliferation through secretion of IL-15 (104).

To summarize, each stage of pregnancy requires a distinct immune environment to sustain and safeguard development. Initial phases necessitate a proinflammatory state for successful implantation and placentation, whereas continued fetal growth mandates increased tolerance and anti-inflammatory cues. T_{reg} cells, M2 macrophages, and uNK cells all play essential roles in in a healthy pregnancy.

3.1.5 Miscarriage

Early pregnancy loss is common in human reproduction with embryonic chromosomal instability accounting for about 50% of cases. Miscarriage, including preclinical losses, is estimated to occur in 50-70% of all pregnancies. Chromosomal errors in human embryos are much more common than in other mammalian species and mostly caused by mitotic non-disjunctions (105). Humans have a high prevalence of subfertility and expressed as monthly fecundity rate (MFR), mere a 20% chance to conceive within one menstrual cycle (106). In sharp contrast, baboons reach a MFR of 80% (107).

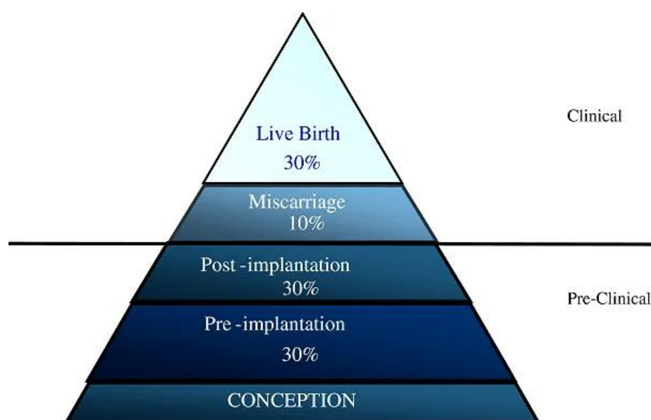


Figure 6. The pregnancy loss iceberg. Estimated rate of human implantation failure and pregnancy loss. Macklon et al. Conception to ongoing pregnancy: the "black box" of early pregnancy loss. Reproduced with permission from Oxford University press.

For the decidua to recognize and support or reject the embryo, it needs to be fully differentiated to secretory phenotype. Hitherto, no single biomarker has been identified to predict receptive endometrium and the mechanisms that disturb responsiveness of decidualizing ESCs to developmentally impaired embryos are poorly understood. Withdrawal of progesterone from decidualizing endometrium induces a menstruation-like bleeding in case of early pregnancy loss. hCG produced by trophoblast contributes to the survival of the corpus luteum while waiting for the placenta to assume progesterone production in gestational week 6-8. Thus, decidual stromal cells harbours an intrinsic selection against compromised embryos that lack the ability to produce sufficient hCG. Decidualizing stromal cells distinguish high from low-quality embryos and respond selectively by shutting down expression of key implantation factors, including IL-1β, IL-6, IL-10, and heparin binding epidermal growth factor (HB-EGF) (16, 108, 109). Further, to minimize maternal investment in a developmentally compromised and invasive embryo, the decidua creates a microenvironment that hampers trophoblast hCG production. That is, proteolytic signals from low-quality human embryos impairs transcription of COX-2 and PGE-2 release, which is essential for implantation. Further, in a human DNA microarray analysis, approximately 449 decidual genes were dysregulated in response to signals from low-quality embryos *in vitro* (105).

ESHRE guidelines 2022 define recurrent pregnancy loss (RPL) as two or more miscarriages before 24 weeks of gestation (110). Couples suffering from infertility are screened for various anatomical and genetic anomalies, as well as endocrine, immunological and coagulopathic disorders. In many cases, no risk factors are found and conversely, many disorders thought to cause miscarriage are prevalent in women with normal pregnancies

making none of them specific (111). In RPL patients, ESCs display a prolonged and disordered proinflammatory phenotype, unable to regulate anti-inflammatory self-limiting responses (16). Further, ESCs that have not been decidualized, do not respond to either developmentally normal or impaired embryos *in vitro* (112). This finding implies that correct decidualization and timing of the limited window of implantation is crucial for pregnancy success. Some RPL patients report reiterated short time to conception, often three months or less (108). This group of infertility patients, estimated to represent 3% of the population, are classified as superfertile and have a MFR of 60% or more (106). Superfertility is characterized by prolonged endometrial receptivity and dysfunctional decidualization which predisposes for late implantation and impaired embryo quality control, causing early placental failure regardless of embryo quality. On the other side of the bell-shaped curve of fertility/infertility are the subfertile, characterized by a highly selective endometrium. Due to an excessive endometrial response, the receptive window is restrained, which leads to conception delay and increased embryo disposal.

Evidence of the relevance of the uterine immune system in miscarriage is accumulating yet not fully explored. Impaired T_{reg} cell augmentation is suggested to be associated with altered IL-6 signalling and increased number of proinflammatory T_H17 in peripheral blood and decidua in women with RPL (113, 114). Further, uNK cells are thought to be dysregulated in RPL patients, with maintained cytotoxicity of uNK cells (115), disturbed ability to interact with HLA expressed by trophoblast (52, 116, 117), impaired ability to regulate spiral artery remodelling (118), and altered cytokine production of uNK cells (119). Decidual macrophages are polarized toward regulatory M2 phenotype in normal pregnancy, but limited polarization has been found for RPL patients (120). An increased presence of M1 macrophages could entail a decreased ability to regulate T_{reg} cells and to produce tolerogenic cytokines. Concerning decidual DCs, studies have shown that a reduced level of tolerogenic DCs in peripheral blood and decidua is associated with RPL (121).

Given the critical role of the maternal local immune system in initiating and maintaining, or terminating normal pregnancy, it is highly plausible that abnormalities in immune function could be contributing to recurrent pregnancy loss in some women. This hypothesis presents the reasoning for proposing that the decidua of women experiencing RPL may not produce the required proinflammatory environment for embryo implantation. To address this, an endometrial biopsy-induced injury was considered to trigger an inflammatory reaction that would boost endometrial receptiveness and improve the likelihood of a successful pregnancy (122). Despite conflicting evidence supportive of this procedure as part of fertility treatment, the use of the procedure in fertility clinics has been widespread. However, Lensen et al. conducted a comprehensive randomized controlled trial to explore whether endometrial scratching enhances the chances of live birth among women undergoing IVF yet found no evidence to support this practice (123).

3.1.6 Menstruation and endometrial repair

In the non-conception cycle, progesterone and estrogen decline initiates a series of interdependent inflammatory events that eventually leads to tissue destruction and simultaneous tissue repair. Progesterone has anti-inflammatory properties in inhibiting prostaglandin synthesis by suppressing expression of COX-2 and increasing expression of the prostaglandin metabolizing enzyme prostaglandin dehydrogenase (DH). Thus, progesterone withdrawal in late luteal phase will lead to a decline in prostaglandin DH which increases the bioavailable pool of prostaglandins and a rapidly elevated expression of COX-2 in endometrial epithelial, perivascular, and stromal cells (124). Superoxide dismutase (CuZn-SOD), which protects cells from oxidative stress (ROS), is highly expressed during decidualization but concentrations decline upon progesterone withdrawal. When ROS levels subsequent increase, the transcription factor NFκB is released from the inhibitory IκB and translocates to the nucleus where it starts transcription of genes encoding COX-2, proinflammatory cytokines and chemokines, as well as MMPs (125). These mediators exert their effect in the extracellular matrix. Prostaglandins have vasoconstrictive effects and furthermore increases vessel leakage. The vasoconstrictive spasms induce tissue ischemic hypoxia, and the secreted cytokines selectively recruit various leukocytes. First, neutrophils enter through the leaky vessels and with their pre-formed granules start the ECM degradation (126). Cytokines secreted by neutrophils attract macrophages that differentiate to M1 phenotype. Differentiation within the tissue is constantly changing as the process evolves. Eosinophils are only found in the endometrium immediately prior to menstruation (127) but their function in balancing the process of menstruation is elusive. Some but not all, eosinophils in the endometrium express MMPs. Other leukocytes, such as local mast cells get activated and releases proteases that aid in the control of excessive cytokine activity, as well as inducing MMP cascades (125). Activated MMPs subsequently result in breakdown of the endometrial *stratum functionalis*. The process of tissue destruction follows a spatial distribution pattern, starting in stromal cells closest to the spiral arteries and spreads to the epithelium. Since hypoxia and tissue degradation are very local, and neutrophils and macrophages simultaneously change phenotype to assist in endometrial reparation, areas of shedding and repair are found close to one another. Endometrial re-epithelialization and scar-free repair is accomplished via secretion of factors such as VEGF, CXCL8 and adrenomedullin, induced by the activated inflammatory pathways (125). Glandular stumps are a source of luminal epithelial cells and stromal cells undergoing mesenchymal-epithelial transition further contribute to a speedy regeneration of the epithelium (128, 129).

Taken together, the physiological cue for menstruation is a third inflammatory state initiated by the withdrawal of progesterone. Local tissue destruction adjoins tissue

remodeling. Progesterone added back within 36 hours of withdrawal leads to a halted tissue breakdown process in the macaque but beyond this time point, addback is pointless (130). This indicates that menstruation is the endpoint of a stepwise process, reversible within a certain timeframe, but once a particular threshold is crossed tissue destruction is inevitable.

4 RESEARCH AIMS

The overarching aim of this thesis is to investigate immune cell composition, phenotype, and function in normal endometrium during the reproductive and postmenopausal periods, and in pregnancy and pregnancy loss. Particular attention is brought to uterine natural killer (uNK) cells, mucosal-associated invariant T (MAIT) cells and overall endometrial cytokine production.

Pre-specified hypotheses:

Study I

1. Cytokines that are known to affect uterine immune cells can be detected in menstrual blood and are affected by endometrial scratching.

Study II

2. Endometrial MAIT cells varies during the menstrual cycle and during different biological stages of the endometrium.
3. Endometrial MAIT cells are transiently tissue resident and affected by environmental factors.
4. MAIT cells can respond to *N. gonorrhoeae*.

Study III

5. The uterine NK cells from vaginal blood display an altered phenotype in women with early pregnancy loss compared to uterine NK cells from women with an ongoing pregnancy.
6. Serum and saliva proteomes are different in women with early pregnancy loss compared to women with an ongoing pregnancy.

The specific objectives addressing the overall aim were:

Study I

1. To assess the cytokine profile in menstrual blood plasma and to investigate the differences in cytokine profiles between menstrual and peripheral blood plasma.
2. To compare the menstrual blood cytokine profile before and after luteal phase endometrial scratching.

Study II

3. To characterize human endometrial MAIT cell phenotype during pre- and postmenopause stages and in pregnancy.

4. To determine the impact of environmental versus genetic factors on endometrial MAIT cells.
5. To determine endometrial MAIT cell tissue residency using transplanted uteri as a model.
6. To assess MAIT cell function and reaction against *Neisseria gonorrhoeae*.

Study III

7. To describe the immune cell composition and uterine NK cell phenotype in early pregnancy bleedings and to correlate this information with pregnancy outcome.
8. To investigate serum and saliva proteome in women with early pregnancy bleeding and correlate this information with pregnancy outcome.

5 MATERIALS AND METHODS

5.1 Table 1. Schematic overview of materials and methods in studies I–III

Study	Hypothesis	Study design	Study sample	Method
I	Cytokines that are known to affect uterine immune cells can be detected in menstrual blood and are affected by endometrial scratching.	Prospective cohort study	19 nulligravidae women donating menstrual and paired peripheral blood for two continuous cycles. Intervention: Endometrial biopsy during "window of implantation".	Selection of 20 cytokines, chemokines and growth factors analyzed in peripheral and menstrual blood. Bead-based multiplex assay (Luminex®)
II	1. Endometrial MAIT cells vary in phenotype and frequency within the menstrual cycle and during different biological stages. 2. Endometrial MAIT cells are transiently tissue resident and affected by environmental factors. 3. MAIT cells can respond to *N. gonorrhoeae*.	Exploratory study	A. Endometrial specimens from hysterectomies in pre- and postmenopausal women. B. Surgical abortion decidua from three women. C. Endometrial specimens from two women after uterus transplantation and peripheral blood from both donor and recipient. D. For the longitudinal parts of the study: Participants in Study I providing endometrial biopsies from two time points and peripheral blood from five time points. E. For environmental and genetic studies: menstrual and peripheral blood from 8 monozygotic twins.	eMAIT frequency and assessment of eMAIT phenotype in different populations: cryopreserved PBMCs and MNCs thawed and stained with pre-selected antibodies. Samples acquired by flow cytometry. Data analyzed by FlowJo. Assessment of MAIT cell reaction against *N. gonorrhoeae* and *E. coli*.
III	1. Immune cells can be detected in vaginal blood from women with early pregnancy bleeding. 2. Vaginal blood immune subset composition and phenotype, as well as serum and saliva proteome, are different in women with early pregnancy loss compared to that of women with continued pregnancy.	Prospective cohort study	26 women presenting at the gynecological emergency clinic with vaginal bleeding (gest. age <12 weeks) donating vaginal and peripheral blood at two time points and saliva at one time point.	Cryopreserved PBMCs and MNCs thawed and stained with pre-selected antibodies. Samples acquired by flow cytometry. Data analyzed by FlowJo. Saliva and serum samples analyzed by bead-based multiplex assay (Luminex®) and proximity extension assay (Olink® Target 96).

