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NATURAL KILLER CELL PROFILING IN WOMEN WITH RECURRENT PREGNANCY LOSS

Denise Habets

NATURAL KILLER CELL PROFILING IN WOMEN WITH RECURRENT PREGNANCY LOSS

DENISE HENRIËTTA JOSEPHINA HABETS

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NATURAL KILLER CELL PROFILING IN WOMEN WITH RECURRENT PREGNANCY LOSS

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. dr. Pamela Habibovic,
volgens het besluit van het College van Decanen,
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op 15 september 2022 om 10:00 uur

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1

GENERAL INTRODUCTION

WHEN PREGNANCIES ARE LOST RECURRENTLY

Being pregnant is very special and many couples will probably be dreaming a lot of how they will love their baby when confronted with a positive pregnancy test, but not how to cope with losing it. No heartbeat, empty sac, incomplete attachment. Pregnancy loss is so personal, so confusing and so devastating, even more so when it happens recurrently. Recurrent pregnancy loss (RPL) is a distressing pregnancy disorder experienced by 1-3% of couples trying to conceive[1]. RPL is differently defined by current guidelines of the Nederlandse Vereniging voor Obstetrie en Gynaecologie[2] (NVOG), the American Society for Reproductive Medicine[3,4] (ASRM) and the European Society of Human Reproduction and Embryology[5] (ESHRE).

RPL generally involves the failure of two or more clinically recognized pregnancies before 20-24 weeks of gestation. However, criteria amongst weeks of gestation and having multiple miscarriages with no previous successful pregnancy or having multiple miscarriages after having experienced at least one successful pregnancy, differ among guidelines (Table 1)[6].

These differences in criteria among guidelines can partly be explained by the fact that guidelines of the NVOG and the ASRM are primarily consensus-based and developed by groups of gynecologists and guidelines of the ESHRE are primarily supported by literature and additional input of experts from other fields such as genetics and internal medicine. However, a lack of standardized definitions, the highly variable clinical interpretation in addition to the uncertainties surrounding the pathogenesis hamper progress in predicting and preventing RPL. Prediction of an ongoing pregnancy is mainly based on maternal age and the number of previous losses. Maternal age is considered to be the strongest predisposing factor for pregnancy losses, as chances of pregnancy loss are 9% for women between 20 and 24 years, but increase to more than 50% for women aged 42 and even up to 75% for 45-year-old women[7]. Moreover, the number of previous losses appears to be an independent factor for pregnancy losses. However, defining RPL as a clinical entity that requires diagnostic testing, not only depends on knowledge of the risk for subsequent pregnancy loss but also on the probability of finding a related etiology[8].

Pathogenesis of pregnancy loss can roughly be classified in four etiologies and include chromosomal abnormalities, anatomical uterine anomalies, thrombophilia and endocrinologic dysfunction. Carriership of a balanced structural chromosome abnormality, such as a translocation or an inversion that does not affect the parent itself, can cause problems when an unbalanced chromosome pattern arises in the fetus. This mainly concerns numerical chromosomal aberrations in 86% of cases such as trisomy, polyploidy and monosomy X, as well as structural chromosomal aberrations in 6% of cases and other

abnormalities such as mosaicism in 8% of cases[9,10]. Balanced structural chromosomal abnormality is partly related to the maternal age at which the second pregnancy loss occurred and to the couple's family history of pregnancy losses[11]. Anatomical uterine anomalies, such as congenital uterine anomalies including septate uterus and bicornuate uterus, have been hypothesized to lead to pregnancy loss as a consequence of implantation taking place at a less vascularized site[12,13,14]. Both acquired and hereditary thrombophilia are related to recurrent pregnancy loss. Acquired thrombophilia referred to as the antiphospholipid syndrome, is characterized by recurrent pregnancy loss, pregnancy-related morbidities such as preeclampsia and fetal growth restriction, and venous or atrial thrombosis in combination with the presence of lupus anticoagulant or cardiolipin antibodies[15]. Women with hereditary thrombophilia factors, such as antithrombin III, protein C and S deficiency, Factor V Leiden, prothrombin mutation and elevated factor VIII, have a higher risk of pregnancy loss than female relatives without hereditary thrombophilia[16,17] as decreased action of these anticoagulants have been postulated to increase the risk of clotting in the developing placenta. Endocrine dysfunction (e.g., hypothyroidism) has been found to be associated with RPL, as thyroid hormones are essential for fetal development and thyroid hormone disorders and the presence of increased thyroid peroxidases antibodies support an important role in pregnancy loss[18]. Unfortunately, despite extensive investigations of these underlying genetic, uterine, trombophilic and endocrinological etiologies, still 50% of RPL cases remain unexplained[19]. However, new insights show that during pregnancy, the maternal immune system needs to make specific adaptations in order to tolerate a semi-allogenic fetus and to support and protect it for growth in the womb[20]. Therefore, RPL might be linked to problems with the way the immune system adjusts to early pregnancy (Figure 1).

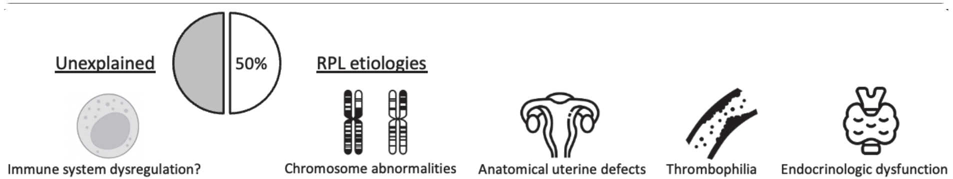

Figure 1 | Schematic representation of RPL etiologies that can explain roughly 50% of RPL cases; including genetic, uterine, thrombophilic or endocrinological factors. Other unexplained cases might be related to immune system dysregulation.

Table 1 | Comparison of (inter)national guidelines on recurrent pregnancy loss

Guideline	**NVOG**
Year of publication	2007
Country/Continent	The Netherlands
Definition	2 or more objectified pregnancy losses
Types of pregnancy	Clinical pregnancies. Extra-uterine, molar and biochemical pregnancies not included in the definition
Weeks of gestation	Up to 20 weeks
Consecutive	Consecutive or nonconsecutive
Risk factors	Maternal age, cytogenetic factors, endocrine factors, uterine factors, antiphospholipid syndrome, thrombophilia, hyperhomocysteinemia, lifestyle factors
Investigations	No thyroid screening -
Considerations	Lifestyle advice on losing weight (examine BMI) and reducing smoking Hyperhomocysteinemia: Vitamin supplementation with B6, B12 and folic acid APS: combination of prophylactic dose of unfractionated heparin and low-dose aspirin

ASRM	ESHRE
2012-2013	2017
United States of America	Europe
2 two or more failed clinical pregnancies	The loss of two or more pregnancies
Clinical pregnancies documented by ultrasonography or histopathological examination	Clinical pregnancies after both spontaneous conception and ART. Ectopic and molar pregnancies as well as implantation failure not included in the definition
Not mentioned	Up to 24 weeks
Consecutive	Consecutive or nonconsecutive
Genetics, hormonal or metabolic factors, anatomical factors, antiphospholipid syndrome, thrombophilia, lifestyle, environmental and occupational factors, infectious factors, male factors, psychological factors, and alloimmune factors	Age (female), genetic factors, endocrine factors, anatomical factors, antiphospholipid syndrome, lifestyle factors preceding pregnancy losses and embryonic factors
Thyroid screening	Thyroid screening (TSH and TPO)
Sperm DNA fragmentation not recommended	Sperm DNA fragmentation can be considered
-	Lifestyle advice on diet, smoking and alcohol
Thyroid dysfunction, diabetes and hyperprolactinemia should be treated	Hyperprolactinemia: Vitamin D supplementation and bromocriptine
APS: combination of prophylactic dose of unfractionated heparin and low-dose aspirin	APS: combination of prophylactic dose of unfractionated heparin and low-dose aspirin

AN IMMUNOLOGICAL PERSPECTIVE ON PREGNANCY

Pregnancy has three stages or trimesters, each of which is marked by specific fetal developments and is confronted with different immunological challenges[20] (Figure 2).

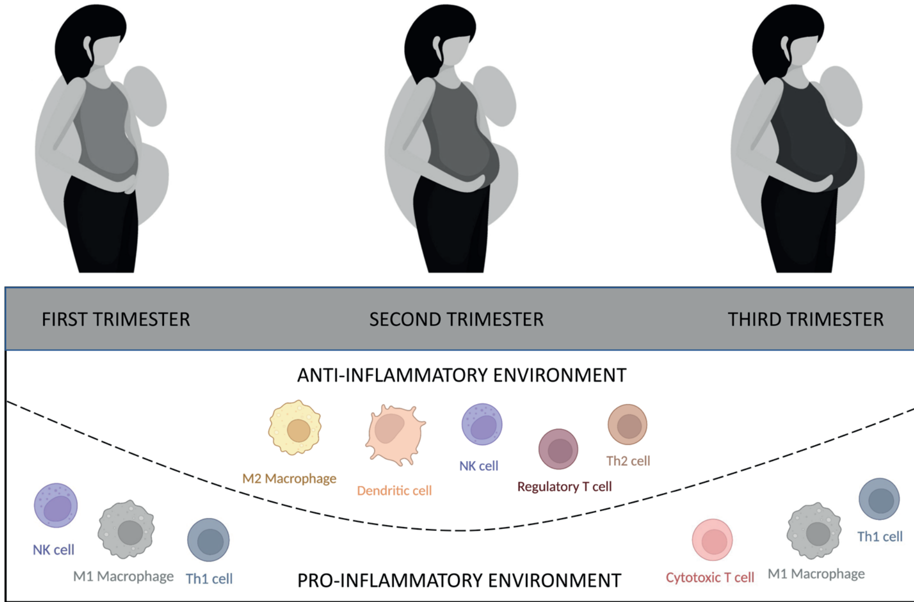

Figure 2 | Three immunological stages of pregnancy and the distribution of their immune cell subsets. The first trimester ensures a balanced pro-inflammatory and anti-inflammatory environment for blastocyst implantation and placentation. The increasingly anti-inflammatory environment is predominantly during the second trimester to ensure placental and fetal growth. The third trimester is characterized by a gradually regaining pro-inflammatory environment which is required for initiating parturition.

During each menstrual cycle the uterus undergoes a series of structural and functional changes tightly regulated by ovarian hormones, and characterized by growth and proliferation (proliferative phase), followed by differentiation and increased secretions (secretory phase) of endometrial tissue. After successful development of a dominant ovarian follicle from one of the ovaries, an ovum is released into the fallopian tube, triggered by a surge in luteinizing hormone (LH) halfway the menstrual cycle. If fertilization does not occur, the ovum moves down the fallopian tube to the uterus, where it degenerates, and passes through the uterus with the next menstrual period[21]. When the ovum is fertilized, it is called a zygote and divides repeatedly as it moves down the fallopian tube into the uterine cavity. The zygote quickly becomes a solid structure of

cells which then transforms in a hollow sphere of cells called a blastocyst. The blastocyst attaches to the lining of the uterus, i.e., the decidua and proceeds into the process of implantation[22]. At the end of the first trimester the inner cells of the implanted blastocyst have developed into the embryo and the outer cells have started to form the placenta[23]. The placenta is of crucial importance as it produces several hormones, such as human chorionic gonadotropin (hCG), that help maintain the pregnancy and stimulate the ovaries to produce estrogen and progesterone continuously[24]. This first trimester is associated with a tightly regulated balance of a pro-inflammatory and anti-inflammatory environment[20] responsible for a receptive endometrium on the one hand and active breakdown and restructuring of the decidua during implantation on the other hand, characterized by the presence of inflammatory cytokines and chemokines such as interleukin (IL)-6, IL-8 and IL-15, MIP1β and tumor necrosis factor (TNF) derived from Th1 cells and macrophages with a M1 phenotype[25]. Although this phase of tissue remodeling requires some degree of inflammation, it needs to be tightly regulated to guarantee sufficient immunological tolerance to enable implantation and subsequent development of the embryo.

The embryo, after 12 weeks called fetus, continues to develop and the placenta forms tiny hairlike projections called villi that extend into the wall of the uterus in a treelike arrangement[26]. This process involves the invasion of fetal trophoblast cells in the decidua and remodeling of the maternal spiral arteries in order to support exchange of nutrients, gas and waste between the mother and the developing fetus[27,28]. Furthermore, it allows placental transfer of maternal immunoglobulin G (IgG) antibodies to the fetus, which is an important mechanism that provides protection to the fetus while his/her humoral response is inefficient[29]. A thin membrane separates the blood of the fetus in the villi from the maternal circulation, that additionally needs to adapt to accommodate increased blood flow to the uterus by increasing cardiac output, stroke volume and heart rate and by decreasing blood pressure and peripheral resistance[29]. Although immunity against pathogens needs to be maintained, the process of ensuring placental and fetal development prior to and during the second trimester is associated with a Type-II and anti-inflammatory dominated microenvironment[20] and previous studies have demonstrated that specifically decidual leukocytes including macrophages, T cells, dendritic cells and natural killer (NK) cells regulate immune balance in the decidua during placentation[30] (Figure 3). Amongst these cells are macrophages with a M2-type phenotype associated with tissue renewal that are present near the spiral arteries, where they induce tissue breakdown and promote vascular remodeling by secreting cytokines, chemokines, proteases, and angiogenic factors[31,32] and prevent detrimental inflammatory conditions by removal of apoptotic cell debris formed during tissue remodeling[33]. Although to a lesser extent, T cells are also present in early pregnancy decidua and they facilitate healthy pregnancy by promoting Th2-type immunity (Th2 cells)[34]. In addition, regulatory T cells are

generated during pregnancy that promote immunological tolerance and prevent excessive inflammation through various mechanisms including the production of suppressive factors like IL-10 and TGFβ. Murine studies demonstrated that these are crucial for fetal survival[35,36]. Dendritic cells are least abundant at this fetal-maternal interface but have been shown to induce Th2 and regulatory T cells contributing to immunological tolerance[38]. In addition, they can promote decidual NK cell activation and proliferation, thereby contributing to enhanced placentation[39,40]. Surprisingly, decidual NK (dNK) cells constitute up to 70% of all leukocytes present in the decidualized endometrium during the first trimester[41] which is in remarkable contrast to peripheral blood where they represent only 5-15% of the circulating leukocytes[42]. In the first and subsequently second trimester, they provide a microenvironment in the decidua that is seemingly pregnancy compatible, by inducing tolerance through direct binding to, amongst others, human leukocyte antigen (HLA)-G, HLA-E and HLA-C on trophoblast cells[43] and by secreting cytokines, chemokines and angiogenic factors, supporting healthy placentation[44].

By 18 to 20 weeks, the placenta is fully formed and in close contact with the maternal circulation, but it continues to grow during the third trimester carrying oxygen and nutrients from mother to the growing fetus and waste materials from fetus to mother until the pregnancy is full term[26]. During this last trimester NK cell numbers decrease[43] and gradually there is a transition to a pro-inflammatory environment, which seems to be driven by the invasion of cytotoxic T cells that release proinflammatory mediators such as perforin and granzyme B[45]. Moreover, this pro-inflammatory environment is preceded by an accumulation of Th1 cells[46] and macrophages with a M1-type phenotype[47] ultimately initiating parturition by the nuclear factor-κB (NF-κB) signaling pathway[48,49].

In conclusion, pregnancy is a process that engages at least three consecutive phases that each have unique inflammatory environments. A successful pregnancy depends on the ability of the immune system to change and adapt to each specific developmental stage[20]. Moreover, it concerns a dynamic interplay between decidual immune cells, stromal cells and trophoblast cells, generating different hormones, enzymes, cytokines and mediators such as prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO)[50] which in turn can influence immune cell function when establishing a successful pregnancy.

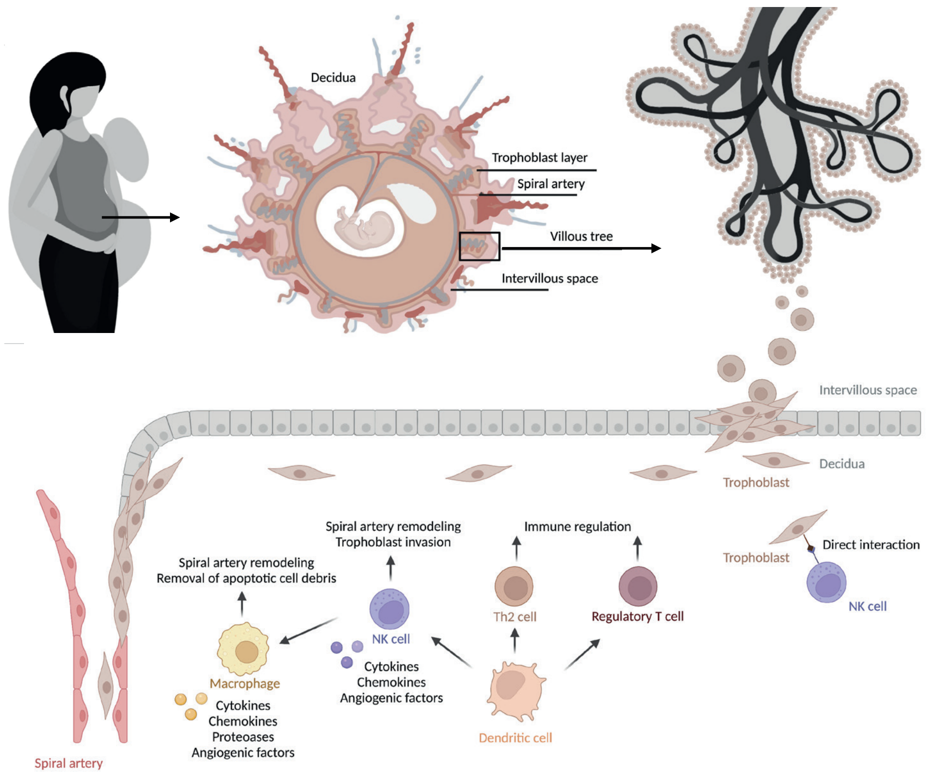

Figure 3 | The crosstalk between invading trophoblasts and decidual immune cells via cell–cell direct interaction (further elaborated for NK cells in chapter 2) and soluble factors contributes to an anti-inflammatory environment in early pregnancy. The anti-inflammatory environment is created by a collaboration between macrophages, T cells, NK cells and dendritic cells, that work together to promote immunological tolerance, in this way ensuring fetal growth and healthy placentation.

NATURAL KILLER CELLS IN EARLY PREGNANCY: TO KILL OR NOT TO KILL

The immune system is of great importance for successful pregnancy. Especially in early pregnancy after successful implantation, the maternal immune system ensures an anti-inflammatory environment for fetal growth and placentation[1]. Of all immune cells, the abundantly present dNK cells have been shown to pose an important task to promote healthy placentation by regulating spiral artery remodeling and trophoblast invasion, in this way contributing to a successful pregnancy[44].

Regulation of spiral artery remodeling by dNK cells

Remodeling of the maternal spiral arteries, transforming the arteries into high conductance vessels with laminar flow, is an essential vascular adaptation to pregnancy[51]. The maternal spiral arteries are closely surrounded by immune cells, in particular dNK cells, that may assist in this remodeling process through secretion of proteolytic matrix metalloproteinases (MMPs)[52]. MMPs contribute to the homeostasis of many tissues by degradation of components of the extracellular matrix and participate in processes such as tissue remodeling and angiogenesis[53]. MMP-7, MMP-9, MMP-19, and MMP-23 have been shown to be expressed by NK cells when measured histologically in human decidual tissue[52,54,31]. Subsequently, remodeling depends on the invasion of extravillous trophoblast (EVT) cells into the vascular wall of the maternal spiral artery which leads to replacement of vascular smooth muscle cells with fibrinoid material, hereby maintaining the maternal spiral artery in a dilated state impeding vasoconstriction[55,56,57]. Although there is great divergence between human and murine pregnancy both anatomical and physiological, mice and rats have provided useful models to study trophoblast and NK cell regulation of placental development in early pregnancy, as all species feature hemochorial placentation[58,59,60]. Essential for spiral artery remodeling in murine pregnancy is interferon gamma (IFN-γ) secreted by dNK cells[61]. Through the utilization of alymphoid mice, which were engrafted with bone marrow from IFN-$\gamma^{-/-}$ mice, or severe immunodeficient mice, lacking T cells and B cells, it has been shown that IFN-γ of dNK cell origin is essential for remodeling of murine spiral arteries but the exact mechanisms have not yet been elucidated[61]. However, as dNK cells produce IFN-γ and macrophage Inflammatory Protein-1 alpha (MIP-1α), which are key cytokines involved in macrophage activation and macrophages are similarly in close proximity of spiral arteries, dNK cells possibly mediate vascular remodeling by stimulating macrophages[62,63]. Furthermore, NK cell-deficient mice consistently have been shown to have narrow vascular lumens, thick vascular walls, and retention of vascular smooth muscle actin, all characteristics of defective decidual vascular remodeling[64,65,66]. This indicates that, even among species that feature hemochorial placentation, there is dependence upon dNK cells and trophoblast cells for remodeling spiral arteries.

Regulation of trophoblast invasion by dNK cells

dNK cells might also contribute to regulation of trophoblast invasion through their influence on EVT cells. A recent study demonstrated that human dNK cells produce the chemokines X-C Motif Chemokine Ligand 1 (XCL1) and C-C Motif Chemokine Ligand 1 (CCL1), and that the receptor for XCL1, X-C Motif Chemokine Receptor 1 (XCR1), is expressed by EVT cells[68]. Furthermore, it has been demonstrated that dNK cells secrete several factors that enhance motility of trophoblast cells in vitro in cell migration and invasion assays, including the chemokines interleukin 8 (IL-8) and interferon-inducible protein (IP-10)[67,68,69,70,71]. Regulation of trophoblast invasion requires establishment of a tight balance between proper invasion without rejection on the one hand and troublous over-invasion into the decidua on the other hand. dNK have also been shown to secrete transforming growth factor beta (TGF-β), which seems to inhibit the invasiveness of trophoblast cells[72,73]. It has also been suggested that, dNK cells prevent EVT cells from over-invading as NK cell depletion has been shown to lead to a decrease of oxygen tension at the placentation site, stabilization of hypoxia-inducible factor 1A protein, and direction of trophoblast differentiation to an invasive phenotype[74]. On the contrary, these mechanisms of inhibiting over-invasion will most likely be mediated by enhancing motility mechanisms such as the production of growth factors including vascular endothelial growth factor (VEGF) and placental growth factor (PLGF)[75,76,77] and human dNK have been shown to secrete several growth factors including VEGF, PLGF and angiopoietin 1 and 2[68,70,73]. In this way, human dNK cells apparently mediate a delicate balance between stimulating and impeding EVT cell invasion.

Regulation of NK cell effector function

It is hypothesized that NK cells have multiple functions during pregnancy. To begin with, NK cells mediate immune tolerance by stimulating angiogenesis for spiral artery remodeling and by stimulating trophoblast invasion. Moreover, they mediate immune responsiveness by inhibiting invasion of trophoblast as a protective mechanism against over-invasion and by providing defense against foreign pathogens. These seemingly different functions of NK cells, immune tolerance and immune responsiveness, are however both active processes of either unresponsiveness or responsiveness to specific antigens that require NK cell activation. How exactly these different functions of NK cells are regulated during pregnancy is unfortunately largely unknown.

We do know that, in the periphery, NK cells have a broad receptor repertoire to regulate their activity by providing activating or inhibiting signals upon binding with a ligand. The balance of these activating and inhibiting signals determines whether NK cells become activated or not[78]. Inhibitory receptors are important to maintain NK cell tolerance for healthy cells as they set the threshold for NK cell activation by providing an inhibitory signaling cascades upon binding to molecules that are typically abundantly expressed on

healthy cells or in tissues characterized by immunological tolerance and that lead to the eventual suppression of NK cell activation[79]. Interaction of activating receptors with their ligands triggers an activating intracellular signaling cascade ultimately leading to cytokine production or cytotoxicity[80]. Activating receptors expressed on peripheral blood NK cells frequently interact with ligands (e.g, stress molecules or virus-associated molecules) that are expressed on low levels on healthy cells while they are abundantly present on diseased cells[79]. In addition, there is the activating CD16 receptor, which mediates antibody-dependent cellular cytotoxicity (ADCC) in response to IgG[81].

NK receptors can be divided into different receptor families, such as the family of killer-cell immunoglobin-like receptors (KIR) including KIR2DL1, KIR2DL2/3, KIR3DL1 and the family of c-type lectin-like receptors including NKG2A, NKG2C and NKG2D[82]. Moreover, there is a family of natural cytotoxicity receptors, a group of activating receptors that are solely expressed on NK cells including NKp30, Nkp44 and NKp46[83]. Additionally, there is a leukocyte immunoglobulin-like receptor family including LILRB1 and a family of immune checkpoint receptors, regulating the degree of immune activation playing a positive role in inducing tolerance, including PD1, TIM3, LAG3, TIGIT and TACTILE[84]. The integrated signals from both activating and inhibitory receptors regulate pNK cell activity, which is manifested by direct cytotoxicity through the release of perforin and granzyme granules, through production of cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF) or by ADCC.

NK cell subpopulations can be defined on basis of the relative expression of CD56 and CD16. Most pNK cells resemble a $CD56^{dim}CD16^{+}$ phenotype with high cytotoxicity capacities, as they contain much more perforin, granzymes and cytolytic granules and the high expression level of CD16 makes them efficient mediators of antibody-dependent cellular cytotoxicity[85,86]. Interestingly, in the uterus, NK cells from non-pregnant endometrial or pregnant decidual tissue are seemingly different from pNK cells both phenotypically and functionally. Unfortunately, our knowledge about endometrial NK (eNK) cells and dNK cells is rather limited as their sampling usually involves invasive sampling of uterine biopsies. Hence, collection of menstrual blood has been explored as an alternative, less invasive method for analysis of eNK cells[87]. Nevertheless, we know that most eNK and dNK cells exhibit a $CD56^{bright}CD16^{-}$ phenotype, which is associated with lower cytotoxicity[85,88]. Although they contain some granules, they are the most efficient cytokine producers and therefore they are hypothesized to have more immunoregulatory properties[89,90]. Thanks to new methods such as single cell RNA sequencing, unique properties of dNK cells have been identified: Recently three tissue resident dNK cell subsets (dNK1, dNK2 and dNK3) were identified by profiling the transcriptomes of about 70,000 single cells from first-trimester placentas with matched maternal blood and decidual cells[90]. These subsets seem to differ in terms of phenotype, as dNK1 cells show the greatest expression of KIRs

(KIR2DS1, KIR2DS4, KIR2DL1, KIR2DL2, and KIR2DL3), NKG2A and LILRB1 compared to dNK2 and dNK3[90]. Probably they also differ in terms of effector functions, for example there might be additional differences in the number of granules, granzymes or perforin these subsets contain or in the production of cytokines. However, robust functional analyses of dNK cells are still lacking.

Slowly it is becoming clearer that there are many more different NK cell subsets than originally thought[91,92]. Possibly, the presence of diverse subsets, each with its own function, is the way that NK cells can fulfill all those seemingly contradictory roles. Furthermore, these different subsets might be dynamically driven by their interaction with other cells and with factors from their micro-environment and might therefore not be present at all times. There might be substantial differences between a non-pregnant and a pregnant state or even between the different trimesters during pregnancy and we can envision that the presence of specialized subsets at specific timepoints is partly the way in which their different functions might be regulated during pregnancy.

NATURAL KILLER CELLS AND RECURRENT PREGANCY LOSS

For pregnancy to be successful, different immune effector functions need to be tightly controlled in order to trigger production of cytokines, chemokines and growth factors and prevent unwanted immune reactivity against cells of fetal origin[93]. Although the contributions of NK cells have been seemingly recognized, their exact role in recurrent pregnancy loss has not been completely elucidated. Possibly, a poorly balanced regulation of the various effector functions of NK cells may play a role in RPL and, amongst other factors, might be dependent on different activating and inhibitory signals regulated by NK cell receptors, as schematically presented in Figure 4.

Partly for this reason, several studies have investigated a relationship between numbers and/or phenotypes of NK cells and RPL. Unfortunately, results of these studies are contradictory, due to heterogeneity by studying distinct subsets or receptors, in various tissues, with different techniques, often using diverse control groups.

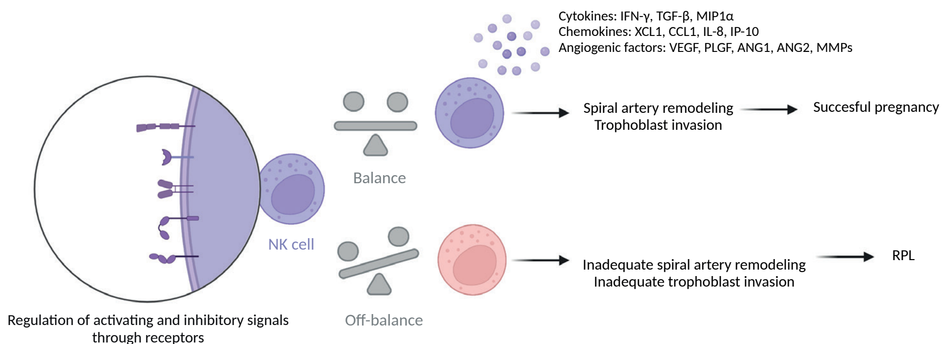

Figure 4 | There needs to be a tight balance between activating and inhibitory signals in order to trigger production of cytokines, chemokines and growth factors and prevent rejection of the fetus. Disruption of this balance might result in RPL.

Additionally, it could be that NK cells need to be investigated in a more comprehensive way, given the complexity of NK cell biology. Furthermore, we might not yet fully know the causal relationships between NK cells and RPL and the rise of new phenotypes and alternative receptors that have not been investigated or only limited, might play a role in RPL of which we are not yet aware. Moreover, pNK cells are different from NK cells in the uterus so their location of sampling might be a very important determinant. Additionally, there might also be factors alongside the immune system that could play an important role in their regulation, such as hormones from the pituitary glands (e.g., luteinizing hormone and follicle-stimulating hormone) and ovaries (e.g., estrogen

and progesterone) or metabolic and cardiovascular factors, particularly abnormal constituents linked to RPL. Altogether, their standard profiling might need to be extended. **Therefore, the aim of this thesis is to profile NK cells in women with RPL.**

Understanding the immunologic pathogenesis of RPL is very much needed for providing appropriate care and development of future treatment strategies and knowing what is the most beneficial way of profiling NK cells in women with RPL, could benefit their diagnosis. In addition, it could provide direction to more mechanistical studies to better understand the exact role of NK cells in RPL and to develop possible new treatment strategies and selecting women whose pregnancy losses may be more likely to respond to specific treatment strategies, as it could very well be that treatment will only work for a subgroup of women with RPL.

SCOPE

The work outlined in this thesis is dedicated to profiling of NK cells in women with RPL. **Chapter 1** provides a general introduction into the clinical problem of RPL, the physiology of the immune system during pregnancy, the importance of NK cells in early pregnancy and the pathology of NK cells in RPL. **Chapter 2** gives a concise overview of available literature on profiling NK cell receptors measured in different tissues by different laboratory techniques in women with RPL versus controls. To investigate whether NK cell phenotypic profiles are influenced by timing of sampling and location of sampling, **chapter 3** examines the influence of the menstrual cycle on peripheral NK cell phenotype profiles and investigates whether phenotypic profiles are different between NK cells in peripheral blood and NK cells sampled in menstrual blood. **Chapter 4** gives a description of the OVIDE protocol which was initiated to assess NK cell phenotype profiles in women with RPL and women with a previous uncomplicated pregnancy. Immune checkpoint receptors have not been thoroughly studied in relation to RPL. But they have proven to be involved in dampening immune responses in the immunosuppressive tumor microenvironment hence they may be important to establish fetal-maternal tolerance in healthy pregnancy as well. Therefore, **chapter 5** investigates whether NK cell phenotypic profiles of women with RPL are different from women with a previous uncomplicated pregnancy through comparing subsets and receptor repertoire, including immune checkpoint receptors, and studying their associations with the severity of RPL. For some receptors it is known that their function is influenced by polymorphisms, which could be of additional value for NK cell profiling. Therefore **chapter 6** studies the p.V176F polymorphism and its relation to CD16 expression and HLA antibody status in RPL, as this could have a possible influence on NK cell antibody-dependent cellular cytotoxicity. **Chapter 7**, researches the additional influence of the metabolic and cardiovascular system in RPL, as NK cell orchestrated spiral artery remodeling during early pregnancy might be indirectly influenced by maternal cardiovascular or metabolic status. As NK cell interventions are being offered to women with RPL **chapter 8**, describes the effectiveness of intravenous immune globulin (IVIG) treatment on live birth rate in women with RPL and an underlying immune condition. Finally, **chapter 9** provides a general discussion followed by a description on the impact of this thesis in **chapter 10** and a summary in **chapter 11**.

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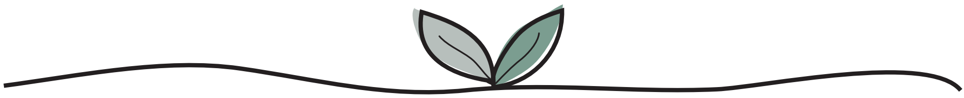

2

NATURAL KILLER CELL RECEPTORS IN RECURRENT PREGNANCY LOSS

Denise Habets, Amber Lombardi, Sanne Claassens,
Marc Spaanderman, Salwan Al-Nasiry, Lotte Wieten

ABSTRACT

Background: Natural Killer cells are important for supporting healthy pregnancy and aberrant receptor expression levels have been associated with recurrent pregnancy loss (RPL).

Design: In the present mini-review, we provide a literature overview of studies investigating the association of activating- and/or inhibitory NK cell receptors with RPL. To this end, we shortly describe the function of NK cell receptors that regulate NK cell function, their possible role in healthy pregnancy and by systematically summarizing literature we outline the outcome of studies comparing receptor expression in RPL vs control groups.

Conclusion: In conclusion, this shows that especially receptors belonging to the killer cell receptor (KIR) family have been studied in the context of RPL. For the activating receptors most studies investigated the association between RPL and natural cytotoxicity receptors (NCR). Although they have been shown to play an important role in maintaining immunological tolerance in other tissues, studies investigating inhibitory immune checkpoint receptors e.g., PD1, TIM3, LAG3 are almost completely lacking.

Keywords: Recurrent Pregnancy Loss, Natural Killer cell, receptor.

2

BACKGROUND

Profiling Natural Killer (NK) cell phenotypic profiles by receptor repertoire may provide concurrent detail on NK cell functional status as the balance of signals from these receptors determines whether an NK cell becomes activated or not[1]. Decidual NK cells (dNK) have an important dual task during pregnancy, maintaining a fine-tuned regulated balance between immune tolerance to the semi-allogenic fetus and still being able to defend pathogens[2]. Concurrently, phenotypic profiling of dNK cells and their precursors, being either peripheral NK (pNK)[3] or endometrial NK (eNK)[4] cells could provide relevant information on NK cell functional status in women with successful pregnancies and recurrent pregnancy loss (RPL). Here, we would like to provide an overview on studies conducted on activating and inhibitory NK cell receptors that regulate NK cell function (Figure 1), focusing on 5 major NK cell receptor families, by shortly describing their function and their possible role in controlling NK cell function in healthy pregnancy. By systematically summarizing studies investigating the association between receptor expression in woman with RPL versus controls, we aim to provide an overview of current knowledge on the association between RPL and NK cell receptor expression.

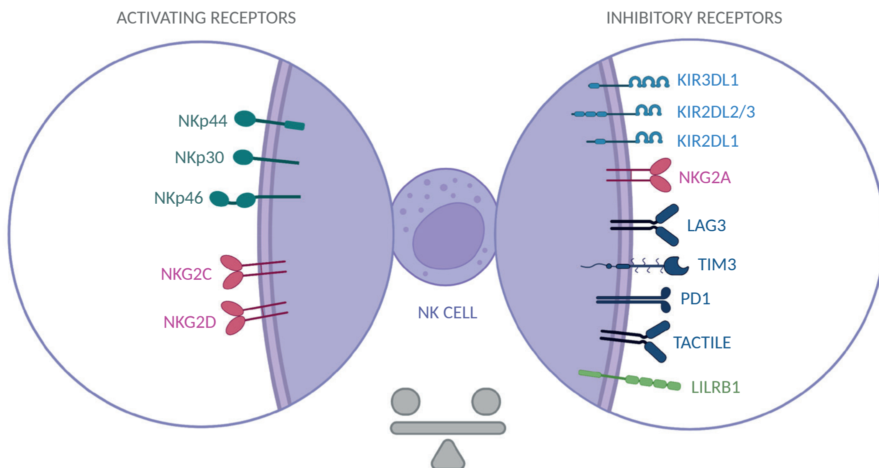

Figure 1 | Both activating and inhibitory receptors, of which a selection relevant for chapter 3 and 5 is depicted here, can transduce positive or negative signals and a fine-tuned balance between these signals regulates NK cell function.

THE KILLER CELL IMMUNOGLOBULIN RECEPTOR FAMILY

Function

The human killer cell immunoglobulin-like receptors (KIR) belong to a family of transmembrane glycoproteins expressed on NK cells that can transduce either activating or inhibitory signals. KIRs contain either two or three immunoglobulin-like domains (2D for two domains, 3D for three domains) with either long or short cytoplasmic tails (L for long-tailed and S for short ones)[5]. Long-tailed receptors possess immunoreceptor tyrosine-based inhibition motifs (ITIMs), which contribute to inhibitory signaling[5]. KIRs that belong to this group of inhibitory receptors are KIR2DL1, KIR2DL2, KIR2DL3 and KIR3DL1 amongst others. In contrast, short-tailed receptors are associated with a transmembrane signaling adaptor protein (DAP12), containing immunoreceptor tyrosine-based activation motifs (ITAMs), including KIR2DS1, KIR2DS2, KIR2DS3 and KIR3DS1[6]. One exception to this is KIR2DL4, which expresses an ITIM but also associates with ITAM-containing FceRI-c adaptors, hereby having the capability to both activate and suppress NK cell function[7].

Role of KIRs in healthy pregnancy

The interactions between KIRs expressed by maternal dNK cells and HLA-C and HLA-G molecules expressed by fetal invading extravillous trophoblast (EVT) cells have been shown to be important for spiral artery remodeling during early pregnancy when regulating a fine balance between sufficient invasion and impeding over-invasion to avoid placental complications[8,9]. KIRs specifically recognize two groups of HLA-C allotypes, that express either C1 epitopes (characterized by an asparagine at position 80) or C2 epitopes (characterized by a lysine at position 80). HLA-C1 subtypes provide the ligand for KIR2DL2 and KIR2DL3 and the HLA-C2 subtypes for KIR2DL1 and KIR2DS1[10,11]. During pregnancy it has been hypothesized that the paternal HLA-C2 epitopes can bind specifically to KIR2DL1 hereby creating a strong inhibitory signal, causing a deleterious effect on dNK cell function. However, in combination with KIR2DS1, the strong inhibitory signal of KIR2DL1 seems to be counter-balanced, hereby creating a protective effect by reducing the risk of pregnancy complications[12] (Figure 2). Nevertheless, more conclusive evidence on the exact mechanism underlying the protective effects of KIR2DS1 in pregnancy is still required.