5.2 Study population and design

This thesis is based on data from three prospective studies, conducted as a collaboration between the Department of Gynecology and Reproductive Medicine at the Karolinska University Hospital, the Center for Infectious Medicine (CIM), Department of Medicine Huddinge, and the Department for Clinical Science, Intervention and Technology (Clintec), Karolinska Institutet. All participants signed a written informed consent to participate in the studies.

5.2.1 Study I: The cytokine profile in menstrual blood

The regulation of the menstrual cycle is dependent on a fine-tuned interplay between endometrial epithelia, endothelia, and the immune system. These cells communicate by secretion of various cytokines. The objective of this study was to investigate if these cytokines could be measured in menstrual blood and if the profile of menstrual blood cytokines was different in menstrual blood compared to peripheral blood. The third objective of the study was to investigate if endometrial scratching could evoke a measurable change of the cytokine profile in menstrual blood.

Study participants were recruited by advertisements in the hospital and at a web page for volunteers (accindi.se formerly known as studentkaninen.se). 28 women were screened and 19 enrolled in the study. Nine women were lost to screening failure or withdrawal of informed consent. The inclusion criteria were: age 18–35 years, a body mass index (BMI) of 19–25kg/m^2, a regular menstrual cycle and no known history of pregnancy. Women who smoked, had undergone previous gynecological surgery, had a chronic disease or continuous medical treatment were excluded from the study. Further, ongoing hormonal contraception and previous or current use of an intrauterine device were considered exclusion criteria. All 19 study participants underwent a physical examination, pelvic exam including transvaginal ultrasound, cervical smear test and antigen testing for Chlamydia. Any pathological findings discovered in the screening were regarded as exclusion criteria. Four of the women included in the study had a multifollicular appearance of the ovaries when examined with transvaginal ultrasound. However, polycystic ovarian syndrome (PCOS) was ruled out by hormone analyses (estrogen, progesterone, 17-hydroxyprogesterone, testosterone, sex hormone binding globulin, prolactin, LH, FSH, and thyroid-stimulating hormone) and absence of menstrual disturbances. 11 out of 19 participants, donated blood twice. The net menstrual volumes varied from 5.5–40 ml.

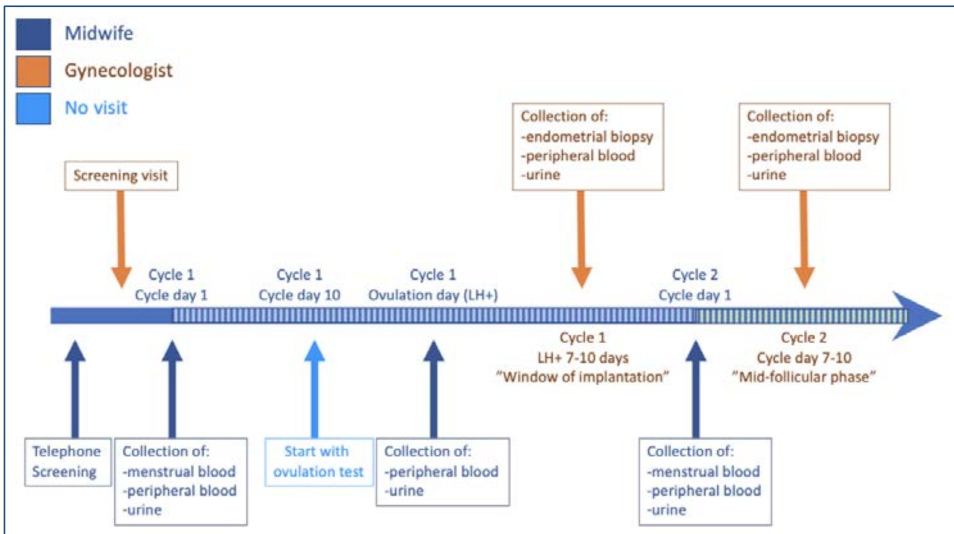


Figure 7. Timeline of intervention and sample collection events (Study I and II)

Table 2. Characterization of study participants (Study I and II)

Clinical parameters	**Healthy study participants (n=19)**
Age (years), median (range)	28 (19-32)
BMI (kg/m^2)	22.2 (20.3-2.,6)
Menstruation duration (days), median (range)	4 (3-7)
Menstrual cycle length (days), median (range)	29 (25-32)
Sampled longitudinally	11

5.2.2 Study II: Human endometrial MAIT cells are transiently tissue resident and respond to *Neisseria gonorrhoeae*

MAIT cells are present in the female genital tract but in various aspects their function and phenotype remain unexplored. The objectives of this study were to characterize human endometrial MAIT (eMAIT) cell phenotype during different stages of reproduction, to

determine the impact of environmental versus genetics on eMAIT cells, and to investigate whether eMAIT are tissue-resident or replenished from the circulation. In addition, we aimed to assess MAIT cell function in response to bacteria known to infect the genital tract.

For the longitudinal studies, peripheral blood samples and endometrial biopsies from study participants in study I were used. The peripheral blood was sampled at five different time points during the menstrual cycle for two consecutive cycles. The endometrial biopsies were taken at two time points, in luteal phase of the first cycle, and in follicular phase of the second cycle (**Figure 7**). To further characterize eMAIT cell in different reproductive stages of the uterus, a group of healthy pre- and postmenopausal women (n=18) undergoing planned hysterectomy for benign indications, were enrolled. The women were asked to participate by their gynecologist at Karolinska University Hospital, Huddinge, after the decision to perform a hysterectomy was taken. Endometrial specimens were taken in the operating room soon after removal of the uterus.

To investigate MAIT cell phenotype in decidua, three healthy women planned for surgical abortion were asked to participate in the study by midwives specialized in family planning at Karolinska University Hospital, Huddinge. Decidual specimens were collected immediately after surgical abortion. The age of decidual specimens collected for the study was determined by measurement of the fetus post abortion and ranged from week four to eight from conception.

For specific assessment of eMAIT cell tissue-residency, endometrial tissue from two women who had undergone a uterus transplantation was collected and combined with peripheral blood samples from both donor and recipient. The recruitment of these two women were made in cooperation with the Division of Obstetrics and Gynecology, Sahlgrenska Academy at the University of Gothenburg and the department of Obstetrics and Gynecology, Sahlgrenska University Hospital Gothenburg.

To study the environmental versus hereditary impacts on endometrial and peripheral MAIT cell phenotype and frequency, eight pairs of female monozygotic twins were recruited via the Swedish twin registry, Karolinska Institutet. We invited both monozygotic and dizygotic twin pairs to participate in the study, unfortunately only one pair of dizygotic twins was willing to take part. The twin pairs enrolled were healthy, with no previous history of gynecological disease and aged between 25-32 years. The twin pairs donated menstrual blood collected via a menstrual cup and matched peripheral blood at one time point. The peripheral blood sample was taken within 72 hours from the onset of menstruation.

To assess the MAIT cell response to bacteria, peripheral and decidual MAIT cells were stimulated with three laboratory strains, two antibiotic sensitive clinical isolates and two

antibiotic-resistant clinical isolates of *N. gonorrhoeae* obtained from the Department of Clinical Microbiology at the Karolinska University Hospital, Huddinge.

5.2.3 Study III: Immune cell profiling of vaginal blood from patients with early pregnancy bleeding

Proper immune cell function is crucial for successful decidualization and embryo implantation, as well as for the regulation of trophoblast invasion and correct placentation. However, the specific role of immune cells in early pregnancy loss remains poorly understood. This study aimed to shed light on this topic by characterizing the composition, frequency, and phenotype of immune cells in vaginal blood among women who experience bleeding in early pregnancy. Additionally, we correlated serum and saliva proteome with vaginal immune cell phenotype and outcome of pregnancy.

Study participants were recruited among women seeking medical attention at the gynecological emergency clinic at the Karolinska University Hospital, presenting with first trimester vaginal bleeding.

29 women agreed to participate in the study, but one was later excluded due to an extrauterine pregnancy diagnosis. None of the included women had signs of molar pregnancy or symptoms indicating local or general infection. Medical and gynecological history was gathered by the doctor attending the patient and the amount of vaginal bleeding assessed by the patient using a four-grade scale bleeding pictogram.

The inclusion criteria were: intrauterine viable fetus or pregnancy of unknown location confirmed by ultrasound, recent or ongoing vaginal bleeding, age between 18–40 years, BMI 19–35kg/m^2, and knowledge of spoken and written Swedish. The exclusion criteria were: non-viable pregnancy confirmed by ultrasound, extrauterine pregnancy, severe bleeding requiring immediate surgery or clinically unstable vital parameters, signs of genital infection, and chronic illness.

A second follow-up visit, 10-14 days after inclusion in the study, was offered to all study participants. The progress of pregnancy was evaluated by transvaginal ultrasound and a second vaginal blood sample was collected in case of ongoing vaginal bleeding. Venous blood and serum samples were collected for longitudinal comparisons. 17 women attended the follow-up consultation, and a second vaginal blood sample could be collected from eight of them.

The final analysis utilized data from a total of 28 study participants, with a median age of 31 years (range 22–40) and a median BMI of 23.7 kg/m^2 (range 16.5–34.7). The study protocol was violated due to one woman having a BMI of 16.5 kg/m^2 and another being diagnosed with anti-phospholipid syndrome later in pregnancy. However, both were kept

in the analysis as their results did not differ significantly from the rest of the study population.

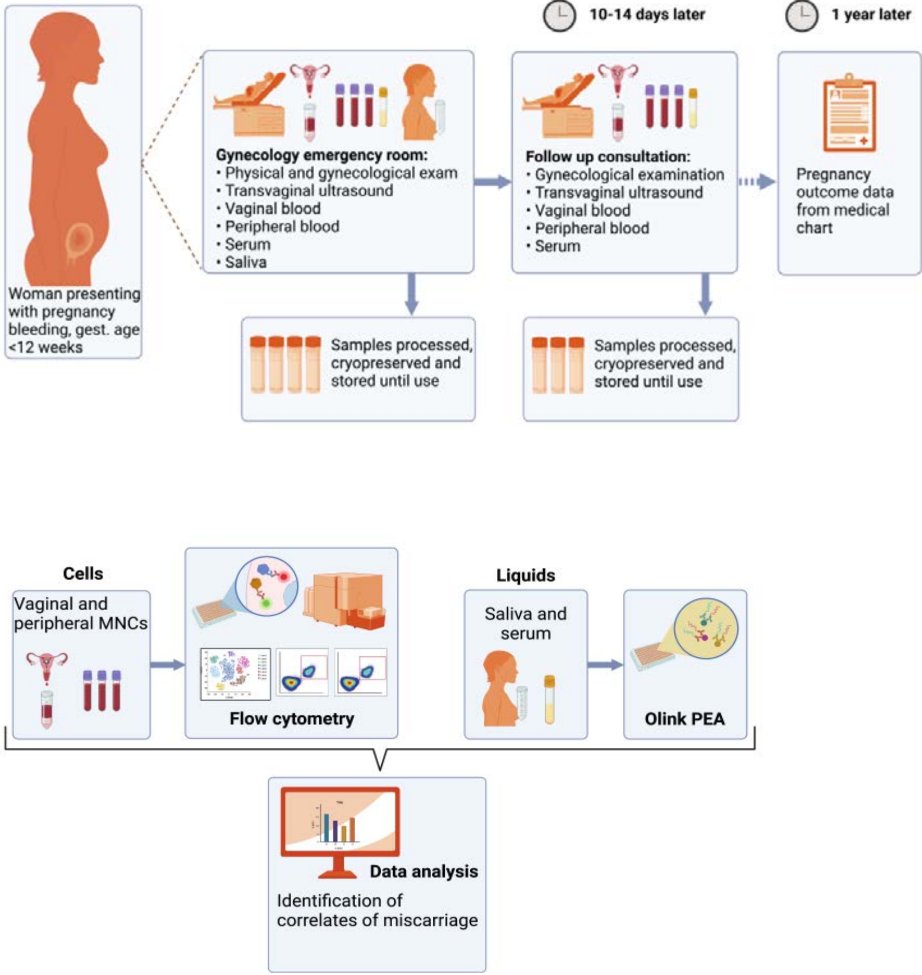


Figure 8. Study and experimental design. Figure created with BioRender.com by Ylva Crona Guterstam.