Other KIRs have been shown to recognize HLA-A and HLA-B alleles with a Bw4 motif[13,14]. However, these interactions might be less relevant as the EVT only expresses HLA-E, HLA-C and HLA-G molecules. KIR2DL4 is a specific KIR that can bind both cell surface HLA-G on EVT cells or soluble HLA-G secreted by EVT cells[15]. Despite low cell surface expression of KIR2DL4 on dNK cells, KIR2DL4 residing in endosomal vesicles has been

shown to mediate activating signaling upon interaction with EVT derived soluble HLA-G molecules and this has been shown to result in strong cytokine secretion in the absence of a killing response hence promoting spiral artery remodeling[15].

Several studies have focused on specific KIR interactions with their MHC-ligands on EVT cells and assessed the impact of subsequent dNK cell activation on successful placentation[16]. However, dNK cell activation is the result of a complex interplay between different receptors both inhibitory and activating, that are stochastically expressed on dNK cells[17]. Therefore, considerably more work needs to be done before we know the impact of all combined KIR-ligand interactions that are important for healthy pregnancy.

Overview of studies comparing KIR genotypes or expression in women with RPL versus controls

Various studies have investigated KIR expression in women with RPL and in controls either by assessing KIR genotypes or by analysis of KIR protein expression on the NK cell surface (SI Table 1). The majority of studies determining genotypes shows that maternal KIR repertoires do not seem to be associated with RPL[18-25]. However, two different studies from Faridi et al. indicated significantly less inhibitory- and more activating KIRs in a small cohort of women with RPL compared to controls[26,27]. In contrast, Hiby et al found significantly less activating KIRs in a large group of women with various types of reproductive failure including RPL[28]. Ay et al found all KIRS to be significantly different between women with RPL and controls, but have reported debatable low values for their control group[29].

KIRs are acquired stochastically and pNK cells as well as dNK cells can completely lack expression of KIRs or express combinations of 1 or more activating and/or inhibitory KIRs. Moreover, individuals can differ in percentages of NK cells expressing KIRs, or combinations of KIRs, making it relevant to study not only the presence of KIR genes but also their expression on the cell surface of NK cells. In RPL women, differences in expression level or percentage of positive NK cells have been reported for KIR2DL1, KIR2DL2 and/or KIR2DL3 in different studies[30,31,32]. On the contrary, Emmer et al and Ntrivalas et al did not observe a difference between RPL and control women in percentage KIR2DL1 or KIR2DL2/3 positive NK cells RPL[33,34]. However, the study of Ntrivalas consisted of only 15 patients including both women with RPL and infertility problems and only 7 controls which might be underpowered. Another relevant point to keep in mind when comparing the different studies is that KIR expression levels were measured on different NK cell subsets. For example, Emmer et al have only looked at $CD56^{+}$ cell populations and saw no difference in KIR expression in women with RPL versus controls[34]. A study by Wang et al has extended this with an additional CD16 marker, by looking at $CD56^{+}CD16^{-}$ cell populations[32]. Other studies have also differentiated between $CD56^{bright}$ ($CD56^{++}$) and $CD56^{dim}$ ($CD56^{+}$) populations[30,31,33]. NK cells

in the periphery are mainly CD56^{dim}, however NK cells in the uterus are characterized by a bright expression of CD56[35,36]. Both studies looking at CD56^{bright} NK cells did not show a difference in KIR expression when comparing women with RPL versus controls, but both studies had small controls groups (n=11 and n=7)[30,33]. Moreover, it is also important to keep in mind that some studies investigated protein expression levels in diverse tissues, which could be relevant as endometrial and decidual NK cells possible better represent the unique uterine environment than peripheral blood. In decidual tissue of women with RPL, less KIR2DL1 positive NK cells were found[32]. Although these data are of great value, considerably more research has to be done in order to obtain conclusive results.

In conclusion most studies show contradictory results. This might partly be explained by the fact that most studies investigating KIR expression in RPL and controls, have included a small number of women and/or controls and used different selection criteria which is important for comparison over studies.

Figure 2 | On the left HLA-C1 epitopes bind KIR2DL2 and KIR2DL3 causing weak inhibition on dNK cell function, whereas on the right HLA-C2 epitopes strongly bind KIR2DL1 causing increased inhibition on dNK cell function that can be counter-acted by a strong activation signal of KIR2DS1, together resulting in proper dNK cell function.

THE NKG2 RECEPTOR FAMILY

Function

NKG2 receptors can be expressed by NK cells and a subset of cytotoxic T cells. Structurally, they are part of the C-type lectin-like receptor superfamily. Every subunit consists of an extracellular C-type lectin-like domain and transmembrane/cytoplasmic segments. Seven different types of NKG2 receptors have been identified, namely NKG2A, -B, -C, -D, -E, -F and -H. NKG2A, -B, -C, -E and -H form heterodimers with CD94, whereas NKG2D forms homodimers[37]. NKG2A and B are both products from alternative splicing from one single gene and have two immunoreceptor tyrosine-based inhibitory motifs (ITIM)[38]. NKG2C, -E and -H lack ITIMs but have a positively charged transmembrane residue that is associated with immunoreceptor tyrosine-based activating motif (ITAM) containing adaptor molecule DAP12[39].

Role of NKG2 receptors in healthy pregnancy

NKG2 receptors primarily interact with the non-classical HLA class I molecule HLA-E and the affinity for HLA-E is higher for NKG2A than for NKG2C[40]. Interaction of HLA-E expressed on EVTS with the inhibitory NKG2A on dNK cells has shown to inhibit cytotoxicity[41]. NKG2C has been shown to be expressed on 30-50% of first-trimester dNK cells and on a few pNK cells (in CMV negative individuals) and the expression levels of NKG2A have been shown to be similar between pNK and dNK cells[12]. Interestingly, expression levels of NKG2A and NKG2C on pNK cells are influenced by viral status, which is most clearly illustrated by the cytomegalovirus (CMV) where especially the percentage NKG2C has been shown to be higher in CMV seropositive individuals[42,43]. Moreover, this also seems to be the case for endometrial NKG2C expression[44].

NKG2D interacts with MHC class I chain-ligand related (MIC) proteins A and B, and the UL-16 binding proteins (ULBPs1-5) which are ligands that are upregulated on cells upon experiencing cellular stress (e.g., upon activation of the DNA damage response in malignantly transformed cells). Hence, NKG2D is one of the most important receptors mediating NK cell activation and anti-tumor reactivity upon interaction with tumor-target cells. As a result of ADAM10/17 mediated proteolytic release, NKG2D ligands can also occur in a soluble form and the release of soluble NKG2D ligands has been observed by EVT cells during pregnancy[45]. Placenta-derived MICs and ULBPs have been associated with down regulation of the NKG2D receptor as they cause internalization of the receptor and therefore indirectly downregulation of NKG2D-mediated NK cell cytotoxicity hence contributing to immunotolerance[46].

Overview of studies comparing NKG2 receptor expression in women with RPL versus controls

Very little research has been conducted on NKG2A receptor expression in RPL vs controls (SI Table 2) and outcomes of the few studies are very diverse and primarily performed on peripheral blood. In decidual biopsies, lower expression of NKG2A was observed on NK cells isolated from RPL women[47].

Also NKG2D has not been studied extensively in RPL and some studies report enhanced expression levels or percentages of positive NK cells while other studies did not observe an association between NKG2D and RPL[30,31,47,48]. Differences between the studies might be attributed to tissue specificity as expression levels were sometimes determined in endometrial or decidual biopsies of RPL women[47] or in peripheral blood[30,31,34,48]. Interestingly, the study that included biopsies saw no differences in either NKG2D expression levels.

THE NATURAL CYTOTOXICITY RECEPTOR FAMILY

Function

The natural cytotoxicity receptor (NCR) family is a group of activating receptors that consists of three type I transmembrane receptors (TM), namely NKp46, NKp44 and NKp30, which are encoded by *NCR1, NCR2* and *NCR3* respectively[49]. Although not so commonly investigated as NKp46, NKp44 and NKp30, there is additionally an activating NKp80 receptor, a c-type lectin-like receptor encoded by *KLRF1*[50]. NKp46 is characterized by two extracellular C2-type-Ig-like domains followed by a stalk region[51]. These two Ig-like domains of NKp46 are arranged in a V-shape conformation that is similar to that of KIRs. The cytoplasmic domain lacks an ITAM, but instead it contains a charged arginine residue in the hydrophobic TM domain that can form a salt bridge with a corresponding aspartate residue in the TM domain of the ITAM adaptor CD3ζ or Fc receptor common γ[52]. NKp46 is expressed by almost all human $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{-}$ NK cells, independent of their activation status[52]. In contrast to NKp46, NKp44 only has a single extracellular V-type Ig domain, followed by a long stalk region[53]. It also has a hydrophobic TM domain however containing a charged lysine residue that can form a salt bridge with a corresponding aspartate residue in the TM domain of the ITAM adaptor DAP12[53]. Unlike the constitutively expressed NKp46, the expression of NKp44 is normally non-existent on resting NK cells in peripheral blood but can be upregulated by IL-2, IL-1β or IL-15, particularly on the $CD56^{bright}$ subset[54,55]. Moreover, NKp44 is constitutively expressed on dNK cells. NKp30 is, similarly to NKp46, expressed on almost all resting and activated NK cells but expression increases following activation with for example IL-2[56]. NKp30 has one extracellular IgV domain and a charged arginine residue in the hydrophobic TM domain that, like NKp46 can interact with an aspartate residue in the TM domain of the ITAM adaptor CD3ζ or Fc receptor common γ[56]. NCRs are classically known as activating receptors mediating NK cell activation and cytotoxicity in upon interaction with virus-infected cells or tumor cells. Typical activating NCR ligands are viral-proteins, e.g., viral-haemagglutinins or -sialic acids, abundantly expressed on the cell surface of virally infected cells but not on healthy cells making those cells sensitive to killing by NK cells. Likewise, ligands like B7-H6 expressed on tumor cells promote NK cell anti-tumor immunity upon interaction with NKp30. Hence, through their pathogen-/disease associated ligands, NCRs play a critical role in distinguishing healthy- vs diseased cells. Our understanding from the NCRs ligands is not complete and more recently it became clear that some of the NCR ligands may also lead to inhibition of NK cell activity, and this has especially been shown for NKp44 also harboring an ITIM motif in its cytoplasmic domain. In addition, in certain tissues alternative splicing of NCR transcripts, induced by the local tissue microenvironment and cytokines, has been shown to result in isoforms of NKp30 and NKp44 that act as inhibitory receptors instead of activating receptors, as summarized by Shemesh et al[57] and Pazina et al[58], illustrating the complexity of NCR mediated control of NK cell immunity.

Role of NCRs in healthy pregnancy

NKp46, NKp44 and NKp30 have been shown to be constitutively expressed on dNK cells[3,59], but the exact nature of the ligands for these receptors on EVT cells are far from completely understood. Several mechanisms for NCR mediated control of NK cell function in pregnancy have been suggested: Upon interaction with viral-ligands, NCRs on dNK cells may promote antiviral immunity in the uterus through the mechanisms described for pNK cells. Moreover, engagement of NKp44 and a yet uncharacterized ligand on EVT has been shown to induce secretion of IL-8, IP10, VEGF and PGF by dNK cells, hence contributing to EVT recruitment and invasion, neoangiogenesis and spiral artery remodeling[3]. In addition to the above-mentioned activating effects, NCRs may inhibit NK cell cytotoxicity against invading EVT, for example via the interaction between NKp44 and its inhibitory ligand PCNA, expressed on first trimester decidual trophoblasts[60]. Or by the expression of inhibitory NCR isoforms as has been shown for NKp30 and NKp44[61]. Both activating and inhibitory NKp30 and NKp44 isoforms have been shown to be expressed on dNK cells and the expression of inhibitory isoforms has been shown to be induced by factors from the decidual microenvironment (e.g., hypoxia, TGFβ and IL-15)[61] making it tempting to speculate that a disturbance in the decidual microenvironment and/or expression levels of inhibitory- versus activating NCR ligands and isoforms may be involved in the pathogenesis of pregnancy disorders.

Overview of studies comparing NCR expression in women with RPL versus controls

Fukui et al have conducted two studies looking into NKp30, NKp44 and NKp46 expression in women with RPL and controls. Results of their first study in 2009, indicated no differences in expression between women with RPL and controls for either one of the receptors when measured in peripheral blood[62]. In their second study in 2017 they found that the percentage of NKp46 positive endometrial NK cells was lower in RPL women compared to controls. For NKp30 and NKp44, no differences in percentage of positive cells were found[63]. Strobel et al also showed a lower percentage of NKp46 positive NK cells in women with RPL compared to controls but found these differences in peripheral blood[48] and Comins-Boo et al only found lower expression levels on CD56^{bright} pNK cells[64]. Nevertheless, results seem conflicting as higher numbers of NKp46 positive NK cells[65] and no difference in expression levels[30] have also been reported by others. Although Fukui et al did not find a difference in expression levels of NKp30 in both endometrial tissue and peripheral blood[62,63], two other studies did report higher levels of NKp30 positive NK cells in women with RPL compared to controls[30,64]. However, it should be noted that in the study of Zhang et al no difference was found looking at the percentage of NKp30 positive NK cells, however the mean fluorescent intensity (MFI) of NKp30 was significantly higher expressed by pNK cells of women with RPL[30]. This may suggest that NK cells from RPL women upregulate NKp30 receptor expression on their membrane, but that the number of cells expressing NKp30 does not increase.

In conclusion, only a few studies have been performed (SI Table 3). As sometimes differences in positive number of cells are hard to detect when for example almost all NK cells are positive and a difference between 98% and 99% is therefore less detectable, it could be relevant to also look at MFI levels in addition to the percentage of positive cells. Moreover, it could be very relevant to take information on splice variants into account when studying NKp30 and NKp44 receptors as they could favor inhibitory signaling during early pregnancy.

THE IMMUNE CHECKPOINT REGULATING RECEPTOR FAMILY

Function

The immune checkpoint (IC) receptor family includes a variety of non-HLA-class I-specific inhibitory receptors such as programmed death protein 1 (PD1) and T cell immunoglobulin and mucin-domain-containing molecule 3 (TIM3), and HLA-class II specific inhibitory receptors such as lymphocyte activation gene-3 (LAG-3)[66-68]. One of the most investigated inhibitory IC receptors is PD1, which contains an extracellular variable immunoglobulin (IgV) domain, a transmembrane region and an intracellular tail with multiple phosphorylation sites[69]. One of these sites is a tyrosine-based switch motif that can interact with SHP-2[70]. TIM3 has extracellular regions which contain an extracellular single membrane-bound distal IgV domain, an extracellular glycosylated mucin domain and an intracellular domain that consists of a C-terminal cytoplasmic tail with five tyrosine residues[71]. The cytoplasmic tail of LAG3 consists of a serene phosphorylation site, a glutamic-acid proline repeat and an inhibitory KIEELE motif[72]. In addition, two recently identified inhibitory IC receptors are T cell immunoreceptor with Ig and ITIM domains (TIGIT) and TACTILE[73]. TIGIT and TACTILE have been suggested to compete with the activating DNAM1 receptor hereby inhibiting IFNγ production of NK cells[74]. Most IC receptors are absent or expressed on low levels in resting NK cells in peripheral blood and their potential effects in controlling NK cell functions have been primarily investigated in the context of NK cell anti-tumor responses.

Role of IC receptors in healthy pregnancy

The exact role of IC receptors in controlling NK cell function in pregnancy is not yet known, we do know that the receptors are particularly expressed in tissues where tolerance is essential and/or on cells that have been activated for a longer period of time, hence providing a type of negative feedback mechanism[75]. IC receptor expression has mostly been described on T cells[75]. Therefore, one could hypothesize that their inhibitory mechanism might also be relevant for NK cell tolerance during early pregnancy. Only recently support for this hypothesis was provided by a study showing that TIM3 positive dNK cell subsets were identified at the maternal-fetal interface of human miscarriage material and murine abortion-prone models, that showed diminished cytotoxicity and produced more Th2-typical cytokines and less Th1 associated cytokines compared to TIM3 negative dNK cells[76]. These findings might indicate that TIM3 could possibly be a regulator of NK cell cytotoxicity during early pregnancy. Moreover, recent single cell RNA seq and mass cytometry data also observed expression of IC receptors on dNK cells or their ligands on EVT or decidual stromal cells[77] and it will be very relevant to confirm the functional relevance of this expression in mechanistic follow up studies.

Overview of studies comparing IC expression in women with RPL versus controls

To date, only two studies have investigated inhibitory immune checkpoint receptors on NK cells in women with RPL and controls. Both studies indicated lower TIM3 expression on pNK cells in women with RPL compared to controls[78,79]. Expression of other inhibitory immune checkpoint receptors on NK cells have not yet been extensively studied in women with RPL and controls as most studies have focused on T cell expression levels.

In conclusion, clinical studies on inhibitory immune checkpoint receptor expression levels of NK cells in women with RPL and controls is scarce (SI Table 4). However, as they have been extensively investigated because of their immune tolerogenic role in cancer, their inhibitory mechanisms might also be very relevant during early pregnancy.

THE LEUKOCYTE IMMUNOGLOBULIN-LIKE RECEPTOR FAMILY

Function

The human leukocyte immunoglobulin-like receptors (LILR) are part of a family of receptors that consists of 11 functional genes coding for five activating, five inhibitory and one soluble form. These different forms are highly homologous in their extracellular regions but very different in their intracellular regions[80]. Inhibitory LILRs (LILRBs) consist of two or four immunoglobulin-like domains and a long cytoplasmic tail with tyrosine-based inhibition motifs as opposed to activating LILRs (LILRAs) that have a short cytoplasmic tail with FcRγ chain containing tyrosine-based activation motifs. Soluble forms of LILRs are generated by alternative splicing and do not possess transmembrane or cytoplasmic regions, and serve as negative regulators[81]. Although multiple other LILR family members exist, amongst them LILRB1-6 as inhibitory receptors and LILRA1-3 as activating receptors, LILRB1 and- 5 and LILRA2 are the only receptors expressed on the cell membrane of NK cells[82].

Role of LILRs in healthy pregnancy

LILRB1 can recognize a broad variety of HLA class I alleles as well as pathogen associated molecules, e.g., the CMV encoded UL18 protein, LILRB5 interacts with HLA-B27 and LILRA2 can interact with degraded immunoglobulins[83,84]. The binding affinity of LILRB1 for HLA-G is many times higher than for other HLA class I alleles[85]. LILRB1 is expressed by a dNK subset that is present in both the decidua basalis and decidua parietalis[86]. Binding of dNK bound LILRB1 to HLA-G results in impairment of cytotoxicity and secretion of IL-6, IL-8 and TNFα[87-89]. Engagement of LILRB1 by HLA-G on decidual macrophages results in secretion of the same cytokines, however, the effect is much stronger[89]. LILRB1 is also expressed on B cells. Recent findings showed that HLA-G blocked B cell proliferation, differentiation and Ig release in a T-cell independent matter of B cell activation[90]. Although very few B cells are present during pregnancy, it may contribute to prevention of unwanted B cell effector functions at the maternal-fetal interface. LILRB1 has also been described as one of the markers for pregnancy-trained decidual NK (PTdNK) cells. NKG2C^{high} LILRB1^{+} NK (PTdNK) cells are increased in secondary and subsequent pregnancies[91]. PTdNK cells possess transcriptomic and epigenetic profiles that promote IFNγ and VEGF-A production[92]. Additionally, LILRB1, which is normally regarded as an inhibitory receptor, has been described to function as an activating receptor in uNK cells in some studies[89,92].

Overview of studies comparing LILRB1 expression in women with RPL versus controls

To date, there are no clinical studies comparing LILR receptor expression levels on NK cells in women with RPL and controls. However, because of their suggestive role during pregnancy, it would also be interesting to examine LILRB1 receptor expression in women with RPL and controls.

CONCLUSION

The main goal of this chapter was to provide an overview of literature on studies investigating NK cell receptors in women with RPL and controls after briefly describing their function and their possible role during pregnancy. KIRs are mostly studied in the context of RPL, both genotypically and phenotypically, while the other receptors have been studied to a lesser extent in smaller cohorts of women. Nevertheless, NKG2 receptors might be interesting in context to CMV status, the NCR receptor splice variants might provide novel insights in inhibitory receptor function and the IC receptors could be very relevant as they are mainly involved in immune tolerance. Moreover, studying these receptors both in the peripheral and uterine environment, could be important for their function during early pregnancy and provides lots of exciting possibilities for novel studies in a well-defined group of women with RPL and controls.

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2

SUPPLEMENTARY FILES

SI Table 1 | Killer-cell immunoglobulin-like receptors (KIRs)

Study	Technique + tissue used	Definition RPL	Definition Control
Ay et al, 2019[29]	PCR-SSP, blood	RPL (≥2 losses) n=70	C (≥2 healthy pregnancies) n=70
*Dambaeva et al, 2016[22]	PCR-SSO, blood	RPL (≥2 losses) n=139	C (database for immune gene frequencies in worldwide populations) n=195
Faridi et al, 2009[26]	PCR-SSP, blood	RPL (≥3 losses) n=205	C (≥2 healthy pregnancies) n=224

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Receptor expression presented as	Receptor	RPL	Control	Results
Percentage of positive individuals in the group	KIR2DL1	30.0	5.7	P<0.05
	KIR2DL2	82.9	8.6	P<0.05
	KIR2DL3	80.0	14.3	P<0.05
	KIR2DL4	68.6	21.4	P<0.05
	KIR2DS1	12.9	0	P<0.05
	KIR2DS2	17.1	0	P<0.05
	KIR2DS4	27.1	0	P<0.05
	KIR2DS5	14.3	0	P<0.05
Percentage of positive individuals in the group	KIR2DL1	97.1	96.9	P>0.05
	KIR2DL2	49.6	49.2	P>0.05
	KIR2DL3	89.2	88.7	P>0.05
	KIR2DL4	100	100	P>0.05
	KIR3DL1	89.9	94.9	P>0.05
	KIR2DS1	45.3	37.4	P>0.05
	KIR2DS2	49.6	49.7	P>0.05
	KIR2DS3	31.7	28.2	P>0.05
	KIR2DS4	93.5	94.9	P>0.05
	KIR2DS5	38.1	35.9	P>0.05
	KIR3DS1	44.6	39.5	P>0.05
	KIR2DL5	56.8	52.8	P>0.05
Percentage of positive individuals in the group	KIR2DL1	68.8	95.9	P<0.0001
	KIR2DL2	53.7	49.6	P>0.05
	KIR2DL3	82.4	83.5	P>0.05
	KIR2DL4	100	100	P>0.05
	KIR2DL5	61.9	67.8	P>0.05
	KIR3DL1	61.5	85.2	P<0.0001
	KIR3DL3	100	100	P>0.05
	KIR2DS1	44.9	39.3	P>0.05
	KIR2DS2	50.7	35.3	P<0.05
	KIR2DS3	45.9	29.5	P<0.05
	KIR2DS4	53.1	72.7	P<0.0001
	KIR2DS5	59.5	54.5	P>0.05
	KIR3DS1	79.0	51.8	P<0.0001
	KIR2DP1	70.2	84.8	P<0.05
	KIR3DP1	100	100	P>0.05
	KIR3DX1	43.9	50.0	P>0.05

SI Table 1 | Continued

Study	Technique + tissue used	Definition RPL	Definition Control
Faridi et al, 2011[27]	PCR-SSP, blood	RPL (≥3 losses) n=177	C (≥2 healthy pregnancies) N=200
Hiby et al, 2010[28]	PCR-SSP, blood	RPL (≥3 losses) amongst other pregnancy disorders n=975	C (≥1 healthy pregnancies) n=592
Hong et al, 2008[23]	PCR-SSP, decidual tissue	RPL (≥3 losses) n=16	C (≥1 healthy pregnancies) n=41
**Nowak et al, 2011[18]	PCR-SSP, blood	RPL (≥3 losses) n=85	C (≥2 healthy pregnancies) N=117

Receptor expression presented as	Receptor	RPL	Control	Results
Percentage of positive individuals in the group	KIR2DL1	69.0	95.0	P<0.0001
	KIR2DL2	54.0	49.0	P>0.05
	KIR2DL3	82.0	84.0	P>0.05
	KIR2DS1	45.0	39.0	P>0.05
	KIR2DS2	50.0	35.0	P<0.05
	KIR2DS4	53.0	73.0	P<0.01
	KIR2DS5	60.0	54.0	P<0.05
Percentage of positive individuals in the group	KIR2DL1	97.1	96.1	P>0.05
	KIR2DL2	48.3	52.9	P>0.05
	KIR2DL3	91.0	89.5	P>0.05
	KIR2DL5	44.7	55.7	P<0.01
	KIR3DL1	95.9	94.3	P>0.05
	KIR2DS1	32.1	43.1	P<0.001
	KIR2DS2	48.8	53.5	P>0.05
	KIR2DS3	25.3	29.6	P>0.05
	KIR2DS5	27.9	36.1	P<0.05
	KIR3DS1	33.0	44.3	P<0.001
Percentage of positive individuals in the group	KIR2DL1	50.0	50.6	P<0.05
	KIR2DL2	100	53.2	P>0.05
	KIR2DL3	38.8	43.7	P>0.05
	KIR2DL5	33.9	28.4	P>0.05
	KIR2DS1	3.2	10.3	P>0.05
	KIR2DS2	6.5	6.3	P>0.05
	KIR2DS3	13.4	6.3	P>0.05
	KIR2DS4	50.0	55.8	P>0.05
	KIR2DS5	25.0	20.4	P>0.05
Percentage of positive individuals in the group	KIR2DL1	98.0	98.0	P>0.05
	KIR2DL2	52.0	52.0	P>0.05
	KIR2DL3	86.0	86.0	P>0.05
	KIR2DL5	50.0	53.0	P>0.05
	KIR3DL1	95.0	93.0	P>0.05
	KIR2DS1	35.0	41.0	P>0.05
	KIR2DS2	54.0	52.0	P>0.05
	KIR2DS3	27.0	38.0	P>0.05
	KIR2DS4	39.0	30.0	P>0.05
	KIRDS4d	86.0	82.0	P>0.05
	KIR3DS1	30.0	38.0	P>0.05

SI Table 1 | Continued

Study	Technique + tissue used	Definition RPL	Definition Control
Ozturk et al, 2012[19]	PCR-SSO, blood	RPL (clinical evaluation of RPL history) n=40	C (≥2 healthy pregnancies) n=90
Su et al, 2018[25]	PCR-SSP, blood	RPL (≥2 losses) n=110	C (≥1 healthy pregnancies) n=105

Receptor expression presented as	Receptor	RPL	Control	Results
Percentage of positive individuals in the group	KIR2DL1	100.0	98.9	P>0.05
	KIR2DL2	65.0	45.5	P>0.05
	KIR2DL3	92.5	82.2	P>0.05
	KIR2DL4	100	100	P>0.05
	KIR2DL5	80.0	56.7	P>0.05
	KIR3DL1	90.0	90.0	P>0.05
	KIR3DL2	97.5	100	P>0.05
	KIR3DL3	100	100	P>0.05
	KIR2DS1	52.5	34.4	P>0.05
	KIR2DS2	65.0	45.5	P>0.05
	KIR2DS3	42.5	32.2	P>0.05
	KIR2DS4	90.0	91.1	P>0.05
	KIR2DS5	55.0	38.9	P>0.05
	KIR3DS1	40.0	41.1	P>0.05
	KIR2DP1	97.5	98.9	P>0.05
	KIR3DP1	97.5	100	P>0.05
Percentage of positive individuals in the group	KIR2DL1	99.1	100	P>0.05
	KIR2DL2	19.1	16.2	P>0.05
	KIR2DL3	98.2	100	P>0.05
	KIR2DL4	100	100	P>0.05
	KIR2DL5A	37.3	35.2	P>0.05
	KIR2DL5B	9.1	9.5	P>0.05
	KIR3DL1	91.8	96.2	P>0.05
	KIR3DL2	100	100	P>0.05
	KIR3DL3	100	100	P>0.05
	KIR2DS1	40.0	38.1	P>0.05
	KIR2DS2	18.2	16.2	P>0.05
	KIR2DS3	23.6	21.9	P>0.05
	KIR2DS4	73.6	76.2	P>0.05
	KIR2DS5	28.2	21.9	P>0.05
	KIR3DS1	38.2	33.3	P>0.05
	KIR2DP1	99.1	100	P>0.05

SI Table 1 | Continued

Study	Technique + tissue used	Definition RPL	Definition Control
Wang et al, 2007[20]	PCR-SSP, blood	RPL (≥3 losses) n=73	C (≥2 healthy pregnancies) n=68
Witt et al, 2004[21]	PCR-SSP, blood	RPL (≥3 losses) n=51	C (≥2 healthy pregnancies) n=55
Yang et al, 2020[24]	PCR-SSO, blood	RPL (≥2 losses) n=160	C (database) n=255
,*Emmer et al, 2000[34]	Flowcytometry, blood	RPL (≥2 losses) n=142	C (non-pregnant healthy control women with ≥1 successful pregnancy and no history of RPL) n=37

Receptor expression presented as	Receptor	RPL	Control	Results
Percentage of positive individuals in the group	KIR2DL1	100	100	P>0.05
	KIR2DL2	30.1	33.8	P>0.05
	KIR2DL3	98.6	98.5	P>0.05
	KIR2DL4	100	100	P>0.05
	KIR2DL5	47.9	41.2	P>0.05
	KIR3DL1	100	98.5	P>0.05
	KIR3DL2	100	100	P>0.05
	KIR3DL3	100	100	P>0.05
	KIR2DS1	60.3	41.2	P<0.05
	KIR2DS2	30.1	26.5	P>0.05
	KIR2DS3	34.3	29.4	P>0.05
	KIR2DS4	98.6	95.6	P>0.05
	KIR2DS5	52.1	38.2	P>0.05
	KIR3DS1	52.1	47.1	P>0.05
	KIR2DP1	100	100	P>0.05
Percentage of positive individuals in the group	KIR2DL1	100	100	P>0.05
	KIR2DL2	56.0	50.0	P>0.05
	KIR2DL3	91.0	85.0	P>0.05
	KIR2DL4	100	100	P>0.05
	KIR2DL5	30.0	37.0	P>0.05
	KIR3DL1	96.0	88.0	P>0.05
	KIR2DS1	40.0	45.0	P>0.05
	KIR2DS2	52.0	47.0	P>0.05
	KIR2DS3	31.0	28.0	P>0.05
	KIR2DS4	35.0	38.0	P>0.05
	KIR2DS5	20.0	33.0	P>0.05
	KIR3DS1	33.0	37.0	P>0.05
Percentage of positive individuals in the group	KIR2DL1	98.0	95.0	P>0.05
	KIR2DL2	53.0	53.0	P>0.05
	KIR2DL3	81.0	87.0	P>0.05
	KIR2DS1	43.0	41.0	P>0.05
	KIR2DS2	53.0	53.0	P>0.05
	KIR2DS4	92.0	95.0	P>0.05
	KIR2DS5	26.0	35.0	P>0.05
Percentage of $CD56^{+}$ cells	KIR2DL1	18	20	P>0.05
	KIR2DL2/3	32	25	P>0.05

SI Table 1 | Continued

Study	Technique + tissue used	Definition RPL	Definition Control
,**Ntrivalas et al, 2005[33]	Flowcytometry, blood	RPL (>3 losses) and infertility n=15	C (fertile women) n=7
***Wang et al, 2014[32]	Flowcytometry, decidual tissue	RPL (≥2 losses) n=60	C (≥1 healthy pregnancies) n=30
***Zhang et al, 2021[30]	Flowcytometry, blood	RPL (≥2 losses) n=49	C (≥1 healthy pregnancies) n=11
***Zhu et al, 2018[31]	Flowcytometry, blood	RPL (≥3 losses) n=31	C (healthy donors) n=21

*Two databases were presented in the original article only one is presented here, both databases of controls were not significantly different for all included markers.

**Data from original paper was extracted from figures.

***Authors report the inhibitory KIR but the used antibody also binds to activating KIR (e.g., in case of KIR2DL1 to KIR2DS1).

Receptor expression presented as	Receptor	RPL	Control	Results
Percentage of $CD56^{dim}CD16^{+}$ cells	KIR2DL1	13.9	9.0	P>0.05
	KIR2DL2	21.0	26.0	P>0.05
Percentage of $CD56^{bright}CD16^{-}$ cells	KIR2DL1	2.3	4.0	P>0.05
	KIR2DL2	6.2	12.0	P>0.05
Percentage of $CD56^{+}CD16^{-}$ cells	KIR2DL1	53.1	63.7	P<0.05
	KIR2DL2	62.4	62.4	P>0.05
$CD56^{+}$ cells				
MFI	KIR2DL1	2060.7	1509.7	P<0.01
	KIR2DL2	5584.9	4850.4	P<0.005
Percentage	KIR2DL1	42.8	45.0	P>0.05
	KIR2DL2	36.8	40.1	P>0.05
$CD56^{bright}$ cells				
Percentage	KIR2DL1	29.1	14.9	P<0.05
$CD56^{dim}$ cells				
MFI	KIR2DL1	2030.2	1580.8	P<0.05
	KIR2DL2	5477.4	4813.2	P<0.01
$CD56^{+}$ cells				
Absolute counts per mL	KIR2DL1	44.0	25.0	P<0.05
	KIR2DL2	82.0	29.0	P<0.05
Percentage	KIR2DL1	19.0	19.0	P>0.05
	KIR2DL2	32.0	35.0	P>0.05
$CD56^{dim}CD16^{+}$ cells				
Absolute counts per mL	KIR2DL1	43.0	25.0	P<0.05
	KIR2DL2	81.0	48.0	P<0.05
Percentage	KIR2DL1	19.0	19.0	P>0.05
	KIR2DL2	32.0	34.0	P>0.05

SI Table 2 | NKG2 C-type lectin receptors (NKG2s)

Study	Technique + tissue used	Definition RPL	Definition control
*Emmer et al, 2000[34]	Flowcytometry, blood	RPL (≥2 losses) n=142	C (non-pregnant healthy control women with ≥1 successful pregnancy and no history of RPL) n=37
Strobel et al, 2021[48]	Flowcytometry, blood	RPL (≥2 losses) pRPL n=47 sRPL n=24	C (previous life birth yes or no) pCtrl n=60 sCtrl n=60
Sotnikova et al, 2014[47]	Flowcytometry, decidual tissue, endometrial tissue	RPL (≥2 losses) n=26	C (healthy pregnancy, voluntary abortion) n=37 C (non-pregnant, improved fertility) n=13
Zhang et al, 2021[30]	Flowcytometry, blood	RPL (≥2 losses) n=49	C (≥1 healthy pregnancies) n=11
Zhu et al, 2018[31]	Flowcytometry, blood	RPL (≥3 losses) n=31	C (healthy donors) n=21

p; pregnant, np; non-pregnant.
sRPL; at least one live- birth before the pregnancy losses, pRPL; no live- birth before the pregnancy losses, sC; already had one or more live-births, pC; no live-births before.
*Data from original paper was extracted from figures.

Receptor expression presented as	Receptor	RPL	Control	Results
Percentage of CD56⁺ cells	NKG2A	44.0	50.0	P>0.05
Percentage of CD56^bright^CD16^dim^	NKG2D	92.3 (pRPL) 92.5 (sRPL)	91.2 (pCtrl) 96.0 (sCtrl)	P>0.05 P>0.05
Percentage of CD56^dim^CD16^bright^	NKG2D	95.6 (pRPL) 95.3 (sRPL)	96.9 (pCtrl) 97.2 (sCtrl)	P>0.05 P<0.05 (sRPL vs sCtrl and pRPL vs sCtrl)
Percentage of CD56⁺ cells	NKG2A NKG2D	44.8 64.1	82.0 (p) 58.8 (p)	P=0.001 P>0.05
Percentage of CD56⁺ cells	NKG2A NKG2D	44.8 64.1	14.3 (np) 12.4 (np)	P=0.001 P=0.001
CD56⁺ cells				
MFI	NKG2D	685.1	641.3	P>0.05
Percentage	NKG2D	67.9	68.4	P>0.05
CD56^bright^ cells				
MFI	NKG2D	730.7	958.6	P<0.001
Percentage	NKG2D	91.1	74.8	P<0.001
CD56^dim^ cells				
MFI		548.4	644.6	P<0.05
Percentage		89.7	73.3	P<0.001
CD56⁺ cells				
Absolute counts per mL	NKG2A	108.0	67.0	P<0.01
	NKG2D	188.0	105.0	P<0.01
Percentage	NKG2A	46.0	49.0	P>0.05
	NKG2D	72.0	38.0	P>0.05
CD56^dim^CD16⁺cells				
Absolute counts per mL	NKG2A	94.0	55.0	P<0.01
	NKG2D	176.0	95.0	P<0.01
Percentage	NKG2A	38.0	40.0	P>0.05
	NKG2D	66.0	67.0	P>0.05

SI Table 3 | Natural cytotoxicity receptors (NCRs)

Study	Technique + tissue used	Definition RPL	Definition control
Fukui et al, 2017[63]	Flowcytometry, endometrial tissue	RPL (not specified) n=28	C (not specified) n=74
Fukui et al, 2009[62]	Flowcytometry, blood	RPL (≥2 losses) n=22	C (fertile) n=15
Giuliani et al, 2014[65]	Flowcytometry, blood	RPL (≥2 losses) n=21	C (not specified) n=10
Strobel et al, 2021[48]	Flowcytometry, blood	RPL (≥2 losses): pRPL n=47 sRPL n=24	C (previous life birth yes or no) pCtrl n=60 sCtrl n=60
Zhang et al, 2021[30]	Flowcytometry, blood	RPL (≥2 losses) n=49	C (≥1 healthy pregnancies) n=11
Comins-boo et al, 2021[64]	Flowcytometry, blood	RPL (≥2 losses) n=58	C (≥2 live births) n=31

sRPL; at least one live- birth before the pregnancy losses, pRPL; no live- birth before the pregnancy losses, sC; already had one or more live- births, pC; no live-births before.

Receptor expression presented as	Receptor	RPL	Control	Results
Percentage of CD56^{+} cells	NKp46	58.0	68.9	P<0.05
	NKp44	6.6	9.1	P>0.05
	NKp30	18.7	17.8	P>0.05
Percentage of CD56^{dim} cells	NKp46	28.4	43.5	P<0.05
	NKp44	3.3	6.6	P>0.05
	NKp30	11.8	11.7	P>0.05
Percentage of CD56^{bright} cells	NKp46	80.9	89.8	P<0.05
	NKp44	9.2	10.0	P>0.05
	NKp30	31.2	35.8	P>0.05
Percentage of CD56^{dim} cells	NKp46	62.0	68.7	P>0.05
	NKp44	1.1	1.6	P>0.05
	NKp30	46.1	51.2	P>0.05
Percentage of CD56^{bright} cells	NKp46	94.0	98.1	P>0.05
	NKp44	12.5	22.4	P>0.05
	NKp30	80.3	66.3	P>0.05
Percentage of CD56^{+} cells	NKp46	3.4	1.9	P<0.05
Percentage of CD56^{bright}CD16^{-} cells	NKp46	96.3 (pRPL) 95.6 (sRPL)	97.2 (pCtrl) 98.2 (sCtrl)	P<0.05 (pRPL vs sCtrl) P<0.05
Percentage of CD56^{dim}CD16^{+} cells	NKp46	63.7 (pRPL) 56.3 (sRPL)	77.1 (pCtrl) 81.5 (sCtrl)	P<0.001 (pRPL vs pCtrl and pRPL vs sCtrl) P<0.001 (sRPL vs sCtrl and sRPL vs pCtrl)
CD56^{+} cells				
MFI	NKp30	1966.3	2052.3	P>0.05
	NKp46	1795.1	1809.2	P>0.05
Percentage	NKp30	45.3	52.1	P>0.05
	NKp46	53.6	55.2	P>0.05
CD56^{bright} cells				
MFI	NKp30	3220.5	2338.0	P<0.01
CD56^{dim} cells				
MFI	NKp30	2734.0	2053.4	P<0.001
Percentage of CD56^{dim}CD16^{+} cells	NKp46	60.1	60.0	P>0.05
	NKp30	83.0	68.8	P<0.05
Percentage of CD56^{bright}CD16^{-} cells	NKp46	98.2	99.0	P<0.05
	NKp30	97.1	94.8	P<0.001

SI Table 4 | Immune checkpoint receptors (ICs)

Study	Technique + tissue used	Definition RPL	Definition control
Li et al, 2017[78]	Flowcytometry, blood	RPL (2 or ≥2 losses) n=20	C (pregnant women) n=20
*Sun et al, 2016[79]	Flowcytometry, blood	RPL (not specified) n=20	C (pregnant) n=30 (not pregnant) n=30

p; pregnant, np; non-pregnant.
*Data from original paper was extracted from text and figures.

Receptor expression presented as	Receptor	RPL	Control	Results
MFI of $CD56^+$ cells	TIM-3	600	250	P<0.001
Percentage of $CD56^+CD3^-$ cells	TIM-3	48.0	50.0 (np) 69.0 (p)	P>0.05 P<0.05

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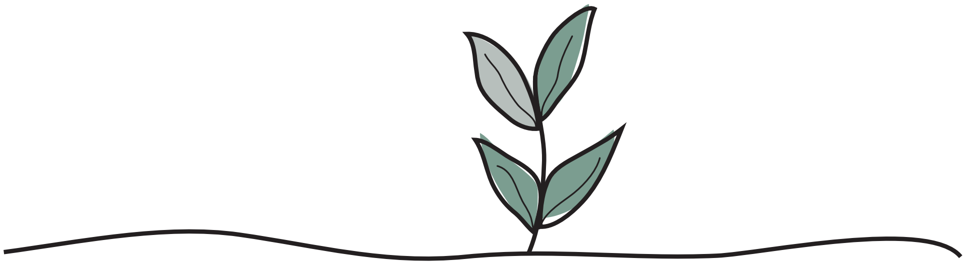

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PERIPHERAL BLOOD NATURAL KILLER CELL PROFILES ARE NOT INFLUENCED BY THE MENSTRUAL CYCLE AND ARE DISTINCTLY DIFFERENT IN MENSTRUAL BLOOD

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SUBMITTED

ABSTRACT

Introduction: NK cells have a dynamic role in controlling tissue-homeostasis and immune-mediated protection against disease. Given their suggested importance for successful pregnancy, NK cell phenotypes have been associated with reproductive success versus failure. Since timing and location of sampling may influence the outcome of such studies, we studied the impact of the menstrual cycle on peripheral blood NK (pNK) cell phenotypic profiles and compared them with menstrual blood (MB).

Methods: MB and pNK cell phenotypic profiles (days 1,7,21 of the menstrual cycle), were obtained by flowcytometry (n=6 healthy females and n=6 males). Staining panels included well-established inhibitory- and activating NK receptors (e.g., KIRs, NKG2A, LILRB1, NKG2D, NCRs and DNAM1), and inhibitory immune checkpoint receptors (e.g., PD1, LAG3, TACTILE, TIM3) not studied before on NK cells from the non-pregnant uterus.

Results: Frequencies of lymphocyte subsets and expression of activating- and inhibitory receptor on pNK was comparable on the different days of the menstrual cycle. As compared to pNK cells, NK cells in MB were primarily CD56^{bright}CD16^{negative} and had a higher expression of KIRs, NKG2A, TACTILE and TIM3, lower levels of LILRB1 and high levels of the tissue-residency markers CD49a and CD103.

Conclusion: Our study demonstrates that, with respect to the menstrual cycle, timing of sampling is not critical for analysis of individual NK receptors. This is informative for the design of studies investigating the clinical relevance of pNK cell phenotypic profiles in e.g., reproductive success, cancer or autoimmunity. Moreover, MB is an attractive source of tissue-resident NK cells, enabling future studies on their role in reproductive success.

Keywords: natural killer cell, immune checkpoint, menstrual cycle, menstrual blood.

INTRODUCTION

The menstrual cycle is regulated by an endocrine axis that prepares the endometrium for potential implantation of the embryo and induces shedding of the lining layer if this implantation did not take place[1]. Cycling is conventionally divided into two phases, the follicular and luteal phase, followed by the menstrual bleeding and governed by a cyclic pattern of hormones from the pituitary glands (e.g., luteinizing hormone and follicle-stimulating hormone) and ovaries (e.g., estrogen and progesterone)[2]. With each menstrual cycle, the endometrium undergoes marked changes that facilitate decidualization when embryo implantation occurs[3]. Immune cells express receptors for cycling-associated hormones, and cyclic fluctuation of hormonal levels has been shown to be important for regulation of immune cell function in the uterus enabling it to tolerate and facilitate implantation, placentation and early fetal growth[4].

In the endometrium of the non-pregnant uterus and in the decidua of the pregnant uterus, a unique population of lymphocytes, Natural Killer (NK) cells, has been proposed to play a very important role at the time of- and following implantation[5,6,7]. Here, they do not live up to their name as killers, but rather contribute to a microenvironment that is pregnancy favorable and supports the process of implantation and placentation[8]. NK cells constitute up to 70% of all leukocytes present in the decidualized endometrium[7] and up to 20-30% of all leukocytes when endometrial immune cells are shed with the menstruum[9] which is in contrast to peripheral blood (PB) where they represent only 5–15% of circulating leukocytes[10]. Moreover, altered phenotypes or dysregulation of peripheral NK (pNK), endometrial NK (eNK) and/or decidual NK (dNK) cells have been associated with reproductive problems such as Recurrent Implantation Failure (RIF)[11,12,13] and Recurrent Pregnancy Loss (RPL)[11,14,15,16,17].

Although uterine-associated NK cells (eNK or dNK) may be most relevant to investigate the role of NK cells in reproduction, their sampling remains a major challenge as it usually involves invasive uterine biopsies before or during pregnancy. Hence, collection of menstrual blood (MB) has been explored as an alternative and less invasive method for analysis of eNK cells[9]. pNK cells on the other hand, can more easily be obtained and monitored over a period of several days and have been used as marker for reproductive success[18]. Since NK cells can express hormone receptors, insight in the influence of the menstrual cycle on NK cells could be relevant to harmonize NK cell sampling.

Several studies addressed the influence of the menstrual cycle on pNK cell numbers and function: In naturally cycling women, some studies observed an increase in pNK cell numbers during the luteal compared to the follicular phase[19,20], while other studies

observed an increase in the periovulatory follicular phase[21] or even no changes at all[22]. Similarly, there is still contradiction about NK cell activity during the menstrual cycle. Some studies described increased pNK cell activity via cytotoxicity assay in women with a normal menstrual cycle during the luteal phase[19], others described increased activity during the follicular phase[23] or decreased activity limited to the periovulatory follicular phase[24]. In addition to analysis of NK cell numbers and effector function, NK cell phenotypic profiling may be informative but this has not been done in a comprehensive manner in relation to the menstrual cycle.

NK phenotypes may provide information on the maturation- and activation status of NK cells. For example, based on the analysis of CD56 and CD16 two functionally distinct NK cells subsets can be identified as $CD56^{dim}$ NK cells, known for their cytotoxic function and $CD56^{bright}$ NK cells, that are known as cytokine producers[25]. Additional information on the functional capacity of NK cells can be obtained by extended analysis of the broad array of inhibitory- and activating receptors that regulate NK cell activation status[26]. Such extended repertoires could include for example the Killer Immunoglobulin-Like Receptor (KIR) family, the NK group 2 (NKG2) receptor family, the Natural Cytotoxicity Receptor (NCR) family and the immune checkpoint (IC) receptor family[27]. The latter family comprises inhibitory receptors like PD1, TIM3, LAG3 and TACTILE that are involved in maintenance of immune tolerance[28] and for which surprisingly, little data is available on their functional relevance for NK cells in reproductive success.

In this study, we aimed to investigate the influence of the menstrual cycle on pNK cell phenotypic profiles, including novel immune checkpoint receptors, in healthy females and male controls. In addition, we obtained MB as a minimally invasive source of eNK cells and compared NK cell phenotypic profiles between pNK and NK cells in MB.

MATERIAL AND METHODS

Study population

Six healthy, non-pregnant females with a regular menstrual cycle and six healthy male controls were included for characterization of PB mononuclear cells. In addition, females were also included for characterization of MB mononuclear cells. In order to sample during a natural menstrual cycle, none of our females used hormonal anticonceptives. Exclusion criteria were chronic medical conditions such as high blood pressure, kidney failure or auto-immune disease, current or recent symptomatic infection and for females a previous vascular complicated pregnancy. Upon written informed consent, according to the Medical Ethical Committee of the Maastricht University Medical Centre (Maastricht UMC+) NL70964.068.10, information on baseline characteristics and cells were obtained.

Collection and isolation of MB and PB mononuclear cells

All PB samples were collected on day 1, 7 and 21 of the menstrual cycle in females and in similar time intervals in male controls. Each female volunteer was asked to collect her MB at night for 8-hours on the first day of the menstrual cycle using the OrganiCup (OrganiCup ApS, Copenhagen, Denmark). The MB sample was decanted from the cup into a 50mL tube containing 10mL buffer (10% human pooled serum and 90% RPMI 1640 GlutaMAX medium supplemented with 1% sodium pyruvate, 0.1% penstrep and 0.3% sodium citrate). MB and PB samples were processed immediately after collection. Mononuclear cells derived from PB and MB samples were isolated by means of density gradient centrifugation using Lymphoprep (Alere Technologies AS, Oslo, Norway). After isolation the layer with mononuclear cells was taken off and washed twice with PBS. For MB samples an additional step was included to remove clots of mucus before the isolation procedure, by using a 30 second program B (relatively mild conditions for the dissociation of soft tissue) according to manufactures instructions on the gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Viability of cells and cell numbers were determined by trypan blue staining in a counting chamber.

Antibodies and flowcytometric analysis

PB and MB mononuclear cells were stained with conjugated monoclonal antibodies (SI Figure 1) for 30 minutes at 4°C in order to determine NK cell phenotypic profiles. After washing twice, samples were measured on a FACS Canto II (BD Biosciences San Jose, CA) and analyzed with the BD FACSDiva Software v.8.0.2 (BD Biosciences, San Jose, CA). In order to reduce inter-experimental variations and to enable comparison of the data of cells obtained on different days, settings of the flowcytometer were standardized by using application settings and CST beads (BD FACSCanto II, BD Biosciences, San Jose, CA) according to manufactures instructions.

Statistical analysis

Data was analyzed with a non-parametric Wilcoxon Signed Rank test for paired samples (PB samples from females on different time points in the menstrual cycle and PB versus MB samples from females) and with a Mann-Whitney-U test for independent samples (PB samples from females versus male controls). Data of males on day 1, 7 and 21 was averaged as males were included as non-cycling controls. Data are presented as median with interquartile range or as percentage. P-values below 0.05 and 0.017 (Bonferroni correction) in case of repeated comparison, were considered statistically significant. All statistical analyses were conducted with IBM SPSS statistics version 25 (IBM Corp, Los Angeles, USA).

RESULTS

There were no significant differences between females with a regular menstrual cycle and male controls in terms of age, BMI, smoking or use of alcohol, drugs or medication (Table 1).

Table 1 | Baseline characteristics of study population

BASELINE CHARACTERISTICS	FEMALES (n=6)	MALE CONTROLS (n=6)	P
Age (years)	28 [26-32]	28 [27-29]	0,871
BMI (kg/m^2)	21 [18-25]	23 [23-26]	0,368
Smoking	0%	0%	-
Alcohol use	67%	83%	0,211
Drug use	0%	33%	0,287
Medication use	0%	17%	0,296
Menarche	14 [13-14]	-	
Days of menstrual bleeding	4 [4-5]	-	
Days of menstrual cycle	27 [27-28]	-	
Gravida	0 [0-1]	-	
Para	0 [0-1]	-	
Abortus	0 [0-1]	-	

Baseline characteristics of study population, data are presented as median [interquartile range] or as percentage.

Peripheral blood NK cell phenotypic profiles remain constant over the menstrual cycle

We determined whether there was a cycling-associated difference in distribution of the main lymphocyte subsets by analysis of T cells ($CD3^+CD56^-$), T helper cells ($CD3^+CD56^-CD4^+$), cytotoxic T cells ($CD3^+CD56^-CD8^+$), regulatory T cells ($CD3^+CD56^-CD4^+CD25^+CD127^-$), NKT cells ($CD3^+CD56^+$) and NK cells ($CD3^-CD56^+$). We observed no differences in percentages of lymphocyte subsets of paired female samples obtained on day 1, 7 and 21 of the menstrual cycle (Figure 1A, SI Table 1). In addition, we did not observe difference between sampling days in percentages $CD56^{dim}$ NK cells ($CD3^-CD56^+CD16^+$) and $CD56^{bright}$ NK cells ($CD3^-CD56^{++}CD16^-$) (Figure 1A, SI Table 1). Furthermore, there were no differences in any of the populations when comparing female samples of the different cycle days with the average of three sampling points in male controls (Table 2).

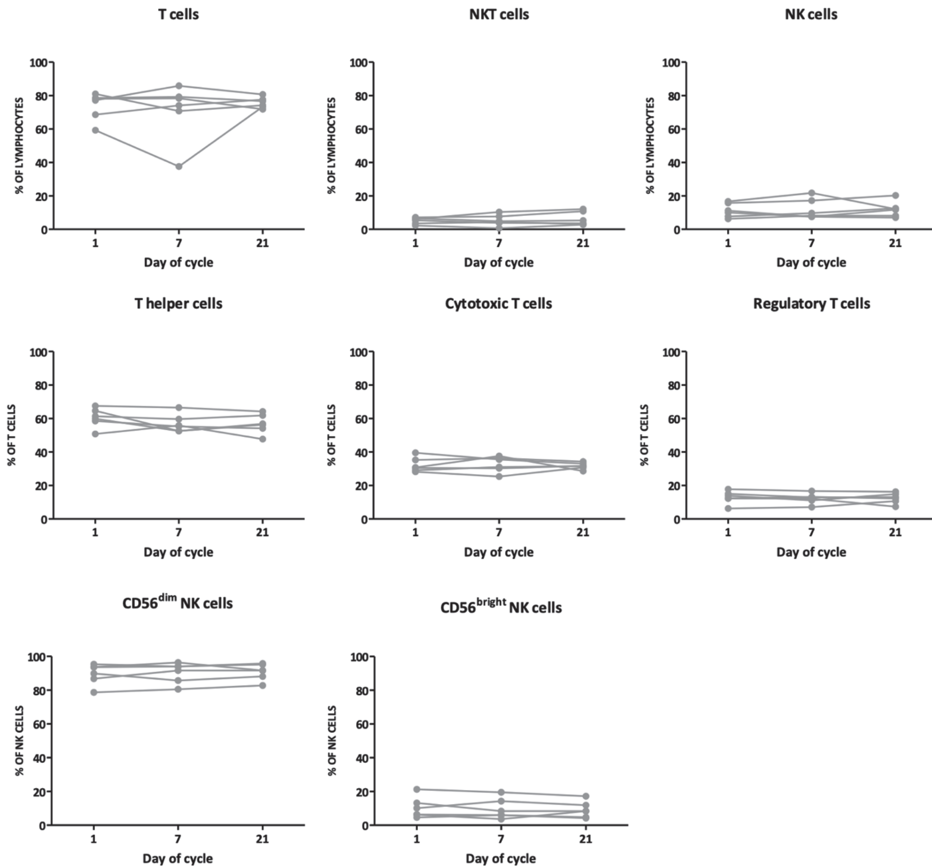

Figure 1A | Frequencies of cell populations in peripheral blood of females with a natural menstrual cycle. Cell populations are presented as percentage of lymphocytes (NKT cells ($CD3^+CD56^+$), T cells ($CD3^+CD56^-$) and NK cells ($CD3^-CD56^+$)) or as percentage of T cells (T helper cells ($CD3^+CD56^-CD4^+$), cytotoxic T cells ($CD3^+CD56^-CD8^+$) and regulatory T cells ($CD3^+CD56^-CD4^+CD25^+CD127^-$)) or as percentage of NK cells ($CD56^{dim}$ NK cells ($CD3^-CD56^+CD16^+$) and $CD56^{bright}$ NK cells ($CD3^-CD56^{++}CD16^-$)). All populations were measured by flow cytometry in peripheral blood females with a regular natural menstrual cycle at day 1, 7 and day 21 of their menstrual cycle. Dots depict individuals, lines depict paired samples.

Next, activating receptors were analyzed to investigate the potential influence of the menstrual cycle on the percentage of pNK cells positive for NKG2C (CD159c) or on the level of expression on pNK cells of DNAM1 (CD226), NKG2D (CD159d), NKp46 (CD335), NKp44 (CD336) and NKp30 (CD337). NKG2C, NKG2D, DNAM1, NKp46 and NKp30 were expressed on pNK cells and we did not find an influence of the menstrual cycle on their expression levels when comparing paired female samples on different cycle days or when comparing female samples with male controls (Figure 1B, SI Table 2, Table 3). One female had missing data on NKG2D expression on cycle day 1. On none of the cycling days, we observed cell surface expression of NKp44 on pNK cells in both females and male controls.

Moreover, we compared expression levels of inhibitory receptors between cycling days and between females and male controls. No differences in percentage of positive pNK cells were observed for KIR2DL1 (CD158a), KIR2DL2/3 (CD158b), KIR3DL1 (CD158e), NKG2A (CD159a) and in MFI of LILRB1 (CD85j) were found on different days of the cycle in females (Figure 1C, SI Table 2). One female had missing data on KIR2DL1 expression on cycle day 21. Inhibitory receptors also did not differ when comparing female samples with male controls (Table 3). Comparison of inhibitory IC receptor family members showed that TACTILE (CD96) was expressed on pNK cells and that expression levels remained constant during the period of sampling and did not clearly differ from the expression levels observed in male controls (Figure 1C, SI Table 2). On none of the sampling days PD1 (CD279), LAG3 (CD223) or TIM3 (CD366) expression was detected on the cell surface of female- or male pNK cells (Figure 1C).

NK cell phenotypic profiles are distinct when comparing PB and MB

As NK cells from MB possibly give a better representation of the local uterine environment than pNK cells, we also studied NK cell phenotype in MB by using a similar approach described for analysis of the influence of the menstrual cycle on pNK phenotype.

When comparing the main lymphocyte subsets of T cells, NK cells and NKT cells, we observed a lower frequency of T cells (P=0,028) in MB. We did not find a difference in T cell subsets of T helper cells, cytotoxic T cells or regulatory T cells when comparing PB and MB. However, we observed a lower frequency of $CD56^{dim}$ NK cells (P=0,028) and a higher frequency of $CD56^{bright}$ NK cells (P=0,028) in MB (Figure 2A, SI Table 3).

Table 2 | Frequencies of cell populations in females and male controls

CELL POPULATIONS	MALE CONTROLS	FEMALES (day 1)
T cells	71,9% [59,3-79,1]	77,7% [66,3-79,2]
NKT cells	7,1% [1,8-10,9]	5,8% [3,2-6,7]
NK cells	12,1% [9,7-21,8]	10,6% [7,4-16,0]
T helper cells	51,3% [43,0-69,2]	60,7% [56,6-65,3]
Cytotoxic T cells	34,0% [27,2-52,6]	30,7% [28,9-36,4]
Regulatory T cells	8,9% [6,9-12,2]	13,1% [10,8-15,7]
$CD56^{dim}$ NK cells	92,7% [90,3-95,6]	91,8% [84,8-94,6]
$CD56^{bright}$ NK cells	7,4% [4,4-9,7]	8,3% [5,9-15,2]

Frequencies of cell populations were measured by flowcytometry and depicted as percentage of lymphocytes (NKT cells ($CD3^+CD56^+$), T cells ($CD3^+CD56^-$) and NK cells ($CD3^-CD56^+$)) or as percentage of T cells (T helper cells ($CD3^+CD56^-CD4^+$), cytotoxic T cells ($CD3^+CD56^-CD8^+$) and regulatory T cells ($CD3^+CD56^-CD4^+CD25^+CD127^-$)) or as percentage of NK cells ($CD56^{dim}$ NK cells ($CD3^-CD56^+CD16^+$) and

Table 3 | Expression of activating and inhibitory receptors on peripheral blood NK cells in females and male controls

ACTIVATING RECEPTORS	MALE CONTROLS	FEMALES (day 1)
NKG2C	8,4% [5,2-12,5]	8,2% [2,9-16,2]
NKG2D	4922 [4353-5307]	3937 [3802-4700]
DNAM1	1210 [1088-1256]	1019 [917-1170]
NKp46	5282 [4498-6085]	4060 [3480-6584]
NKp30	2462 [1670-2938]	2254 [968-3643]
INHIBITORY RECEPTORS	**MALE CONTROLS**	**FEMALES (day 1)**
KIR2DL2/3	22,1% [19,9-31,2]	28,0% [17,6-34,6]
KIR3DL1	16,2% [9,1-25,0]	7,2% [2,0-17,7]
KIR2DL1	14,1% [10,1-19,1]	7,7% [3,2-30,0]
NKG2A	52,9% [46,7-65,1]	48,1% [40,4-64,4]
LILRB1	265 [176-351]	586 [275-860]
TACTILE	392 [304-518]	433 [227-459]

Receptor expression is depicted as percentage positive cells of total NK cells ($CD3^-CD56^+$) for NKG2C, KIR2DL2/3, KIR3DL1, KIR2DL1, NKG2A) or as normalized mean fluorescent intensity of total NK cells ($CD3^-CD56^+$) for NKG2D, DNAM1, NKp46 and NKp30.

P	FEMALES (day 7)	P	FEMALES (day 21)	P
0,617	76,3% [62,5-81,0]	0,617	75,5% [73,0-78,4]	0,207
0,317	4,7% [3,1-8,4]	0,617	4,5% [3,0-11,2]	0,617
0,617	9,0% [7,5-18,4]	0,614	12,0% [7,8-14,5]	0,801
0,317	55,6% [52,5-61,3]	0,130	56,6% [52,5-62,5]	0,317
0,617	33,3% [29,1-36,6]	1,000	31,7% [30,3-33,3]	0,317
0,313	12,5% [10,1-14,1]	0,317	12,7% [9,9-15,3]	0,317
1,000	92,8% [84,4-94,6]	1,000	91,6% [86,8-95,4]	0,614
1,000	7,2% [5,4-15,6]	1,000	8,4% [4,7-13,2]	0,614

CD56^{bright} NK cells (CD3^{-}CD56^{++}CD16^{-})) in peripheral blood of male controls (n=6) and females with a regular natural menstrual cycle (n=6) at day 1, day 7 and day 21 of their menstrual cycle, data are presented as median [interquartile range].

P	FEMALES (day 7)	P	FEMALES (day 21)	P
1,000	6,7% [3,1-16,5]	0,617	12,1% [3,2-21,8]	1,000
0,380	4652 [3448-5694]	1,000	4667 [4189-5125]	0,617
0,317	1091 [895-1353]	0,617	1113 [842-1167]	0,317
0,617	3731 [3165-6390]	0,617	4149 [3290-6078]	0,617
1,000	2219 [1123-3465]	1,000	2349 [1139-3267]	1,000
P	**FEMALES (day 7)**	**P**	**FEMALES (day 21)**	**P**
0,617	29,8% [16,0-37,8]	0,617	27,6% [17,1-35,8]	0,617
0,317	6,3% [1,2-17,5]	0,317	5,1% [1,5-17,2]	0,317
0,617	8,0% [4,4-23,8]	0,617	18,8% [7,5-48,2]	1,000
0,617	47,4% [38,2-65,4]	0,617	47,2% [41,3-64,8]	0,617
0,317	206 [171-588]	0,617	321 [181-958]	0,617
0,617	458 [280-646]	1,000	279 [267-448]	0,617

All receptors were measured by flow cytometry in peripheral blood in male controls (n=6) and females with a regular natural menstrual cycle (n=6) at day 1, day 7 and day 21 of their menstrual cycle, data are presented as median [interquartile range].

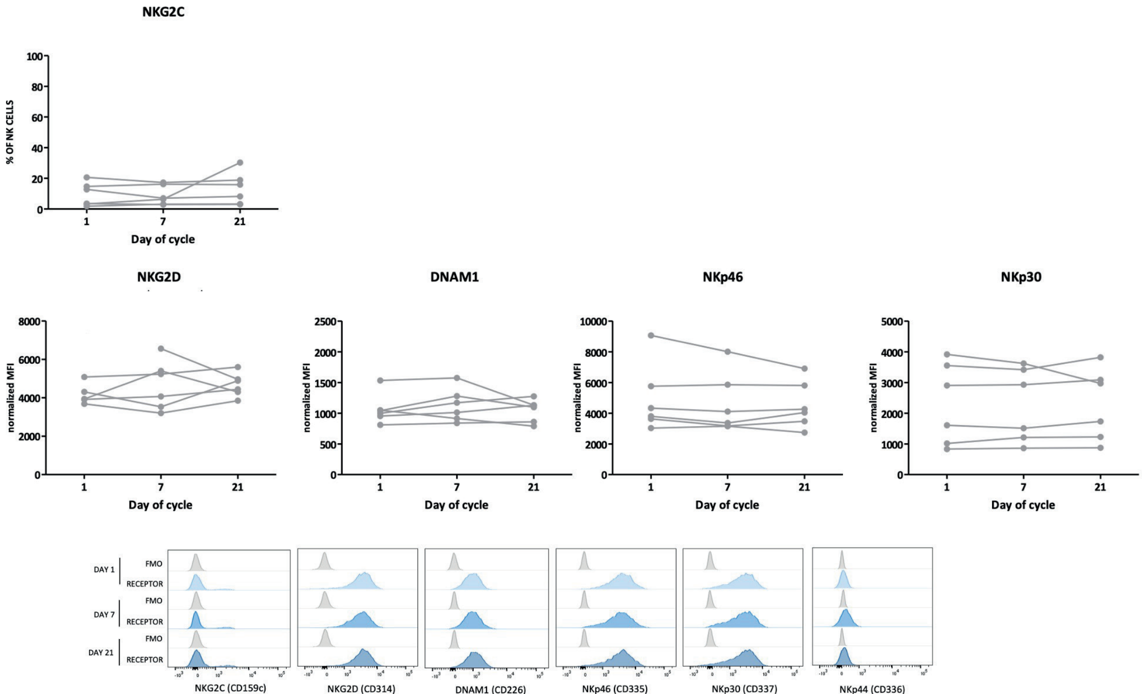

Figure 1B | Expression of activating receptors on peripheral blood NK cells of females with a natural menstrual cycle. Percentage of NKG2C ($CD159c^{+}$) positive cells of total NK cells ($CD3^{-}CD56^{+}$) and normalized mean fluorescent intensity (MFI) of NKG2D ($CD159d^{+}$), DNAM1 ($CD226^{+}$), NKp46 ($CD335^{+}$) and NKp30 ($CD337^{+}$) of total NK cells ($CD3^{-}CD56^{+}$) were measured by flow cytometry in peripheral blood of females with a regular natural menstrual cycle at day 1, 7 and day 21 of their menstrual cycle. Dots depict individuals, lines depict paired samples, one female had missing data on NKG2D expression at cycle day 1. Bottom: representative histograms of activating receptors on peripheral NK cells, grey histograms represent the FMO (no receptor staining) and blue histograms represent receptor staining on different days of the menstrual cycle.

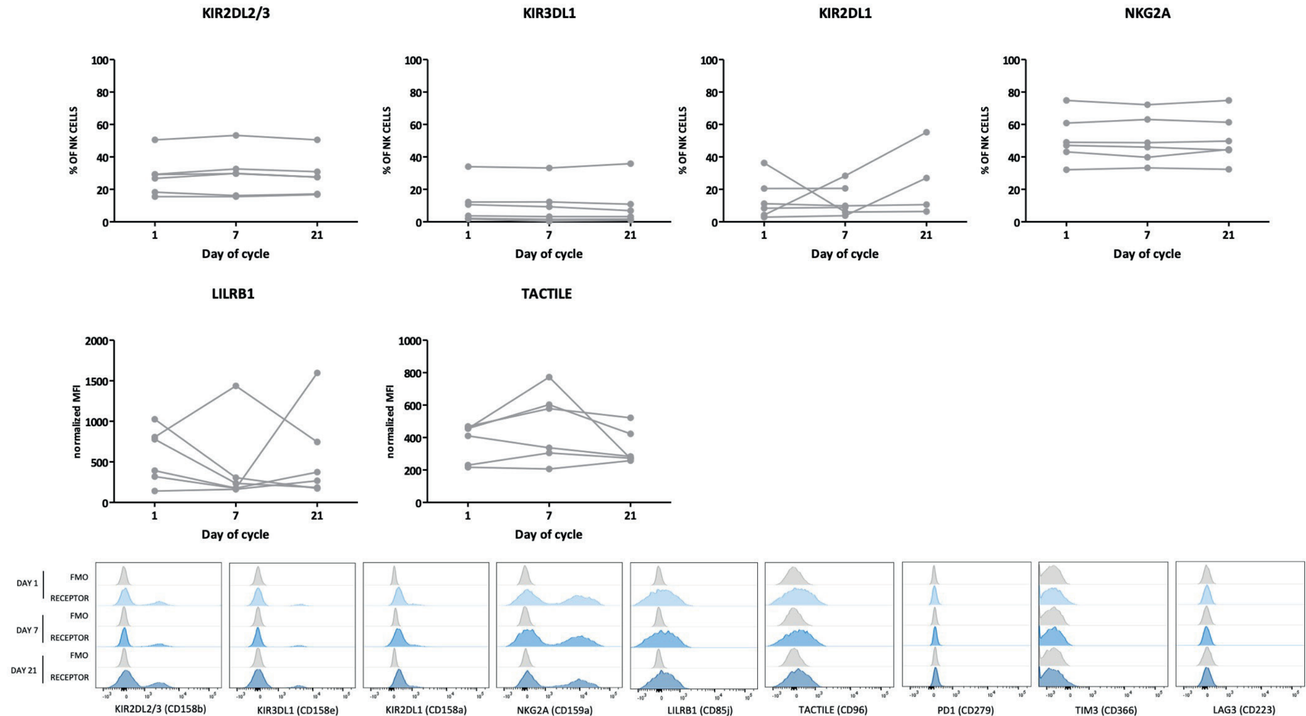

Figure 1C | Expression of inhibitory receptors on peripheral blood NK cells in females with a natural menstrual cycle. Percentage of KIR2DL2/3 ($CD158b^+$), KIR3DL1 ($CD158e^+$), KIR2DL1 ($CD158a^+$), NKG2A ($CD159a^+$) positive cells of total NK cells ($CD3^-CD56^+$) and normalized mean fluorescent intensity of LILRB1 ($CD85j^+$) and TACTILE ($CD96^+$) of total NK cells ($CD3^-CD56^+$) were measured by flow cytometry in females with a regular natural menstrual cycle at day 1, 7 and day 21 of their menstrual cycle. Dots depict individuals, lines depict paired samples, one female had missing data on KIR2DL1 expression at cycle day 21. Bottom: representative histograms of inhibitory receptors on peripheral NK cells, grey histograms represent the FMO (no receptor staining) and blue histograms represent the receptor staining on different days of the menstrual cycle.

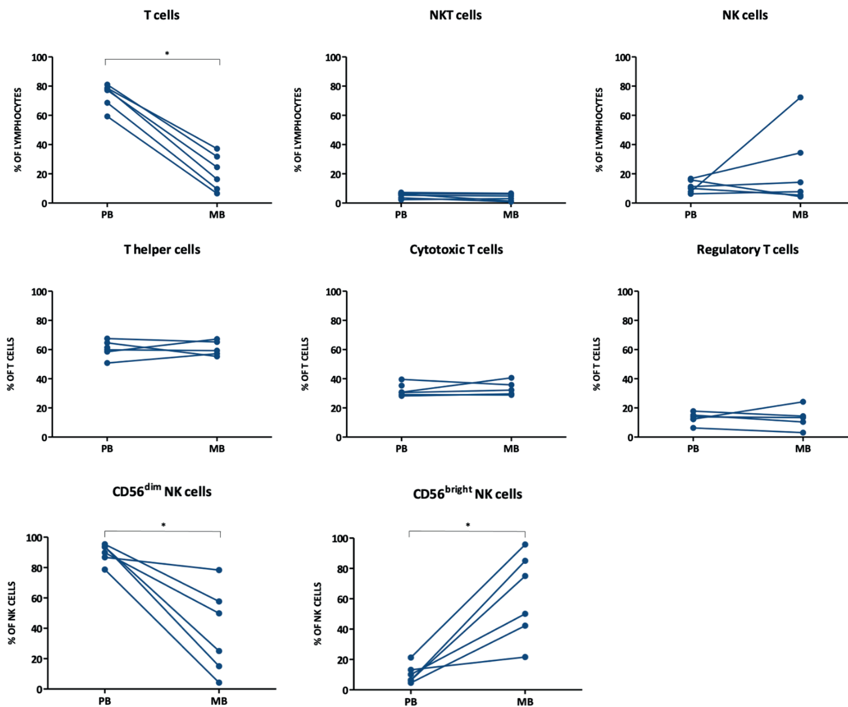

Figure 2A | Frequencies of cell populations in female peripheral blood (PB) and menstrual blood (MB). Cell populations are presented as percentage of lymphocytes (NKT cells ($CD3^{+}CD56^{+}$), T cells ($CD3^{+}CD56^{-}$) and NK cells ($CD3^{-}CD56^{+}$)), as percentage of T cells (T helper cells ($CD3^{+}CD56^{-}CD4^{+}$), cytotoxic T cells ($CD3^{+}CD56^{-}CD8^{+}$) and regulatory T cells ($CD3^{+}CD56^{-}CD4^{+}CD25^{+}CD127^{-}$)) or as percentage of NK cells ($CD56^{dim}$ NK cells ($CD3^{-}CD56^{+}CD16^{+}$) and $CD56^{bright}$ NK cells ($CD3^{-}CD56^{++}CD16^{-}$)). All populations are measured by flow cytometry in females with a regular natural menstrual cycle at day 1 of their menstrual cycle. Dots depict individuals, lines depict paired samples.

By analysis of expression of the activating receptors on NK cells from MB vs PB, we observed a lower MFI for DNAM1 (P=0,046) and a higher MFI for NKp44 in MB (P=0,027). No differences between MB and PB were observed in percentage NK cells positive for NKG2C or in the MFI of NKG2D, NKp46 and NKp30 (Figure 2B, SI Table 4). In MB, higher percentages of NK cells expressing inhibitory receptors were detected for KIR2DL1 (P=0,028), KIR2DL2/3 (P=0,046) and NKG2A (P=0,028) while the MFI of LILRB1 was lower on NK cells in MB (P=0,046) (Figure 2C, SI Table 4). There was no difference in expression of the inhibitory KIR3DL1. The IC receptors TACTILE and TIM3 were expressed on NK cells in MB of all individuals and the MFI was higher than the MFI on pNK cells (P=0,028 for TACTILE and P=0,028 for TIM3). LAG3 was not expressed on NK cells isolated from PB or NK cells from MB. PD1 was not expressed on pNK cells, however in 2 out 6 females PD1 expression was observed on NK cells isolated from MB.

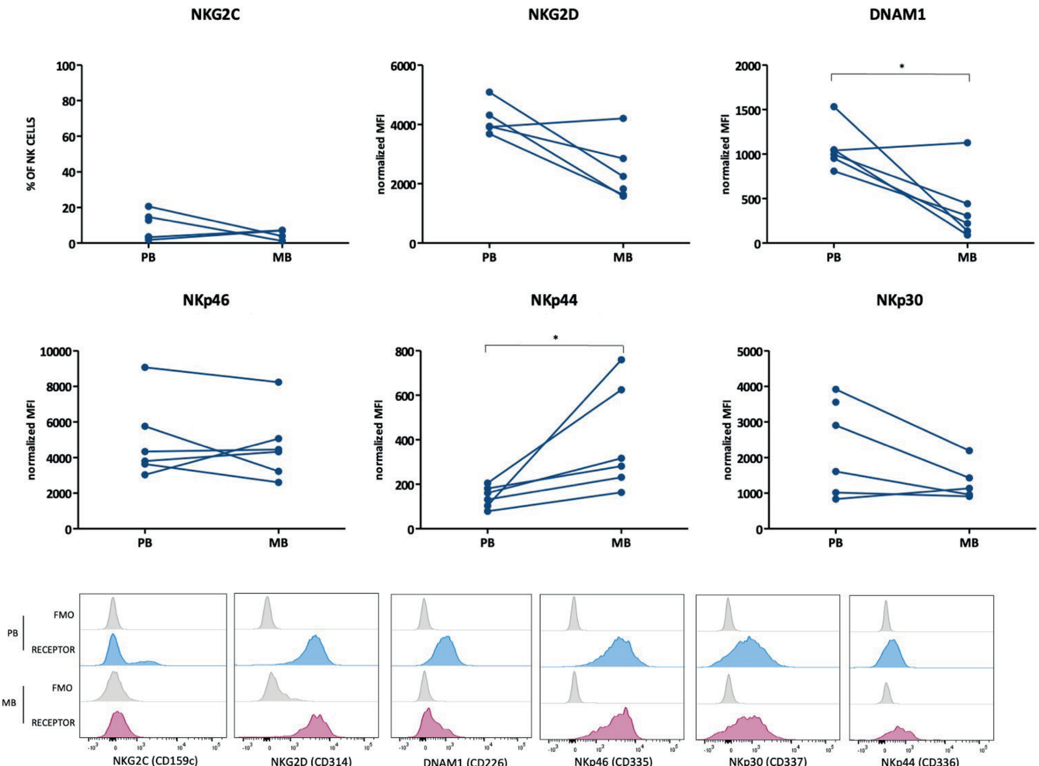

Figure 2B | Expression of activating receptors in female peripheral blood (PB) and menstrual blood (MB). Percentage of NKG2C positive cells of total NK cells ($CD3^-CD56^+$) or normalized mean fluorescent intensity of total NK cells ($CD3^-CD56^+$) for NKG2D, DNAM1, NKp46, NKp44 and NKp30 were measured by flow cytometry in females with a regular natural menstrual cycle at day 1 of their menstrual cycle. Dots depict individuals, lines depict paired samples. Bottom: Representative histograms depicting the FMO in grey and receptor expression on peripheral blood NK cells in blue and menstrual blood NK cells in pink.