Table 3. Clinical characteristics of early pregnancy bleeding patients n=28

		Miscarriage group	Non– miscarriage group
Group size	n	14	14
General characteristics	Age, median (range)	30 (25 – 40)	31 (22 – 39)
	BMI, median (range)	25,4 (21,3 – 34,7)	23,1 (16,5 – 29,7)
	Systolic blood pressure mmHg, median (range)	120 (100 – 136)	119 (96 – 138)
	Diastolic blood pressure, mmHg, median (range	77 (64 – 86)	74 (60 – 87)
	Pulse bpm, median (range)	84 (74 – 105)	79 (65 – 111)
	Temperature Celsius, median (range)	37,0 (36,8 – 37,7)	37,1 (36,3 – 37,4)
	CRP mg/L, median (range)	<5 (<5)	<5 (<5 – 12)
	Hemoglobin g/L, median (range)	127 (117 – 142)	123 (101 – 148)
	u– HCG positive, n (%)	14 (100)	14 (100)
Gynecological history			
	Menstrual cycle length days, median (range)	29 (26 – 34)	29 (23 – 30)
	Menstrual duration days, median (range)	5 (2 – 6)	5 (4 – 10)
	Previous pregnancies n (%)		
	0	4 (29)	3 (21)
	1	2 (14)	3 (21)
	2	2 (14)	2 (14)
	≥3	6 (43)	6 (43)
	Previous miscarriages n (%)		
	0	8 (57)	8 (57)
	1	2 (14)	2 (14)
	2	3 (21)	1 (7)
	≥3	1 (7)	3 (21)
Current medication	n (%)		
	Low molecular heparin	0	1 (7)
	ASA	0	2 (14)
	Prednisolone	0	2 (14)
	Vaginal progesterone	2 (14)	2 (14)
	Asthma medication	3 (21)	0
	Iron supplements	2 (14)	2 (14)
	Levothyroxine	2 (14)	1 (7)
	No medication	9 (64)	10 (71)
Conception			
	spontaneous, n (%)	12 (86)	11 (79)
	IVF, n (%)	2 (14)	3 (21)
Current pregnancy characteristics			
	Gestational age days (last menstrual period), median (range)	54 (38 – 70)	55 (40 – 84)
	Gestational age days (CRL), median (range)	44 (37– 56)	58 (40 – 82)
	Pregnancy location		
	Intrauterine, n (%)	10 (71)	13 (93)
	Pregnancy of unknown location, n (%)	4 (29)	1 (7)
	Yolk sac visible, n (%)	7 (54)	12 (86)
	Subchorionic hemorrhage, n (%)	2 (14)	6 (43)
Bleeding pictogram	n (%)		
	Spotting	2 (14)	4 (29)
	Light	6 (43)	7 (50)
	Moderate	3 (21)	3 (21)
	Heavy	3 (21)	0
	Vaginal bleeding no. of days, median (range)	2 (1 – 8)	1 (1 – 10)
Course of pregnancy			
	Delivery at term, n (%)	n/a	11 (76)
	Uneventful pregnancy, n (%)	n/a	7 (50)
	Premature delivery (<37 weeks of gestation), n (%)	n/a	2 (14)
	Stillborn, n (%)	n/a	0
	IUGR, n (%)	n/a	2 (14)
	Preeclampsia, n (%)	n/a	2 (14)

5.3 Sample collection

5.3.1 Study I and II

The study participants were asked to collect menstrual blood for two consecutive cycles using a menstrual cup (Lunette™). To obtain samples from the start of endometrial shedding, the collection of menstrual blood took place during the first 24 hours from the start of menstruation. The women transferred the menstrual blood themselves to a 50 ml Falcon tube prefilled with 5 ml Roswell Park Memorial institute (RPMI) cell medium containing 10% Fetal Calf serum (FCS), heparin, penicillin/streptomycin, gentamicin, and fungizone/amphotericin B. The instructions provided advised the study participants to keep the tubes refrigerated until use and deliver it to the research midwives as soon as the sampling was completed. Matched venous blood and serum samples were taken at the same day as the participants handed in the menstrual blood samples. To detect the LH-surge, a urinary ovulation test was used. To test if endometrial scratching would alter the cytokine profile in menstrual blood in study I, an endometrial biopsy was taken at cycle day 7–9 post ovulation in between the two collection times. For comparisons of eMAIT cell characteristics during proliferative and secretory phase in study II, an additional endometrial biopsy was taken at cycle day 7-10 in the second menstrual cycle. The endometrial biopsies were taken using an aspiration biopsy cannula (Endorette™, Medscand®).

The twin pairs in study II, collected menstrual blood for the first 24 hours of menstruation as described above. Matched peripheral blood and serum samples were taken via venipuncture.

Decidual material for study II, was collected during the surgical abortion and carried on ice to the laboratory at Center for Infectious Medicine, Karolinska Institute for immediate processing. Processing was performed as described in section 3.4.1.2.

For hysterectomies, both for benign indications and after uterus transplantation, endometrial tissue was obtained soon after removal of the uterus in the operating theater. The samples were transferred to tubes containing RPMI medium and placed on ice during transport to the laboratory at Center for Infectious Medicine, Karolinska Institute and processed within 24 hours.

5.3.2 Study III

During the gynecological examination, the speculum was used to collect a sample of vaginal blood, which was then transferred to a 50 ml Falcon tube. The tubes were prefilled with 10 ml of RPMI medium containing 10% FCS, heparin, penicillin/streptomycin, gentamicin, and fungizone/amphotericin B.

Venous blood samples and serum were collected via venipuncture within 30 minutes of vaginal blood sample collection.

To obtain saliva samples, study participants were instructed to rinse their mouths with water to eliminate food residues and wait for 10 minutes to allow saliva to pool in the mouth before providing the sample.

Vaginal blood and saliva were kept cool at 4°C, while venous blood and serum were kept at room temperature until processed.

All biological material was cryogenically stored until grouped analysis was performed.

5.4 Cell isolation

5.4.1.1 Blood samples (Study I, II and III)

Venous and menstrual blood samples were processed by adding the sample to tubes containing density gradient media (LymphoPrep/Ficoll). Then, the tubes were centrifuged, and mononuclear cells could be isolated and thereafter washed with phosphate-buffered saline (PBS). The samples were cryopreserved for future analysis. The plasma fraction was aliquoted and frozen to -80°C for subsequent batched analysis.

5.4.1.2 Tissue samples (Study II and III)

Tissue samples were collected in complete RPMI medium containing 10% FCS, 2mM L-glutamine and penicillin/streptomycin. Larger parts of tissue were manually dissected into smaller pieces. Enzymatic digestion was used to isolate cells from all endometrial tissue samples i.e., from hysterectomies, endometrial biopsies, and decidua. Since some of the vaginal blood samples also contained fragments of tissue, all vaginal blood samples were enzymatically digested to ensure complete extraction of cells and to treat all samples consistently. The samples were transferred to tubes containing RPMI medium supplemented with collagenase II and DNAse Type I, then incubated in a water bath with a magnetic stir at 37°C until the tissue was dissolved or for a maximum of 40 minutes. To stop the reaction, fivefold the volume of complete RPMI medium was added, allowing the FCS to quench remaining enzyme activity. Next, the suspension was passed through a 70 μm strainer to remove any remaining cell aggregates. Mononuclear cells were obtained with density gradient centrifugation, washed with PBS, and frozen down using Mr. Frosty for slow temperature decline to -80°C. The cells were subsequently transferred to liquid nitrogen for storage.

5.5 Saliva isolation (Study III)

To prevent bacterial growth and protein degradation, the saliva samples were kept at 4°C through collection and processing. To remove cellular and food debris, the samples were centrifuged at 4.000 rpm for 20 minutes in the cold, whereafter saliva supernatants were isolated. The saliva supernatants were aliquoted into 1.5 ml tubes, supplemented with a protease inhibitor (cOmplete, Roche) to prevent further protein degradation and frozen down to -80°C.

5.6 Flow cytometry (Study II and III)

In this thesis, the flow cytometry technique has been central for my studies of immune cells. The principle of flow cytometry is fluorochrome-coupled antibodies that specifically targets protein expressed on the surface or inside the cell of interest. These antibodies are mixed with cell suspensions to stain the cells. During analysis, the flow cytometer uses sheath fluid to create a laminar flow, in which single stained cells travel, and pass one by one through laser beams. Each laser beam causes fluorochromes to be excited and subsequently to emit light of a corresponding wavelength. The photons generated are converted to an electrical signal using photomultiplier tubes. The resulting emitted light from a given fluorochrome is proportional to the amount of antibody-bound antigen, which can be visualized and quantified by software with a computer.

Frozen peripheral blood mononuclear cells (PBMC) and mononuclear cells from menstrual blood/vaginal blood/tissue were thawed in complete medium containing RPMI, 10% FCS, and 2 mM L-glutamine. They were washed twice and resuspended in PBS solution containing 2% FCS and 2 mM EDTA. Antibody staining for flow cytometry was performed in the dark at room temperature with a preselected panel of antibodies different for each study. To be able to distinguish between live and dead cells, samples were also stained with Live/Dead Aqua (Invitrogen). To acquire analysis, samples were fixed with BD FIX/PERM and then evaluated using an 18 parameter BD LSR Fortessa flow cytometer for study II and 29 parameter BD A5 Symphony flow cytometer for study III. The data obtained were analyzed using FlowJo (BD, Treestar) with the use of plugins Downsample, UMAP, and Phenograph. Also phyton and R-scripts implementing clustering and UMAP algorithms were used.

5.7 Luminex multiplex assay (Study I and III)

In order to determine the cytokine profile in menstrual and peripheral blood in study I, the bead-based Luminex multiplex assay was used. This is an immunoassay which allows measurements of up to 50 analytes in samples as small as 50 μl and it is validated for

measurements in serum, plasma, and cell culture supernatants. In brief, the method can be described as follows. Analyte-specific antibodies coupled with magnetic beads distinguished by its fluorescence signature, are incubated with the sample of interest. During incubation, the analytes bind to their respective antibodies on the surface of the beads. Next, a detection antibody labeled with a fluorescent tag is added. This detection antibody binds to the analyte captured on the bead surface, thereby creating a sandwich complex. Finally, a streptavidin-phycoerythrin (Streptavidin-PE) conjugate which binds to the detection antibody is added. The bead-antibody-analyte complex is then analyzed using a Luminex instrument that utilizes two laser beams to excite the fluorescent beads and detection antibodies. A magnet holds the beads in a monolayer while one laser excites the bead fluorescent dye and the second excites the PE. The signal emitted from the bead determines the analyte being measured and the PE signal is proportional to the amount of analyte present. The fluorescent emissions are converted to a signal with photomultiplier tubes and analyzed by a computer (**Figure 9**)

In study I, a panel of 20 biomarkers was selected based on their known effects on the endometrium during the menstrual cycle and pregnancy. All samples from a given individual were assayed on the same plate and only one 96-well assay plate was run. Plasma aliquots from menstrual and peripheral blood were thawed in one batch and analyzed using the human Magnetic Luminex assay (R&D Systems). The assay was run according to the manufacturer's instruction. In brief, plasma samples were diluted with Assay Calibrator diluent RD6-52 in volumes 75 μl of sample and 75 μl of diluent. 150 μl of diluted plasma sample was loaded on a 96-well plate and mixed with 50 μl of beads. The mixture was incubated in room temperature in the dark on a microplate shaker for 2 hours at 800 rpm. Next, 100 μl Wash Buffer was added and then removed while the beads and analytes were kept fixed using a magnet. This procedure was repeated twice. Then 50 μl of PE-conjugated Streptavidin was added to each well and the plate incubated at room temperature for 30 minutes on a shaker. The liquids were removed, and the plate washed two times with 100 μl of Wash Buffer. The last step was to re-suspend the beads with 100 μl of Wash Buffer and incubate on the shaker for 2 minutes before samples were analyzed using a Magplex instrument (Merck Millipore, Germany).