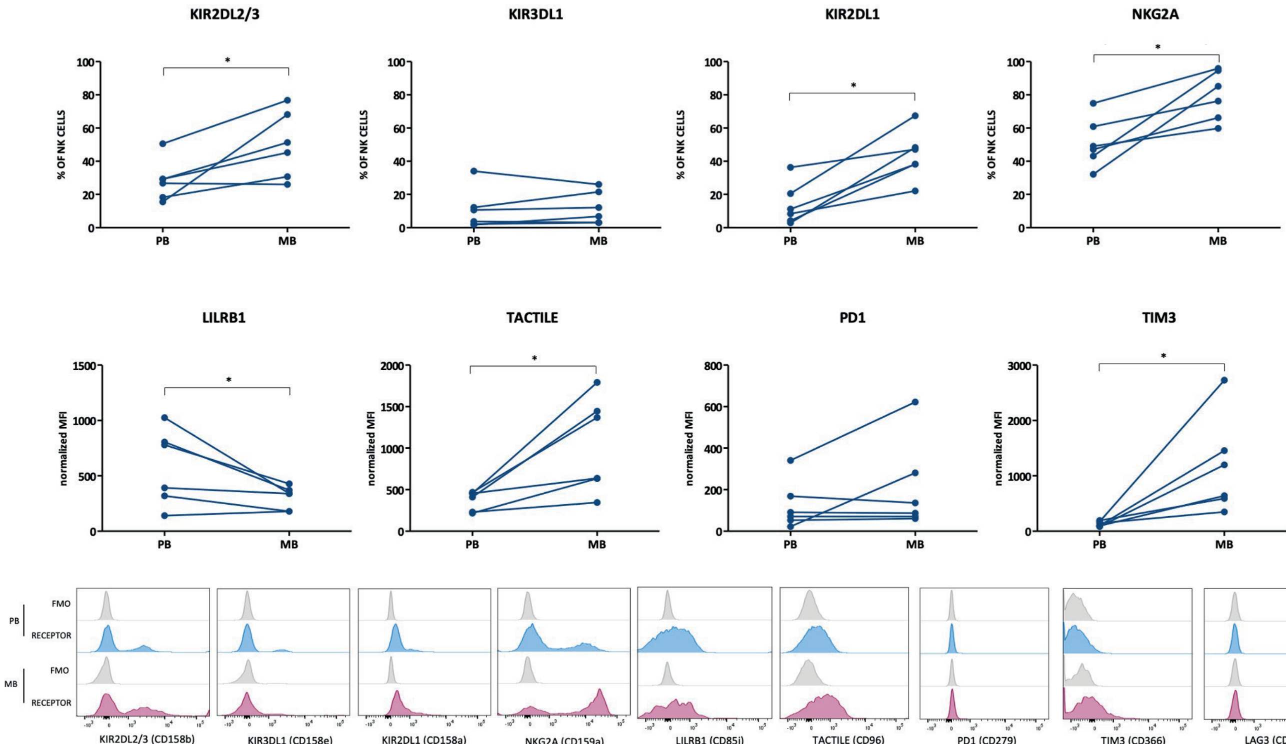

Figure 2C | Expression of inhibitory receptors on female peripheral blood (PB) and menstrual blood (MB) NK cells. Percentage of KIR2DL2/3, KIR3DL1, KIR2DL1, NKG2A positive cells of total NK cells ($CD3^{-}CD56^{+}$) and normalized mean fluorescent intensity of total NK cells ($CD3^{-}CD56^{+}$) for LILRB1, TACTILE, PD1 and TIM3 measured by flow cytometry in females with a regular natural menstrual cycle at day 1 of their menstrual cycle. Dots depict individuals, lines depict paired samples. Bottom: representative histograms of inhibitory receptors on NK cells, depicting FMO in grey and receptor expression in peripheral blood in blue and in menstrual blood in pink.

NK cells isolated from menstrual blood express markers for tissue residency

To further characterize the phenotypic profile of NK cells isolated from MB, we studied expression of markers previously associated with tissue-resident NK cells and observed three recently identified and functionally different NK cell subsets in the decidua (CD49a, CD103, CD39 and CD18). We applied the gating used to define these three decidual NK (dNK) subsets[29] (Figure 3A).

In MB from all six females, we could detect CD49a positive NK cells, and the average percentage of $CD56^+CD16^-CD49a^+$ NK cells in MB was 67% (n=6). In PB from those women sampled at the same time, we did not observe CD49a positive NK cells. Further analysis of the $CD56^+CD16^-CD49a^+$ subset showed that, on average, 3% of the cells was $CD103^-CD39^+CD18^-$, 21% was $CD103^-CD39^-CD18^+$ and 58% was $CD103^+CD18^+$ (Figure 3B).

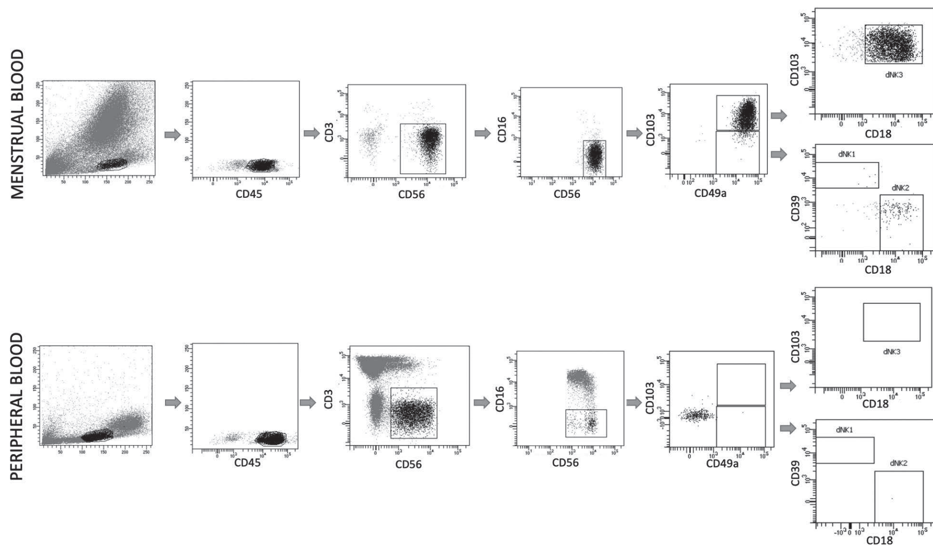

Figure 3A | Representative gating strategy for determining tissue resident dNK subpopulations in female menstrual blood (MB) and peripheral blood (PB) at cycle day 1.

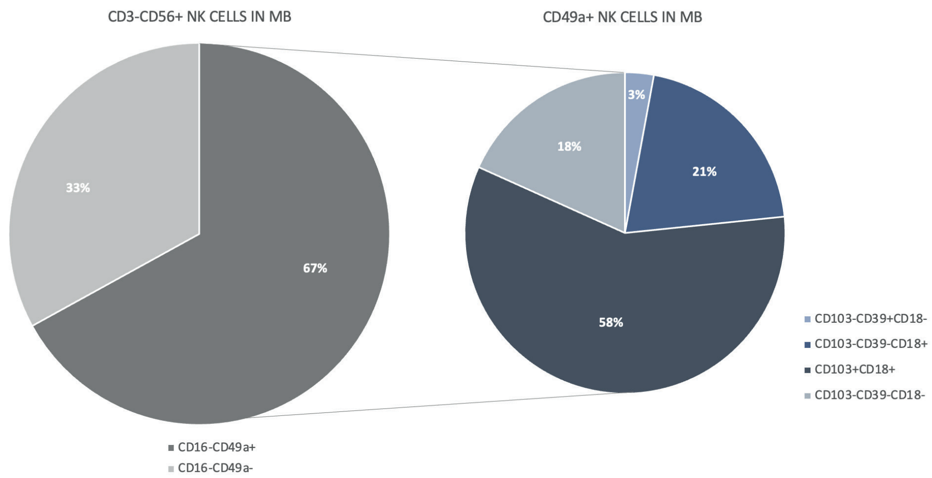

Figure 3B | Tissue resident NK cell subpopulations in menstrual blood (MB). Markers of tissue residency were measured by flow cytometry in females (n=6) at day 1 of the menstrual cycle.

DISCUSSION

The aim of this explorative study was to study the influence of the menstrual cycle on pNK cell phenotypic profiles and to compare these phenotypic profiles of pNK with NK cells from MB.

No differences were found in the major lymphocyte subsets and in repertoires of activating-, and inhibitory receptors on pNK cells throughout the menstrual cycle. Moreover, the receptor repertoires in females are highly comparable to male controls. Although we analyzed the expression of a broader panel of subsets and receptors, this is in line with a previous study of Northern et al[22] who also found no influence of the menstrual cycle on pNK cells together emphasizing that timing of sampling does not seem to have a major influence on pNK cell phenotype. In addition, our results show that the location of sampling does matter as the phenotype of NK cells obtained from MB was profoundly different from pNK cells. The high numbers of NK cells and higher frequencies of CD56^{bright} NK cells and KIR expressing NK cells in MB are in agreement with what has been found by others that studied NK cells in MB[9].

A properly balanced uterine immune system is essential to establish immunological tolerance for the semi-allogeneic fetus while simultaneously providing protection against pathogens. Although the critical role of IC receptors in establishing and maintaining immune tolerance is becoming more and more clear, their role in reproductive success is relatively unexplored. We observed a higher expression of TACTILE and TIM3 on NK cells isolated from MB as compared to pNK cells. In addition to earlier findings regarding CD56^{bright} and CD56^{dim} distinction[9], these findings also underscore the different function of eNK cells as compared to their pNK cell counterparts. Especially the higher expression of TIM3 is very interesting as TIM3 has been described to be a crucial regulator of NK cells in pregnancy by reducing their cytotoxicity and modulating cytokine production in the context of gestation[30]. The potential role of TACTILE on eNK cells has not been studied before. Comparable to TIM3, TACTILE can suppress NK cell cytotoxicity and the production of proinflammatory cytokines although these effects on NK cells have been mainly studied in the context of cancer immunity[31]. The hypothesis that eNK cells have a more tolerogenic and potentially more proangiogenic phenotype than pNK cells is also supported by the relatively high expression of NKp44 on NK cells isolated from MB. As previous studies showed that through the interaction with NKp44, trophoblast cells trigger the production by dNK cells of chemokines promoting trophoblast invasion and of VEGF[32,33,34]. Altogether supporting the hypothesis that NK cells in the uterus have a different and more immunosuppressive phenotype.

Collection of eNK cells from MB provides ample of interesting opportunities for future studies on the role of NK cells in reproduction. One of the main advantages of sampling MB to obtain shed eNK cells is that it is less invasive than uterine biopsies. Although eNK cells sampled from MB may not fully represent the cells at the time of implantation, as they are collected when the endometrium is breaking down[9], we were able to obtain viable eNK cells that could be used for phenotypic profiling. When sampling MB we cannot rule out the possibility of contamination with pNK cells. However, we anticipate the pNK fraction to be very small as almost all NK cells harvested from MB expressed the tissue residency markers CD49a, CD103, CD39 and CD18 and we did not observe these markers on pNK cells. Moreover, we were able to detect NK cells in MB that phenotypically, based on CD56, CD16, CD49a, CD103, CD18 and CD39, resemble the dNK1, dNK2 and dNK3 subsets recently identified by single cell RNAseq in the decidua by Vento-Tormo et al[29]. In our study the majority of the NK cells was $CD56^{+}CD16^{-}CD49a^{+}CD103+$, a marker combination also present on the dNK3 subset. Although, our panels were not designed to measure co-expression of KIRs and the markers used to identify dNK1-3 subsets, the high percentage of KIR positive cells in MB suggests that the CD103+ cells found in MB are different from the dNK3 subset which lacks KIRs. In line with the data on decidual NK cells, our data suggest that in MB multiple phenotypically different subsets of NK cells are present and it would be interesting to investigate their functional capacities in a follow up study.

In conclusion, we showed that NK cell phenotypic profiles that are based on the expression of key activating- and inhibitory NK cell receptors can be studied on pNK cells without an influence of the menstrual cycle, meaning it does not matter on which day of the cycle pNK cells are sampled in females. Our study also showed that NK cell subsets and receptor expression are distinctly different when studied on NK cells isolated from MB. These cells are predominantly positive for the tissue residency markers CD49a and CD103, providing exciting new possibilities for further investigations exploring their role in reproductive problems such as recurrent pregnancy loss.

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SUPPLEMENTARY FILES

PANEL 1	Clone	Fluorochrome
CD3	REA613	APC-Vio770
CD158b	DX27	PE
CD56	REA196	PE-Vio770
CD158e	DX9	PerCP
CD159a	REA110	APC
CD158a	REA284	FITC

PANEL 2	Clone	Fluorochrome
CD3	REA613	Vio Blue
CD4	REA623	APC-Vio770
CD8	BW135/80	PerCP-Vio700
CD25	4E3	VioBright-FITC
CD45	REA747	PE-Vio770
CD127	REA614	PE

PANEL 3	Clone	Fluorochrome
CD3	REA613	Vio Blue
CD16	REA423	FITC
CD56	REA196	APC-Vio770
CD85j	GHI/75	PE-Vio770
CD159c	REA205	APC

PANEL 4	Clone	Fluorochrome
CD3	REA613	Vio Blue
CD56	REA196	APC-Vio770
CD337	REA823	PE-Vio770
CD336	2.29	VioBright-FITC
CD335	REA808	PE
CD314	REA797	APC

PANEL 5	Clone	Fluorochrome
CD3	REA613	PerCP-Vio700
CD56	REA196	APC-Vio770
CD279	PD1.3.1.3	VioBright-FITC
CD226	DX11	PE
CD96	REA195	PE-Vio770
CD366	REA635	APC
CD223	REA351	Vio Blue

PANEL 6	Clone	Fluorochrome
CD3	REA613	PerCP-Vio700
CD56	REA196	APC-Vio770
CD45	REA747	VioGreen
CD16	REA423	VioBlue
CD49a	REA1106	PE
CD103	REA803	APC
CD18	REA1112	PE-Vio770
CD39	REA739	VioBright-FITC

SI Figure 1 | Conjugated antibodies divided over 6 panels to determine NK cell phenotype by means of cell populations, activating receptors and inhibitory receptors.

SI Table 1 | Frequencies of cell populations in females at different days of their menstrual cycle

CELL POPULATIONS	PB	MB	P
T cells	77,7% [66,3-79,2]	20,4% [8,8-33,2]	**0,028**
NKT cells	5,8% [3,2-6,7]	3,9% [1,2-6,2]	0,172
NK cells	10,6% [7,4-16,0]	11,0% [5,2-43,9]	0,463
T helper cells	59,9% [54,7-66,1]	59,3% [56,3-66,2]	0,893
Cytotoxic T cells	30,5% [28,7-35,2]	32,2% [29,3-38,2]	0,500
Regulatory T cells	13,9% [9,3-16,4]	13,4% [6,7-19,3]	0,500
CD56^{dim} NK cells	91,8% [84,8-94,1]	37,5% [12,3-62,9]	**0,028**
CD56^{bright} NK cells	8,3% [5,9-15,2]	62,6% [37,1-87,7]	**0,028**

Frequencies of cell populations were measured as percentage of lymphocytes (NKT cells (CD3^{+}CD56^{+}), T cells (CD3^{+}CD56^{-}) and NK cells (CD3^{-}CD56^{+})), as percentage of T cells (T helper cells (CD3^{+}CD56^{-}CD4^{+}), cytotoxic T cells (CD3^{+}CD56^{-}CD8^{+}) and regulatory T cells (CD3^{+}CD56^{-}CD4^{+}CD25^{+}CD127^{-})) or as percentage of NK cells (CD56^{dim} NK cells (CD3^{-}CD56^{+}CD16^{+}) and CD56^{bright} NK cells (CD3^{-}CD56^{++}CD16^{-})) by flow cytometry in females with a regular natural menstrual cycle (n=6) at day 1, day 7 and day 21 of their menstrual cycle, data are presented as median [interquartile range].

SI Table 2 | Expression of activating and inhibitory receptors in females at different days of their menstrual cycle

ACTIVATING RECEPTORS	**DAY 1 (females)**	**DAY 7 (females)**
NKG2C	8,2% [2,9-16,2]	6,7% [3,1-16,5]
NKG2D	3937 [3802-4700]	4652 [3448-5694]
DNAM1	1019 [917-1170]	1091 [895-1353]
NKp46	4060 [3480-6584]	3731 [3165-6390]
NKp30	2254 [968-3643]	2219 [1123-3465]
INHIBITORY RECEPTORS	**DAY 1 (females)**	**DAY 7 (females)**
KIR2DL2/3	28,0% [17,6-34,6]	29,8% [16,0-37,8]
KIR3DL1	7,2% [2,0-17,7]	6,3% [1,2-17,5]
KIR2DL1	7,7% [3,2-30,0]	8,0% [4,4-23,8]
NKG2A	48,1% [40,4-64,4]	47,4% [38,2-65,4]
LILRB1	586 [275-860]	206 [171-588]
TACTILE	433 [227-459]	458 [280-646]

Expression of activating receptors was measured as percentage of NKG2C ($CD159c^+$) positive cells of total NK cells ($CD3^-CD56^+$) and NKG2D ($CD159d^+$), DNAM1 ($CD226^+$), NKp46 ($CD335^+$) and NKp30 ($CD337^+$) normalized mean fluorescent intensity of total NK cells ($CD3^-CD56^+$). Expression of inhibitory receptors was measured as percentage of KIR2DL2/3 ($CD158b^+$), KIR3DL1 ($CD158e^+$), KIR2DL1 ($CD158a^+$),

SI Table 3 | Frequencies of cell populations in peripheral blood and menstrual blood of females at cycle day 1

CELL POPULATIONS	**DAY 1 (females)**	**DAY 7 (females)**
T cells	77,7% [66,3-79,2]	76,3% [62,5-81,0]
NKT cells	5,8% [3,2-6,7]	4,7% [3,1-8,4]
NK cells	10,6% [7,4-16,0]	9,0% [7,5-18,4]
T helper cells	60,7% [56,6-65,3]	55,6% [52,5-61,3]
Cytotoxic T cells	30,7% [28,9-36,4]	33,3% [29,1-36,6]
Regulatory T cells	13,1% [10,8-15,7]	12,5% [10,1-14,1]
$CD56^{dim}$ NK cells	91,8% [84,8-94,6]	92,8% [84,4-94,6]
$CD56^{bright}$ NK cells	8,3% [5,9-15,2]	7,2% [5,4-15,6]

Frequencies of cell populations were measured as percentage of lymphocytes (NKT cells ($CD3^+CD56^+$), T cells ($CD3^+CD56^-$) and NK cells ($CD3^-CD56^+$)), as percentage of T cells (T helper cells ($CD3^+CD56^-CD4^+$), cytotoxic T cells ($CD3^+CD56^-CD8^+$) and regulatory T cells ($CD3^+CD56^-CD4^+CD25^+CD127^-$)) or as percentage.

DAY 21 (females)	P (day 1-7)	P (day 1-21)	P (day 7-21)
12,1% [3,2-21,8]	0,753	0,917	0,115
4667 [4189-5125]	0,893	0,043	0,917
1113 [842-1167]	0,173	0,917	0,345
4149 [3290-6078]	0,116	0,249	0,917
2349 [1139-3267]	0,600	0,345	0,345
DAY 21 (females)	**P (day 1-7)**	**P (day 1-21)**	**P (day 7-21)**
27,6% [17,1-35,8]	0,138	0,786	0,116
5,1% [1,5-17,2]	0,056	0,249	0,893
18,8% [7,5-48,2]	0,917	0,715	0,068
47,2% [41,3-64,8]	0,463	0,500	0,600
321 [181-958]	0,345	0,753	0,753
279 [267-448]	0,116	0,753	0,075

NKG2A (CD159a$^+$) positive cells of total NK cells (CD3$^-$CD56$^+$) and LILRB1 (CD85j$^+$) and TACTILE (CD96$^+$) normalized mean fluorescent intensity of total NK cells (CD3$^-$CD56$^+$). All receptors were measured by flow cytometry in females with a regular natural menstrual cycle (n=6) at day 1, day 7 and day 21 of their menstrual cycle, data are presented as median [interquartile range].

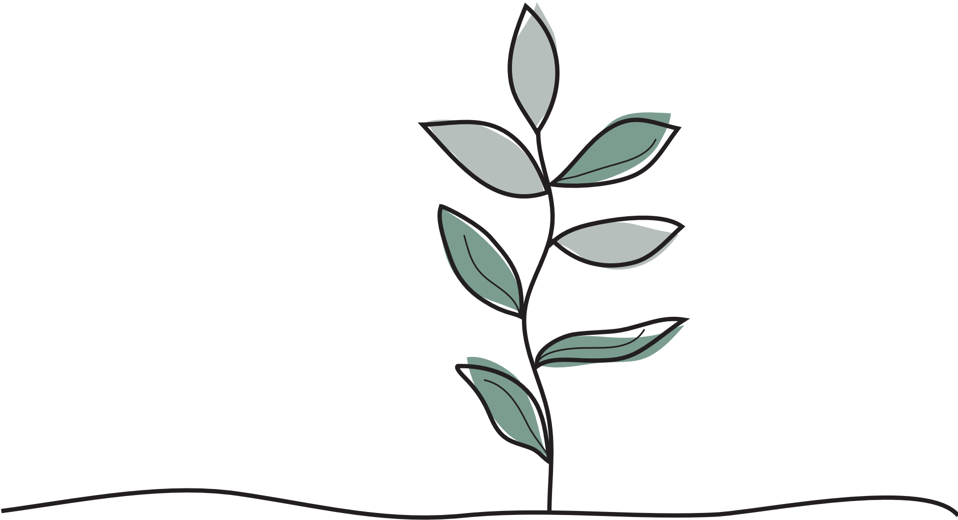
DAY 21 (females)	P (day 1-7)	P (day 1-21)	P (day 7-21)
75,5% [73,0-78,4]	0,917	0,600	0,917
4,5% [3,0-11,2]	0,752	0,600	0,173
12,0% [7,8-14,5]	0,753	0,600	0,753
56,6% [52,5-62,5]	0,173	0,056	0,833
31,7% [30,3-33,3]	0,917	0,917	0,600
12,7% [9,9-15,3]	0,249	0,500	0,917
91,6% [86,8-95,4]	0,463	0,463	0,500
8,4% [4,7-13,2]	0,463	0,463	0,500

of NK cells (CD56^{dim} NK cells (CD3$^-$CD56$^+$CD16$^+$) and CD56^{bright} NK cells (CD3$^-$CD56^{++}CD16$^-$)) by flow cytometry in females with a regular natural menstrual cycle (n=6) at day 1 of their menstrual cycle in peripheral blood (PB) and menstrual blood (MB), data are presented as median [interquartile range]

SI Table 4 | Expression of activating and inhibitory receptors in peripheral blood and menstrual blood of females at cycle day 1

ACTIVATING RECEPTORS	PB	MB	P
NKG2C	9,0% [2,2-19,1]	5,5% [1,9-7,2]	0,465
NKG2D	3937 [3802-4700]	2037 [1620-3190]	0,080
DNAM1	1019 [917-1170]	264 [125-613]	**0,046**
NKp46	4060 [3480-6584]	4384 [3066-5853]	0,600
NKp44	146 [97-187]	299 [214-658]	**0,027**
NKp30	1606 [923-3409]	1136 [932-1808]	0,138
INHIBITORY RECEPTORS	**PB**	**MB**	**P**
KIR2DL2/3	28,0% [17,6-34,6]	48,3% [29,5-70,3]	**0,046**
KIR3DL1	7,2% [2,0-17,7]	9,5% [3,0-22,6]	0,345
KIR2DL1	9,8% [3,9-24,5]	42,7% [34,1-53,1]	**0,028**
NKG2A	48,1% [40,4-64,4]	80,7% [64,6-94,9]	**0,028**
LILRB1	586 [275-860]	340 [179-385]	**0,046**
TACTILE	433 [227-459]	1003 [562-1533]	**0,028**
PD1	81 [45-212]	112 [68-366]	0,345
TIM3	124 [92-178]	920 [530-1775]	**0,028**

Expression of activating receptors was measured as percentage of NKG2C ($CD159c^+$) positive cells of total NK cells ($CD3^-CD56^+$) and NKG2D ($CD159d^+$), DNAM1 ($CD226^+$), NKp46 ($CD335^+$), NKp44 ($CD336^+$) and NKp30 ($CD337^+$) normalized mean fluorescent intensity of total NK cells ($CD3^-CD56^+$). Expression of inhibitory receptors was measured as percentage of KIR2DL2/3 ($CD158b^+$), KIR3DL1 ($CD158e^+$), KIR2DL1 ($CD158a^+$), NKG2A ($CD159a^+$) positive cells of total NK cells ($CD3^-CD56^+$) and LILRB1 ($CD85j^+$), TACTILE ($CD96^+$), PD1 ($CD279^+$) and TIM3 ($CD366^+$) normalized mean fluorescent intensity of total NK cells ($CD3^-CD56^+$). All receptors were measured by flow cytometry in females with a regular natural menstrual cycle (n=6) at day 1 of their menstrual cycle in peripheral blood (PB) and menstrual blood (MB), data are presented as median [interquartile range].



4

A REPRODUCTIVE IMMUNOLOGY STUDY ON NATURAL KILLER CELL PHENOTYPIC PROFILES IN WOMEN WITH UNEXPLAINED RECURRENT PREGNANCY LOSS (THE OVIDE STUDY): PROTOCOL

Denise Habets, Marc Spaanderman, Lotte Wieten, Salwan Al-Nasiry

ABSTRACT

Background: A healthy pregnancy commences with successful implantation, placentation and development of the embryo within the uterine cavity. In particular, the immunological environment of the uterus is of great importance, since it must be able to tolerate foreign paternal antigens expressed by the embryo while also maintaining the ability to defend against pathogens. Earlier research has shown that the immune cell population in the uterus is unique and that particularly natural killer cells play a special role in creating a predominantly tolerant immunological environment of the uterus during early pregnancy regulating spiral artery remodeling, a process associated with the cardiovascular system to accommodate vascular growth during early pregnancy. Despite exciting advances in understanding the reproductive role of natural killer cells, considerably more work needs to be done to establish a significant association of natural killer cells with unexplained recurrent pregnancy losses. Therefore, in this study we aim to investigate phenotypic profiles of natural killer cells in peripheral blood and menstrual blood and the influence of cardiovascular fitness on natural killer cells in unexplained recurrent pregnancy losses.

Design: Prospective, single-center, non-intervention, cohort study. Non-pregnant women with a history of unexplained recurrent pregnancy loss (group 1) or with a previous successful pregnancy (group 2) will be included. Women will first be asked to fill out a lifestyle questionnaire after which peripheral blood will be sampled for flowcytometric analysis. Next, cardiovascular fitness levels will be evaluated by measuring exercise tolerance during a maximal test on a cycle ergometer and participants will be asked to return samples of urine and menstrual blood upon their first following menstrual cycle.

Ethics and dissemination: The protocol is approved by the local medical ethical review committee at the Maastricht University Medical Centre. Findings from this study will be shared with the academic and medical community and patient organizations to optimize medical care of women with unexplained recurrent pregnancy losses.

Trial registration: NTR 8287, registered January 15th, 2020.

Keywords: natural killer cells, phenotype, recurrent pregnancy loss, cardiovascular fitness.

BACKGROUND

Recurrent pregnancy loss (RPL), defined as the loss of two or more pregnancies, is an unsolved problem in reproductive medicine affecting 1-3% of women who are trying to conceive[1]. In 50% of women an explanation is found in traditional fertility investigations. Unfortunately, no specific cause can be identified in the other 50% and is therefore referred to as unexplained RPL[1]. A tightly regulated balance of the immunological environment in the uterus is essential for successful implantation, placentation and development of the embryo during early pregnancy; on the one hand tolerating semi-allogenic antigens of the embryo while also being able to activate against pathogens on the other hand[2]. Previous research has shown that the immune cell population in the uterus is truly unique and that particularly natural killer (NK) cells play a special role in creating a tolerant immunological environment during early pregnancy[3]. NK cells make up for 5-15% of circulating lymphocytes in peripheral blood. These peripheral NK (pNK) cells are named after their effector function, killing target cells[4]. However, during early pregnancy, NK cells with poor killer function populate the uterine lining during the time of and following implantation, increasing dramatically in numbers accounting for 80% of lymphocytes in early pregnancy[5]. The exact function of these uterine NK (uNK) cells is still unknown but recent studies suggest uNK are functionally different from pNK cells and play an important role in regulating invading trophoblast cells and spiral artery remodeling during early pregnancy[5].

Inadequate invasion and remodeling have been described to be related to RPL[6]. Moreover, inadequate function of spiral arteries has been related to several pregnancy complications such as RPL[7]. Successful reproduction hinges upon vascular growth and accommodations throughout the menstrual cycle and pregnancy, in order to promote subsequent fetal growth and development[8]. Therefore, there might also be an influence of the cardiovascular system on successful spiral artery remodeling. Both cardiovascular fitness and NK cell status might then be important determinants related to inexplicable losses of early pregnancies.

NK cells seem to be very important for a healthy pregnancy and several studies have investigated a possible association between numbers and/or phenotypes of NK cells and RPL. To date, results are controversial and often based on a partly redundant receptor repertoire measured in different tissues by different techniques. Therefore, in this innovative study we aim to explore NK cell phenotypic profiles, including novel immune checkpoint receptors, by studying them systemically in peripheral blood and locally in menstrual blood, as menstrual blood-derived NK cells have been shown to resemble endometrial NK cells in the non-pregnant uterus[3]. Furthermore, this study will provide novel information on cardiovascular fitness in unexplained RPL and its relation to NK cell phenotypic profiles.

STUDY OBJECTIVES

Aim

The main goal of this study is to analyze NK cell phenotypic profiles in women with unexplained recurrent pregnancy losses in relation to their cardiovascular fitness.

Primary Objectives

To compare the percentage of pNK cells with activating or inhibitory receptors between women with unexplained recurrent pregnancy loss (group 1) and women with a previous successful pregnancy (group 2) in peripheral blood.

Secondary Objectives

To compare the percentage of uNK cells with activating or inhibitory receptors between women with unexplained recurrent pregnancy loss (group 1) and women with a previous successful pregnancy (group 2) in menstrual blood.

To analyze cardiovascular fitness by lifestyle questionnaire and measurements of maximum oxygen uptake (VO2max) and total body water for women with unexplained recurrent pregnancy loss (group 1) and women with a previous successful pregnancy (group 2).

METHODS

Design

This study will be cross-sectional: the study population will consist out of women with unexplained RPL (group 1) and women with a previous uncomplicated pregnancy (group 2). Measurements will be performed during a scheduled visit at the outpatient clinic of Gynecology & Obstetrics in the Maastricht University Medical Centre+ (Maastricht UMC+).

Measurements will be performed according to the following general protocol. Women who are willing to participate in the study will first be asked to fill out a baseline characteristics questionnaire, next peripheral blood will be sampled with a venapunction and last VO2max will be measured during cycling. On the same day, participants will be asked to sample their menstrual blood during their next menstrual cycle and return it to the hospital. The duration of the study for one participant is, depending on the moment of their menstrual cycle, maximal + 5 weeks from the visit when initial measurements take place. The duration of the total study is approximately 24 months.

- Months 01-18 Patient inclusion and sampling
- Months 06-18 NK cell phenotype analysis
- Months 12-24 Data analysis and writing

Eligibility criteria

We will include non-pregnant women between the age of 18 and 36 whom speak Dutch.

- Group 1: unexplained RPL

  Women will be recruited at the outpatient clinic of Gynecology & Obstetrics in the Maastricht UMC+, in which approximately 75 couples per year receive specialized investigations and care for RPL. During a visit women will be informed about the study and will be asked for permission to contact them after one week. To ensure that there is no influence of a recent pregnancy loss on the results, women should be 3 months post-partum. Assuming that 10% of the women does not meet the inclusion criteria or decline participation, the estimated source population is 60 women per year. Due to the previously observed willingness of women to contribute to scientific studies which may help them and future women, we expect that the majority of eligible women is willing to participate.
- Group 2: previous uncomplicated pregnancy

  Women will be recruited at our obstetric clinic during postpartum visits after an uncomplicated pregnancy in the Maastricht UMC+, comorbidity is not expected. During a visit women will be informed about the study and will be asked for permission to contact them after one week. To ensure that there is no influence of a previous pregnancy on the results, women should be 3 months post-partum. The estimated source population is 150 women per year of which 60 women will be included.

Additionally, women will be recruited through advertisement by means of a flyer on the Facebook page of the Maastricht UMC+ department of obstetrics and gynecology.

Inclusion criteria

- Group 1: Recurrent (≥2) unexplained pregnancy losses, defined by normal parental karyotype, no maternal thrombophilia and no uterine abnormality.
- Group 2: Previous uncomplicated pregnancy, defined by healthy live birth after 37 weeks of gestation without major obstetric complications.

Exclusion criteria

- Current or recent (<3 months ago) pregnancy
- Current or recent (<2 weeks) symptomatic genital infection such as chlamydia or gonorrhea
- Younger than 18 or older than 36 years
- Unable to give consent in Dutch

Outcome measures

Main study parameter

- Percentage of NK cells with activating or inhibitory receptors in peripheral blood

Secondary study parameters

- Percentage of NK cells with activating or inhibitory receptors in menstrual blood
- VO2 max: measured in milliliters per kilogram of body weight per minute

Data collection

After obtaining informed consent, women will be screened systematically on the same day as they visit the outpatient clinic in the Maastricht UMC+. Screening includes a baseline characteristics questionnaire, blood sampling and VO2 max testing. Additionally, an instruction will be given about sampling menstrual blood with the menstrual cup.

- Baseline characteristics questionnaire
  Women will be asked to fill in a baseline characteristics questionnaire (see SI Figure 1 for more details). The questionnaire contains questions about age, BMI, medical history, intoxications such as smoking and previous gynecological and obstetric history. By exploring baseline characteristics in both group 1 and group 2, we will be able to determine their (confounding) contribution.
- Laboratory screening
  Blood samples are taken at the Maastricht UMC+ by a well-trained nurse or medical doctor and will be processed using standard automated laboratory techniques. In total 15 ml blood will be taken. Laboratory screening includes NK cell typing by flow

cytometry with 20 markers for killer cell immunoglobulin receptors, NKG2 receptors, natural cytotoxicity receptors and immune checkpoint receptors. See SI Figure 2 for more details.

- VO2max
  Cardiovascular fitness levels will be defined as the peak oxygen uptake (VO2max in mL/min/kg) during a maximal test on a cycle ergometer. Tests will be performed in the morning after a light breakfast ad libitum by an experienced investigator. The initial workload will be set at 10Watt for 1 minute, followed by 10Watt increments every minute until exhaustion. Breath by breath oxygen uptake will be measured using spiro-ergometric equipment. An electrocardiography (ECG) continuously will record heart rate and rhythm.
- Menstrual blood collection
  Each participant will be asked to collect her menstrual blood in one consecutive menstrual cycle using the menstrual cup during one 8 hours shift and bring it back to the Maastricht UMC+ for analysis. Menstrual blood is very similar to the uterine immune microenvironment and is a validated source for studying non-pregnant uNK cells from the endometrium[3]. See SI Figure 3 for more details.
- Sample storage
  A part of the obtained blood samples will be stored at the Transplantation Immunology and Tissue Typing LAB in Maastricht UMC+ for future studies, of which the nature is not yet known. Any future study analysis on this banked human material that is in line with this present study will only be conducted when approved by the METC.

Sample size

Based on previous research on the activating marker NKp46 we expect a difference of 5% between group 2 (mean 90% ± 10%) and group 1 (mean 85%)[5]. When comparing two independent study groups for a continuous endpoint with an alpha of 0.05 and a beta of 0.2 (power is 1 minus beta thus 0.8), a total number of 63 women in each group is needed to detect this 5% difference and show discriminatory levels of NK cell phenotypes. It seems feasible to include 63 women in group 1 and 63 in group 2 in 1.5 years, considering the estimated source population is 60 for group 1 and 150 for group 2 per year and expecting that the majority of eligible women is willing to participate.

However, if it is not feasible to realize this sample size due to circumstances, an attempt will be made to determine the phenotypic profiles based on groups of receptors, rather than using the receptors individually. New algorithms for analysis will have to be developed for this, but do offer an opportunity to reduce sample size when needed.

Statistical analysis

SPSS statistics version 24 (IBM corp, Amonk, NY, USA) will be used to perform statistical analysis. Descriptive statistics will be used to analyze the outcome variables and compare them between the different study groups. Data of each parameter will be presented as means +/- standard deviation or medians +/- interquartile range based on normality using histograms and the Kolmogorov- Smirnov test. Differences between women with unexplained RPL (group 1) and women with a previous uncomplicated pregnancy (group 2) will be tested with the independent t test or Mann Whitney U test depending on whether or not data are normally distributed. A probability (p) value of less than 0.05 will be considered statistically significant. Confounders such as age (the higher the age, the higher the risk of pregnancy loss), the number of losses (the higher the number, the higher the risk of another pregnancy loss) or potential disturbing variables can be checked with linear regression models.

DATA MANAGEMENT

Data management plan

Data will be collected in a secured database by the coordinating investigator. Data handling will be done anonymously. Missing data will be mentioned along with the reason. Data will be preserved for 15 years in compliance with the Dutch Personal Data Protection Act (in Dutch: De Wet Bescherming Persoonsgegevens). There are no restrictions in data access for trial investigators.

Data monitoring

Data monitoring will be performed in compliance with Good Clinical Practice rules and regulations to achieve high quality research and ensure safety of participants. A certified independent party of the Maastricht UMC+ will monitor the study. The monitor is approved by the local medical ethical committee. Given the low risk of adverse events, a safety surveillance by a safety monitoring board is not indicated. Interim analysis is not planned.

Patient and public involvement

Patients and/or the public will not be involved in the design, conduct or dissemination plans of this study. However, the overall results of the study will be shared with the patient organization (FREYA) and patients will be informed about the ability to participate in this study via a specialized outpatient clinic for women with RPL in the Maastricht UMC+.

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SUPPLEMENTARY FILES

Studienummer ....

Datum afspraak |__|__|-|__|__|-|__|__|__|__|

ALGEMENE VOORGESCHIEDENIS

Voor- en achternaam vrouw: .... Geboortedatum: |__|__|-|__|__|-|__|__|__|__|
Leeftijd |__|__| jaar **Lengte** |__|__|__|cm **Gewicht** |__|__|__|kg **BMI**|__|__|kg/m²
Eigen geboortegewicht |__|__|__|__| gram **bij** |__|__|+|__|weken + dagen

Voor- en achternaam partner: .... Geboortedatum: |__|__|-|__|__|-|__|__|__|__|
Leeftijd |__|__| jaar **Lengte** |__|__|__|cm **Gewicht** |__|__|__|kg **BMI**|__|__|kg/m²

Etniciteit
☐ 0. Kaukasisch (Europees) ☐ 3. Latino-Amerikaans ☐ 6. Caribbisch
☐ 1. Midden-Oosten + Noord-Afrikaans ☐ 4. Sub-Sahara Afrikaans ☐ 7. anders
☐ 2. India/Pakistan/Bangladesh ☐ 5. Aziatisch

Sociaal economische status
Scholing ☐ 0. basis ☐ 1. VMBO ☐ 2. HAVO/VWO/MBO ☐ 3. HBO/WO
Inkomen ☐ 0. minimum ☐ 1. modaal ☐ 2. tweemaal modaal
Gemiddelde werkuren .... uur/week
Beroep ....

MEDISCHE VOORGESCHIEDENIS

Hypertensie	☐ 0. nee	☐ 1. ja	Nierziekte	☐ 0. nee	☐ 1. ja
Beroerte	☐ 0. nee	☐ 1. ja	Auto-immuun ziekte	☐ 0. nee	☐ 1. ja
Hart- en vaatziekte	☐ 0. nee	☐ 1. ja	Aangeboren afwijkingen	☐ 0. nee	☐ 1. ja
Stollingsstoornis	☐ 0. nee	☐ 1. ja	Schildklieraandoening	☐ 0. nee	☐ 1. ja
Trombose	☐ 0. nee	☐ 1. ja	Longziekte	☐ 0. nee	☐ 1. ja
Diabetes	☐ 0. nee	☐ 1. ja	Neurologische ziekte	☐ 0. nee	☐ 1. ja
Metabool syndroom	☐ 0. nee	☐ 1. ja			

Anders ....

Buikoperaties ☐ 0. nee ☐ 1. ja: ....

Medicatie		*naam*	*dosering*
Bloeddrukverlagers	☐ 0. nee ☐ 1. ja	....	|__|dd|__|
Vitaminen/foliumzuur	☐ 0. nee ☐ 1. ja	....	|__|dd|__|
Progestagenen	☐ 0. nee ☐ 1. ja	....	|__|dd|__|
Aspirine	☐ 0. nee ☐ 1. ja	....	|__|dd|__|
Calcium	☐ 0. nee ☐ 1. ja	....	|__|dd|__|
Heparine/LMWH	☐ 0. nee ☐ 1. ja	....	|__|dd|__|
Immunosuppressiva	☐ 0. nee ☐ 1. ja	....	|__|dd|__|
Anticonceptiemiddelen	☐ 0. nee ☐ 1. ja	....	|__|dd|__|
Anders	☐ 0. nee ☐ 1. ja	....	|__|dd|__|

Intoxicaties

Vrouw	Roken	☐ 0. nee	☐ 1. voormalig	☐ 2. huidig: |__|__|sigaretten/dag
	Alcohol	☐ 0. nee	☐ 1. voormalig	☐ 2. huidig: |__|__|glazen/week
	Cafeïne	☐ 0. nee	☐ 1. voormalig	☐ 2. huidig: |__|__|kopjes/dag
	Drugs	☐ 0. nee	☐ 1. voormalig	☐ 2. huidig
Partner	Roken	☐ 0. nee	☐ 1. voormalig	☐ 2. huidig: |__|__|sigaretten/dag
	Alcohol	☐ 0. nee	☐ 1. voormalig	☐ 2. huidig: |__|__|glazen/week
	Cafeïne	☐ 0. nee	☐ 1. voormalig	☐ 2. huidig: |__|__|kopjes/dag
	Drugs	☐ 0. nee	☐ 1. voormalig	☐ 2. huidig

Allergieën ☐ 0. nee ☐ 1. ja: ....

FAMILIE VOORGESCHIEDENIS

Trombose ☐ 0. nee ☐ 1. ja: partner/patiënt
Pre-eclampsie ☐ 0. nee ☐ 1. ja: partner/patiënt
Anders ....

Miskramen in de familie ☐ 0. nee ☐ 1. ja

Zo ja, gelieve te specificeren:					
	Moeder van patiënt	☐ 1	☐ 2	☐ >2	☐ N.V.T.
	Broer of zus van patiënt	☐ 1	☐ 2	☐ >2	☐ N.V.T.
	Moeder van partner	☐ 1	☐ 2	☐ >2	☐ N.V.T.
	Broer of zus van partner	☐ 1	☐ 2	☐ >2	☐ N.V.T.

GYNAECOLOGISCHE VOORGESCHIEDENIS

Eerste menstruatie |__|__| leeftijd in jaren
Regelmatige cyclus ☐ 0. nee ☐ 1. ja
Zo ja, gelieve te specificeren: |__|__|dagen bloeden elke |__|__|dagen
Bloedverlies tijdens menstruatie ☐ 0. licht ☐ 1. matig ☐ 2. Zwaar
Na gemeenschap ☐ 0. nee ☐ 1. ja
Tussendoor ☐ 0. nee ☐ 1. ja
Pijn tijdens menstruatie ☐ 0. licht ☐ 1. matig ☐ 2. Zwaar
Na gemeenschap ☐ 0. nee ☐ 1. ja
Polycysteus ovarium syndroom ☐ 0. nee ☐ 1. ja

Klachten van onvruchtbaarheid ☐ 0. nee ☐ 1. ja
Zo ja, gelieve te specificeren: Duur |__|__| maanden
Oorzaak ☐ 0. onbekend ☐ 1. eisprong ☐ 2. anatomisch ☐ 3. partner

OBSTETRISCHE VOORGESCHIEDENIS

Aantal keer:
Zwanger |__| Bevallen |__| Moeder |__| Miskraam |__|
Buitenbaarmoederlijke zwangerschap |__| Mola zwangerschap |__|

OBSTETRISCHE VOORGESCHIEDENIS | BEVALLING (1)

Geboortedatum |__|__|-|__|__|-|__|__|__|__| Geslacht ☐ 0. J ☐ 1. M
Zwangerschapsduur |__|__|+|__|weken + dagen Prematuur ☐ 0. nee ☐ 1. ja
Geboortegewicht |__|__|__|__| gram
Neonatale uitkomst ☐ 0. levend ☐ 1. intra-uteriene vruchtdood ☐ 2. neonatale dood
Afwijkingen ☐ 0. nee ☐ 1. ja, specificeer: ....
Huidige vader ☐ 0. nee ☐ 1. ja Tijd tot zwangerschap |__|__| maanden
Conceptie ☐ 0. spontaan ☐ 1. ovulatie-inductie ☐ 2. IUI ☐ 3. IVF ☐ 4. ICSI ☐ 5. donor
Bevalling ☐ 0. spontaan ☐ 1. inleiding ☐ 2. geplande keizersnede ☐ 3. spoed keizersnede
NICU opname ☐ 0. nee ☐ 1. ja, dagen ....

Zwangerschapscomplicaties
Pre-eclampsie ☐ 0. nee ☐ 1. ja
Eclampsie ☐ 0. nee ☐ 1. ja
Hypertensie ☐ 0. nee ☐ 1. ja
Groeivertraging ☐ 0. nee ☐ 1. ja
HELLP ☐ 0. nee ☐ 1. ja
Abruptio placenta ☐ 0. nee ☐ 1. ja
Diabetes ☐ 0. nee ☐ 1. ja

Andere gegevens, specificeer: ....

OBSTETRISCHE VOORGESCHIEDENIS | BEVALLING (2)

Geboortedatum |__|__|-|__|__|-|__|__|__|__| Geslacht ☐ 0. J ☐ 1. M
Zwangerschapsduur |__|__|+|__|weken + dagen Prematuur ☐ 0. nee ☐ 1. ja
Geboortegewicht |__|__|__|__| gram
Neonatale uitkomst ☐ 0. levend ☐ 1. intra-uteriene vruchtdood ☐ 2. neonatale dood
Afwijkingen ☐ 0. nee ☐ 1. ja, specificeer: ....
Huidige vader ☐ 0. nee ☐ 1. ja Tijd tot zwangerschap |__|__| maanden
Conceptie ☐ 0. spontaan ☐ 1. ovulatie-inductie ☐ 2. IUI ☐ 3. IVF ☐ 4. ICSI ☐ 5. donor
Bevalling ☐ 0. spontaan ☐ 1. inleiding ☐ 2. geplande keizersnede ☐ 3. spoed keizersnede
NICU opname ☐ 0. nee ☐ 1. ja, dagen ....

Zwangerschapscomplicaties
Pre-eclampsie ☐ 0. nee ☐ 1. ja
Eclampsie ☐ 0. nee ☐ 1. ja
Hypertensie ☐ 0. nee ☐ 1. ja
Groeivertraging ☐ 0. nee ☐ 1. ja
HELLP ☐ 0. nee ☐ 1. ja
Abruptio placenta ☐ 0. nee ☐ 1. ja
Diabetes ☐ 0. nee ☐ 1. ja

Andere gegevens, specificeer: ....

OBSTETRISCHE VOORGESCHIEDENIS | MISKRAAM (1)

Datum |__|__|-|__|__|__|__| (MM-JJJJ)
Conceptie ☐ 0. spontaan ☐ 1. ovulatie-inductie ☐ 2. IUI ☐ 3. IVF ☐ 4. ICSI ☐ 5. donor
Zwangerschapsduur ☐ 0. <10 weken ☐ 1. 10-14 weken ☐ 2. >14 weken
Uitkomst ☐ 0. vroege miskraam $_{<10}$ ☐ 1. foetale miskraam $_{10\text{-}14}$ ☐ 2. late miskraam $_{>14}$ ☐ 3. beëindiging (sociaal of medisch)
Bij vroege miskraam Zwangerschapstest + ☐ 0. ja ☐ 1. nee
Indien ja, echo gehad ☐ 0. ja ☐ 1. nee (BM)
- Hartactie ☐ 0. aanwezig (FM) ☐ 1. afwezig
- Embryo ☐ 0. aanwezig (EM) ☐ 1. afwezig
- Vruchtzak ☐ 0. aanwezig (VZM) ☐ 1. afwezig (PULM)

Methode ☐ 0. spontaan ☐ 1. medicatie ☐ 2. instrumentaal
Andere gegevens, specificeer: ....

OBSTETRISCHE VOORGESCHIEDENIS | MISKRAAM (2)

Datum |__|__|-|__|__|__|__| (MM-JJJJ)
Conceptie ☐ 0. spontaan ☐ 1. ovulatie-inductie ☐ 2. IUI ☐ 3. IVF ☐ 4. ICSI ☐ 5. donor
Zwangerschapsduur ☐ 0. <10 weken ☐ 1. 10-14 weken ☐ 2. >14 weken
Uitkomst ☐ 0. vroege miskraam $_{<10}$ ☐ 1. foetale miskraam $_{10\text{-}14}$ ☐ 2. late miskraam $_{>14}$ ☐ 3. beëindiging (sociaal of medisch)
Bij vroege miskraam Zwangerschapstest + ☐ 0. ja ☐ 1. nee
Indien ja, echo gehad ☐ 0. ja ☐ 1. nee (BM)
- Hartactie ☐ 0. aanwezig (FM) ☐ 1. afwezig
- Embryo ☐ 0. aanwezig (EM) ☐ 1. afwezig
- Vruchtzak ☐ 0. aanwezig (VZM) ☐ 1. afwezig (PULM)

Methode ☐ 0. spontaan ☐ 1. medicatie ☐ 2. instrumentaal
Andere gegevens, specificeer: ....

SI Figure 1 | Case record file (CRF) of the OVIDE study.

Datum: |____________________|
Studienummer: |____________________|
Uitvoerder: |____________________|
Materiaal: 2x EDTA-buis en 1x stolbuis

Lyseren
Giet 5mL bloed EDTA over in een 50mL Falcon vul aan met 45mL lysisbuffer
Vortex 5 seconden
Incubeer 10 minuten - check translucentie
Centrifugeer op 300G voor 10 minuten
Giet supernatant af en voeg 10mL PBS toe en resuspendeer pellet
Pool beide buizen en vul aan tot 25mL met PBS
Centrifugeer op 300G voor 5 minuten
Giet supernatant af en was met 25mL PBS en resuspendeer pellet
Centrifugeer op 300G voor 5 minuten
Giet supernatant af en voeg 1mL PBS toe en resuspendeer pellet en zet op ijs!

Tellen
Maak een 1:10 verdunning voor cel telling met behulp van POCH-100i: 50µL cel suspensie + 450µL PBS,
Operator: 105 - WB
Uit POCH-100i volgt aantal cellen in $*10^9$/L, omzetten in $*10^6$/mL en vermenigvuldigen met 10 i.v.m. 1:10 verdunning in bovenstaande stap
Totaal |_____|$*10^6$/mL

Vriezen
Werk op ijs en zet ampullen klaar op ijs met sticker
Benodigd aantal cellen overbrengen in 15mL buis met 5mL PBS
Centrifugeer op 300G voor 5 minuten
Giet supernatant af en resuspendeer pellet in medium A
Voeg al zwenkend medium B toe aan cel suspensie in medium A
Vul ampullen: per ampul 2mL
Plaats ampullen in mister frosty en bewaar bij minus 80 graden
Na 24 uur verplaats ampullen van mister frosty naar stikstof

Kleuren
Verdun de suspensie met FACS-buffer tot een concentratie van $4.0*10^6$ cellen/mL (staat gelijk aan 200.000 cellen per well van 50uL): |______|uL cel suspensie + |______|uL FACS-buffer
Verdun 1µL LD-marker met 1000µL PBS (LD) en werk op ijs en in het donker (licht flow uit)
Voeg cellen, life-dead marker en benodigde antilichamen toe en vortex zachtjes
Incubeer 30 minuten in het donker
Was de cellen met 200µL FACS-buffer
Centrifugeer 5 minuten 1730 RPM
Giet af en was de cellen nogmaals met 200µL FACS-buffer
Centrifugeer 5 minuten 1730 RPM
Giet af en resuspendeer pellet in 200µL fixatiebuffer en pipeteer over in buisjes
Bewaar buisjes in het donker bij 4 graden

SI Figure 2 | Standard operating procedure (SOP) for NK cell phenotyping.

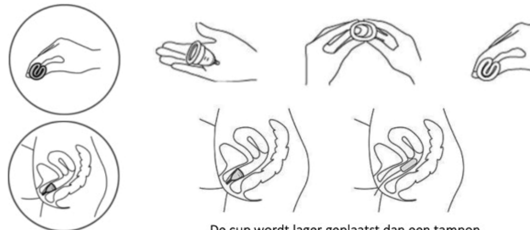
Beste mevrouw,
U heeft aangegeven vrijwillig deel te willen nemen aan de OVIDE studie met de ORGANICUP.

De ORGANICUP is een zacht medisch siliconen menstruatie cup ontworpen voor vrouwen als een handig, veilig en milieuvriendelijke alternatief voor tampons, inlegkruisjes en maandverband.

Hoewel velen de cup gemakkelijk kunnen inbrengen, kan het zijn dat de eerste keer een beetje onwennig voelt of dat u misschien een paar keer moet oefenen voordat u het inbrengen beheerst.
Om u hierbij te helpen volgen een paar stappen om het inbrengen te vergemakkelijken.
Belangrijk: voor gebruik moet u de cup eerst 3-5 minuten in kokend water steriliseren!

Stap 1 **Was uw handen en de cup met lauwwarm water en milde zeep**

Stap 2 **Vouw de cup, ontspan en breng deze in gevouwen toestand laag in uw vagina in**



De cup wordt lager geplaatst dan een tampon

Wanneer de gevouwen cup ingebracht is en u laat deze los dan kan u een "plop" of "zuig" geluid horen, dit betekent dat de cup helemaal uitgevouwen is.
Als de cup op zijn plaats zit, probeer dan een beetje aan het steeltje te trekken. Als u weerstand voelt, is het vacuüm gecreëerd en is de cup succesvol geplaatst.

Stap 3 **Draag de cup maximaal 8 uur aaneengesloten**

Stap 4 **Verwijder de cup en leeg hem in het bijgeleverde buisje**
Trek met schone handen aan het steeltje tot u de onderkant van de cup voelt, knijp zachtjes in de onderkant zodat het vacuüm loslaat en verwijder de cup voorzichtig door deze rechtop te houden.
Leeg de cup in het bijgeleverde (OVIDE) buisje, zwenk de buis en bewaar deze op kamertemperatuur.

Stap 5 **Was de cup met lauwwarm water en milde zeep**
De cup kunt u vervolgens voor eigen gebruik opnieuw inbrengen.

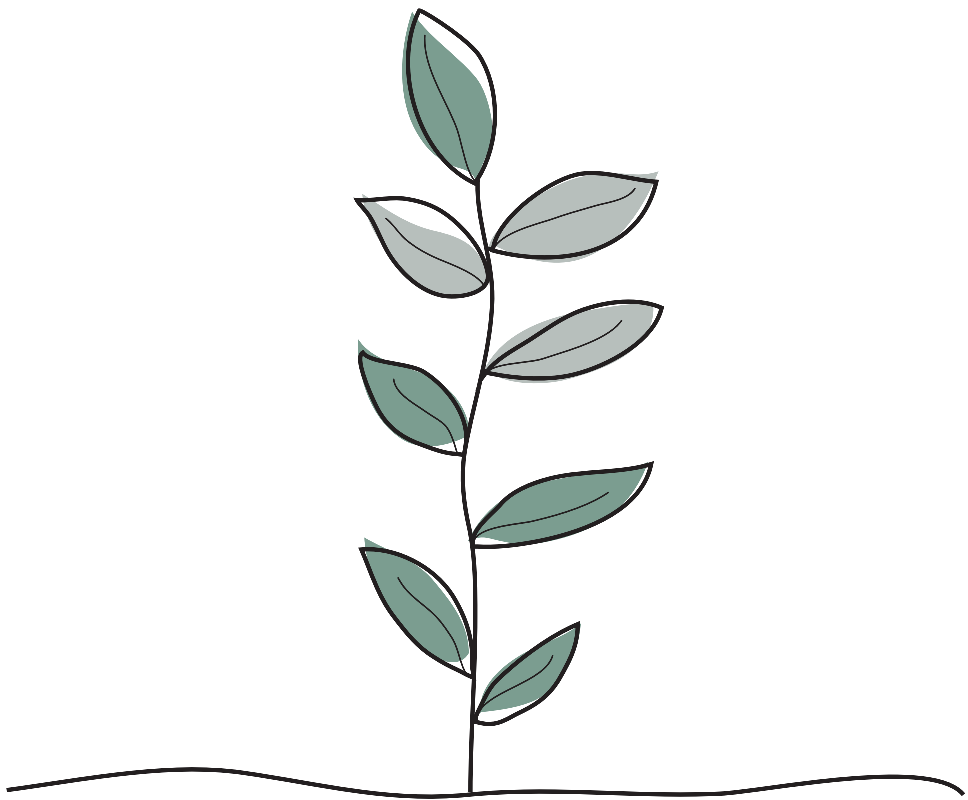
Stap 6 **Lever het buisje de eerstvolgende werkdag in**
Bij het secretariaat van: Transmuraal Vrouwen Dagcentrum in het Maastricht UMC+.

Transmuraal Vrouwen Dagcentrum
Het dagcentrum in het Maastricht UMC+ bevindt zich op niveau twee aan de zuidzijde van het ziekenhuis: route D, niveau 2, kleur groen. Het centrum is van maandag tot en met vrijdag geopend van 7:00 tot 15:30 uur.

Heeft u vragen of opmerkingen?
Mail dan gerust naar: ovide.verloskunde@mumc.nl of neem telefonisch contact op via 043-3874152.

Hartelijk dank voor uw deelname aan het onderzoek met de ORGANICUP.

SI Figure 3 | Instructions for menstrual blood collection.



5

NATURAL KILLER CELL PROFILES IN RECURRENT PREGNANCY LOSS: INCREASED EXPRESSION AND POSITIVE ASSOCIATIONS WITH TACTILE AND LILRB1

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Marc Spaanderman, Salwan Al-Nasiry, Lotte Wieten

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ABSTRACT

Introduction: NK cells are important for healthy pregnancy and aberrant phenotypes or effector functions have been associated with RPL. We compared expression of a broad panel of NK cell receptors, including immune checkpoint receptors, and investigated their clinical association with RPL as this might improve patient stratification and prediction of RPL.

Methods: Peripheral blood mononuclear cells were isolated from fifty-two women with RPL and from twenty-two women with an uncomplicated pregnancy for flowcytometric analysis and plasma was used to determine anti-CMV IgG antibodies.

Results: Between RPL and controls, we observed no difference in frequencies of T-, NKT or NK cells, in CD56dimCD16+ or CD56brightCD16- NK cell subsets or in the expression of KIRs, NKG2A, NKG2C, NKG2D, NKp30, NKp44, NKp46 or DNAM1. NK cells from women with RPL had a higher expression of LILRB1 and TACTILE and this was associated with the number of losses. The immune checkpoint receptors PD1, TIM3 and LAG3 were not expressed on peripheral blood NK cells. In RPL patients, there was a large variation in NKG2C expression and higher levels could be explained by CMV seropositivity.

Conclusion: Our study identified LILRB1 and TACTILE as NK cell receptors associated with RPL. Moreover, we provide first support for the potential role of CMV in RPL via its impact on the NK cell compartment. Thereby our study could guide future studies to confirm the clinical association of LILRB1, TACTILE and NKG2C with RPL in a larger cohort and to explore their functional relevance in reproductive success.

Keywords: recurrent pregnancy loss, natural killer cell, immune checkpoint, cytomegalovirus.

INTRODUCTION

Recurrent pregnancy loss (RPL), defined by the Dutch national guidelines as two or more pregnancy losses below twenty weeks of gestation, affects 1-2% of women trying to conceive[1,2]. RPL imposes a heavy burden on the affected couple and often results in a variety of emotional feelings, such as grief, guilt and anxiety[3]. Until now, the cause of RPL, such as chromosomal abnormalities, thrombophilia, endocrine or uterine anomalies, is known in about half of the cases, whereas the other half of RPL cases cannot be explained yet[4]. It has been hypothesized that a significant proportion of these unexplained pregnancy losses may have an immune-related etiology leading to defective placentation[5].

During placentation, extravillous trophoblast cells (EVTs) from the fetus invade the decidua and spiral arteries[6,7]. Inadequate invasion and remodeling have been described to be related to obstetric complications, such as RPL and pre-eclampsia[8,9]. From an immunological point of view, invading EVTs are a unique challenge to the maternal immune system, as the fetus is genetically half different from the mother. Hence, a tight balance needs to be established between proper invasion of the EVTs without rejection and impeding over-invasion of the trophoblast cells into the decidua of the mother on the one hand as well as being able to adequately respond to pathogens on the other hand[10]. Natural killer (NK) cells have been proposed to play a critical role in maintaining this balance[10]. NK cells are present in high numbers in the decidua during early pregnancy, in which they surround the invading trophoblast cells and the spiral arteries[6]. During early pregnancy, they secrete a variety of cytokines, chemokines and growth factors, among vascular endothelial growth factor A/C and placental growth factor, which modulate invasion and homing of trophoblasts to the spiral arteries[7,10]. The production of these factors peaks during gestational weeks eight to fourteen, the time during which spiral artery remodeling is maximal[11].

Unlike other lymphocytes, NK cells do not require antigen presentation by human leukocyte antigen (HLA) molecules in order to differentiate between diseased- or foreign cells and healthy cells of self-origin. NK cells have a broad receptor repertoire that regulates their function by providing activating or inhibiting signals upon binding with a ligand and the balance of these signals determines whether NK cells become activated or not[12]. Two of the receptor families most frequently studied in relation to reproductive success are the family of killer-cell immunoglobin-like receptors (KIR), including e.g., the inhibitory KIR2DL1, KIR2DL2/3 and KIR3DL1 that interact with HLA class I, and the family of c-type lectin-like receptors including NKG2A, NKG2C, both interacting with HLA-E, and NKG2D binding to MHC class I chain-related (MIC) proteins and UL16 binding proteins (ULBP)[13]. Besides, there is a family of natural cytotoxicity receptors (NCR), a group of activating receptors that are primarily expressed on NK cells, including NKp30, Nkp44 and NKp46 interacting with diverse

ligands on tumor cells (e.g., B7-H6, NKp44L, vimentin) but also more general with heparan sulfate proteoglycans[13] and a leukocyte immunoglobulin-like receptor family including the NK cell associated LILRB1 receptor with specificity for HLA-G.[14] Additionally, there is a group of immune checkpoint receptors, which regulate the degree of immune activation. The inhibitory receptors in this group are important for immunological tolerance and PD1, TIM3, LAG3 and TACTILE are examples of this group of receptors[15,16,17]. Although the role of this receptor family in pregnancy is not yet known, a recent study has shown that TIM3 positive NK cells are associated with an immunosuppressive phenotype with reduced cytotoxicity and production of anti-inflammatory cytokines[18].

Given their suggested important role in pregnancy, several studies explored the association between NK cell numbers and reproductive success, though the results between these studies are not fully conclusive yet[19]. Moreover, in women with RPL, differences in peripheral blood NK cell receptor expression have been described for example by Fukui et al.[20], Comins et al.[21] and Strobel et al.[22] who primarily focused on activating receptors, and by Zhu et al.[23], Ntrivalas et al.[24] and Emmer et al.[25] focusing on CD16 in combination with inhibitory KIRs and NKG2A. The immune checkpoint receptors have not been thoroughly studied in relation to RPL, except for TIM-3[18] and TIGIT[21]. However, they can dampen immune reactivity against tumors and have been shown to be distinctive for patients versus controls in cancer immunology[26]. The many overlapping mechanisms of immunological tolerance between cancer and pregnancy warrant further evaluation of the impact of these receptors on pregnancy outcome.

In our study we compared NK cell phenotypic profiles, including novel immune checkpoint inhibitors, in women with a history of recurrent pregnancy loss and women with a previous uncomplicated pregnancy and investigated their clinical association with RPL.

MATERIAL AND METHODS

Study population

In this retrospective study, fifty-two women with recurrent pregnancy loss and twenty-two women with a previously vascular uncomplicated pregnancy were included for characterization of peripheral blood (PB) leukocytes. Women with 2 or more reported pregnancy losses before 20 weeks of gestation, were included at least 3 months after pregnancy loss. Women were excluded if outcomes from the clinical RPL evaluation indicated abnormal parental karyotype, thrombophilia (presence of anti-phospholipid antibodies/Factor V Leiden mutation/Prothrombin mutation/Lupus anticoagulant or deficiency of Protein C, Protein S, antithrombin), endocrine abnormality (e.g., thyroid dysfunction) or uterine anomalies. Upon written informed consent, according to the Medical Ethical Committee of the Maastricht University Medical Centre (Maastricht UMC+) NL67368.068.18, information on baseline characteristics and cells were obtained. All PB samples were processed immediately after collection. Plasma was available of 30 women with RPL and of 16 controls.

Isolation of PB leukocytes

Leukocytes derived from ethylene diamine tetra acetic acid (EDTA) blood samples were isolated by incubation of whole blood with red blood cell lysis buffer (0.155 mol/L NH_4Cl, 10 mmol/L $KHCO_3$ and 0.1 mol/L EDTA (Na_2) with pH adjusted to 7.6) in a 1:5 ratio followed by centrifugation. After lysis the pellet with leukocytes was washed twice with PBS. Viability of cells and cell numbers were determined by trypan blue staining in a counting chamber.

Antibodies and flowcytometric analysis

PB leukocytes were stained with conjugated antibodies for 30 minutes at 4°C in order to determine NK cell phenotype (CD3 (REA613), CD56 (REA196), CD158b (DX27), CD158e (DX9), CD159a (REA110), CD158a (REA284), CD4 (REA623), CD8 (BW135/80), CD85j (GHI/75), CD159c (REA205), CD337 (REA823), CD336 (2.29), CD335 (REA808), CD314 (REA797), CD279 (PD1.3.1.3), CD226 (DX11), CD96 (REA195), CD366 (REA635), CD223 (REA351). After washing twice, samples were measured on a FACS Canto II (BD Biosciences San Jose, CA) and analyzed with the BD FACSDiva Software v.8.0.2 (BD Biosciences, San Jose, CA). Acquisition of sample was stopped at 5000 CD3-CD56 NK cells and this criterium was met for all samples. All measured events were included in the analysis. SI Figure 1 shows the gating strategy for determining cell populations and NK receptors. NK cell receptors were analyzed on total population of CD3-CD56+ NK cells and additionally on CD3-$CD56^{dim}$ and CD3-$CD56^{bright}$ NK cells. In order to reduce inter-experimental variations, data acquisition was standardized with application setting and daily calibration with CST beads. In a pilot study, we observed that there was no consistent impact of timing of sampling with respect to the menstrual cycle on peripheral cell populations or NK cell receptors (manuscript submitted).

CMV status

Anti-CMV IgG antibodies in plasma were measured by chemiluminescence immunoassay according to manufactures procedures (CMV IgG elecsys, Roche, Mannheim, Germany).

Statistical analysis

Data was assessed for normality by visual inspection of histograms. Data of baseline characteristics was either presented as average and standard deviation and analyzed with the independent-samples t-test or presented as percentage and analyzed with the Pearson's chi-square test. Data of immune markers from PB samples and CMV status of women with RPL and controls was presented as median with interquartile range and analyzed with the non-parametric Mann-Whitney-U test. Data of right-skewed immune markers was normalized by logarithmic transformation before linear regression analysis. Linear regression analysis was performed to estimate associations between immune markers and having multiple pregnancy losses. The estimated associations were presented as regression coefficient (B) and 95% confidence interval, and were additionally adjusted for BMI. Levene's test was used to test whether standard deviations between groups differed. Moreover, linear regression analysis was performed to estimate correlations, presented as correlation coefficient (*r)*, between specific immune markers and the number of pregnancy losses. A P-value below 0.05 was considered statistically significant. All statistical analyses were conducted with IBM SPSS statistics version 25 (IBM Corp, Los Angeles, USA).

RESULTS

A total of 52 women with RPL was included in the analysis in addition to 22 controls. Women with RPL had on average more previously confirmed pregnancies versus controls (5 versus 2, P<0.001), more pregnancy losses (4 versus 0, P<0.001) and fewer births (0 versus 1 P=0.001). Furthermore, women with RPL had higher body mass index versus controls (26.0kg/m^2 versus 22.8kg/m^2, P=0.021). For all other variables, no significant differences were found (Table 1).

Table 1 | Baseline characteristics of study population

BASELINE CHARACTERISTICS	RPL (n=52)	Controls (n=22)	P
Age	31.3 ± 3.5	31.8 ± 3.1	0.579
Height	168 ± 8	170 ± 5	0.305
Weight	74 ± 18	67 ± 10	0.052
BMI	26.0 ± 6.3	22.8 ± 4.2	**0.021**
Smoking	18	6	0.252
Alcohol use	39	44	0.721
Drugs use	4.8	0	0.374
Medication use	23	19	0.741
Allergies	40	44	0.770
Menarche	13.1 ± 1.7	13.3 ± 1.3	0.703
Regular cycle	90	77	0.214
Gravida	5 ± 2	2 ± 1	**<0.001**
Para	0 ± 1	1 ± 1	**0.001**
Pregnancy losses	4 ± 2	0 ± 0	**<0.001**

Baseline characteristics of study population, data are presented as average ± standard deviation or as percentage.

<u>NK cells from women with RPL have a higher expression in LILRB1 and TACTILE</u>

First, we observed no differences in percentages of the main lymphocyte subsets of T cells ($CD3^+CD56^-$), NKT cells ($CD3^+CD56^+$) and NK cells ($CD3^-CD56^+$) between women with RPL and women with a previous uncomplicated pregnancy (Figure 1, SI Table 1). In addition, we did not observe a difference in T helper cells ($CD3^+CD56^-CD4^+$), cytotoxic T cells ($CD3^+CD56^-CD8^+$), cytotoxic $CD56^{dim}$ NK cells ($CD3^-CD56^+CD16^+$) or cytokine producing $CD56^{bright}$ NK cells ($CD3^-CD56^{++}CD16^-$) (Figure 1, SI Table 1).

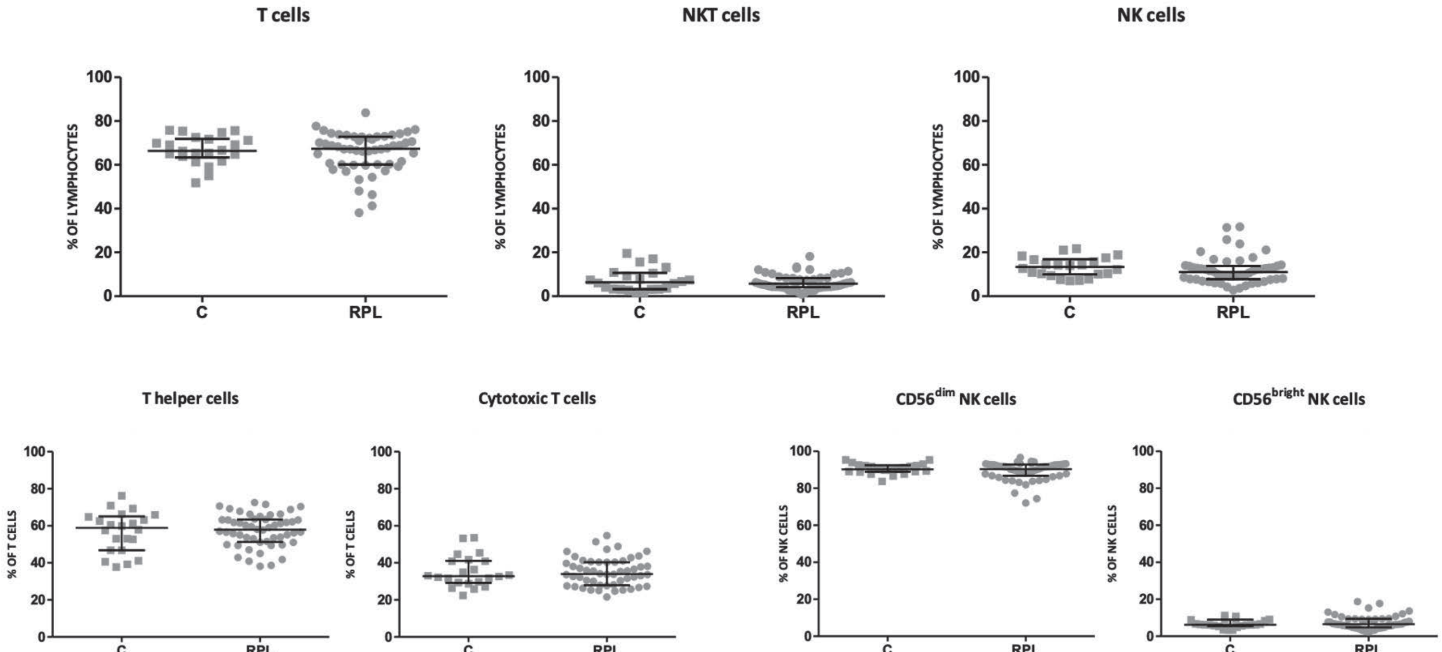

Figure 1 | Frequencies of cell populations in women with a previous uncomplicated pregnancy (C n=22) and women with recurrent pregnancy loss (RPL n=52). Cell populations are presented as percentage of lymphocytes (NKT cells ($CD3^+CD56^+$), T cells ($CD3^+CD56^-$) and NK cells ($CD3^-CD56^+$)), as percentage of T cells (T helper cells ($CD3^+CD56^-CD4^+$) and cytotoxic T cells ($CD3^+CD56^-CD8^+$)) or as percentage of NK cells ($CD56^{dim}$ NK cells ($CD3^-CD56^+CD16^+$) and $CD56^{bright}$ NK cells ($CD3^-CD56^{++}CD16^-$)). All populations were measured by flow cytometry. Dots indicate individuals, lines indicate median and interquartile range.

Second, we analyzed activating receptors to investigate potential differences in the percentage of positive NK cells or in expression levels depicted as mean fluorescent intensity (MFI) of total NK cells ($CD3^{-}CD56^{+}$). There were no differences in expression of the percentage of NK cells positive for NKG2C (CD159c) or in the expression levels of DNAM1 (CD226), NKG2D (CD159c), NKp46 (CD335) and NKp30 (CD337) when comparing women with RPL and women with a previous uncomplicated pregnancy. NKp44 (CD336) was not detected on NK cells (Figure 2, SI Table 1).

Third, expression of inhibitory receptors was compared between both groups of women. No difference in the percentage of positive NK cells was observed for KIR2DL1 (CD158a), KIR2DL2/3 (CD158b), KIR3DL1 (CD158e) and NKG2A (CD159a). We did observe a higher MFI for LILRB1 (CD85j) in women with RPL (RPL 771 versus control 298, P=0.007) (Figure 3, SI Table 1). Fourth, analysis of inhibitory immune checkpoint receptors showed that TACTILE (CD96) was higher expressed on NK cells in women with RPL (RPL 747 versus control 370, P=0.046) (Figure 3, SI Table 1). PD1 (CD279), LAG3 (CD223) or TIM3 (CD366) expression was not detected on the cell surface of NK cells (Figure 3, SI Table 1).

In addition to the analysis of total NK cells, we compared expression of activating and inhibitory receptors between RPL woman vs controls for the $CD56^{dim}$ NK cell subset (SI Figure 2, SI Table 2) and $CD56^{bright}$ NK cell subsets (SI Figure 3, SI Table 2). On $CD56^{dim}$ cells, we observed a higher MFI for TACTILE (RPL 859 versus control 308, P=0.005) and LILRB1 (RPL 673 versus control 363, P=0.016) in RPL as compared to controls (SI Figure 2), while this difference was not observed on the $CD56^{bright}$ subset (SI Figure 3). On the $CD56^{bright}$ subset, we observed a lower percentage of NKG2A positive cells in women with RPL compared to controls (RPL 83.4 versus control 91.9, P=0.042) while this difference was not observed in total CD56 NK cell subset or in the $CD56^{dim}$ subset. On both dim and bright subsets of NK cells, all the other receptors were equivalently expressed in women with RPL and controls (SI Figure 2, SI Figure 3, SI Table 2).