In study III, we used the Luminex assay to detect a pre-selected panel of biomarkers in serum as well as in saliva. The Luminex assays are not validated for use with saliva, and therefore two different dilutions were used to determine a useful range. However, the results were not congruent either for protein levels detected in saliva nor in serum, and we therefore decided to exclude the Luminex assay results from the study.

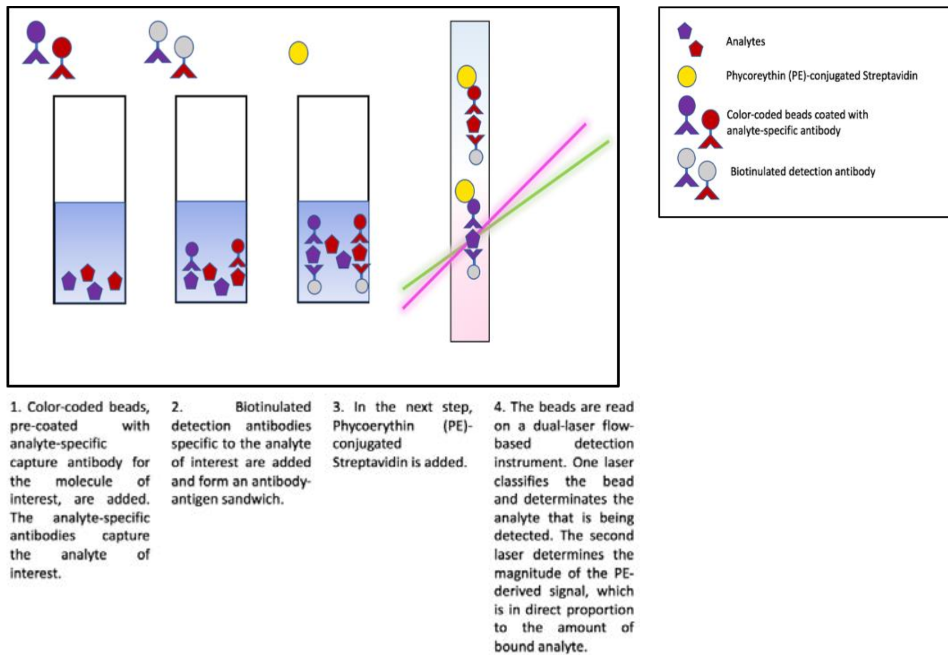


Figure 9. Luminex Multiplex assay principle. © Ylva Crona Guterstam

5.8 OLINK Proximity Extension Assay (Study III)

The OLINK assay platform is used to measure protein biomarkers in biological samples and allows measurement of up to 3000 different proteins in one sample. The Proximity Extension Assay technology works by using pairs of antibodies that are specific to different epitopes of the analyte of interest. The antibodies are coupled to unique cDNA strands which hybridize when coming in sufficient proximity. The DNA ring that is formed is then amplified by a DNA polymerase. The DNA sequence is subsequently amplified and analyzed with qPCR. This provides highly specific and sensitive information on analyte content in a sample. Moreover, the matched DNA strands limits the risk of cross-reactivity in the multiplex assay. The detected amount of each analyte is expressed as a normalized protein expression value in log_2 scale to minimize intra- and inter-assay variation.

In study III, serum and saliva samples were thawed and diluted in PBS, randomized on a 96-well plate, and shipped on dry ice to OLINK Proteomics in Uppsala for analysis (131).

5.9 Functional experiments (Study II)

To conduct the functional experiments on MAIT cells, we received aliquots containing 1*10^9 CFU of bacteria from the clinical microbiology department at Karolinska

Laboratory. These cultures had been collected from patients suspected of infection with *N. gonorrhoeae* and preserved by freezing for later use. To cultivate these fastidious bacteria, we inoculated the bacteria onto two chocolate agar plates. The first plate was supplemented with polymyxin and vancomycin, while the second contained vancomycin, colistin and trimethoprim. After incubating the plates at 36°C for two days in 5-6% CO_2, we identified the microorganism colonies using MALDI-ToF Mass Spectrometry (Bruker Daltonik, Germany). For identification at the species level, a MALDI-ToF log score ≥2.0 was considered adequate.

Thereafter, thawed PBMCs were resuspended in complete RPMI growth medium supplemented with gentamicin. CD107a conjugated FITC was added to the assay to detect degranulation at the end. Cells were then exposed to either *E. coli* or *N. gonorrhoeae* and stimulated for 18 hours. *E. coli* (K-12 mutant strain D21, Coli Genetic Stock Center) was fixed for four minutes in one-fold concentrated BD Cellfix before used at 10 CFU per cell. *N. gonorrhoeae* (CCUG 41811 strain) was fixed for four minutes in one-fold concentrated BD Cellfix before the bacterial suspension was diluted and used at 0.1, 0.5, 1, 5, 10, and 50 bacteria per cell. The various dilutions were selected to explore whether the response exhibited dose-dependent characteristics. Further, three isolates of patient derived *N. gonorrhoeae* cultures were fixed for four minutes in one-fold concentrated BD Cellfix before used at 10 bacteria per cell or 10 ng/ml IL-12 and 100 ng/ml IL-18 for positive control. During the last six hours of incubation, the cytokine secretion inhibitors Monensin and Brefeldin A were added. To assess MR1-specificity, a MR1 blocking antibody and its respective non-blocking isotype were used. Similar results were observed in eight titration experiments, each involving three to four donors. Untreated cells were used as control to distinguish between bacterial stimulation and stimulation due to incubation or other unforeseen factors.

5.10 Statistical analysis

5.10.1 Study I

For comparison of median cytokine concentrations in study I, the SPSS Statistics 25 (Chicago, IL, USA) was used. The data failed the normality test, which is why the Wilcoxon signed rank test was used to compare the difference in median cytokine concentrations between menstrual and peripheral blood plasma and between menstrual blood plasma at the two time points. The correlation analysis was performed using GraphPad Prism 7.0. Correlations between cytokines were tested using Spearman's rank correlation test.

Because of the large number of correlation comparisons, it was decided to reject the null hypothesis only for correlations with a p-value of <0.01.

5.10.2 Study II and III

Analysis of flow cytometry results was conducted with FlowJo (BD, Treestar). GraphPad Prism 7.0 and 9.0 was used to analyze data. D'Agostino-Pearson omnibus normality test was used to test Gaussian distribution of flow cytometry and proteome data. In study II, paired or unpaired t-tests were used to make comparisons for normally distributed data, while in study III, a regression analysis with R version 4.2.2 was conducted using Pearson correlation coefficients for serum proteome data with a normal distribution. Benjamini-Hochberg correction of multiple testing was used to reduce the false discovery rate. Saliva proteome data included very few samples and nonparametric Spearman correlation was used. Mann-Whitney U test or Wilcoxon matched-pairs signed-rank test was used to analyze data that failed the normality test. P-value <0,05 was considered significant. Volcano plots were generated using the *EnhancedVolcano* 1.12.0 package. Hierarchical clustering was done with *pheatmap*. UMAP visualization was done using the umap-learn python script.

5.11 Ethical considerations

The study protocols were designed according to Good Clinical Practice. Prior to the start of each study, the projects underwent ethical evaluation and were approved by the Regional Ethics Committee. The study participants received written and oral information about the studies before signing the informed consent form. They were also informed that they, at any time, could withdraw their informed consent without stating a reason for doing so.

Study participants did not directly benefit from inclusion in the studies. However, participants in study I-II, were financially compensated when donating an endometrial biopsy and they could also keep the menstrual cup that was provided.

The risks of participating were negligible as the performed examinations, tissue- and blood samples are procedures not associated with any common complications. The endometrial biopsy procedure is invasive and can be perceived as painful and/or traumatic for the patient. Before giving the biopsy, the study participants received thorough information about the procedure and were offered local anesthetics. The menstrual cup is a widely used hygiene device which does not pose a risk of physical injury. However, because of cultural values and taboo regarding menstruation, it can be

considered inappropriate to collect and handle menstrual blood. To avoid misunderstandings due to culture clash, a lot of work was put into making the oral and written information as clear as possible. In the third study, women with early pregnancy bleeding seeking care at the gynecological emergency room, were examined with doppler ultrasonography for utero-placental blood flow measurements. The results of that examination are not included in this thesis. Doppler ultrasonography of the utero-placental blood flow is not part of the clinic's standard routines for this group of patients. However, it was not expected to cause discomfort for the woman and was considered safe for the fetus since only the placental blood flow were examined with doppler.

6 RESULTS

6.1 Study I

In study I, we showed that cytokines are measurable in menstrual blood plasma and that the menstrual blood cytokine profile was very similar between individual donors (**Figure 10A**).

When quantifying the levels of cytokines, we found C5/c5a, IL-6, IL-1β and CXCL8 to be expressed with the highest levels in menstrual blood plasma. All being proinflammatory cytokines and chemokines.

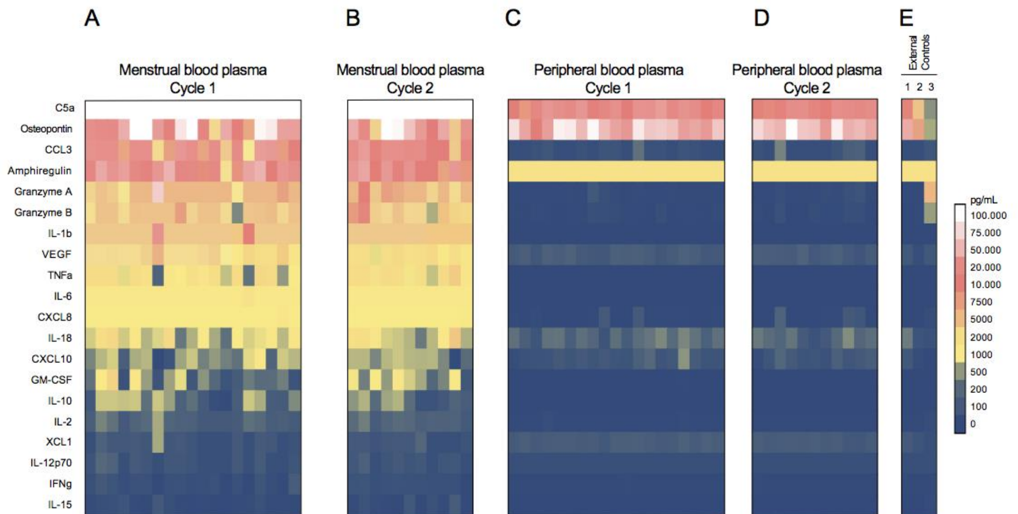


Figure 10. Heatmap visualizing cytokines in menstrual blood in pg/ml. (A) First menstrual cycle and before endometrial scratching. (B) Second menstrual cycle after endometrial scratching. Peripheral blood from cycle one (C), and cycle two (D). (E) Displaying an external male control. © Elsevier.

Furthermore, we found that menstrual blood plasma cytokine profile was distinctly different from the peripheral blood plasma cytokine profile (**Figure 10A, C**). The most pronounced differences were found for C5/c5a, IL-6, IL-1β and CXCL8. Most cytokines showed higher concentrations in menstrual blood than in peripheral blood (**Figure 11 and 12**).