Expression of LILRB1 and TACTILE is associated with RPL and with the number of pregnancy losses

Regression analysis showed that expression levels of TACTILE (B=0.39; P=0.050) and LILRB1 (B=0.64; P=0.020) were associated with RPL (Table 2). After adjustment for BMI, the associations not only persisted but even increased (B=0.60; P=0.006 and B=0.97; P=0.002 respectively, Table 2). Furthermore, regression analysis showed positive associations for both TACTILE (R=0.330; P=0.004) and LILRB1 (R=0.279; P=0.017) with the number of pregnancy losses (Figure 4, SI Table 3). For the other receptors there was no association with the number of pregnancy losses (SI Table 3).

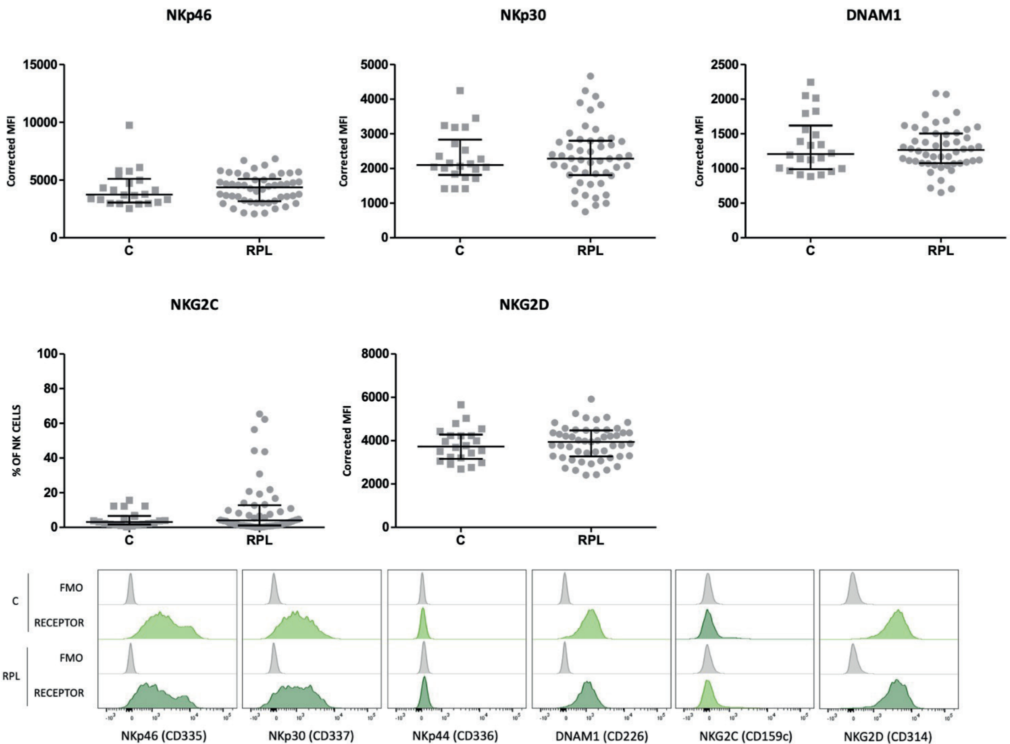

Figure 2 | Expression of activating receptors in women with a previous uncomplicated pregnancy (C n=22) and women with recurrent pregnancy loss (RPL n=52). Data show percentage NKG2C positive cells of total NK cells ($CD3^{-}CD56^{+}$) or corrected mean fluorescent intensity (MFI) of total NK cells ($CD3^{-}CD56^{+}$) for NKG2D, DNAM1, NKp46 and NKp30. Correction was done by subtracting the MFI of the fluorescence minus one (FMO). All data were acquired by flow cytometry. Dots indicate individuals, lines indicate median and interquartile range. Bottom: representative histograms of activating receptors on peripheral NK cells, grey histograms represent the FMO (no receptor staining) and green histograms represent the receptor staining in RPL and C.

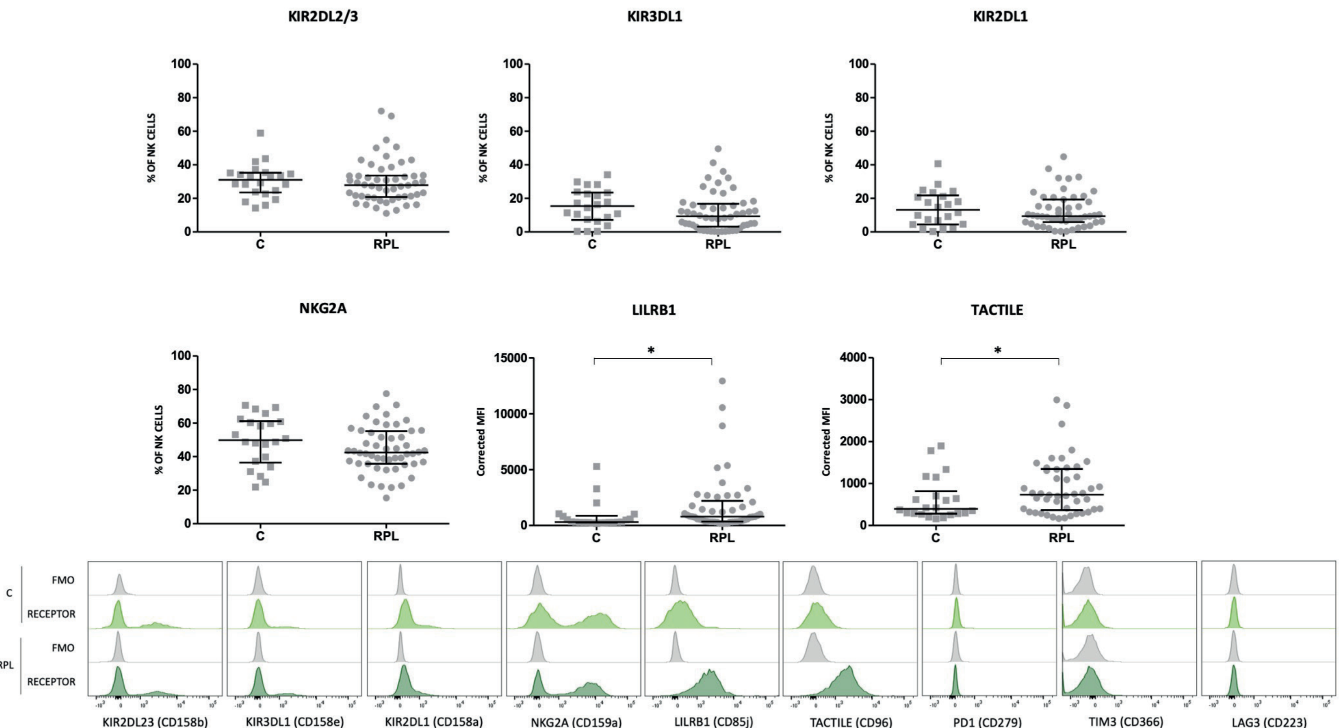

Figure 3 | Expression of inhibitory receptors in women with a previous uncomplicated pregnancy (C n=22) and women with recurrent pregnancy loss (RPL n=52). Data show percentage of KIR2DL2/3, KIR3DL1, KIR2DL1, NKG2A positive cells of total NK cells ($CD3^{-}CD56^{+}$) or corrected mean fluorescent intensity (MFI) of total NK cells ($CD3^{-}CD56^{+}$) for LILRB1 and TACTILE. Correction was done by subtracting the MFI of the fluorescent minus one (FMO). All data were acquired by flow cytometry. Dots indicate individuals, lines indicate median and interquartile range. Bottom: representative histograms of inhibitory receptors on peripheral NK cells, grey histograms represent the FMO (no receptor staining) and green histograms represent the receptor staining in RPL and C.

Table 2 | Regression coefficient table

IMMUNE MARKERS		**CRUDE**		**ADJUSTED FOR BMI**	
		B [95% CI]	P	B [95% CI]	P
CELL POPULATIONS	T cells	-0.02 [-0.10, 0.05]	0.526	-0.04 [-0.13, 0.05]	0.341
	NKT cells	-0.07 [-0.39, 0.26]	0.686	-0.06 [-0.44, 0.32]	0.751
	NK cells	-0.18 [-0.42, 0.06]	0.135	-0.12 [-0.41, 0.16]	0.396
	T helper cells	0.02 [-0.07, 0.11]	0.648	-0.05 [-0.15, 0.05]	0.306
	Cytotoxic T cells	-0.00 [-0.11, 0.11]	0.988	0.08 [-0.04, 0.20]	0.192
	$CD56^{dim}$ NK cells	-0.02 [-0.04, 0.01]	0.216	-0.00 [-0.04, 0.03]	0.801
	$CD56^{bright}$ NK cells	0.04 [-0.18, 0.27]	0.712	-0.05 [-0.31, 0.21]	0.688
ACTIVATING RECEPTORS	NKp46	0.00 [-0.15, 0.16]	0.947	-0.06 [-0.25, 0.12]	0.497
	NKp30	-0.03 [-0.22, 0.17]	0.801	-0.10 [-0.34, 0.13]	0.377
	DNAM1	-0.03 [-0.16, 0.11]	0.665	0.11 [-0.03, 0.25]	0.131
	NKG2C	0.18 [-0.59, 0.96]	0.641	0.36 [-0.55, 1.27]	0.428
	NKG2D	0.02 [-0.09, 0.12]	0.747	0.09 [-0.03, 0.21]	0.153
INHIBITORY RECEPTORS	KIR2DL23	-0.05 [-0.25, 0.15]	0.601	0.03 [-0.21, 0.27]	0.826
	KIR3DL1	-0.41 [-1.19, 0.38]	0.304	-0.44 [-1.36, 0.48]	0.340
	KIR2DL1	-0.07 [-0.66, 0.52]	0.808	-0.60 [-1.23, 0.03]	0.062
	NKG2A	-0.11 [-0.28, 0.06]	0.197	-0.09 [-0.30, 0.11]	0.356
	LILRB1	0.64 [- 0.10, 1.18]	**0.020**	0.97 [0.37, 1.57]	**0.002**
	TACTILE	0.39 [-0.00, 0.78]	**0.050**	0.60 [0.18, 1.03]	**0.006**

All data on immune markers in women with RPL (n=52) and controls (n=22) were acquired by flow cytometry and subsequently normalized by logarithmic transformation for linear regression analysis; data of estimated associations between immune markers and having recurrent pregnancy losses are presented as regression coefficient (unstandardized B) and [95% confidence interval].

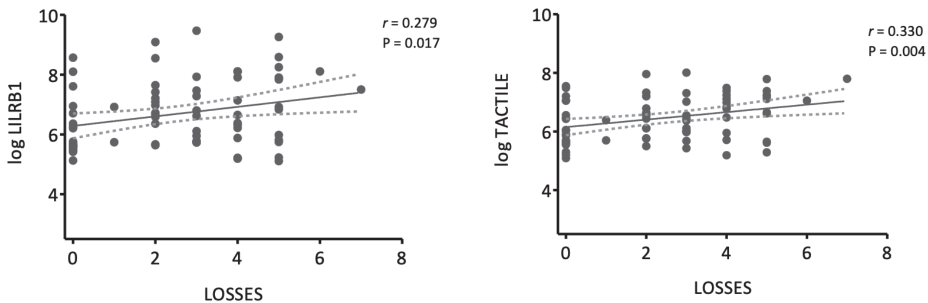

Figure 4 | Linear regression between between logarithmically transformed LIRLB1 and TACTILE expression and number of pregnancy losses (n=74), presented as regression line with correlation coefficient (*r*) and 95% confidence interval (dotted lines).

Percentage of NKG2C positive NK cells is related to CMV seropositivity in RPL

When comparing standard deviations (SD) in cell populations and receptor expression between women with RPL and controls (SI Table 4), SD of NKG2C expression was increased in women with RPL (SD 1.65 versus 1.02; P=0.028). Human Cytomegalovirus (CMV) has been shown to impact the NK cell repertoire, both functionally and phenotypically and an increased expression of NKG2C has been correlated to CMV status[27,28,29]. Hence, we examined anti-CMV IgG antibodies in available sera of 30 women with RPL and of 16 controls. There was no difference in percentages of NKG2C positive cells between CMV^+ RPL women and CMV^+ controls (P=0.407) and no difference between CMV^- RPL women and CMV^- controls (P=0.368, Figure 5). In the control group, percentages of NKG2C positive cells were comparable between CMV^+ and CMV^- controls (P=0.759, figure 5), however the CMV+ group was rather small (n=5). Interestingly, in the group of RPL women, we observed an increased expression of NKG2C in CMV^+ women when compared to CMV^- women (P=0.043).

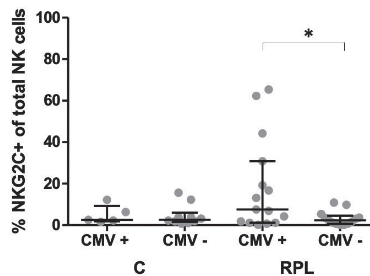

Figure 5 | Frequencies of natural killer (NK) cells expressing NKG2C are shown for women with a previous healthy pregnancy (C n=16) and women with recurrent pregnancy loss (RPL n=30) being either positive of negative for the cytomegalovirus (CMV). Dots represent individuals, lines indicate median and interquartile range.

DISCUSSION

The primary goal of this study was to phenotypically profile NK cells in women with unexplained RPL and women with a previously uncomplicated pregnancy and to study the clinical association with RPL. Our results show that two receptors, LILRB1 and the immune checkpoint receptor TACTILE are differently expressed in RPL women. Furthermore, we show a positive association of LILRB1 and TACTILE with RPL and with the number of pregnancy losses.

As 50% of RPL cases are yet without an identifiable cause, there is a growing need for the development and clinical use of predictive biomarkers. Given the potential importance of NK cells for successful pregnancy, several studies used flow cytometry to compare human NK cell numbers and phenotypes between RPL and pregnancies without complications[18,20-25]. The partly conflicting data from these studies illustrate that comparing pNK cell numbers or pNK cell subsets based on $CD56^{dim}$ and $CD56^{bright}$ will not be sufficiently robust as biomarker. To better capture the breath of the receptor repertoire we designed antibody panels covering several of the NK receptors previously associated with RPL as well as a selection of immune checkpoint receptors, i.e., PD1, LAG3, LIRLB1 and TACTILE, that have not been studied before for this purpose. Although, our analysis was still based on a selection of NK receptors, and a fully comprehensive analysis of the repertoire would require a Cytof-, spectral cytometry- or single cell RNAseq approach, the identification of LILRB1 and TACTILE as interesting candidates for further analysis.

LILRB1 is an immunoglobulin-like receptor that interacts with MHC class I proteins and preferentially binds to HLA-G, a non-classical MHC class I protein expressed on EVTs in the decidua during pregnancy[30]. Interestingly, LILRB1 is an inhibitory receptor for peripheral blood NK (pNK) cells, but it has been shown to act as an activating receptor for NK cells in the decidua hence promoting the production of proangiogenic cytokines, EVT invasion and spiral artery remodeling[31,32,33]. LILRB1 has been shown to be expressed by decidual NK cells present in both the decidua basalis and decidua parietalis[34]. Moreover, in CMV positive woman, LILRB1 expression was expressed at higher levels on NKG2C positive endometrial NK cells from multigravid women as compared to woman without previous pregnancies[35]. In line with this, a second study, showed increased expression of LILRB1 on NKG2C positive endometrial and decidual NK cells from woman with multiple as compared to first time pregnant women[36]. These NK cells, termed ‘pregnancy trained decidual NK cells’ had unique transcriptomic and epigenetic signatures, and secreted IFN-γ and VEGFα upon activation which promoted capillary sprouting. The subsequent observation that secretion could be blocked by monoclonal antibody blockade of LILRB1 clearly illustrated the potential functional relevance of LILRB1 in controlling NK cell function in the uterus[37].

The functional relevance of TACTILE remained obscure for many years, however, in the context of anti-cancer immune responses it has been shown to negatively control cytokine production by NK cells by antagonizing NK cell activation mediated by the DNAM1 receptor[38]. Although the role of TACTILE in the decidua is not known, expression of its ligand, the poliovirus receptor (PVR or CD155), on EVT and maternal endothelial cells, has been demonstrated by single cell RNAseq analysis of first trimester placentas[39]. Moreover, we do know that TIGIT, an immune checkpoint receptor that shares the same ligand as TACTILE, has been found on decidual NK cells and has been assumed to have a putative inhibitory interaction between the decidual NK cell and the EVT and has been suggested to promote the tolerogenic functions of dendritic cells and regulatory T cells at the fetal-maternal interface[39].

Based on our current data, we cannot provide a direct link on the potential relation between LILRB1 or TACTILE expression on peripheral blood NK cells and expression on NK cells in the uterus. Hence, it would be interesting to follow up on our findings with a paired analysis of LILRB1 or TACTILE expression profiles from peripheral blood NK cells and uterine NK cells in RPL versus control women, for example, to assess whether recruitment of LILRB1 or TACTILE positive subsets to the uterus is disturbed in RPL women.

Inhibitory KIRs and NKG2A are the best characterized inhibitory receptors for NK cells, they are highly expressed on dNK cell and have been proposed to regulate dNK cell effector function by interaction with HLA-C and HLA-E[40]. In line with two previous studies[24,25], but in contrast to two other studies[23,41], we did not observe an association between RPL and percentages of pNK cells positive for KIR2DL1, KIR2DL2/3 or NKG2A. Additionally, we could not confirm the reduced TIM-3 and higher NKG2D expression observed on pNK cells in peripheral blood of women with RPL reported by two other studies[18,23]. The lack of consistency of these results may be explained by variation between the studies in the definition of RPL, in patient inclusion criteria, or technical differences in e.g., the used antibody clones and set up of the flow cytometer. Moreover, a weakness of most of these studies, including ours, is the relatively low study power. This emphasizes the importance of confirmatory studies, preferably with large patient cohorts, as well as harmonization of study protocols for adequate comparison of studies, to ultimately obtain truly reliable biomarkers.

Another important point to consider is the location of sampling since it is becoming increasingly clear that NK cells in peripheral blood and in tissues are very different[42]. In peripheral blood, we could not detect the immune checkpoint receptors PD1, LAG3 and TIM3. Our data are in line with the concept that these receptors are primarily expressed on highly activated NK cells or on subsets of tissue-resident NK cells[43]. This underlines the

importance of including NK cells obtained from the (pregnant) uterus in future studies exploring the causal relation between NK cells and pregnancy outcome or NK cell-based targets for intervention. However, for a biomarker to be predictive such a causal relation with the disease is not *perse* required. Moreover, sampling of uterine NK cells, e.g., trough biopsies, is a relatively invasive procedure. Although obtaining NK cells from menstrual blood is an attractive alternative[44] caution should be taken as some of the predictive surface markers may be lost during the isolation procedure. Hence, an advantage of phenotyping pNK cells is that it can be more easily implemented in routine diagnostics for in- or outpatient clinics. Evidently, this is provided that a robust and predictive NK cell phenotype can be identified and confirmed in a standardized setting.

To further assess whether the peripheral blood NK compartment can predict for the phenotype and function of uterine NK cells, a comparison between phenotypes and function of both peripheral blood and uterine NK cells from control vs RPL women will be needed as this cannot be concluded from the current study. Exploring the potential relation between peripheral blood and uterine NK cells is also interesting to better understand the origin of uterine NK cells for which several possibilities have been proposed. The first possibility is that NK cells are recruited from the peripheral blood to the uterus, where they undergo further tissue specific differentiation under the influence of the uterine environment[10,45]. The second possibility is that uterine NK cells already reside in the tissue[46]. The third possibility is that uterine NK cells are likely to be a heterogeneous population arising from both[47].

Because of the complexity of the immune response, the integration of multiple parameters might be necessary for accurate prediction. First of all, this could be done by the analysis of co-expression of receptors, which may reveal additional information on the relation between specific NK cell subsets and pregnancy outcome. Co-expression can be assessed by conventional two-dimensional analysis using a predefined and sequential gating strategy. However, this approach may lead to losses of important information, including for example the interplay between specific cell types or receptors[48]. Hence, more in-depth information can be obtained by multivariate-models (e.g., T-SNE, ViSNE) or a recently developed method based on Principal Component Analysis (PCA), called Discriminant Analysis of Multi Aspect Cytometry (DAMACY), that can quantitatively compare the immune cell composition between groups after the fusion of the data from the different flow cytometry panels[48,49]. For future analysis it would be exciting to analyze if several receptors possibly cluster in women with RPL or women with a previous healthy pregnancy using those more advanced analysis models.

Viral status is another feature to include in future comprehensive NK cell profiles. CMV seropositivity or reactivation is the most prominently studied example of the impact of viruses on the NK cell repertoire, and it has been linked to increased expression of NKG2C, KIR and LILRB1 and the induction of "memory NK cells", characterized by a longer lifespan and enhanced effector function[27,28,29]. Interestingly, a study with 25 healthy controls showed that CMV status was not related to NKG2C expression on menstrual blood NK cells while it was positively associated with higher NKG2C expression on NK cells in peripheral blood[50]. We did not observe a clear association between NKG2C and CMV status in controls but we did observe an increased percentage of NKG2C positive NK cells in CMV^{+} women with RPL when compared to CMV^{-} women with RPL. Although CMV has been suggested to negatively impact reproductive success, its role in pathogenesis of RPL is still quite controversial[51] [52,53,54]. Unfortunately, we determined CMV status only in a limited number of women (30 RPL and 16 controls) since plasma samples were not available for all individuals in our cohort. Especially the CMV positive control group was underpowered (n=5) to strongly conclude that there was no difference in NKG2C expression between CMV positive versus CMV negative control women. Since the epidemiology of CMV infection can be diverse, as various populations might be affected differently, larger cohorts with a broader socioeconomic distribution of RPL cases are needed to get more clear results on the role of CMV in RPL, and it would be highly interesting to integrate evaluation of the NK cell repertoire in those studies as well.

In summary, our study showed that LILRB1 and TACTILE were higher expressed in women with RPL and positively associated with the number of pregnancy losses. Moreover, we showed CMV seropositivity was associated with higher expression of NKG2C in RPL woman, providing a first indication that CMV may influence reproductive success through its impact on the NK cell compartment. Thereby, it provides an interesting starting point for future studies in large- and standardized cohorts to confirm their predictive value for RPL as well as functional studies to investigate a potential causal relation with pregnancy outcome.

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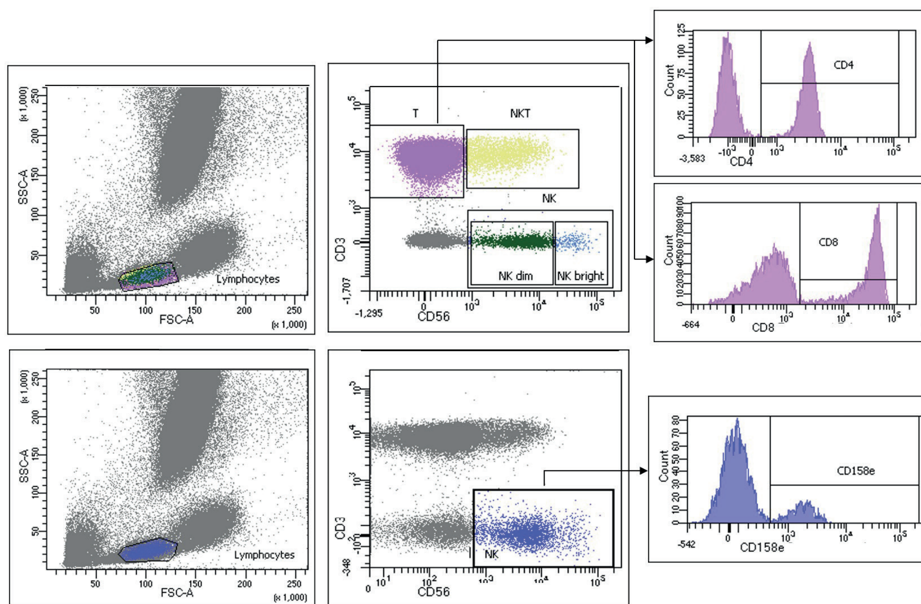
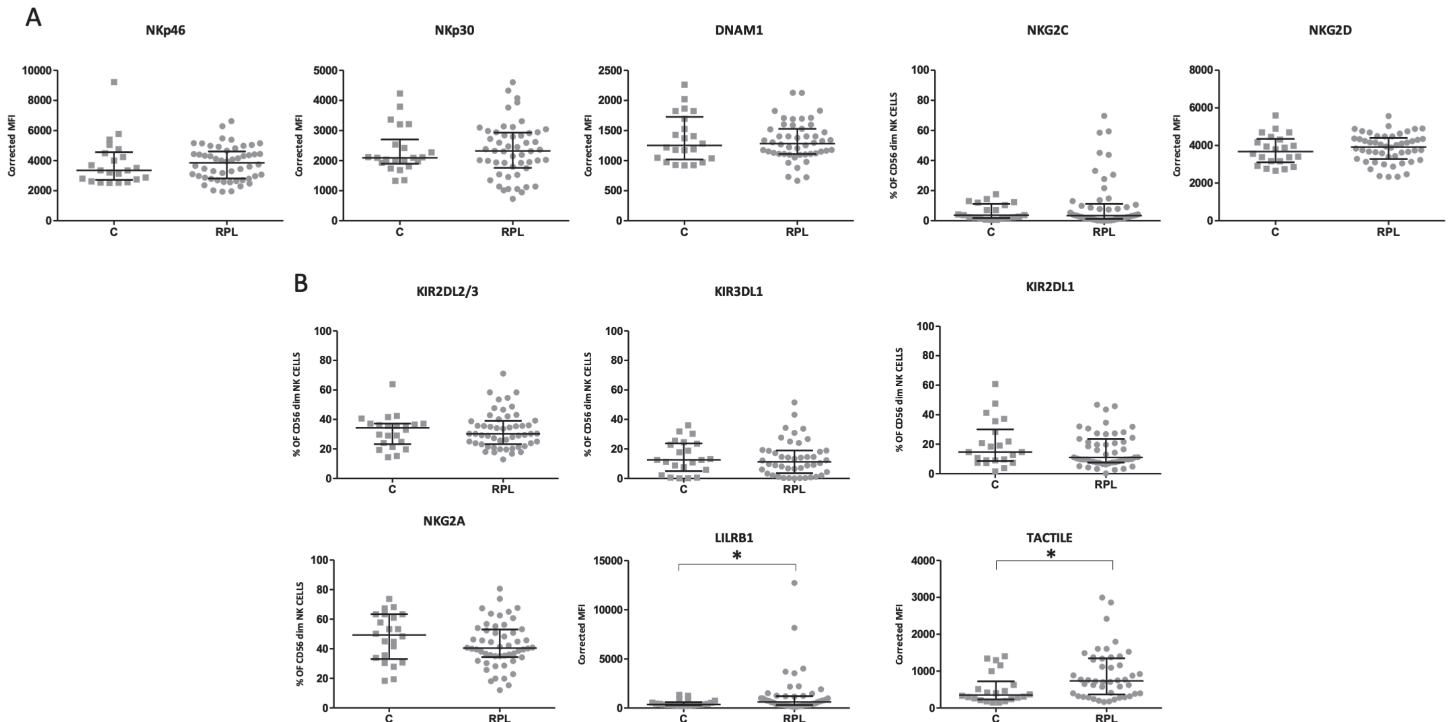
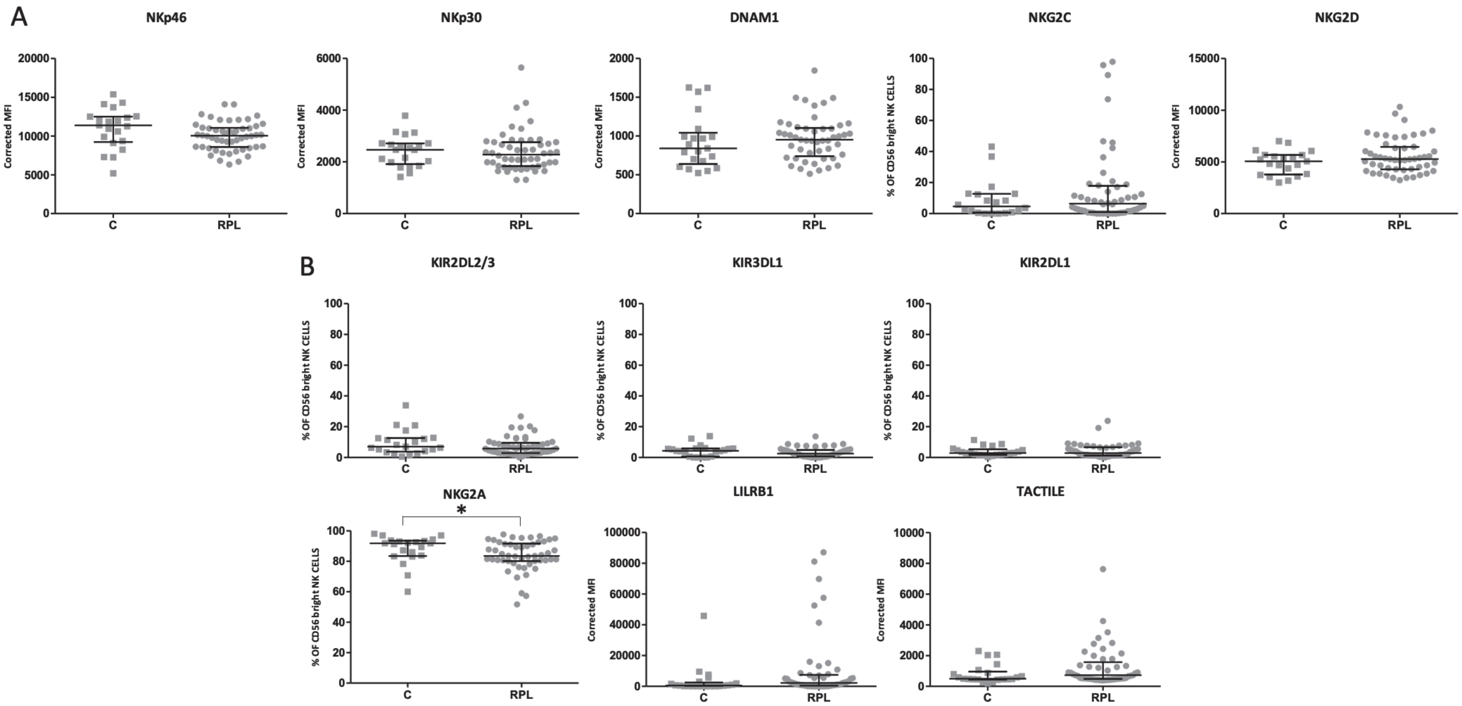
SUPPLEMETARY FILES



SI Figure 1 | Gating strategy for determining cell populations (as percentage of lymphocytes) and receptors on peripheral NK cells (as percentage of NK cells or as mean fluorescent intensity of NK cells) after measuring 5000 NK cell events for each sample, representative histograms of each receptor are shown in Figure 2 and 3.



SI Figure 2 | Expression of activating (A) and inhibitory (B) receptors on CD3-CD56^{dim} NK cells in women with a previous uncomplicated pregnancy (C n=22) and women with recurrent pregnancy loss (RPL n=52). Data show percentage positive cells for NKG2C, KIR2DL2/3, KIR3DL1, KIR2DL1 and NKG2A or corrected mean fluorescent intensity (MFI) for NKp46, NKp30, DNAM1, NKG2D, LILRB1 and TACTILE of CD56^{dim} NK cells. Correction was done by subtracting the MFI of the fluorescence minus one (FMO). All data were acquired by flow cytometry. Dots indicate individuals, lines indicate median and interquartile range.



SI Figure 3 | Expression of activating (A) and inhibitory (B) receptors on CD3-CD56^{bright} NK cells in women with a previous uncomplicated pregnancy (C n=22) and women with recurrent pregnancy loss (RPL n=52). Data show percentage positive cells for NKG2C, KIR2DL2/3, KIR3DL1, KIR2DL1 and NKG2A or corrected mean fluorescent intensity (MFI) for NKp46, NKp30, DNAM1, NKG2D, LILRB1 and TACTILE of CD56^{birght} NK cells. Correction was done by subtracting the MFI of the fluorescence minus one (FMO). All data were acquired by flow cytometry. Dots indicate individuals, lines indicate median and interquartile range.

SI Table 1 | Frequencies of cell populations, inhibitory and activating receptors in women with RPL and controls

IMMUNE MARKERS		RPL (n=52)	Controls (n=22)	P
CELL POPULATIONS	T cells	67.2% [60.1-72.8]	66.2% [62.8-71.5]	0.929
	NKT cells	6.3% [4.4-8.3]	6.7% [3.2-10.7]	0.745
	NK cells	10.7% [7.9-13.0]	14.0% [9.8-17.1]	0.092
	T helper cells	59.0% [53.0-63.4]	57.9% [46.8-65.4]	0.845
	Cytotoxic T cells	33.6% [27.6-38.1]	33.0% [29.1-41.3]	0.878
	$CD56^{dim}$ NK cells	90.3% [86.8-92.8]	90.2% [89.0-92.5]	0.611
	$CD56^{bright}$ NK cells	6.5% [4.4-9.2]	6.2% [5.5-8.7]	0.679
ACTIVATING RECEPTORS	NKp46	4188 [3177-5003]	3761 [3021-5212]	0.651
	NKp30	2285 [1811-2779]	2062 [1787-2619]	0.797
	DNAM1	1282 [1109-1512]	1220 [986-1676]	0.971
	NKG2C	3.5% [1.1-9.8]	3.1% [1.7-6.6]	0.529
	NKG2D	3863 [3288-4483]	3688 [3130-4332]	0.609
INHIBITORY RECEPTORS	KIR2DL23	28.0% [20.7-33.7]	29.3% [23.2-34.8]	0.388
	KIR3DL1	8.9% [2.9-17.1]	14.4% [6.9-23.6]	0.174
	KIR2DL1	9.3% [5.9-20.5]	14.7% [4.5-22.3]	0.588
	NKG2A	41.5% [35.1-47.9]	50.8% [38.5-61.6]	0.124
	LILRB1	771 [333-1662]	298 [268-702]	**0.007**
	TACTILE	747 [398-1375]	370 [276-929]	**0.046**

Cell populations are determined by flowcytometry as percentage of lymphocytes (T cells ($CD3^+CD56^-$), NKT cells ($CD3^+CD56^+$) and NK cells ($CD3^-CD56^+$)), as percentage of T cells (T helper cells ($CD3^+CD56^-CD4^+$) and cytotoxic T cells ($CD3^+CD56^-CD8^+$)) or as percentage of NK cells ($CD56^{dim}$ NK cells ($CD3^-CD56^+CD16^+$) and $CD56^{bright}$ NK cells ($CD3^-CD56^{++}CD16^-$)). Receptor expression was determined as percentage positive cells of CD3-CD56+ NK cells for NKG2C, KIRs and NKG2A or as corrected MFI (MFI receptor minus MFI FMO) for NKp46, NKp30, DNAM1, NKG2D, LILRB1 and TACTILE. Data are presented as median [interquartile range].

SI Table 2 | Frequencies of inhibitory and activating receptors in women with RPL and controls on CD56^{dim} and CD56^{bright} NK cells

IMMUNE MARKERS		**CD56^{dim} NK cells**	
		RPL (n=52)	Controls (n=22)
ACTIVATING RECEPTORS	NKp46	3444 [2756-4399]	3344 [2748-4752]
	NKp30	2307 [1537-2860]	2106 [1923-3202]
	DNAM1	1238 [1109-1401]	1187 [972-1428]
	NKG2C	2.5 [0.8-9.2]	2.7 [1.6-12.0]
	NKG2D	3744 [3207-4341]	3401 [2924-4161]
INHIBITORY RECEPTORS	KIR2DL23	28.9 [21.8-39.4]	29.7 [21.3-36.5]
	KIR3DL1	11.3 [2.0-18.3]	13.1 [7.6-24.5]
	KIR2DL1	17.3 [8.1-27.6]	18.4 [9.0-35.6]
	NKG2A	38.4 [31.4-46.9]	48.4 [33.9-63.4]
	LILRB1	673 [285-1083]	363 [307-543]
	TACTILE	859 [285-1485]	308 [213-511]

Receptor expression was determined by flowcytometry as percentage positive cells of CD3-CD56^{dim} NK cells and CD3-CD56^{bright} NK cells for NKG2C, KIRs and NKG2A or as corrected MFI (MFI receptor minus MFI FMO) for NKp46, NKp30, DNAM1, NKG2D, LILRB1 and TACTILE. Data are presented as median [interquartile range].

P	CD56^{bright} NK cells RPL (n=52)	Controls (n=22)	P
0.652	9954 [8294-11193]	11376 [9103-12488]	0.056
0.819	2111 [1777-2763]	2469 [1983-2715]	0.559
0.886	942 [711-1083]	841 [675-993]	0.279
0.729	5.5 [0.5-11.3]	7.2 [0.9-12.9]	0.560
0.307	5157 [4285-6025]	5054 [3831-5678]	0.237
0.920	6.5 [3.0-11.7]	7.0 [3.4-12.8]	0.270
0.533	2.3 [0.6-4.9]	4.2 [0.5-5.9]	0.296
0.286	3.2 [1.2-7.2]	2.9 [1.7-4.8]	0.729
0.532	83.4 [79.2-92.2]	91.9 [83.8-93.4]	**0.042**
0.016	1572 [339-5437]	574 [126-1854]	0.060
0.005	683 [482-1269]	496 [454-1062]	0.104

SI Table 3 | Correlation coefficient table

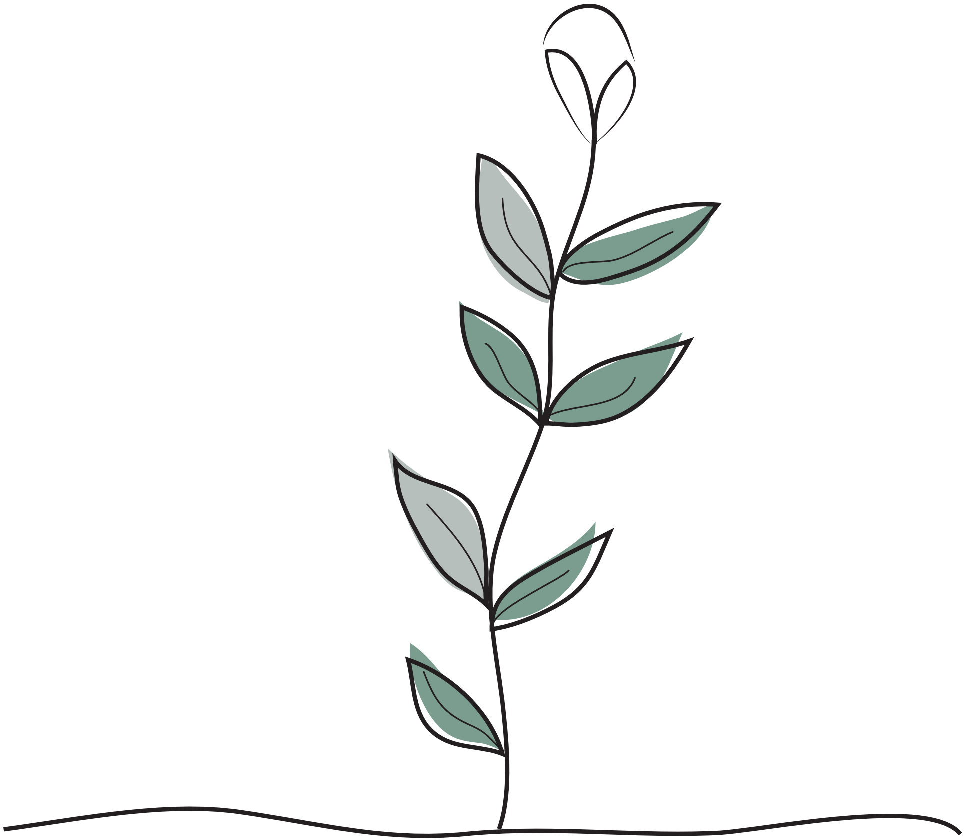
IMMUNE MARKERS		*r*	P
CELL POPULATIONS	T cells	-0.139	0.424
	NKT cells	0.067	0.575
	NK cells	-0.061	0.609
	T helper cells	-0.059	0.620
	Cytotoxic T cells	0.039	0.741
	$CD56^{dim}$ NK cells	-0.047	0.693
	$CD56^{bright}$ NK cells	-0.015	0.897
ACTIVATING RECEPTORS	NKp46	-0.029	0.813
	NKp30	-0.005	0.964
	DNAM1	-0.043	0.719
	NKG2C	0.048	0.693
	NKG2D	-0.028	0.815
INHIBITORY RECEPTORS	KIR2DL23	0.052	0.663
	KIR3DL1	-0.123	0.300
	KIR2DL1	-0.021	0.863
	NKG2A	-0.028	0.815
	LILRB1	0.279	**0.017**
	TACTILE	0.330	**0.004**

All immune markers were measured by flow cytometry in women with RPL (n=52) and controls (n=22) and subsequently normalized by logarithmic transformation for linear regression analysis; data of estimated associations between immune markers and the number of pregnancy losses are presented as correlation coefficient (r).