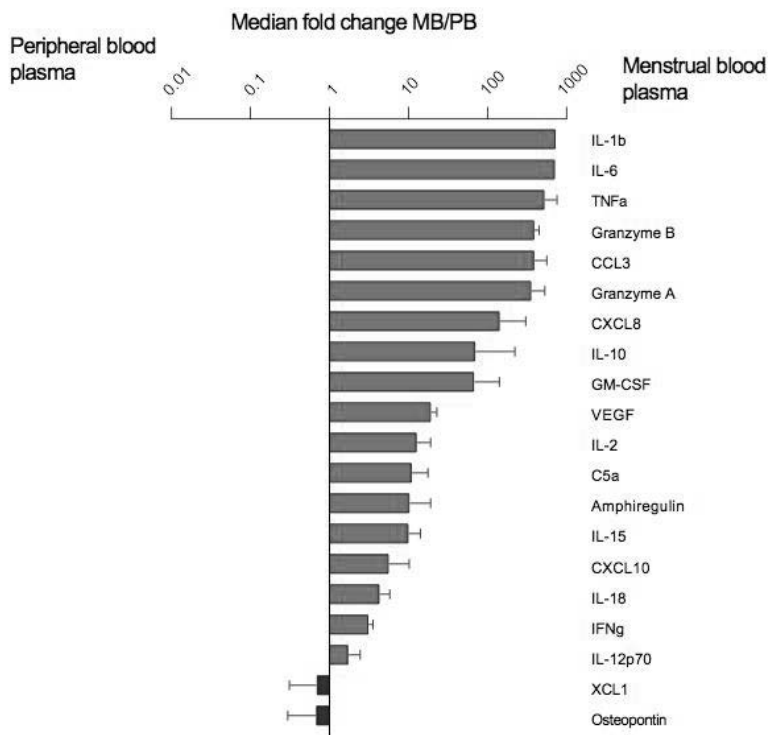
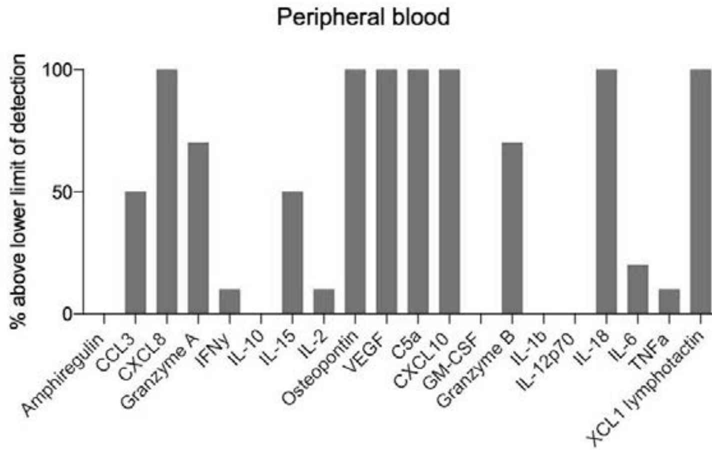


Figure 11. Fold change of indicated cytokines in menstrual blood plasma compared to peripheral blood plasma. Displayed are median values with range on a logarithmic scale. © Elsevier.

To rule out the possibility of procedure errors we used one external male control. A peripheral blood sample was aliquoted to three fractions and subsequently treated as i) a standard peripheral blood sample, and ii) a menstrual blood sample diluted in RPMI medium but kept refrigerated during collection and storing, and iii) a menstrual blood sample diluted in RPMI medium and kept in room temperature for 24 hours. The first and second aliquot did not differ significantly from the other peripheral blood samples (**Figure 10C-D, E1-2**). However, the third sample showed a similarity to menstrual blood with regard to granzyme A and B, and osteopontin (**Figure 10A-B, E3**). This indicates that dilution media and processing did not affect most cytokines measured and that the cytokines measured were of endometrial origin.



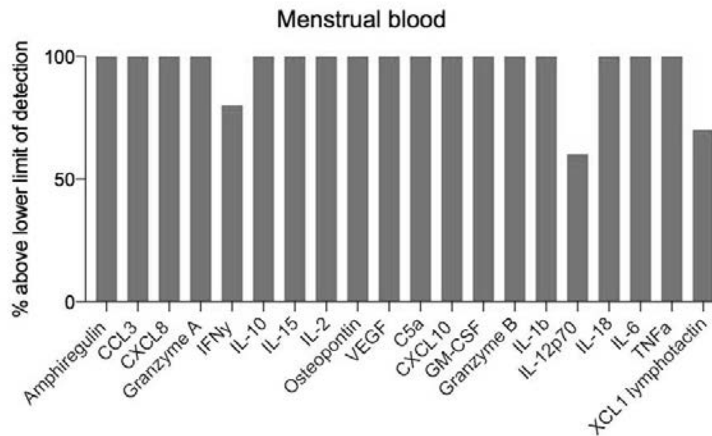


Figure 12. Graph showing values of indicated cytokines within the range of detection in peripheral blood plasma and in menstrual blood plasma. © Elsevier.

We subjected the endometrium to an iatrogenic wounding via an endometrial biopsy cannula, to evaluate a possible inflammatory provocation in the next menstrual cycle. However, the results showed no such difference in cytokine profile when comparing before and after endometrial scratching and the menstrual blood cytokine profile remained stable throughout two subsequent cycles (**Figure 10A, C, and 13**).

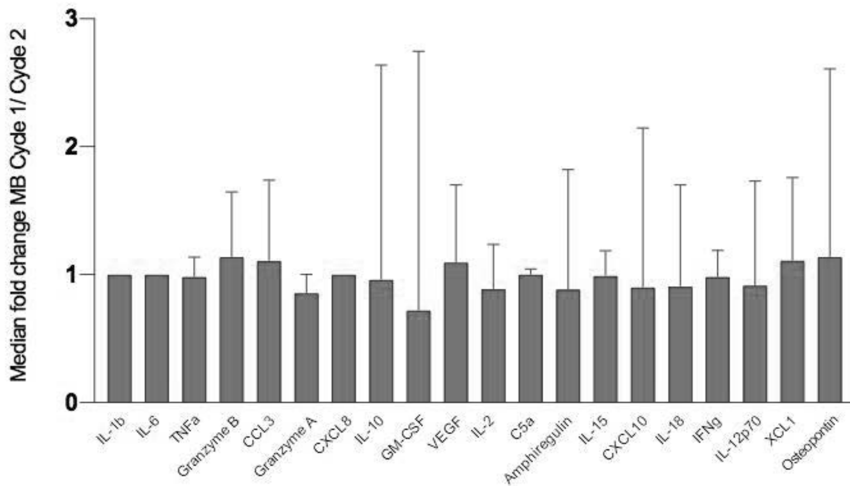


Figure 13. Fold change of indicated cytokine expression before and after endometrial scratching. Median values with range. Only paired samples were included (n=11). © Elsevier.

Correlation analysis of cytokines in menstrual blood plasma revealed 14 positive correlations below the adjusted p-value 0.01 (**Figure 14A**). The most frequent correlations were found for IFN-γ and IL-10. IFN-γ correlated with TNF, CCL3, CXCL10 and IL-12p70. IL-10 correlated with XCL1/Lymphotactin, amphiregulin, IL-2 and IL-15.

In peripheral blood plasma, six significant ($p<0.01$) positive correlations were found (**Figure 14B**). These correlations were not the same as in menstrual blood. GM-CSF and C5/c5a both correlated with two cytokines, TNF, IL-2 and CCL3, IL-18 respectively. This further strengthens the interpretation that menstrual blood cytokine profile is different from peripheral blood cytokine profile.

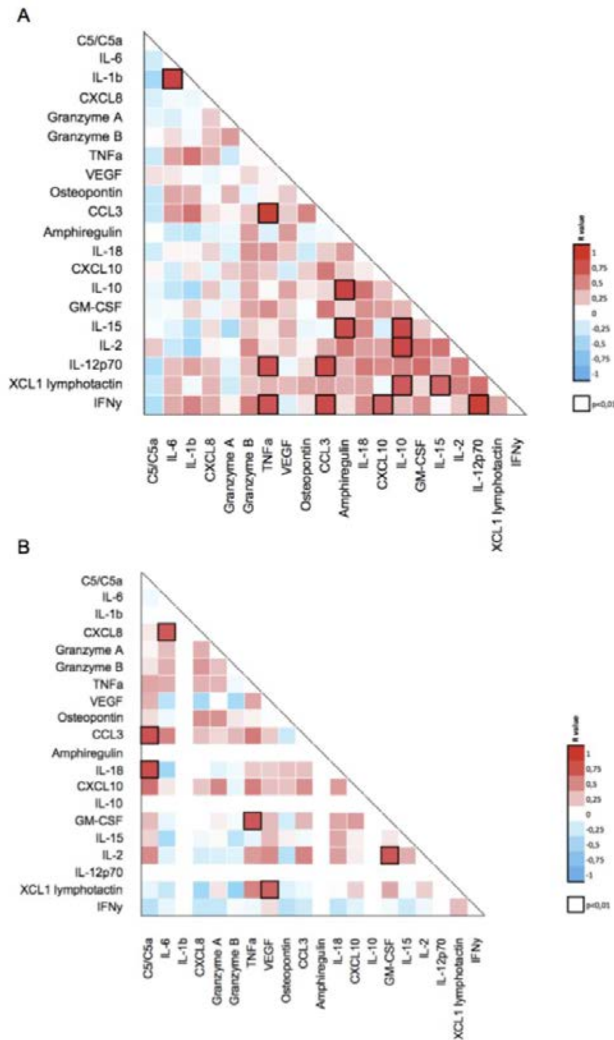


Figure 14. Correlations of cytokines in (A) menstrual blood plasma and (B) in peripheral blood plasma. © Elsevier.

6.2 Study II

In study II, we used flow cytometry to analyze the frequency and phenotype of MAIT cells in different compartments. MAIT cells were defined by the expression of the semi-invariant TCR chain Vα7.2 and CD161 (**Figure 15a**). We found that eMAIT cells are a stable population of immune cells that do not respond to changes in the endometrium during cyclic regeneration, pregnancy, or postmenopausal atrophy. The frequency of MAIT cells in the endometrium from women of fertile age was similar to that in peripheral blood

(**Figure 15b**). There was no variation in the frequency of MAIT cells in endometrium between fertile-aged and postmenopausal women, nor in the first trimester decidua (**Figure 15c**). Like peripheral blood MAIT cells, the eMAIT cells were predominantly CD8⁺ and this was consistent also in postmenopausal endometrium and in decidua. The frequency of eMAIT cells remained stable throughout the menstrual cycle (cycle day 7 vs cycle day 21) (**Figure 15d**) and a stable Ki67-expression in eMAIT cells further supported their lack of proliferation. In line with this, peripheral blood MAIT cells remained stable throughout the menstrual cycle (**Figure 15e**).

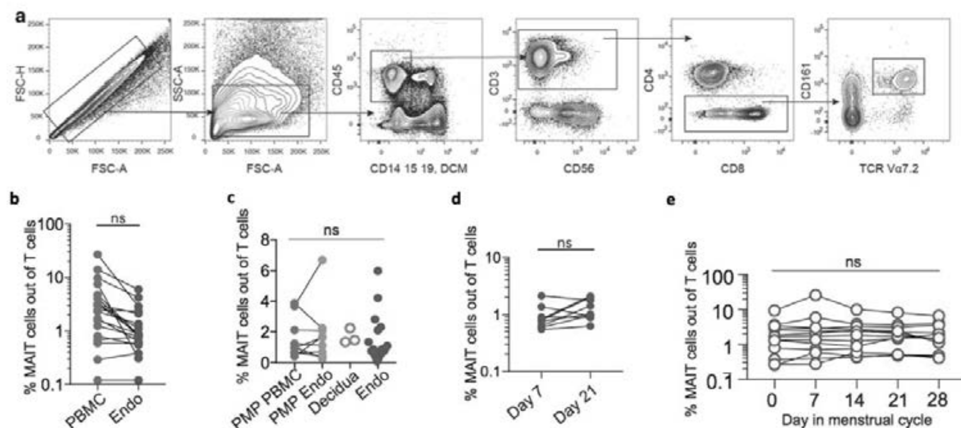


Figure 15. (a) Representative FACS plot identifying endometrial CD3⁺CD161⁺TCRVα7.2⁺ MAIT cells. (b) MAIT cell frequency in peripheral blood and in premenopausal endometrium. (c) Frequency of MAIT cells in peripheral blood, pre-and postmenopausal endometrium, and decidua. (d) frequency of eMAIT cells at day 7 and 21 in the menstrual cycle. (e) Frequency of peripheral blood MAIT cells at five time points in the menstrual cycle. © Elsevier

Further, we found a strong correlation between peripheral blood MAIT cell frequencies in monozygotic twin pairs (**Figure 16**). In contrast, no such correlation was found for the eMAIT cell population. As control, we assessed the circulating and endometrial MAIT cell populations from 19 unrelated premenopausal women, and no correlation was found in either compartment. This suggests that environmental factors impact the size of eMAIT cell compartment, while genetic factors have a greater impact on the peripheral MAIT cell population.

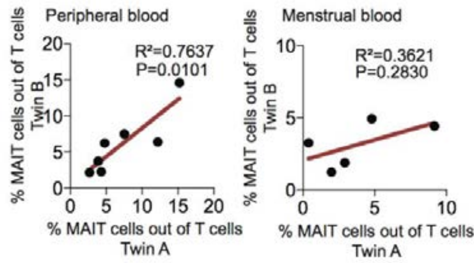


Figure 16. Correlation of peripheral blood and menstrual blood MAIT cell frequencies in monozygotic twins. © Elsevier

When examining tissue-residency markers of eMAIT cells we made three important observations. Endometrial MAIT cells in pre- and postmenopausal endometrium and decidua, demonstrated a high expression of CD69 and CD103 compared to peripheral blood, and eMAIT cells from two women who had undergone uterus transplantation, showed a similar tissue-residency phenotype (**Figure 17a and b**). Our third finding was that the vast majority of eMAIT cells from transplanted uteri expressed recipient HLA molecules, suggesting that they were of recipient origin (**Figure 17c**). This implies that even though eMAIT cells display tissue-residency markers, they are probably a transiently tissue-resident population that gets replenished from the circulation to the endometrial tissue, where they begin to express tissue-residency markers.

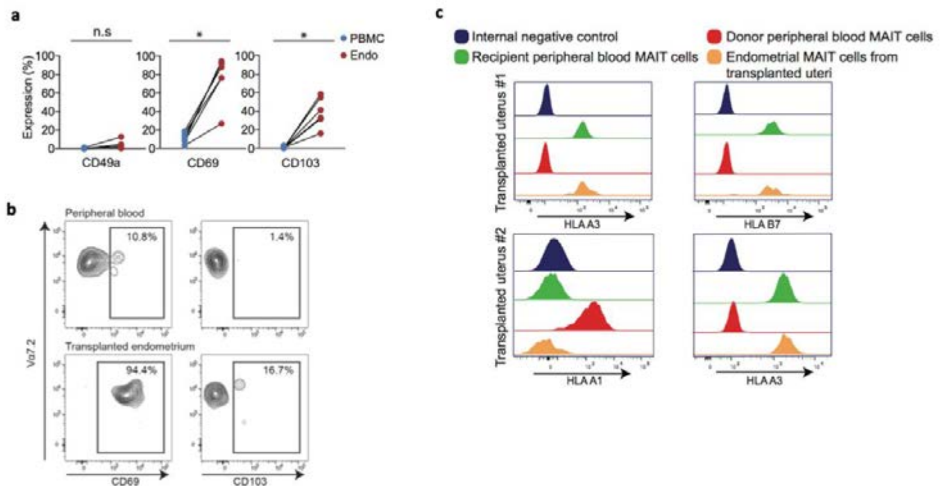


Figure 17. (a) Summary expression of CD49a, CD69 and CD103 on MAIT cells in premenopausal endometrium and matched peripheral blood. (b) FACS plot representing CD69 and CD103

expression on peripheral blood and eMAIT cells from one patient post uterus transplantation. (c) Anti-HLA stainings on MAIT cells from donor and recipient peripheral blood, and endometrium post uterus transplantation in two patients. © Elsevier

Additionally, we observed that eMAIT cells from premenopausal women exhibited a more activated phenotype compared to peripheral blood MAIT cells, expressing CD38, HLA-DR and PD-1 at a significantly higher frequency, but lower expression of CD127. Comparing premenopausal eMAIT phenotype to decidual and postmenopausal eMAIT cells, we saw no difference in most of the aforementioned markers except for higher expression of HLA-DR on eMAIT cells from premenopausal endometrium.

Finally, we found that peripheral MAIT cells responded similarly to the laboratory strain of *N. gonorrhoeae* as they did to *E. coli*, though with overall lower responses and in a dose-dependent manner.

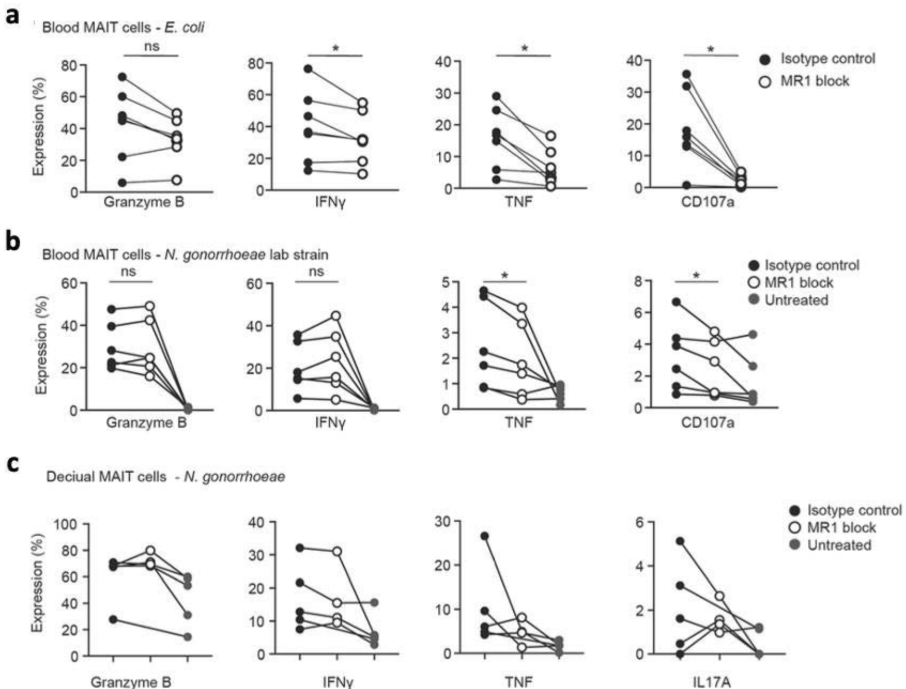


Figure 18. (a) Expression of granzyme B, IFN-γ, TNF, and CD107a on peripheral blood MAIT cells after stimulation with *E. coli* with or without MR1 blockade. (b) Expression of granzyme B, IFN-γ, TNF, and CD107a on peripheral blood MAIT cells after stimulation with a *N. gonorrhoeae* lab strain with or without MR1 blockade as compared to unstimulated. (c) Expression of granzyme B, IFN-γ, TNF, and CD107a on decidual MAIT cells after stimulation with *N. gonorrhoeae* with or without MR1 blockade as compared to unstimulated cells. © Elsevier.

The measured functional readouts were expression of granzyme B, IFN-γ, TNF, and CD107a, as well as upregulation of the activation marker CD69. In addition, the MAIT cell response to the laboratory strain of *N. gonorrhoeae* was less MR-1 dependent than the response to *E. coli*, suggesting that MAIT cell reaction against *N. gonorrhoeae* is induced by both MR-1 dependent and independent pathways (**Figure 18a and b**). Decidual MAIT cells responded similarly to peripheral MAIT cells after stimulation with *N. gonorrhoeae* with the difference of higher basal expression of granzyme B in unstimulated cells (**Figure 18c**). Peripheral MAIT cells showed a comparable and adequate response to three distinct clinical isolates of *N. gonorrhoeae* with significantly higher response rates of granzyme B, TNF, and CD107a towards two antibiotic-sensitive clinical isolates than towards both two antibiotic-resistant clinical isolates and three laboratory strains.

6.3 Study III

In study III, we enrolled 28 women who experienced early pregnancy bleeding with a viable intrauterine fetus confirmed by transvaginal ultrasound. Out of these women, 14 were subsequently diagnosed with miscarriage. We found that vaginal blood collected during early pregnancy bleeding, contained decidual NK (dNK) cells that displayed a tissue-resident phenotype positive for CD49a (**Figure 19a and b**).

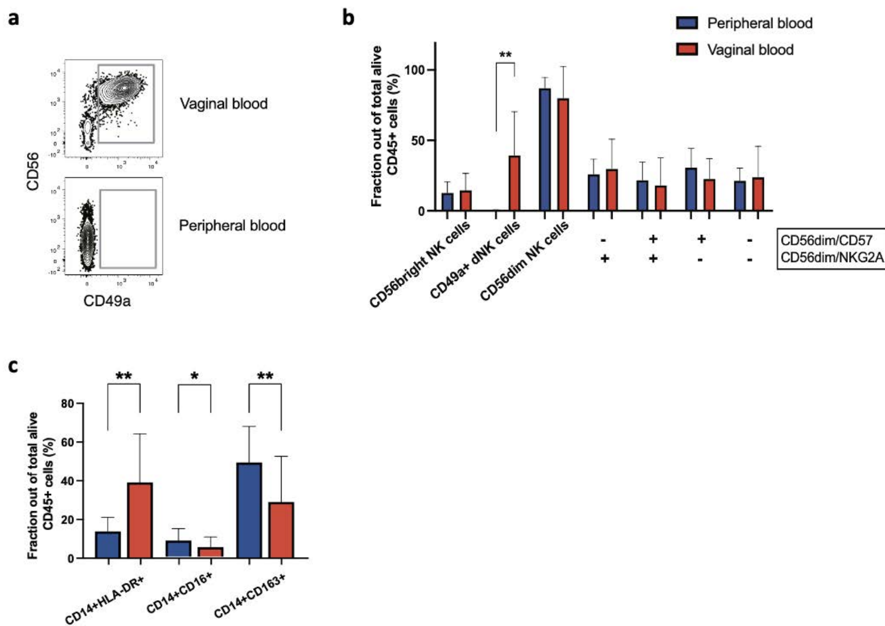


Figure 19. (a-b) Representative FACS plot and aggregated data on frequencies of NK cell subsets in vaginal and peripheral blood. (c) Frequencies of CD14⁺ monocyte/macrophage subsets in vaginal and peripheral blood. Created by Ylva Crona Guterstam.

When comparing vaginal blood and peripheral blood mononucleated cells, we found no difference in composition of B cells, T cells, monocytes, and conventional NK cells. However, CD14⁺HLA-DR⁺ monocytes were significantly more frequent in vaginal blood compared to peripheral blood (**Figure 19c**). Further, the peripheral blood did not contain tissue-resident dNK cells.

Women who subsequently had a miscarriage showed a distinct immune cell profile in vaginal blood different from that of women who continued their pregnancy. Notably, vaginal blood from women in the miscarriage group showed a clear abundance of tissue-resident CD49a⁺ NK cells and an increased frequency of CD4⁺ effector memory T cells (CCR7⁻CD45RA⁺) (**Figure 20a and b**). In addition, the same group of women had an increased frequency of CD49a⁺ T cells in peripheral blood (**Figure 20c**).

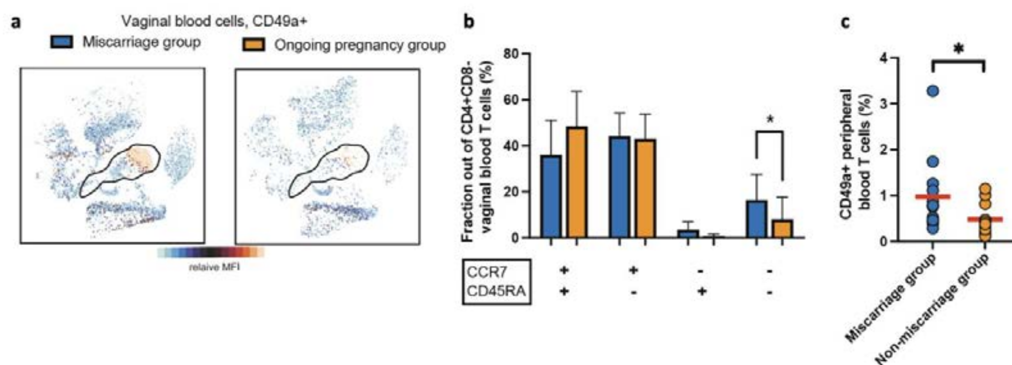


Figure 20. (a) UMAP of vaginal blood cells in the miscarriage group (left) and ongoing pregnancy group (right). The relative level of CD49a on NK cells (circled area) is visible. (b) Frequency of vaginal blood naïve/mature CD4⁺ T cells in the miscarriage group and the ongoing pregnancy group. (c) Frequency of CD49a⁺ T cells in peripheral blood in the miscarriage group and the ongoing pregnancy group. Created by Ylva Crona Guterstam.

Correlation analysis between the serum proteome levels and the frequency of CD49a⁺ NK cells in vaginal blood in the miscarriage group revealed statistically significant negative correlations for CSF1, granzyme A and H, TWEAK, and CAIX, respectively. Further, soluble CD28 was statistically significantly and negatively correlated with the frequency of CD49a⁺ T cells in peripheral blood in the miscarriage group.