SI Table 4 | Table of variances

IMMUNE MARKERS		RPL SD	Controls SD	P
CELL POPULATIONS	T cells	0.16	0.10	0.109
	NKT cells	0.60	0.73	0.148
	NK cells	0.50	0.35	0.163
	T helper cells	0.16	0.20	0.107
	Cytotoxic T cells	0.22	0.23	0.778
	$CD56^{dim}$ NK cells	0.06	0.03	0.052
	$CD56^{bright}$ NK cells	0.48	0.34	0.086
ACTIVATING RECEPTORS	NKp46	0.31	0.32	0.957
	NKp30	0.41	0.30	0.219
	DNAM1	0.25	0.29	0.243
	NKG2C	1.65	1.02	**0.028**
	NKG2D	0.21	0.20	0.873
INHIBITORY RECEPTORS	KIR2DL23	0.41	0.34	0.316
	KIR3DL1	1.54	1.53	0.773
	KIR2DL1	1.13	1.24	0.520
	NKG2A	0.33	0.35	0.616
	LILRB1	1.11	0.93	0.273
	TACTILE	0.76	0.74	0.979

All immune markers were measured by flow cytometry in women with RPL (n=52) and controls (n=22) and subsequently normalized by logarithmic transformation; data are presented as the degree of dispersion relative to its mean (standard deviation).



6

ANALYSIS OF THE HIGH-AFFINITY FCYRIIIA P.176VAL VARIANT AND ITS ASSOCIATION WITH CD16 RECEPTOR EXPRESSION AND ANTI-HLA ANTIBODY STATUS IN WOMEN WITH RECURRENT PREGNANCY LOSS

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SUBMITTED

ABSTRACT

Introduction: Natural Killer (NK) cells have been implicated in recurrent pregnancy loss (RPL) as they are the most abundant decidual lymphocytes in the first trimester of pregnancy. The p.Val176Phe (formerly known as Val158Phe) Single Nucleotide Polymorphism in the *FCGR3A* gene encoding the human IgG receptor FcγRIIIA has been associated with an enhanced affinity for IgG and a stronger NK-mediated antibody-dependent cellular cytotoxicity (ADCC) response. We hypothesized that the high-affinity p.176Val variant in *FCGR3A* might attribute to RPL pathophysiology and might be associated with increased FcγRIIIA expression and related to allo-antibodies such as anti-paternal human leukocyte antigen (HLA) antibodies.

Methods: In an observational pilot study, frequencies of the high- and low-affinity *FCGR3A* polymorphisms were determined for 50 women with RPL and 164 healthy female controls. Additionally, differences in NK cell surface CD16 expression and frequencies of the high- and low-affinity polymorphisms based on anti-HLA antibody status were analyzed.

Results: No significant differences were observed when comparing frequencies of *FCGR3A*-p.176 genotypes in women with RPL and healthy female controls. NK cells from RPL women with the high-affinity genotype (V/V or V/F) showed a higher mean fluorescent intensity for the CD16 receptor than NK cells from RPL women with the low-affinity genotype (F/F). Lastly, no difference in frequencies of the high-affinity or low-affinity *FCGR3A*-p.176 polymorphisms were detected when comparing women with or without class I and class II anti-HLA antibodies.

Conclusion: Our study does not provide strong evidence for an association between the high-affinity FcγRIIIA p.176Val variant and RPL.

Keywords: Recurrent Pregnancy Loss, Natural Killer cell, FCGR3A, CD16, Human Leukocyte Antigen antibodies.

INTRODUCTION

Recurrent Pregnancy Loss (RPL) is a disruptive problem for couples trying to conceive. Despite observed clinical associations, the current knowledge on etiological factors in RPL is still incomplete and more than 50% of cases remains unexplained[1]. As the fetus is genetically semi-allogenic to the mother, tight regulation of complex interplay between a large array of immune cells and molecules with immunomodulatory properties is essential for the proper establishment and continuation of successful pregnancy[2]. Emerging evidence now suggests that dysregulation of Natural Killer (NK) cells could be responsible for several cases of unexplained RPL[3].

NK cells are innate lymphocytes and peripheral NK cells (pNK) are primarily known as potent killers of virally-infected or tumor cells through the release of granzyme and perforin containing granules or via death receptors[4]. Moreover, they can produce cytokines contributing to Th1 polarization and CD8 T cell function[5]. Two important lineages of NK cells have been described that can be classified according to the expression level of CD56: CD56^{dim} NK cells predominantly express the FcγRIIIA receptor CD16 and constitute around 90% of NK cells in peripheral blood and mediate cytotoxicity. Conversely, CD56^{bright} NK cells lack expression of CD16 and while they are mostly known for their capacity to produce cytokines, they are poorly cytotoxic and form merely 5-10% of NK cells in peripheral blood[6]. During early pregnancy and in the secretory phase of the menstrual cycle, these CD56^{bright}CD16^{negative} NK cells constitute the predominant lymphocyte subset present in the decidual or else endometrial layer of the uterus[7]. This illustrates the divergent functions of NK cells, in which uterine NK (uNK) cells regulate trophoblast invasion and promote placental vasculature hence contributing to adequate placentation[8,9]. The considerable interest in the role of uNK cells in pregnancy complications, particularly in RPL, indicates the perceived importance of these cells in early pregnancy. Although some controversy still exists, overall results indicate that RPL is associated with several abnormalities in NK cell number and activation[10].

NK cell activation is controlled by a broad panel of inhibitory- and activating receptors, and the balance between signaling via these receptors determines the level of NK cell activation[11]. CD16 or FcγRIIIA, is one of the most powerful activating receptors on NK cells and it enables NK cells to respond to antibody-coated target cells and to exert antibody-dependent cellular cytotoxicity (ADCC)[12]. The process of ADCC starts with the binding of an antibody to its cognate antigen expressed on the target cell surface. The Fc domain of these antibodies is then bound by FcγRIIIA expressed on NK cells, triggering the release of cytotoxic granules and subsequent lysis and death of the target cell[13]. The strength of NK cell mediated ADCC is determined by factors like the isotype- or fucosylation status of

the antibody[14,15]. Moreover, the rs396991 c.526G>T single nucleotide polymorphism (SNP) in the *FCGR3A* gene, results in expression of either valine or phenylalanine at amino acid position 176 (also known as position 158 in the mature protein excluding signal peptides) and has been reported to influence human IgG_1 binding and ADCC activity[16]. Functional studies have shown that the presence of a p.176-valine (V/V or V/F) results in a FcγRIIIA receptor with a higher affinity for IgG1 and IgG3 antibodies compared to the homozygous p.176-phenylalanine (F/F) genotype[17]. ADCC is a major mechanism of action of therapeutic monoclonal antibodies (mAbs) such as Rituximab and a higher ADCC response to Rituximab as well as increased CD16 expression have been reported to correlate with the genotypes encoding a p.176-valine (V/V and V/F) in the *FCGR3A* gene[18].

The percentage of uNK cells that express CD16 under physiological conditions is low, however, elevated CD16 expression levels have been observed in woman with pregnancy complications[19,20]. In a mouse study, uNK cells have been shown to mediate ADCC against invading trophoblast cells of the fetus in an FcγRIIIA dependent manner[21]. Furthermore, 30% of women develop antibodies against paternal human leukocyte antigen (HLA) allo-antigens expressed by the fetus[22]. These allo-antibodies are considered a harmless phenomenon during most pregnancies primarily because the fetal cells most closely in contact with the immune system almost completely lack expression of polymorphic HLA class I and class II molecules[23]. Nevertheless, several studies suggested that these anti-HLA antibodies were associated with RPL as a higher incidence of anti-HLA antibodies has been observed in women with RPL compared to women without[24]. Although the potential underlying mechanism is not very well explored, these allo-antibodies may contribute to RPL via complement fixation and/or ADCC resulting in damage of the invading trophoblast cells showing aberrant expression of polymorphic HLA molecules.

Given the impact of *FCGR3A* polymorphism on the strength of the ADCC response, we hypothesized that the high-affinity *FCGR3A* genotype is enhanced in women with RPL, see Figure 1. As such, we identified *FCGR3A*-p.176 polymorphisms in women with RPL and healthy individuals. In addition, we sought to delineate differences in NK cell surface CD16 expression in women with RPL with the high-affinity and low-affinity receptor genotype. Since we expect the effect of *FCGR3A* polymorphism to be most relevant in women with antibodies against fetal allo-antigens, we also determined HLA antibody status in women with RPL and studied distribution of the high-affinity and low-affinity polymorphic subgroups.

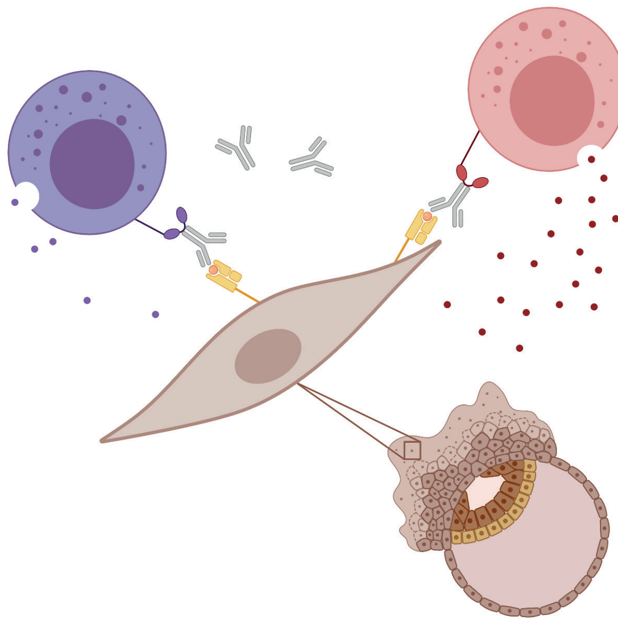


Figure 1 | Illustrative representation of antibody dependent cellular cytotoxicity (ADCC) of Natural Killer (NK) cells in the uterus with a low affinity CD16 receptor depicted in purple and a high affinity CD16 receptor depicted in red. We hypothesize that a high affinity receptor induces a stronger ADCC response and an increased CD16-mediated degranulation capacity of NK cells against the trophoblast in women with recurrent pregnancy loss (RPL).

MATERIALS AND METHODS

Sample collection

Women with RPL: Blood samples of 50 women with RPL were available for genotyping the p.V176F region of *FCGR3A*. Of these 50 samples, 41 were available for additional flowcytometric analysis and 32 sera samples were available for anti-HLA antibody testing. All women gave informed consent and participated in the preconceptional cardiovascular assessment program (PCVS). The PCVS evaluation is performed according to Dutch national guidelines (www.nvog.nl) of RPL at least 3 months after pregnancy loss and consists of parental karyotypic screening, thrombophilia screening, endocrine screening and gynecological ultrasound. Women with 2 or more reported pregnancy losses before 24 weeks of gestation, were included. Women were excluded if outcomes from the PCVS evaluation indicated abnormal parental karyotype, thrombophilia (for example, presence of anti-phospholipid antibodies/Factor V Leiden mutation/Prothrombin mutation/Lupus anticoagulant or deficiency of Protein C, Protein S, antithrombin), endocrine abnormality (e.g., thyroid dysfunction) or uterine anomalies. Leukocytes were isolated from ethylene diamine tetra acetic acid (EDTA) blood samples and directly used for flowcytometry and additionally for DNA extraction using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). This study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre (MUMC+) (14-4-118).

Healthy controls: Previously genotyped DNA samples of 164 healthy female volunteers from Sanquin Amsterdam served as a control group for the genotyping assay[25].

Genotyping the p.V176F region of *FCGR3A*

Genotyping of the 50 women with RPL for *FCGR3A* (p.V176F) was performed using an *FCGR3A* gene-specific forward primer and a generic reverse primer, producing a 9654 bases long polymerase chain reaction (PCR) product, following a previously described protocol[26]. In addition, amplicons obtained were purified by ExoSAP-IT (Affymetrix, Santa Clara, California) and then sequenced using ABI BigDye Terminator Chemistry (Life Technologies) and an ABI 3730 sequencer (Life Technologies) with a specific forward and reverse sequencing primer. Data were analyzed using DNASTAR Lasergene SeqMan Pro (DNASTAR Lasergene, Madison, Wisconsin). Data were analyzed using Genemarker version 1.40 (Soft Genetics LLC, State College, PA).

Genotyping of 164 healthy female volunteers for the SNP in the *FCGR3A* gene (p.V176F) was performed using an *FCGR*-specific multiplex ligation-dependent probe amplification (MLPA) assay (MRC-Holland, Amsterdam, The Netherlands), The genotypes of the healthy controls obtained by MLPA assay were available from a previous study[25]. Genetic data was tested for Hardy-Weinberg equilibrium.

Flowcytometry

Freshly isolated leukocytes were stained with conjugated antibodies anti-CD3 (VioBlue, REA613, Miltenyi Biotec, Germany), anti-CD56 (APC-Vio770, REA196, Miltenyi Biotec, Germany) and anti-CD16 (FITC, REA423, Miltenyi Biotec, Germany) for 30 min at 4°C in the dark. After two wash cycles, samples were measured on a FACS Canto II (BD Biosciences, San Jose, CA) and analyzed with the BD FACSDiva Software v8.0.2 (BD Biosciences, San Jose, CA). In order to reduce inter-experimental variations, all samples were measured with application settings to standardize voltage and compensation settings.

Anti-HLA antibody testing

Serum anti-HLA antibodies were tested using single antigen beads (LABScreen, One Lambda Inc/ThermoFisher, Canoga Park, CA), according to the manufacturer's instructions. Anti-HLA antibody profiles were analyzed using the HLA Fusion software v4.2 (One Lambda Inc/ThermoFisher, Canoga Park, CA). Antibodies with a mean channel fluorescence intensity (MFI) lower than 1000 were considered negative and antibodies with an MFI higher than 1000 positive.

Statistical analysis

Data was tested for normality with Shapiro-Wilk. Dichotomous data was analyzed with Chi Square and was compared between women with RPL and controls (percentages of frequencies of polymorphisms) and between women with RPL categorized as either being positive or negative for class I, class II and class I and/or class II anti-HLA antibodies (percentages of frequencies of polymorphisms), respectively. Continuous data among women with RPL was analyzed with Mann Whitney U or Kruskal-Wallis (percentages of NK cells ($CD3^{negative}CD56^{positive}$), and of the $CD56^{dim}CD16^{positive}$ and $CD56^{bright}CD16^{negative}$ NK cell subsets and percentages plus MFI of CD16 expression on NK cells). Overall, a P-value below 0.05 was considered statically significant and all statistical analyses were conducted with IBM SPSS statistics version 25 (IBM Corp, Los Angeles, USA).

RESULTS

Frequencies of *FCGR3A*-p.176 genotypes are comparable between women with RPL and healthy women

To study frequencies of the high-affinity and the low-affinity *FCGR3A* genotype in women with RPL, single-nucleotide changes in position 526 (G→T) were detected by sanger sequencing. Based on position 176, the following subgroups of *FCGR3A* genotypes were identified: valine/valine (V/V), valine/phenylalanine (V/F) and phenylalanine/phenylalanine (F/F). High-affinity genotypes were subsequently identified as V/F or V/V and low-affinity genotypes as F/F. To compare frequencies in women with RPL with frequencies in the normal population, a cohort of healthy blood bank donors (n=164) was used that had been genotyped for *FCGR3A* p.V176F SNP. The frequencies in both cohorts were as follows: 31 out of 50; 62% (high-affinity genotype) and 19 out of 50; 38% (low-affinity genotype) for women with RPL, versus 87 out of 164; 53% (high-affinity genotype) and 77 out of 164; 47% (low affinity-genotype) for controls (Table 1). No significant deviation from the Hardy-Weinberg expectation was observed. There was no significant difference between women with RPL and controls (P=0.265). In addition, there was no significant difference (P=0.374) when frequencies were compared for VV (20%; 10 out of 50), V/F (42%; 21 out of 50) and F/F (38%; 19 out of 50) in women with RPL, versus respectively 13.4%; 22 out of 164 (V/V), 39.6%; 65 out of 164 (V/F) and 47%; 77 out of 164 (F/F) in controls (Table 1).

Table 1 | Frequencies of FcγRIIIA-p.176 genotypes in women with RPL and healthy female controls

	RPL (n=50)	**Controls (n=164)**
High affinity (VV+VF)	62.0% (31/50)	53.0% (87/164)
Low affinity (FF)	38.0% (19/50)	47.0% (77/164)
VV	20.0% (10/50)	13.4% (22/164)
VF	42.0% (21/50)	39.6% (65/164)
FF	38.0% (19/50)	47.0% (77/164)

Frequencies of FcγRIIIA-p.176 genotypes were based on the valine and phenylalanine SNP on amino acid position 176.

Analysis of the influence of the p.V176F polymorphism on CD16 positive NK cells

In addition to qualitative differences, the p.V176F SNP has been shown to result in quantitative differences in the level of receptor expression on NK cells. To study if these quantitative differences also occurred in our cohort of RPL women, the percentage of CD16 positive NK cells and the level of expression were determined by flowcytometry (for gating strategy see SI Figure 1) and compared between the high- vs the low-affinity subgroups of women with RPL. First, we compared distribution of total NK cells, defined as $CD3^{negative}CD56^{positive}$ and of the two main

NK cell subsets; the $CD56^{bright}CD16^{negative}$ mostly known as cytokine producers and the cytotoxic $CD56^{dim}CD16^{positive}$. No significant differences were found between the high-affinity genotype and the low-affinity genotype when comparing percentage of total $CD3^{negative}CD56^{positive}$ NK cells (median high-affinity 10,8 [7,5-17,4] versus low-affinity 10,0 [8,0-19,6] P=0.708), the percentage of $CD56^{dim}CD16^{positive}$ NK cells (median high-affinity 88,1 [85,3-92,4] versus low-affinity 87,8 [81,5-90,8] P=0.612), or the percentage of $CD56^{bright}CD16^{negative}$ NK cells (median high-affinity 4,1 [2,6-5,9] versus low-affinity 4,0 [3,3-7,4] P=0.470) (Figures 2A-C).

Additionally, there was no significant difference in percentage of total NK cells (P=0.120) when percentages were compared between V/V (8,6 [5,2-10,8]), V/F (14,3 [9,5-18,3]) and F/F (10,0 [8,0-19,6]). Neither when percentage of $CD56^{dim}CD16^{positive}$ NK cells (P=0.123); V/V (83,0 [79,0-88,5]), V/F (89,3 [86,2-92,7]), F/F (87,8 [81,5-90,8), nor when percentage of $CD56^{bright}CD16^{negative}$ NK cells (P=0.094); V/V (6,1 [4,1-6,6]), V/F (3,9 [2,3-4,9), F/F (4,0 [3,3-7,4]) was compared between V/V, V/F and F/F (Figures 2D-F).

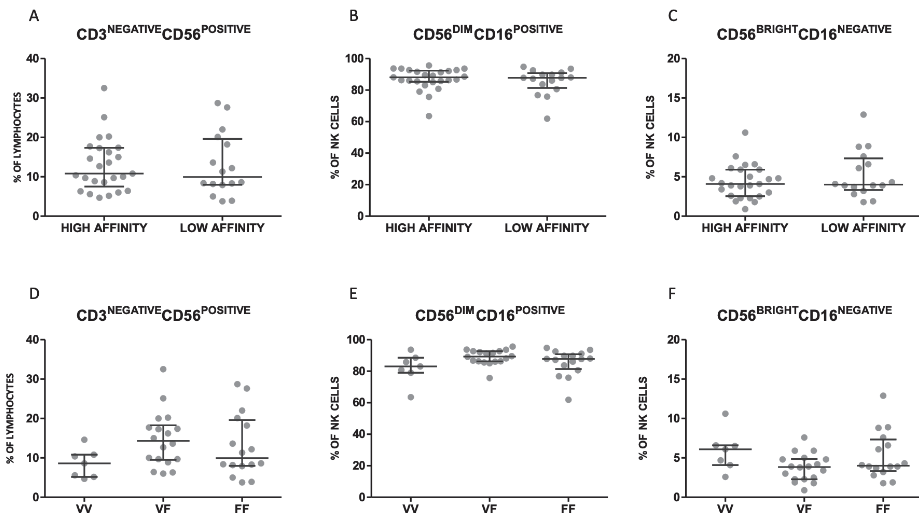


Figure 2 | Frequencies of NK cell subsets in women with RPL genotyped for FcγRIIIA. Percentage of total $CD3^{negative}CD56^{positive}$ NK cells from lymphocytes (A), $CD56^{dim}CD16^{positive}$ NK cells (B) and $CD56^{bright}CD16^{negative}$ NK cells (C) as measured by flow cytometry in women whose genotyping demonstrated FcγRIIIA-p.176 high affinity (V/V or V/F) and low affinity (F/F) or VV, VF and FF respectively (D, E, F). Dots depict individuals, lines depict median and inter quartile range per group.

Next, the percentage of CD16 on all NK cells, irrespective of the expression level of CD56, was compared between the high-affinity and low-affinity groups among women with RPL. No significant difference (P=0.329) was observed between both groups (median high-

affinity 94,8 [93,0-96,7]) versus low-affinity: 94,6 [90,7-95,5]) (Figure 3A). Similarly, there was no significant difference between polymorphic subgroups when percentages of CD16 on total CD3^{negative}CD56^{positive} NK cells (P=0.116) were compared for V/V (93,1% ([92,6-94,9]), V/F (95,0% [94,3-97,0]) and F/F (94,6% [90,7-95,5]) (Figure 3C).

However, we observed a higher MFI for CD16 in RPL women with the high-affinity genotype (median 23575 [19782-26220]) versus the low-affinity genotype (median 17367 [13257-19730]) (P=0.001, Figure 3B and 3E). In line with those data, the CD16 MFI was higher for RPL women with the V/V or V/F genotype than for RPL women with the F/F genotype: MFI for V/V was 22575 [18731-24607], for V/F was 24294 [20157-26637] and for F/F was 17367 [13257-19730] (P=0.003) (Figure 3D).

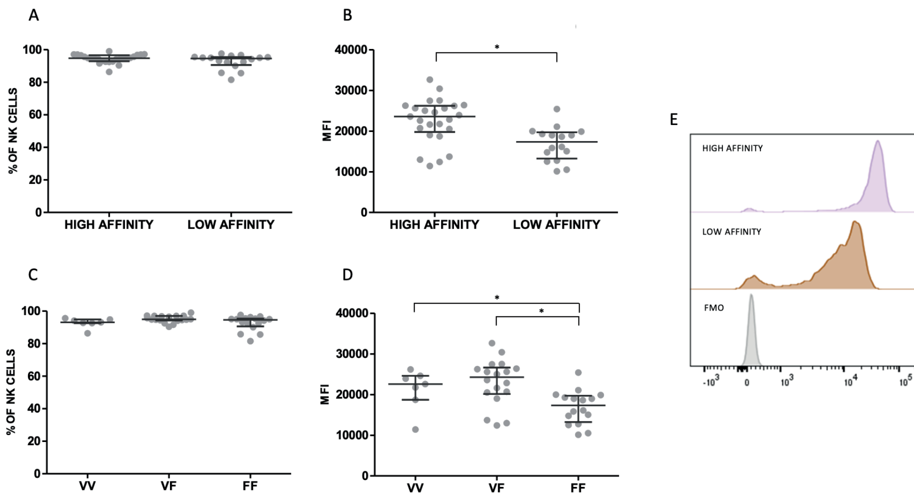


Figure 3 | CD16 expression on NK cells in women with RPL genotyped for FcγRIIIA. Percentage of CD16 positive cells of total CD3^{negative}CD56^{positive} NK cells (A/C) and MFI (B/D) of CD16 expression measured by flow cytometry in women with high-affinity (V/V or V/F) or low-affinity (F/F) FcγRIIIA-p.176 genotypes (A and B) or VV, VF and FF genotypes (C, D). Dots depict individuals, lines depict median and interquartile range per group, *P<0.05. Representative histogram of CD16 expression on NK cells (E) depicting FMO (no anti-CD16) in grey and CD16 expression for the high affinity genotype in purple and the low affinity genotype in brown.

No obvious association between HLA antibody status and frequencies of *FCGR3A*-p.176 genotypes

The impact of the *FCGR3A*-p.176 polymorphism will primarily be relevant in combination with maternal IgG allo-antibodies with the potential to trigger NK cell mediated ADCC against fetal cells expressing paternal allo-antigens. In the transplantation setting, the

highly polymorphic family of HLA molecules (HLA-A/-B/C and HLA-DR/-DQ/-DP) represents the most important allo-antigens and anti-HLA antibodies can provoke complement- and/or cell-mediated cytotoxicity against cells of the allograft[27]. Even in normal pregnancies, approximately 30% of women develop anti-HLA antibodies[22]. To obtain initial evidence for a potentially synergistic role of anti-HLA antibodies and *FCGR3A*-p.176 polymorphism, we determined, as a small pilot, the presence and specificity of anti-HLA antibodies by Luminex-single antigens in 32 women from our RPL cohort. Anti-HLA antibodies were present in 26 out of 32 women (Figure 4). Antibodies were directed against HLA class I in 11/32 women, against HLA class II in 3/32 women or against both, class I and class II in 12/32 women (Figure 4). Under physiological conditions, HLA-C is the only polymorphic HLA molecule that is expressed on fetal trophoblast cells that are in contact with the maternal immune system and a higher incidence of especially anti-HLA-C antibodies has been observed in RPL[23]. Hence, we further defined specificity of the HLA class I and -class II antibodies found in the 32 tested women with RPL (Figure 4). Out of 23 women who had antibodies against class I, 7 had antibodies against HLA-B, 3 against HLA-A, 2 against HLA-C, 1 against HLA-A+C, 2 against HLA-B+C, 3 against HLA-A+B and 5 against HLA-A+B+C. Out of 15 women who had antibodies against class II, 1 had antibodies against HLA-DP, 4 against HLA-DQ, 1 against HLA-DQ+DRB1, 1 against HLA-DQ+DRB3/4/5, 4 against HLA-DQ+DRB1+DRB3/4/5, 1 against HLA-DRB1+DRB3/4/5, 1 against HLA-DP+DRB3/4/5 and 2 against HLA-DP+DQ+ DRB1+DRB3/4/5 (Figure 4).

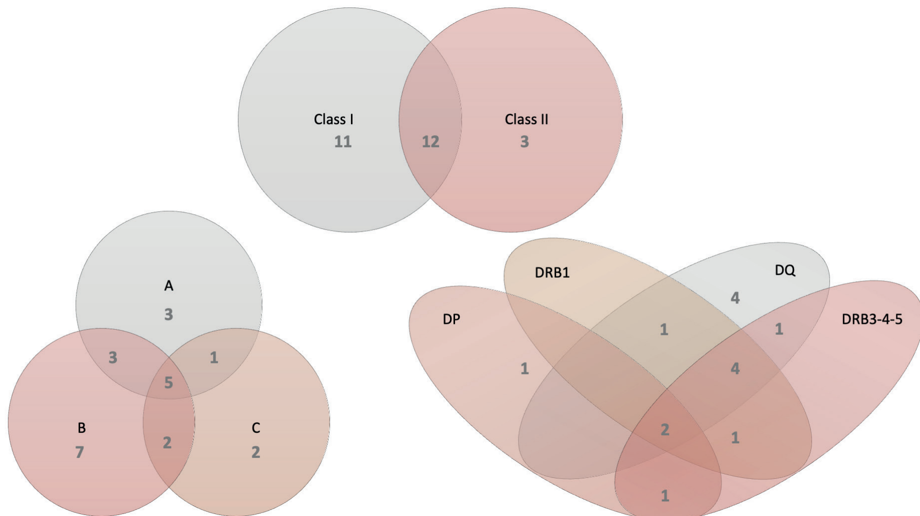


Figure 4 | Specificity of anti-HLA antibodies. Anti-HLA antibodies, including HLA-A, B, C, DP, DQ, and DR, were detected in sera of 32 women with RPL. Specificity and number of individuals that were positive for anti-HLA antibodies are indicated in a venn diagram representation.

Next, we determined the frequencies of high- and low-affinity FcγRIIIA in RPL women and compared them between women with and without antibodies. Frequencies of the high-affinity receptor in women with antibodies were 57,7% (class I and/or class II Ab positive), 60,9% (class I Ab positive), 46,7% (class II Ab positive) compared to 66,7%% in women who completely lacked anti-HLA antibodies, 55,5% in women without class I antibodies and 70,6% in women without HLA class II antibodies (Table 2). In this small group of patients, we did not observe a significant difference in receptor high-affinity versus low-affinity genotypes based on antibody status (class I and/or class II (P=0.687), class I (P=0.783) and class II (P=0.169)). Likewise, we did not observe a difference between women with- or without anti-HLA antibodies in frequencies of the V/V, V/F and FF genotypes (class I and/or class II (P=0.083), class I (P=0.285) and class II (P=0.190)) (Table 2). Due to the low power, we did not evaluate FcγRIIIA in groups defined based on specificity of the anti-HLA antibodies.

Table 2 | Frequencies of FcγRIIIA-p.176 genotypes in RPL women with or without class I and class II antibodies

	Class I		**Class II**		**Class I and/or class II**	
	+ (n=23)	- (n=9)	+ (n=15)	- (n=17)	+ (n=26)	- (n=6)
High affinity (VV+VF)	60,9% (14/23)	55,5% (5/9)	46,7% (7/15)	70,6% (12/17)	57,7% (15/26)	66,7% (4/6)
Low affinity (FF)	39,1% (9/23)	44,5% (4/9)	53,3% (8/15)	29,4% (5/17)	42,3% (11/26)	33,3% (2/6)
VV	13,1% (3/23)	33,3% (3/9)	6,7% (1/15)	29,4% (5/17)	11,5% (3/26)	50% (3/6)
VF	47,8% (11/23)	22,2% (2/9)	40,0% (6/15)	41,2% (7/17)	46,2% (12/26)	16,7% (1/6)
FF	39,1% (9/23)	44,5% (4/9)	53,3% (8/15)	29,4% (5/17)	42,3% (11/26)	33,3% (2/6)

Class I and class II anti-HLA antibodies were tested in sera from 32 RPL women by Luminex single antigens and represented as positive (+) when antibodies were detected or negative (-) when antibodies were not detected. Women were subsequently grouped based on FcγRIIIA-p.176 frequencies of high affinity (VV+VF) and low affinity (FF) or FcγRIIIA-p.176 VV, VF and FF.

DISCUSSION

The present explorative observational pilot study was conducted to investigate occurrence of the p.V176F polymorphism in the *FCGR3A* gene in women with RPL. Moreover, we studied the association with NK cell surface CD16 expression and performed a small pilot to explore a potential synergy of the p.V176F SNP and anti-HLA antibodies.

In our study, no significant differences were found when comparing frequencies of *FCGR3A*-p.176 polymorphisms between RPL women and our control group of women. In our women with RPL the VF genotype is the most common (42%) followed by FF (38%) and VV (20%) and in our control group the FF genotype is most common (47%) followed by VF (40%) and VV (13%). This is in line with results from previous studies that investigated the presence of the *FCGR3A*-p.176 polymorphism and reported VF or FF as most frequent genotype in different populations including ethnic groups from the Netherlands, Great Britain, Austria, Australia, China, Africa[28] Norway[29], Singapore[30] and Japan[31]. Though moderate in size (n=50), our study included a well characterized and relatively homogenous cohort of women with RPL, as the risk for clinical confounders was kept to a minimum by pre-screening the women by means of the PCVS evaluation program. By doing so cases of pregnancy loss caused by abnormal parental karyotype, thrombophilia, aberrant endocrine factors and abnormalities in ultrasound examination were excluded. Unfortunately, obstetric history was not recorded in the healthy female controls and no additional blood samples were available from these women for analysis of CD16 expression and anti-HLA antibody status, making it impossible to attribute differences to previous successful or unsuccessful pregnancies.

We observed a higher MFI for CD16 on NK cells from RPL women with the high-affinity genotype (median 23575) than on NK cells from RPL women with the low-affinity genotype (median 17367). However, there was no significant difference in the percentage of NK cells expressing CD16. In our study, we used the REA423 Miltenyi antibody that recognizes the same epitope as the frequently used anti-CD16 clone 3G8. By using multiple anti-CD16 clones binding to different epitopes, three studies concluded that differences in CD16 expression levels between the high- and low affinity receptor variants were the result of enhanced affinity of the 3G8 clone for *FCGR3A*-p.176V rather than a true difference in the number of molecules[33,34,35]. However, two other studies that used a 3G8 clone reported similar MFI's for CD16 for both homozygous F and V genotypes[17,36]. Although we cannot completely rule out that the difference in CD16 MFI that we observed was caused by a difference in affinity of the antibody, our data are in line with a study that showed that the absolute number of CD16 receptors per NK cell was significantly higher, in addition to higher expression of CD16 both at mRNA

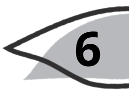
expression and at cell surface expression level, in individuals who expressed V/V at FcγRIIIa-p.176 versus F/F[32]. Additionally, it would be relevant to take copy number variation (CNV) in the *FCGR3A* gene into account in future analysis as it has been described to correlate with FcγRIIIa expression levels[37].

In our study and previous studies assessing the impact of the p.176 SNP, CD16 expression levels were measured on pNK cells. To study the impact on RPL, it would be relevant to also determine CD16 expression on uNK cells, since these are in close proximity of the invading trophoblast and can directly interact with these cells during early pregnancy[38]. Although feasible protocols exist, uNK cells are more difficult to obtain and require menstrual blood or endometrium biopsy sampling[39,40]. The link between *FCGR3A*-p.176 polymorphisms and uNK cell CD16 expression levels has not been studied yet. However, previous studies showed that uNK cells predominantly lack CD16 expression and have a $CD56^{bright}CD16^{negative}$ phenotype which is in contrast to pNK cells where 85-95% of NK cells is $CD56^{dim}CD16^{positive}$[41,42,43]. Despite their low frequencies, $CD16^{positive}$ NK cells have been described to be present in decidua of 81.4% of women with a history of antiphospholipid antibody syndrome experiencing RPL[44]. Also, a significantly higher absolute cell count of $CD16^{postive}$ endometrial NK cells has been described in infertile women when compared with fertile women[45]. This suggests that CD16 may be an important marker of failed immunological adaptation during the implantation phase of early pregnancy. Furthermore, CD16 expression on uNK cells could be increased by local factors. For example, decidual NK cells have been shown to acquire CD16 expression and a more cytotoxic effector function upon interaction with cytomegalovirus (CMV) infected fibroblast[46], and CMV seropositivity has been described to be more frequently present in women with RPL as compared to control women with a healthy pregnancy[47]. To further investigate the potential impact of *FCGR3A*-p.176 polymorphism, genotyping including CNV and analysis of CD16 expression levels on uNK cells could be combined with analysis of viral status.

Since we anticipated that the hypothesized effect of *FCGR3A* polymorphism would be most pronounced in women with allo-antibodies, anti-HLA antibody status was determined. In our small cohort, we did not observe an association between HLA antibody status and frequencies of the receptor genotypes. HLA-antibodies are known to play an important role in organ transplantation as the presence of pre-transplantation donor-specific HLA-antibodies is associated with rejection and impaired organ survival[48]. In pregnancy, the presence of HLA antibodies is presumably largely a harmless phenomenon since they occur in relatively high numbers in normal pregnancy[49]. However, their role could be debated as also harmful effects of anti-HLA antibodies on pregnancy outcome have been described[50]. A higher incidence of anti-HLA antibodies has been observed in women with RPL compared

to women without RPL[24]. The exact mechanism behind increased HLA antibody formation in women with RPL is currently unclear but increasing gravidity[51,52] and the fetal and maternal HLA phenotype combination[53] may be important determinants responsible for the higher incidence of anti-HLA antibodies in women with RPL.

In our group of women with RPL, anti-HLA antibodies were present in 81% (26 out of 32). This is higher than seen in the previous mentioned study where anti-HLA antibodies were detected in 32% of RPL cases[24]. Since none of our women had reported a previous blood transfusion or transplant in their medical history, antibodies present were most likely from a previous pregnancy. Unfortunately, HLA-typing of the partner was unknown so it was not entirely certain that the antibodies present were directed against paternal antigens of the current- or a previous conceptus. In our Luminex assays, we assigned a MFI signal of >1000 as anti-HLA antibody positive which was based on our experience with these assays in routine diagnostics for kidney- and stem cell transplantation. However, it remains to be elucidated whether this is a clinically relevant cut off for RPL. In addition, there are other ADCC-inducing (allo-)antibodies, aside from HLA, that might play an important role during pregnancy. Anti-platelet antigen antibodies have been associated with pregnancy loss, as an increased maternal ADCC immune response to fetal platelet antigens has shown to cause pregnancy loss in a murine model[21]. However, we did not determine anti-platelet antigen antibodies in our study. In addition, anti-phospholipid antibodies have been highly associated with recurrent pregnancy loss[54]. The women in our cohort did not have anti-phospholipid antibodies as they were tested for during the PCVS thrombophilia screening.