Finally, in the miscarriage group, ANGPT2 and MMP12 showed a significant fold change, as determined by serum protein ratios and fold change analysis.

7 DISCUSSION

In this thesis, I have characterized uterine immune cells and cytokines in different phases of a woman's life. In healthy cycling endometrium, during pregnancy, after menopause and in pregnancy loss.

7.1 Cytokines in the cycling endometrium

Cytokines are signaling peptides that regulate the responses and developmental fate of immune cells. The expression of cytokine genes is strictly regulated, however, their transcription can be influenced by various stimuli. The endometrium is known to be highly active in cytokine production, and cytokines are important regulators of the menstrual cycle and decidual preparation for pregnancy.

In study I, we conducted a broad analysis of the cytokines present in menstrual blood in a cohort of healthy nulliparous women and found that the menstrual blood cytokine profile was very similar among the individuals participating in the study. Further, we found no variation in the menstrual blood cytokine profile before and after endometrial scratching and over subsequent cycles. Only a few studies, reporting contradicting results, have been performed previously to evaluate cytokines in menstrual blood. Furthermore, to my knowledge, there has been no investigation into cytokines present in menstrual blood following endometrial scratching. Tortorella et al. assessed IL-6, IL-1β and TNF in menstrual blood aspirated from the cervical os during menstrual period day two-three using ELISA (132). Study participants were women seeking treatment for infertility diagnosed with chronic endometritis. They found significantly increased levels of all measured cytokines in menstrual blood in the chronic endometritis group compared to controls. However, no comparison with peripheral blood was performed. In contrast, concentrations of TNF and VEGF in peripheral blood and menstrual blood aspirates, did not show any variance when comparing infertility patients with endometriosis to a control group without endometriosis (133). In line with our results, Naseri et al. recently performed a 62-plex analysis of menstrual blood cytokines in comparison to peripheral blood, and found 35 of them to be significantly elevated in menstrual blood including all aforementioned cytokines (134). Our results indicate that sample processing has minimal influence, which was confirmed by examining a control sample of peripheral blood that was treated in the same way as a menstrual blood sample, with only two of the cytokines showing variation between the two samples. Thus, strongly indicating that the cytokine profile of menstrual blood can be reliably measured and differs significantly from peripheral blood.

Due to the fact that menstruation is an inflammatory process, it was anticipated that there would be an increase in proinflammatory markers in menstrual blood. Consistent with this expectation, C5/C5a, IL-6, IL-1β and CXCL8, which are all proinflammatory cytokines and

chemokines, showed the highest levels of expression in menstrual blood. Signal peptides recognized for their ability to activate and increase proliferation of NK cells, such as IL-12p70 and IL-15, as well as XCL1/Lymphotactin and IFN-γ, which are known to be secreted by activated NK cells, were present at lower levels, but still more abundant than in peripheral blood. This finding is in line with the observation that uNK cells peak in abundance during mid-secretory phase, and subsequently decline towards the onset of menstruation (135, 136).

The local immune environment of the uterus is known to react and change when it encounters external stimuli. In response to semen after unprotected intercourse, the woman's cervix is marked by the influx of macrophages, dendritic cells and memory T cells that infiltrate the epithelial and stromal tissues. This infiltration of leukocytes is accompanied by increased expression of the inflammatory cytokines GM-CSF/CSF2, IL-6, IL-8 and IL-1A (137). Intrauterine devices, both copper and levonorgestrel, are known to induce an increase in classical proinflammatory cytokines in cervicovaginal secretions (138). Furthermore, there is some evidence to support the idea that a particular subset of uNK cells in women who have previously been pregnant acquire a memory phenotype after exposure to trophoblast antigens HLA-E and HLA-G. This memory phenotype is believed to promote improved placentation in subsequent pregnancies (139). Therefore, cytokines serve as means of communication for immune cells and endometrial cells, with both responding to various stimuli by releasing new sets of cytokines. Menstrual blood has shown to be an important biological sample, offering insight into the uterine environment during the last stage of the menstrual cycle. Given the hypothesis that uterine receptivity is mediated by the expression of proinflammatory cytokines and that such an inflammatory milieu is not generated in some women with infertility, it was believed that an iatrogenic injury to the endometrium could possibly induce a sufficient inflammatory response able to bridge the insufficient decidua and aid implantation after assisted fertility treatment. Gnainsky et al. reported a rise in proinflammatory cytokines (TNF, IL-15, GROα, MIP-1β/CCL4) when endometrial scratching was performed in mid-to-late proliferative phase (122, 140). Nevertheless, others reported an increased implantation rate after endometrial scratching in both proliferative and secretory phase of the endometrium, and after endometrial scratching in both the preceding cycle and the same cycle as embryo transfer (141). Thus, there were different opinions on the right timing of the procedure. For study I, we postulated that such an induced inflammatory response would be measurable in menstrual blood following endometrial scratching during the "window-of-implantation" LH+ 7–10 days. However, we found no detectable difference in menstrual cytokine profile when comparing before and after endometrial scratching and the menstrual blood cytokine profile remained stable throughout two subsequent cycles. Thus, our results were consistent with the randomized controlled study of Lensen et al., which shows no benefit either for women undergoing IVF or those trying to conceive spontaneously, to be treated with an endometrial biopsy (123, 142). Additionally, any

increase in proinflammatory cytokines that occurred immediately after the procedure had normalized in time for the start of menstruation.

One limitation to the use of menstrual blood as proxy for the endometrial microenvironment is that it only provides us with a snapshot. It does not provide us with information about the proliferative phase leading up to the window-of-implantation in secretory phase. For cytokine studies in the early phases of the menstrual cycle, we are still dependent on invasive methods for sampling. Furthermore, the study cohort in study I and II all reported normal menstrual bleeding and regular cycles. However, some of the study participants donated as much as 40 ml of menstrual blood collected during the first 24 hours of menstruation. When considering that the typical volume of menstrual blood over the entire period is no more than 80 ml, the occurrence of 40 ml within the first 24 hours may suggest abnormal uterine bleeding. None of the included women hade signs of genital infection, polyps, adenomyosis or uterine fibroids as possible causes of heavy bleeding, and it is difficult to interpret what impact this may have had on our results. Yet, when comparing the result of these individuals to those of the others, no significant difference in cytokine profile was observed. Another limitation is the number of study participants. Possibly by expanding this in future studies, differences not detected here may be revealed.

7.2 Dynamics of the endometrial MAIT cell compartment

MAIT cells are innate-like non-classical T cells that play a crucial role in defending mucosal tissues against microbes. They are activated by APCs presenting microbe derived riboflavin metabolites in the non-polymorphic MHC-like receptor 1 (MR1), but can also respond to inflammatory cues, such as IL-12 and IL-18 (143) in a non-MR1 dependent manner. The role of human MAIT cells in the endometrium during regenerative cycles and postmenopause, as well as their significance in sexually transmitted infections is not well understood. Furthermore, MAIT cells are postulated to be of importance in the fetomaternal interface, where they sustain tissue homeostasis without exposing the semi-allogenic fetus and placenta to harmful immune responses, and at the same time safeguarding the fetus from bacterial pathogens (100). While most peripheral MAIT cells are $CD8^+$, some are double-negative (DN) lacking both CD8 and CD4, and a small portion express CD4. $CD8^+$ MAIT cells are reported to exhibit superior proinflammatory functions and greater cytotoxic potential (144). Nevertheless, all major subpopulations of MAIT cells display a memory phenotype and are prompt to respond upon activation.

In study II, we showed that eMAIT cells represent a stable population of immune cells that do not respond to changes in the endometrium during cyclic regeneration, early pregnancy, or postmenopausal atrophy. Additionally, the levels of peripheral blood MAIT cells remained remarkably stable throughout the menstrual cycle. This is in contrast to

uNK cells, which are known to fluctuate in numbers due to hormonal changes during the menstrual cycle (52). Unlike uNK cells which proliferates in response to IL-15 secreted by the endometrium when progesterone levels increase (145), we showed that the eMAIT population is not affected by progesterone changes suggesting that their regulation must be controlled by hitherto unknown factors. Solders et al. reported a higher frequency of MAIT cells in intervillous blood compared to peripheral blood from healthy term pregnancies (146). This contrasts our results that showed very little difference in peripheral blood MAIT cell and decidual MAIT cells frequency. However, migration assays in Solders report, showed that MAIT cells migrate toward conditioned medium from placental explants. Together, this suggests that in contrast to early decidual MAIT cells, term pregnancy MAIT cells get recruited from the circulation and homes to the placental intervillous space.

According to our results, the phenotype of eMAIT cells remain consistent in pre- and postmenopausal endometrium and in early pregnancy decidua. Similar to peripheral blood, the majority of eMAIT cells were CD8^{+}. However, eMAIT cells exhibited a more activated phenotype and expressed tissue-residency markers to a higher extent compared to their peripheral counterparts. The increased expression of activation markers such as HLA-DR and PD-1 by eMAIT cells, is in concordance with previous studies showing an activated phenotype in mucosal sites (147). The response of CD56^{+}MAIT cell subsets to innate cytokine stimulation has been found to be stronger, as reported by Dias et al. (148). Moreover, a decrease in the number of CD56^{+}MAIT cells has been observed in first term decidua. This reduction has been interpreted as an immunomodulatory mechanism that aims to prevent immune responses that could potentially harm the fetus (149). However, our investigation found no significant difference in MAIT cell frequency in peripheral blood or in non-pregnant endometrium compared to early pregnancy decidua. Hence, the immune-tolerogenic properties of endometrial MAIT cells in early pregnancy needs to be further investigated.

The endometrium undergoes cyclic shedding and regeneration, which is a unique feature of human reproductive physiology. This process results in a continuous renewal of tissue and local immune cells. The source of monthly immune cell regeneration is not yet clear, as evidence exists for both proliferation of local basal layer precursors and bone-marrow-derived immune cell recruitment. Our study demonstrated that eMAIT cells in pre- and postmenopausal endometrium and decidua express high levels of tissue-residency markers CD69 and CD103, indicating that they are likely to be resident within the endometrium. Previous studies using solid organ transplantations across HLA-barriers has provided evidence of persisting T cell tissue residency in lung and intestine (150, 151). However, our analysis of eMAIT cells in human uterus transplantations resulted in very few, if any, donor MAIT cells remaining in the endometrial mucosa. The same has been found regarding NK cells in uterus transplants, showing that the majority of uNK cells were of

recipient origin (54). This indicates that the recipient's circulating MAIT cells replenish the endometrium over time. One possible explanation for this phenomenon may be that tissue-resident MAIT cells have a shorter lifespan compared to conventional MAIT cells or that they lack the ability to self-replenish. Further, it has been suggested that decidual MAIT cells exhibit a higher level of Ki67 expression *in vivo* compared to peripheral blood MAIT cells, indicating that they have the capacity for *in situ* proliferation.

The non-invasive approach to study endometrial immune cells from menstrual blood collected with a menstrual cup yields reliable results (152, 153), and when we studied menstrual and peripheral blood MAIT cells from monozygotic twin pairs, we found a strong correlation between peripheral blood MAIT cell frequencies. In contrast, no such correlation was found for the eMAIT cell population. Previous studies has shown strong genetic regulation of quantitative levels of CD39 expressing T cells and NK cells (154) and Strunz et al. found that the uNK cell population size were under genetic control (54). This suggests that the uterine MAIT cell compartment more than other local immune cells, is more influenced by environmental factors. However, the mechanisms behind this are still not clear and renders a need for further investigation.

MAIT cells in the female genital tract are known to react to stimulation by *E. coli*. Levels of IL-17 and Il-22, cytokines expressed by female genital tract MAIT cells, increase in genital secretions during infections such as chlamydia and gonorrhea (85, 155, 156). However, the source of these cytokines is unknown. The responsiveness of MAIT cells to relevant pathogens in the female genital tract had not been studied previously. We found that peripheral MAIT cells were functional and responded similarly to the laboratory strain of *N. gonorrhoeae* as they did to *E. coli*. The response was not entirely dependent on MR1 presenting riboflavin metabolites, even though *N. gonorrhoeae* similarly to *E. coli* has a riboflavin synthesis pathway, suggesting partial activation through cytokines. Antibiotic resistance to *N. gonorrhoeae* is a growing problem that has the potential to cause disseminated infection and long-term damage, increasing the risk for extrauterine pregnancy and infertility. In line with the results of Buolouis et al. (157), our study showed that although MAIT cells responded to antibiotic-resistant strains, the response level was significantly lower than that for antibiotic-sensitive strains. Nevertheless, a clear positive response was detected, and a potential future scope for studies could be to investigate the use of MAIT cells as an immunotherapeutic alternative treatment against multi-resistant *N. gonorrhoeae* infections. Another future study could explore MAIT cell responses to other clinically relevant pathogens, such as *Chlamydia trachomatis*. This intracellular bacterium is notoriously difficult to culture, making accurate models for experimentation complicated to develop. However, this was not within the scope of this thesis.

7.3 Vaginal blood from early pregnancy bleedings contains tissue-resident decidual NK cells

Vaginal bleeding during early pregnancy is a common condition, and unfortunately, a large part of those pregnancies end in miscarriage. While embryonic chromosomal disorders are thought to cause around 50% of all miscarriages, and immunological factors are believed to be significant contributors to miscarriage, the cause in the individual case often remains unknown. To date, no adequate examination or test can predict which woman, with a live embryo detected by ultrasound and vaginal bleeding, will ultimately miscarry or continue their pregnancy. This uncertainty leads to many unnecessary doctor's visits, increasing healthcare costs and anxiety in expectant mothers. Therefore, there is a pressing need for translational research to explore the mechanisms behind miscarriage, as well as studies aimed at improving the ability of healthcare providers to predict the outcome of impending miscarriage. By enhancing the understanding of this complex condition and finding new ways to correctly diagnose and thus shorten the time to treatment can help alleviate women's anxiety and reduce the burden on healthcare systems.

Study III is part of a broader investigation integrating serum and saliva proteome, and the hemodynamics of the utero-placental blood flow within the uterine arteries with the characteristics of fetomaternal interface immune cells, as observed in vaginal blood samples collected during early pregnancy bleeding. In this pilot study, we initially set out to identify the types of immune cells that can be found in vaginal blood and discovered that all major immune cell subsets were represented. Further, when comparing the frequencies of vaginal blood immune cell subsets to the frequencies of their counterparts in peripheral blood, we found an increased level of tissue-resident CD49a^{+} dNK cells in vaginal blood. Decidual NK cells are proven to be functionally and phenotypically distinct from peripheral blood NK cells, expressing CD56^{bright}CD16^{-}. They are considered to have a regulatory role rather than being primarily cytotoxic. Previous studies have shown that dNK cells are involved in sustaining immune tolerance at the fetomaternal interface, promoting spiral artery formation and regulating trophoblast invasion (52). Tissue-residency marker CD49a is a cell adhesion integrin that binds to collagen and laminin. It has been established as a marker for identifying tissue-resident NK cells in both mice and humans (158, 159). In normal first trimester decidua, CD49a^{+} NK cells make up the majority of all NK cells and they are thought to secrete growth-promoting factors facilitating fetal growth (160). Their role in early pregnancy loss is still elusive, but lower frequencies of CD49a^{+} NK cells have been found in menstrual blood in women with recurrent miscarriage (161).

We found that women who subsequently miscarried had a significantly increased level of tissue-resident CD49a^{+} dNK in their vaginal blood, compared to those who carried their pregnancies beyond fetal viability. This indicates that in the group of patients that will

experience miscarriage, tissue-resident dNK cells may leak from the disrupted fetomaternal interface, and that such leakage can be detected during the first emergency visit while the fetus is still viable. Thus, tissue-resident dNK cells in vaginal blood has the potential as prognostic factor in the prediction of pregnancy outcome in women with early pregnancy bleedings.

Moreover, the vaginal blood from the women who subsequently miscarried showed an elevated frequency of $CD4^{+}CCR7^{-}CD45RA^{-}$ effector memory T cells and a rise in the levels of $CD49a^{+}$ T cells in their peripheral blood. During early pregnancy, T cells account for approximately 10-20% of decidual leukocytes, with around 30-45% of them being $CD4^{+}$ T cells (68). Zeng et al. identified the majority of $CD4^{+}$ T cells in early pregnancy decidua as having an effector memory phenotype (162). Transcriptomic studies of first-trimester decidua have demonstrated an upregulation of CD49a transcription in decidual T cells, but not in peripheral blood T cells (163). Our findings, when compared to this information, suggests that there may be some leakage of these cells from the disrupted fetomaternal interface during miscarriage, both into the vaginal blood and the circulation. However, despite T_{reg} cells being another significant T cell subgroup enriched in early pregnancy decidua compared to peripheral blood (69), we did not observe higher levels of T_{reg} cells in the vaginal blood. Importantly, we did not include markers specifically targeting T_{reg} cells, such as the transcription factor FOXP3. Thus, there is room for improvement here and future studies by us and others should focus more on this. It is also important to note the possibility of contamination of other immune cells in the lower female genital tract, which has a high concentration of microorganisms in the genital tract flora. Immune cell composition differs substantially between decidua and the lower female genital tract, however, within the cervical $CD4^{+}$ T cells, the vast majority exhibit an effector memory phenotype (164). Therefore, we conducted a hierarchical clustering analysis of the flow cytometry data to determine the significance of each immune cell subset in relation to its correlation with miscarriage. The results showed that the frequency of $CD49a^{+}$ dNK cell in vaginal blood exhibited the strongest association with the miscarriage group. Future studies should include additional T cell markers to reveal more about their phenotype in vaginal and peripheral blood.

The correlation analysis between serum proteome levels and the frequency of $CD49a^{+}$ dNK cells in vaginal blood within the miscarriage group revealed significant negative correlations for several markers. However, the small number of samples included in the analysis is a limitation that could make our results difficult to interpret. Additionally, proteome analysis of biological samples may not assume that all observations are independent of each other. Despite these limitations, I employed statistical methods to reduce the false discovery rate of multiple testing, making our results intriguing to explore in future studies where serum proteins can be combined with flow cytometry data to develop more precise diagnostic tools.

7.4 Methodological considerations

The studies incorporated in this thesis address important knowledge gaps pertaining to the expression of cytokines in the endometrium, the dynamics of endometrial MAIT cells, and the immune cells in decidua during early pregnancy loss. I have maintained a consistent approach throughout my research utilizing human samples and establishing well-defined study cohorts. In study II, we compared both laboratory strains of bacteria and samples obtained from clinical patients with infection. This approach allowed us to obtain results that are applicable to a larger population, ultimately enhancing the generalizability of my findings.

There are, however, some methodological considerations to be discussed. An initial power calculation was not performed for any of the studies. This is related to the inherent difficulty in defining clear primary and secondary endpoints in explorative research. However, I tried to address statistical significance and sample sizing by choosing proper statistical analysis for the type of data, reducing type I errors by adjusted p-values and adjusted significance levels.

The study population of study I and II providing menstrual blood and endometrial tissue samples, was meticulously chosen and represents a significant proportion of women of reproductive age. To ensure the integrity of the results, women who had previously been pregnant or had ever used an IUD were excluded from the study, as they may have an altered inflammatory cell and cytokine composition. However, it should be noted that it is not possible to rule out that some study participants may had been pregnant unknowingly. The assessment of tissue-residency in the two women who had undergone uterus transplantation is complicated by the fact that they were on immunosuppressant medication, and that the time point for evaluation was relatively long after the transplantation procedure. It is possible that this could have affected our findings. Nevertheless, despite this limitation, the clinical setting presented a unique opportunity to gain novel insights into the origin of eMAIT cells.

The inclusion of women with pregnancy of unknown location (PUL) in our study population for study III may potentially compromise its accuracy, as some of them may have already undergone an abortion prior to the emergency visit. This could result in an inaccurate representation of the intended study population. Another factor that can cause bleeding in early pregnancy is extrauterine pregnancy, which could have been an interesting separate group to include in our study.

The use of a menstrual cup as a collection method for menstrual blood may result in potential contamination of the sample with cells, cytokines, and other substances from the vaginal tract. Nevertheless, we chose to collect menstrual blood within the first 24 hours of menstruation, as previous research has demonstrated that the highest number of live mononuclear cells are typically observed during this time frame. This method has

also shown strong consistency with regards to immune cell composition and cytokine levels in the endometrium during the late secretory phase and early menstruation (165, 166).

We used the bead-based Luminex immunoassay when detecting cytokines in menstrual blood. An alternative method would have been to use enzyme linked immunosorbent assay (ELISA). ELISA and Luminex are both immunoassay techniques used to detect the presence of specific antigens in a biological sample. However, there are some key differences between the two methods. ELISA is an enzyme-linked immunosorbent assay that uses a colorimetric substrate to detect a target molecule. ELISA is generally simpler and less expensive than Luminex, but it is also less sensitive and less flexible in terms of the number of targets that can be measured simultaneously. Luminex, on the other hand, is a bead-based multiplex immunoassay that uses fluorescent microspheres to detect multiple target molecules simultaneously. Luminex is more sensitive and flexible than ELISA, now allowing the detection of up to 100 targets in a single sample. Luminex is also faster than standard ELISA, with results typically available within a few hours, compared to the overnight incubation required for ELISA. The time, sensitivity and multiplexing aspects were key when we selected the Luminex technique.

The use of enzymatic tissue digestion has been recognized as a confounding factor in studies involving tissue-resident cells, resulting in a reduction of certain molecule's surface abundance (167). In both study II and III, we resorted to enzymatic disintegration of endometrial tissue and vaginal blood to obtain sufficient quantities and high-quality cell numbers that could not be acquired through mechanical means alone. However, all endometrial tissue from study II and the vaginal blood samples from study III, were treated equally. While it is possible that enzymatic digestion could have caused bias when comparing endometrial to peripheral blood cell phenotype, it seems less probable that this factor can account for similarities or differences in phenotype between endometrial tissue from pre- and postmenopausal women or decidua, or in vaginal blood between women who subsequently miscarried compared to those who did not.

8 CONCLUSIONS

Based on the studies included in this thesis, I have made the following conclusions.

- The cytokine profile of menstrual blood is markedly distinct from that of peripheral blood and demonstrates consistency across repeated menstrual cycles.

- Endometrial MAIT cells exhibit a phenotypically activated state and demonstrate a tissue-resident profile.

- Endometrial MAIT cells represent a population that is transiently tissue-resident and are replenished from the circulation over time.

- The phenotype and size of the endometrial MAIT cell population remain largely unchanged during endometrial regeneration throughout the menstrual cycle and pregnancy, as well as endometrial atrophy following menopause.

- Endometrial MAIT cells are capable of responding to *N. gonorrhoeae*.

- Vaginal blood from women with early pregnancy bleeding that subsequently miscarry, contain significantly higher levels of tissue-resident decidual NK cells.

9 POINTS OF PERSPECTIVE

Despite a growing interest in the immunology of the uterine mucosa and its significance for health and disease in this compartment, our understanding of this intricate system remains limited.

Based on the current knowledge in this field, the proinflammatory process of decidualization is necessary for sufficient implantation of an embryo. However, in the course of the first trimester, all immune cell types within the decidua undergo profound phenotypic changes to maintain immune tolerance. The precise mechanisms behind this modulation are not yet fully understood, but likely involve immunosuppressive signaling from T cells, macrophages, trophoblast, and stromal cells. Deviations from these processes may contribute to pathophysiological conditions like recurrent implantation failure and miscarriage. Non-invasive collection of vaginal blood from women with threatened miscarriage has provided valuable information on cells from the fetomaternal interface, revealing clear differences between those who miscarried and those who continued with their pregnancy. Future research should focus on characterizing vaginal blood NK cells, macrophages, and T cells with more detailed phenotypical markers and conducting functional experiments to identify potential immune cell pool aberrations in women who experience miscarriage.

Furthermore, this thesis demonstrates that utilizing menstrual blood for the analysis of cytokines and immune cells in the secretory endometrium is a reliable and non-invasive method. Therefore, this approach should be considered for future studies with the aim to compare menstrual blood cytokine and immune cell profiles among women with conditions such as endometriosis, abnormal uterine bleeding, or recurrent miscarriages, who are likely to exhibit an abnormal cytokine and/or immune cell profile. The cytokine profile in menstrual blood holds significant potential as diagnostic tool, particularly for conditions like endometriosis where women currently undergo surgery for proper diagnosis. Implementation of menstrual blood testing for diagnosis could save healthcare systems considerable resources and minimize the risk of complications for patients.

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