To study the potential impact of humoral rejection, including ADCC, in RPL in more detail several additional factors could be included in future analysis. Antibodies can only trigger ADCC if their cognate (paternal) antigens are sufficiently expressed on fetal cells. Under physiological conditions, trophoblast cells that are in close proximity with the maternal immune system lack expression of classical HLA class Ia and class II antigens while they do express HLA class Ib antigens which are known to dampen immune responses at the feto-maternal interface[23]. The almost complete lack of expression of paternal polymorphic HLA molecules would normally protect trophoblast cells from binding to anti-HLA antibodies and thus from complement- or cell mediated cytotoxicity. Extravillous trophoblast cells do, however, express polymorphic HLA-C molecules and mismatches in highly immunogenic HLA-C*07 and -C*17 have been associated with RPL[55]. Interestingly, a higher incidence of antibodies specific for HLA-C was previously found in women with recurrent pregnancy loss illustrating that more in-depth analysis of antibody specificity may help to unravel their role in the pathophysiology of unexplained recurrent pregnancy loss. Another factor to consider is inflammatory status of the uterus, as in early pregnancy a disturbed balance between tolerogenic- and inflammatory immune reactivity may lead to aberrant expression

of paternal HLA molecules contributing to humoral rejection. Although this has not been studied in the uterus yet, inflammation-induced expression of HLA has been associated with humoral rejection in the transplantation setting[56]. In addition, human islets do not express HLA class II under normal conditions, but under inflammatory conditions there is induced expression of HLA class II[57]. Increased expression of paternal allo-antigens like HLA together with increased CD16 expression and a more cytotoxic profile of uNK cells could then possibly lead to ADCC in early pregnancy, while under normal conditions no ADCC would occur, and the high affinity FcγRIIIA variant may aggravate the response.

In summary, with the current set of experiments, we did not observe an association between the high affinity variant of the FcγRIIIA receptor and RPL. To further investigate the role of *FCGR3A*-p.176 polymorphisms and their possible association to functional uterine NK cell ADCC activity in RPL pathophysiology, genomic analysis can be combined with more in-depth analysis of (allo-)antibody profiles and viral status.

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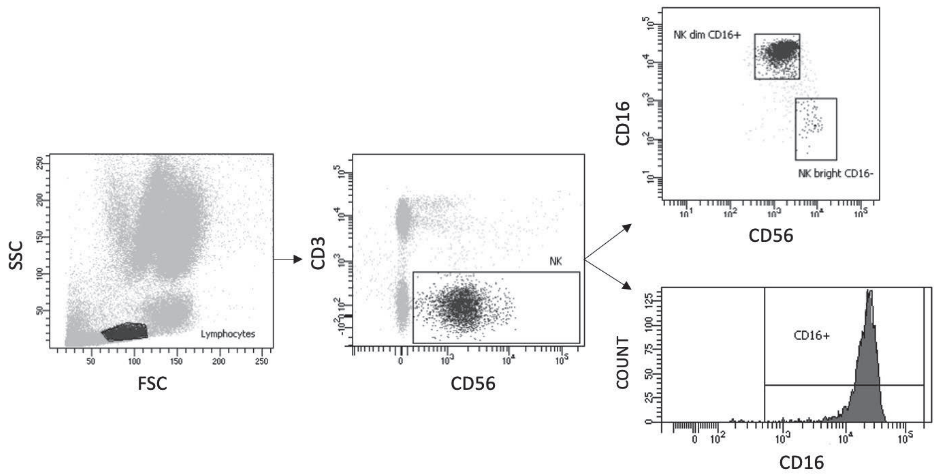
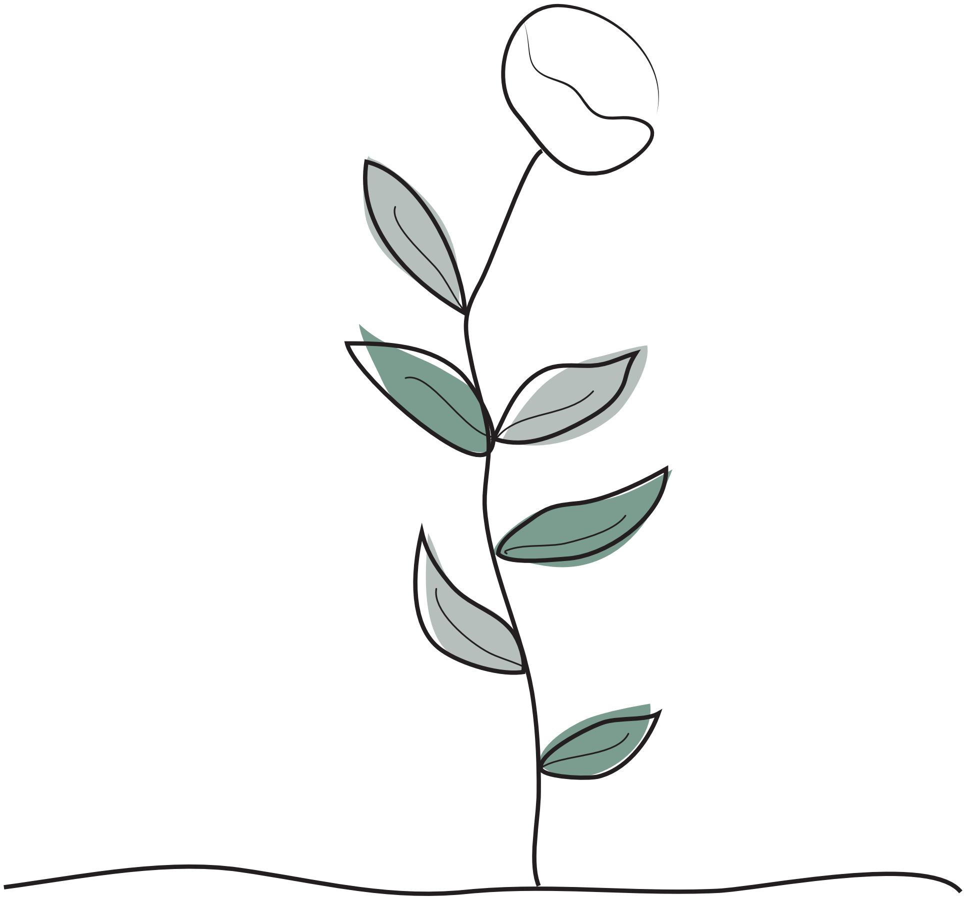
SUPPLEMENTARY FILE



SI Figure 1 | Representative flow cytometric gating strategy to identify NK cells in peripheral blood of women with RPL. NK cells were gated as CD3^{negative}CD56^{positive} and delineated by expression of CD16.



7

PRECONCEPTIONAL EVALUATION OF WOMEN WITH RECURRENT PREGNANCY LOSS: THE ADDITIONAL VALUE OF ASSESSING VASCULAR AND METABOLIC STATUS

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ABSTRACT

Introduction: A majority of recurrent pregnancy loss cases (RPL) remains unexplained. We hypothesized that complications in vascular and metabolic status may guide towards underlying problems that also predispose to RPL and that the number of pregnancy losses is related.

Methods: A retrospective study in 123 women with either a history of low-order RPL (2-3 pregnancy losses) or high-order RPL (≥ 4 pregnancy losses) and 20 women with a history of uncomplicated pregnancy (controls) was performed. Vascular status was assessed by measuring hemodynamic parameters, determining abnormal parameters and analyzing their contribution to the circulatory risk profile (CRP). In a similar way, metabolic status was assessed. Metabolic parameters were measured, used to determine abnormal parameters and analyzed for their contribution to the metabolic syndrome (MetS).

Results: No major differences were observed in vascular or metabolic parameters between women with RPL and controls. There was no relation with the number of pregnancy losses. However, when analyzing the presence of abnormal constituents, more than 80% of women with RPL had at least one abnormal constituent of the CRP. While only 27% had one or more abnormal constituent of the MetS.

Conclusion: The presence of abnormal circulatory factors prior to pregnancy, and to lesser extent constituents of the metabolic syndrome, may predispose to RPL and offer new insights to its pathophysiology.

Keywords: Recurrent Pregnancy Loss, Cardiovascular Disease, Metabolic Syndrome.

INTRODUCTION

Recurrent pregnancy loss (RPL) is a heterogeneous condition and a devastating complication of early pregnancy affecting approximately 1 in 50 couples who are trying to conceive. Although there is no consensus on its definition, the diagnosis of RPL is considered after two or more consecutive pregnancy losses from the time of conception until 24 weeks of gestation, according to most guidelines, such as that of the European Society for Human Reproduction and Embryology (1). Although a few conditions have been causatively linked to the occurrence of RPL, such as antiphospholipid syndrome, uterine malformations and parental chromosomal aberrations, the exact pathophysiology is still elusive and a majority of RPL cases remains without an identifiable cause (1,2). In addition, several risk factors such as increasing maternal age, smoking, alcohol use, overweight and stress have been associated with the prevalence and prognosis of RPL (3). Next to these associated conditions and risk factors, the number of previous pregnancy losses is relevant to the definition of RPL and the prediction of live birth in subsequent pregnancies. However, the contribution of the number of previous pregnancy losses to understanding the pathophysiology of RPL remains unclear (4,5). It is clear that the current diagnostic workup is not sufficient enough and new factors involved in this adverse obstetric complication are very desirable.

Women with obstetric complications, such as preeclampsia and gestational diabetes have been shown to have an increased risk of cardiovascular diseases (CVD) later in life (6,7,8). Leading the American Heart Association to update its guidelines in 2011 to incorporate obstetric complications as risk factors for development of cardiovascular disease in women (9). Moreover, women with RPL have an increased risk of ischemic stroke mortality (10) and a twofold higher risk of coronary heart disease (11) later in life. Hypotheses have been proposed for the association between RPL and CVD. First, a joint underlying genetic, thrombogenic, metabolic or immune defect may contribute to both RPL and CVD (12,13). The genetic predisposition to RPL is suggested by the finding that a positive family history of CVD is associated with a 1.6 higher risk of RPL (14). Second, RPL itself could trigger or augment a cascade of inflammatory responses or other mechanisms causing endothelial dysfunction, that could lead to CVD if persistent (13,15).

Next to this association with CVD, RPL is also associated with characteristics of the Metabolic Syndrome (MetS) such as high body mass index (BMI) (16). In addition, the beneficial effect of metabolic interventions, such as metformin on early pregnancy outcomes, suggest the presence of a metabolic pathway among RPL cases (17).

Collectively, the above lines of evidence suggest that cardiovascular and metabolic complications may guide towards underlying problems that also predispose to RPL and that different vascular and metabolic phenotypes may be associated with RPL. There is limited knowledge on the prevalence and coincidence of vascular and metabolic complications in women with RPL. The present pilot study first aims to analyze vascular and metabolic status in non-pregnant women with RPL and women with a previous uncomplicated pregnancy. Secondly, this study aims to investigate the contribution of the number of pregnancy losses.

METHODS

Study Design

This retrospective study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre (Maastricht UMC+) (14-4-118). Data was available for analysis from 123 women, who gave informed consent and participated in the preconceptional cardiovascular assessment program (PCVS) between 2015 and 2019. This program consists of a structured evaluation performed at least 3 months after miscarriage according to Dutch national guidelines (www.nvog.nl) of RPL and comprises thrombophilia screening, parental karyotypic evaluation, endocrine screening and ultrasound examination for uterine anomalies. Women of reproductive age, with 2 or more reported pregnancy losses before 24 weeks of gestation according to guidelines of the European Society for Human Reproduction and Embryology (1), were included. Women with additional pregnancy complications (e.g., stillbirth, intrauterine growth restriction or preeclampsia in previous pregnancy) or medical complications (e.g., autoimmune disease or kidney disease) were excluded. Women were subsequently divided into two subgroups; having 2 or 3 pregnancy losses (low-order RPL) or having ≥4 pregnancy losses (high-order RPL). Healthy women with at least one previous uncomplicated pregnancy, recruited after advertisement, served as a control group.

Baseline characteristics

Maternal characteristics of age, weight, height and BMI (weight/height^2) were reported. Furthermore, information on obstetric and medical history (number of pregnancies, parity, number pregnancy losses, family history of RPL, smoking habits and the use of coffee, alcohol, drugs and medication) was collected using standardized questionnaires.

Vascular and metabolic status

- Hemodynamic parameters

  Plasma volume (PV) was measured by means of the indicator dilatation technique (18). PV was standardized for body surface area (BSA) according to the Mosteller formula; multiplying the square root of the height (cm) by the weight (kg) divided by 3600. Mean arterial pressure (MAP), systolic and diastolic blood pressure were taken as the median out of eleven measurements that were measured every three minutes in half an hour (Carescape V100, GE Healthcare, Eindhoven, the Netherlands). Heart rate (HR), cardiac output (CO) and stroke volume (SV) were measured during cardiac ultrasonography according to the American Society of echocardiography (ECG) guidelines (19). All images were acquired in left lateral position, after 10 min of rest to ensure stable hemodynamic variables and timed at the end of expiration. Images were recorded as ECG-gated digital loops (MAC 5500, GE Healthcare, Eindhoven, the Netherlands) and stored for offline analysis.

Data was collected and analyzed offline using specific software (Xcelera, Philips, Best, the Netherlands) after completing all measurements. HR was calculated by measuring the time interval between two consecutive R peaks on the ECG. SV was calculated using the following formula: SV = π (OTD/2)2*VTI; three VTI traces were used to determine SV. CO was calculated as CO = HR*SV. Total peripheral vascular resistance (TPVR) was calculated as R = 80*MAP/CO. Uterine artery resistance was measured with a transvaginal probe (GRIC5-9D, GE Healthcare, Eindhoven, the Netherlands) by means of a previously published technique (20). In brief, the velocity in the selected artery had to be 60cm/s or higher to meet the standard of being the uterine artery instead of other paracervical vessels. The angle of insonation had to be as close as possible to 0° and pulsatility index (PI), calculated as peak systolic velocity - end diastolic velocity / time averaged velocity, was measured over at least three cardiac cycles in both left and right uterine artery. Mean uterine artery PI was calculated from the left and right uterine artery PI.

- Circulatory risk profile (CRP)

  The CRP was previously described by Scholten et al (21) and was defined as 1) hypertension: systolic blood pressure of 140mmHg or higher or diastolic blood pressure of 85mmHg or higher or the use of antihypertensive medication; 2) reduced PV: PV less than 1405mL/m^2; 3) increased TPVR: TPVR more than 1600 dyne.sec/cm^5. Women who used antihypertensive medication were not included in the calculations detailed concerning plasma volume and vascular resistance in order to prevent any confounding effect of medication; 4) increased left or right uterine artery PI: right uterine artery PI (> 2.66), or left uterine artery PI (> 2.33) were considered abnormal as these non-pregnant cut-off values were shown to be discriminatory between healthy and complicated pregnancies as previously described by Spaanderman et al (22).
- Metabolic parameters

  Total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were analyzed using an enzymatic colorimetric assay (Cobas 8000 instrument, Roche Diagnostics, Mannheim, Germany). Glucose was analyzed with an enzymatic Spectrophotometric assay (Cobas 8000 instrument, Roche Diagnostics, Mannheim, Germany) and insulin with a chemiluminescent immunometric assay on the Immulite XPi instrument (Siemens Healthcare Diagnostics, New Orleans, USA).
- Metabolic Syndrome (MetS)

  The MetS was defined according to the National Cholesterol Education Program - Adult Treatment Panel III (23) as having 3 or more of the following: 1) abdominal obesity, defined as BMI >30kg/m^2 (24); 2) hypertriglyceridemia, serum level of triglycerides of ≥1.7 mmol/L; 3) low HDL cholesterol, ≤1.29 mmol/L 4) elevated blood pressure, systolic/diastolic blood pressure of ≥130/85 mmHg or use of antihypertensive medication; 5) hyperglycemia, fasting plasma glucose level of ≥6.1 mmol/L or the use of anti-diabetic medication.

Statistics

Baseline characteristics, vascular and metabolic parameters were tested for normality by the Shapiro-Wilk test. Data are presented as median with interquartile range (continuous data) or as percentage (dichotomous data) and compared between subgroups (low- and high-order) RPL and control group by the Kruskal Wallis test (continuous data) or by the Chi-squared test (dichotomous data). To specify which group was significantly different from other groups, the Mann-Whitney-U test was used for continuous data. To test the correlation between continuous vascular and metabolic parameters and the number of pregnancy losses (trend analysis), a Pearson correlation analysis was performed. A P-for trend value below 0.05 was considered statistically significant. Abnormal parameters were additionally compared between subgroups RPL by Mann-Whitney-U tests. Overall, a P-value below 0.05 was considered statistically significant. All statistical analyses were conducted with IBM SPSS statistics version 25 (IBM Corp, Los Angeles, USA).

RESULTS

Study population

A total of 123 women with RPL were included in the analysis; 65 with low-order RPL and 58 with high-order RPL, in addition to 20 women with previously uncomplicated pregnancies included as controls. A flowchart of inclusion is shown in Figure 1. There were no missing values in either RPL groups, however, the control group (n=20) had some missing values in baseline characteristics (Table 1). Both groups of women with RPL had more previously confirmed pregnancies vs. controls (3 [3-4] and 5 [5-7] vs. 2 [1-2]), more pregnancy losses (3 [2-3] and 5 [4-5] vs. 0 [0-1]) and fewer births (0 [0-1] and 0 [0-1] vs. 2 [1-2]). Furthermore, both groups of women with RPL had higher body mass index vs. controls (24.4 [21.4-27.3] and 24.4 [21.7-27.3] vs. 21.8 [19.5-24.1]) kg/m^2 (p=0.028). For all other variables, no statistically significant differences were found between groups.

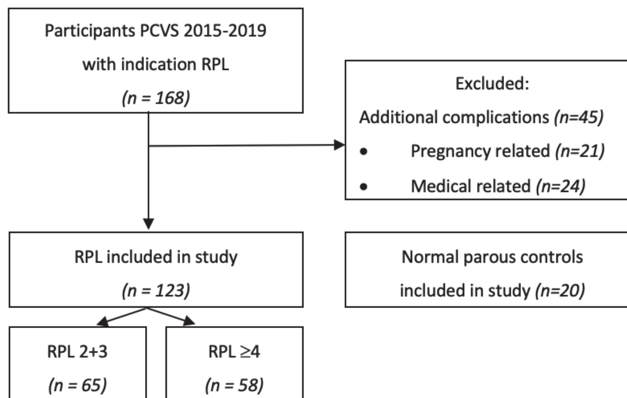

Figure 1 | Flowchart of inclusions in study and sub-analysis PCVS = preconceptional cardiovascular assessment program RPL = recurrent pregnancy loss.

Vascular status

Table 2 describes the hemodynamic parameters in women with low-order RPL (2-3 pregnancy losses) versus women with high-order RPL (≥ 4 pregnancy losses) versus controls. Both groups of women with RPL differed from controls by lower mean arterial pressure (81 [76-87] and 84 [79-89] vs. 88 [81-93]), lower systolic blood pressure (107 [102-115] and 111 [105-118] vs. 116 [110-122]) and lower diastolic blood pressure (66 [59-73] and 69 [64-74] vs. 73 [68-79]). Although differences were not significant, both groups of women with RPL tended to have lower plasma volume (1433 [1313-1540] and 1405 [1307-1508] vs. 1471 [1321-1622]), higher cardiac output (4.5 [3.7-5.2] and 4.6 [4.3-5.4] vs

4.5 [4.2-4.7]), higher peripheral resistance (1425 [1275-1656] and 1474 [1231-1615] vs. 1332 [1243-1502]) and higher uterine artery PI (2.6 [2.3-3.5] and 2.7 [2.1-3.1] vs 2.5 [1.8-2.8]) compared to controls. There was no clear tendency for differences in heart rate and stroke volume between the three study groups.

Table 1 | Baseline characteristics of study population

Baseline characteristics	**RPL = 2+3 (n=65)**	**RPL ≥ 4 (n=58)**	**Controls (n=20)**	**P**
Age (years)	32.8 [29.5-35.4]	33.7 [30.4-36.5]	32 [29.5-38]	0.390
BMI (kg/m^2)	24.4 [21.4-27.3]	24.4 [21.7-27.3]	21.8 [19.5-24.1]	**0.028**
Gravida	3 [3-4]	5 [5-7]	2 [1-2]	**<0.001**
Para	0 [0-1]	0 [0-1]	2 [1-2]	**<0.001**
Pregnancy losses	3 [2-3]	5 [4-5]	0 [0-1]	**<0.001**
History of				
RPL in family of patient	21.9%	35.1%	-	
RPL in family of partner	20.0%	14.0%	-	
Smoking	20.6%	24.1%	16.7%#	0.810
Alcohol use	34.9%	50.9%	41.7%#	0.210
Medication use	28.1%	25.9%	25.0%#	0.950

Data are presented as median [interquartile range] or as percentage #9 controls had missing values
- data was not available.

Trend analysis for the number of pregnancy losses in RPL showed no statistical significance in hemodynamic parameters. However, with an increasing number of pregnancy losses, a lower PV (P for trend=0.150) and CO (P for trend=0.346) and higher SV (P for trend=0.462) and mean uterine artery PI (P for trend=0.490) were observed.

Figure 2 describes the prevalence of the CRP in the total group of women with RPL (n= 123) and the prevalence of individual parameters of abnormal vascular status in women with low- vs. high-order RPL. Within women with RPL, 83% had at least one abnormal parameter contributing to the CRP and 42% had at least 2 abnormal parameters (Figure 2a). There were no significant differences between the two RPL groups in individual abnormal parameters (data from controls are not shown because of missing values). Overall, hypertension was not frequently observed in women with RPL (1/65 and 4/58). Increased mean uterine artery PI was, however, commonly present (41/65 and 30/58) in women with RPL (Figure 2b).

Table 2 | Comparison of non-pregnant hemodynamic parameters

Hemodynamic parameters	**2-3 RPL (n=65)**
Plasma volume (mL/m^2 BSA)	1433 [1313-1540]
Mean arterial pressure (mmHg)	81 [76-87]
Systole (mmHg)	107 [102-115]
Diastole (mmHg)	66 [59-73]
Heart rate (bpm)	69 [63-77.5]
Cardiac output (L/min)	4.5 [3.7-5.2]
Stroke volume (mL/m^2)	67.0 [60.1-76.3]
Peripheral resistance (dyne·s/cm^5)	1425 [1275-1656]
Mean uterine artery PI	2.6 [2.3-3.5]

Comparison of non-pregnant hemodynamic parameters between low-order RPL (2-3 pregnancy losses) high-order RPL (≥ 4 pregnancy losses) and controls PI = pulsatility index data are presented as median [interquartile range] #15 controls had missing values.

Table 3 | Comparison of non-pregnant metabolic parameters

Metabolic parameters	**2-3 RPL (n=65)**
Cholesterol total (mmol/L)	4.5 [4.0-5.1]
Cholesterol HDL (mmol/L)	1.6 [1.4-1.8]
Triglycerides (mmol/L)	0.8 [0.7-1.1]
Glucose fasting (mmol/L)	5.0 [4.75-5.4]
Insulin (pmol/L)	39.7 [26.9-57.6]

Comparison of non-pregnant metabolic parameters between low-order RPL (2-3 pregnancy losses) high-order RPL (≥ 4 pregnancy losses) and controls HDL = high density lipoprotein data are presented as median [interquartile range\ #12 controls had missing values.

≥ 4 RPL (n=58)	Controls (n=20)	P	P for trend
1405 [1307-1508]	1471 [1321-1622]	0.483	0.150
84 [79-89]	88 [81-93]	**0.006**	0.837
111 [105-118]	116 [110-122]	**0.003**	0.519
69 [64-74]	73 [68-79]	**0.002**	0.712
71 [65-78]	69 [62-75]	0.475	0.857
4.6 [4.3-5.4]	4.5 [4.2-4.7]#	0.311	0.346
71.8 [61.8-78.3]	71.0 [69.5-73.5]#	0.363	0.462
1474 [1231-1615]	1332 [1243-1502]#	0.634	0.655
2.7 [2.1-3.1]	2.5 [1.8-2.8]	0.204	0.490

≥ 4 RPL (n=58)	Controls (n=20)	P	P for trend
4.5 [4.0-5.1]	4.5 [4.3-5.1]#	0.919	0.328
1.6 [1.3-1.9]	1.4 [1.2-1.7]#	0.317	0.466
0.9 [0.6-1.1]	0.7 [0.6-0.8]#	0.252	0.940
4.95 [4.8-5.3]	4.9 [4.75-5.15]#	0.730	0.657
36.4 [24.3-65.7]	46.5 [29.5-49.3]#	0.939	0.418

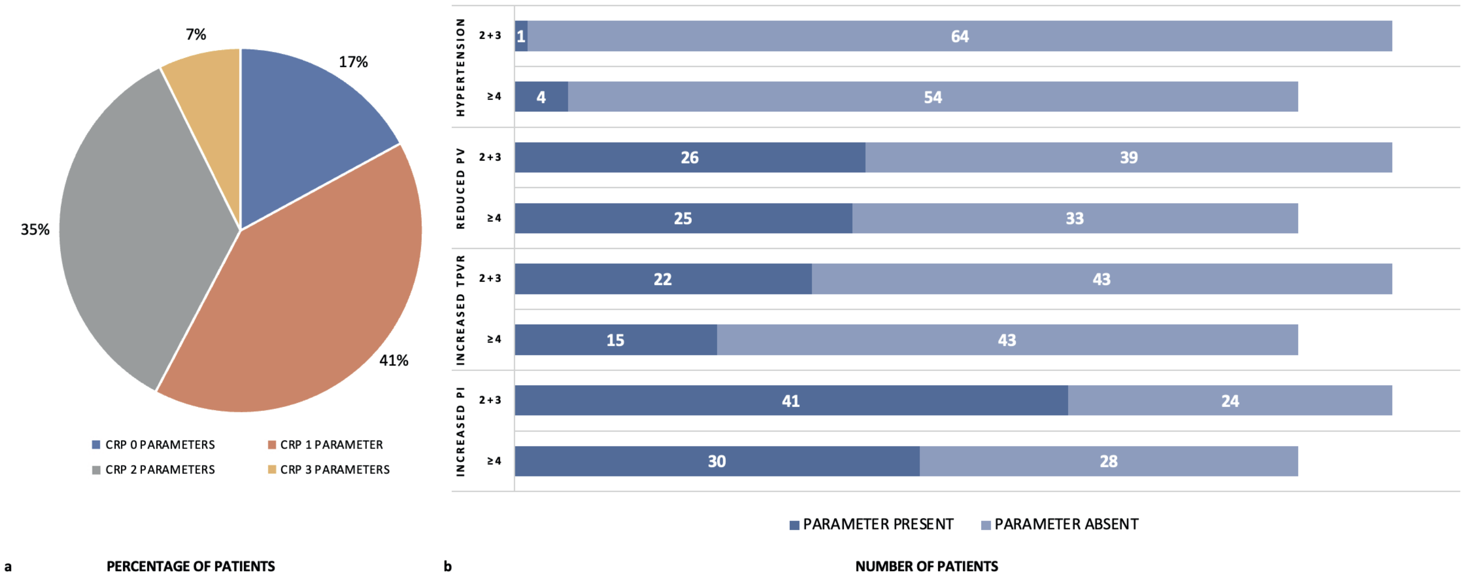

Figure 2 | Prevalence of the circulatory risk profile in women with RPL (n= 123) (a) and its individual abnormal parameters in low-order (n=65) and high-order (n=58) RPL (b) CRP= circulatory risk profile PV= plasma volume TPVR= total peripheral vascular resistance PI= pulsatility index data is presented as percentage of patients (a) or as absolute number of patients (b) data of controls is not shown.

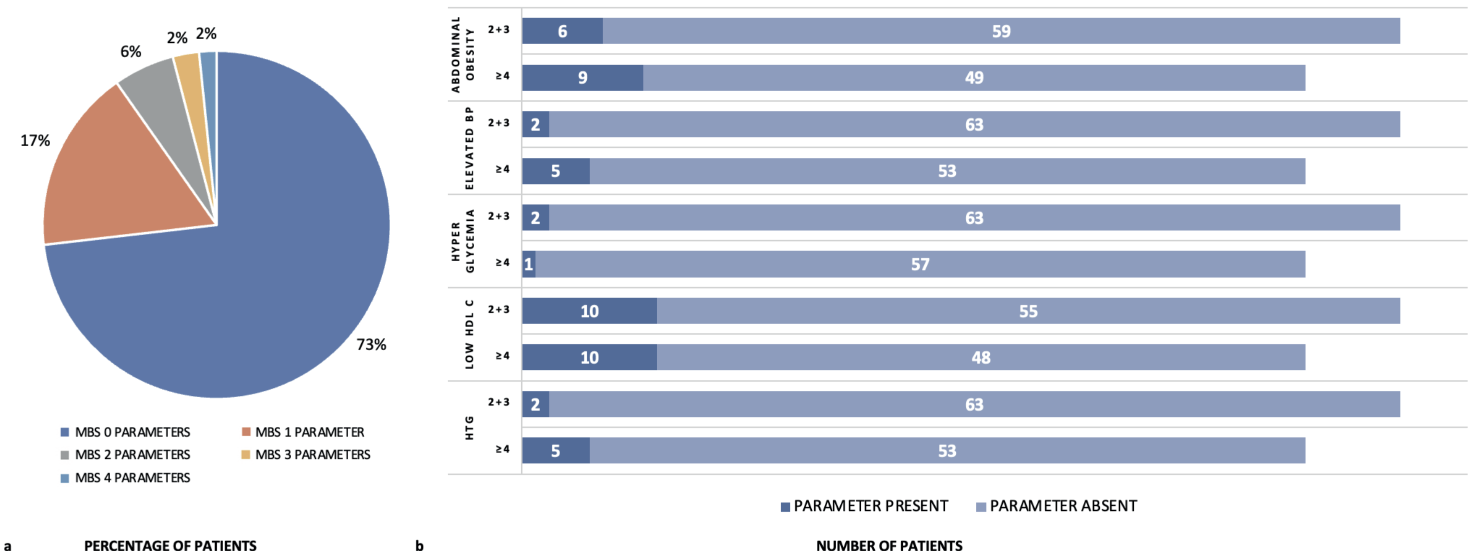

Figure 3 | Prevalence of the metabolic syndrome in women with RPL (n= 123) (a) and its individual abnormal parameters in low-order (n=65) and high-order (n=58) RPL (b) MBS = metabolic syndrome BP= blood pressure HDL C= high density lipoprotein cholesterol HTG= hypertriglyceridemia data is presented as percentage of patients (a) or as absolute number of patients (b) data of controls is not shown.

Metabolic status

Table 3 describes the metabolic parameters in women with low-order RPL versus women with high-order RPL versus controls. Although there were no statistically significant differences between the three groups, women with RPL tended to have higher HDL cholesterol (1.6 [1.4-1.8] and 1.6 [1.3-1.9] vs 1.4 [1.2-1.7]), higher triglycerides (0.8 [0.7-1.1] and 0.9 [0.6-1.1] vs 0.7 [0.6-0.8]) and lower insulin (39.7 [26.9-57.6] and 36.4 [24.3-65.7] vs 46.5 [29.5-49.3]) levels compared to controls. For total cholesterol and glucose levels there was no clear tendency. Trend analysis for the number of pregnancy losses in RPL showed no statistically significance in metabolic parameters. However, total cholesterol (P for trend=0.328) and HDL cholesterol (P for trend=0.252) tended to be higher and insulin (P for trend=0.418) tended to be lower as the number of losses increased.

Figure 3 describes the prevalence of the MetS in the total group of women with RPL (n=123) and the prevalence of individual parameters of abnormal metabolic status in women with low- vs. high-order RPL. Within women with RPL, 73% had none of the parameters of the MetS and 4% had 2 or 3 parameters. Therefore, 5 women with RPL were diagnosed with MetS (Figure 3a). There were no significant differences between the two RPL groups in individual abnormal metabolic parameters (data from controls are not shown because of missing values). Overall, hyperglycemia was not frequently observed in women with RPL (2/65 and 1/58). Low HDL cholesterol was, however, more present (10/65 and 10/58) in women with RPL (Figure 3b).

DISCUSSION

Main findings

In this moderate-size retrospective analysis of 123 women with RPL undergoing extensive preconceptional assessment program there were no major differences in vascular or metabolic parameters compared to controls with previously uncomplicated pregnancies, nor among women with low- vs. high-order RPL. The number of previous pregnancy losses did not relate to vascular nor metabolic parameters. Importantly, the majority of women with RPL (83%) had at least one abnormal vascular parameter contributing to the CRP and 42% had at least 2 abnormal vascular parameters, while only 27% had an abnormal metabolic parameter of the MetS. Although high resistance in the uterine arteries was observed in the majority of women with RPL (71/123), overt hypertension was not frequently reported (5/123). These findings do not confirm our hypothesis of a dose-dependent biological relation between the number of miscarriages and vascular or metabolic status in women with RPL. The high prevalence of the CRP in these women does warrant further investigation into the role of cardiovascular system in RPL.

Strengths and limitations

The strength of our study is the extensive assessment program of various parameters pertaining to the vascular and metabolic system and subsequently using previously described cut-off values to define abnormal parameters in these systems. In this way risk profiles were composed to facilitate interpretation of screening results and documentation of the presence of abnormal circulatory and metabolic profiles. Additionally, we took into consideration the important co-variable: the number of previous pregnancy losses, to link the severity of this adverse pregnancy complication to the vascular and metabolic system. Furthermore, in order to study a homogenous population of RPL, we excluded women with additional medical and obstetric complications as this could be an important confounder on the studied parameters. However, as hemodynamic and metabolic factors could also influence explained RPL, generalizability is limited. Finally, our analysis of the circulatory profile included data on uterine artery PI as an indication of uterine perfusion, hereby assessing not only systemic but also local vascular status, which is arguably more relevant to the pathogenesis of RPL.

There were a few limitations of this study. First, the control group was modest in size and data were not available for analysis on some parameters, decreasing the power of the study and increasing possible selection bias. However, results did not differ after stochastic regression imputation. Secondly, the indication of repeated pregnancy loss was self-reported and lacked important detailed information (e.g., ultrasound features or gestational age) to characterize well-defined subgroups of women with RPL.

Interpretation

Preconceptional assessment of maternal vascular status might be informative on the projected success of subsequent pregnancy and is therefore a potential supplement to current investigations for women with unexplained RPL. Early pregnancy is characterized by a wide range of substantial hemodynamic and vascular changes, such as increases in maternal cardiac output associated with an increase in stroke volume and heart rate, increases in plasma volume, decreases in blood pressure and a reduction in peripheral resistance (25). Importantly, women with reduced plasma reserves are less capable of mobilizing volumes of blood in response to increased arterial demands, as is the case in pregnancy, which in turn can be insufficient for the growing demands of the developing placenta and fetus. Our data is not consistent, though tends to be in line with previous results from a study by Donckers et al (26), showing low plasma volumes in women with unexplained RPL. The observed trend of lower plasma volume with increasing number of previous RPL observed in our study, although not significant, supports the hypothesis that women with RPL may need sustained increased sympathetic tone to meet the arterial demands of early pregnancy. As failing to do so could be a detrimental factor contributing to pregnancy loss (26). Alternatively, the lack of statistical significance in our study could be attributed to differences in plasma volume measurements or the choice of control group. Higher values of plasma volume were described in the nulliparous control group in the study by Donckers et al, compared to our multiparous control group. Future studies utilizing non-invasive, reproducible and readily methods for measuring plasma volume are greatly anticipated.

In addition to measurements of plasma volume, complete assessment of maternal hemodynamic status takes into account the complex interaction between the cardiac, vascular and local uterine compartments. We observed high prevalence of the CRP (83%), with a predominance of increased uterine artery PI in the majority of women with RPL, in the absence of overt hypertension. This suggests that subtle changes in uterine perfusion could be linked to inadequate adaptation of the local uterine environment in early pregnancy and could be an additional pathway to explore in the research on unexplained RPL. Although we failed to demonstrate a dose-dependent gradient between the number of miscarriages and circulatory abnormalities in women with RPL, larger studies taking into account the genetic constitution of the embryo and the gestational age at miscarriage, are better suited to elucidate the contribution of the number of previous losses to various phenotypes of RPL.

Next to vascular status, assessment of metabolic status can yield valuable information on the background of maternal constitutional factors and risks in subsequent pregnancy. Early pregnancy can be viewed as an anabolic state with an increase in maternal fat stores and small increases in insulin sensitivity, accordingly nutrients are stored to meet the

demands of late pregnancy (27). In contrast, late pregnancy is better characterized as a catabolic state with decreased insulin sensitivity and increased insulin resistance, which in turn results in increases in maternal glucose and free fatty acid concentrations, allowing greater substrate availability for fetal growth (27). Our data implies that women with RPL do not differ significantly in preconceptional levels of cholesterol, triglycerides, glucose and insulin from women whom had a previous successful pregnancy. Furthermore, only 5 out of 123 women in our study fulfilled the criteria for the diagnosis of the MetS. This is not in line with a recent study from Hilali et al (16) were the percentage of the MetS or of at least having one of its components was 24.4% in patients with unexplained RPL and this was associated with increasing numbers of previous pregnancy losses. The difference in our findings could be attributed to differences in average BMI: this was 26 in the patient population of Hilali et al (considered overweight) versus 24 (considered normal weight) in our study population.

BMI was significant higher in our women with RPL and this might have had a confounding effect on hemodynamic and metabolic parameters, as plasma volume, cardiac output and peripheral resistance have proven to be positively correlated with BMI. However, as the BMI of our women was within clinical ranges of normal weight, standard deviations were similar to controls and when plasma volume, cardiac output and peripheral resistance were adjusted for body composition results remained similar, confounding contribution was considered to be minimal.

The relevance of our findings to clinical practice can be highlighted by observations from the several studies showing that vascular and metabolic abnormalities in pregnant and non-pregnant women can be treated with help of lifestyle interventions, which are relatively easy to implement in practice (28, 29). Improved lifestyle will result in more favorable parameters and therefore increase the chance for a healthy pregnancy (29). The risk of the MetS and CVD later in life could decrease simultaneously and therefore might have the dual benefit of improving women’s general health and fulfilling the aspiration of a healthy ongoing pregnancy.

CONCLUSION

In this retrospective study we assessed vascular and metabolic status of women with a history of RPL. Although no major differences were observed in vascular or metabolic parameters between women with RPL and controls and there was no relation with the number of pregnancy losses, more than 80% of women with RPL had at least one abnormal parameter of the CRP, while only 27% had an abnormal parameter of the MetS. We discuss the findings in the context of abnormal adaptation as a potential mechanism in RPL and emphasize the relevance of preconceptional vascular and metabolic assessment and prospective trials in women with RPL.

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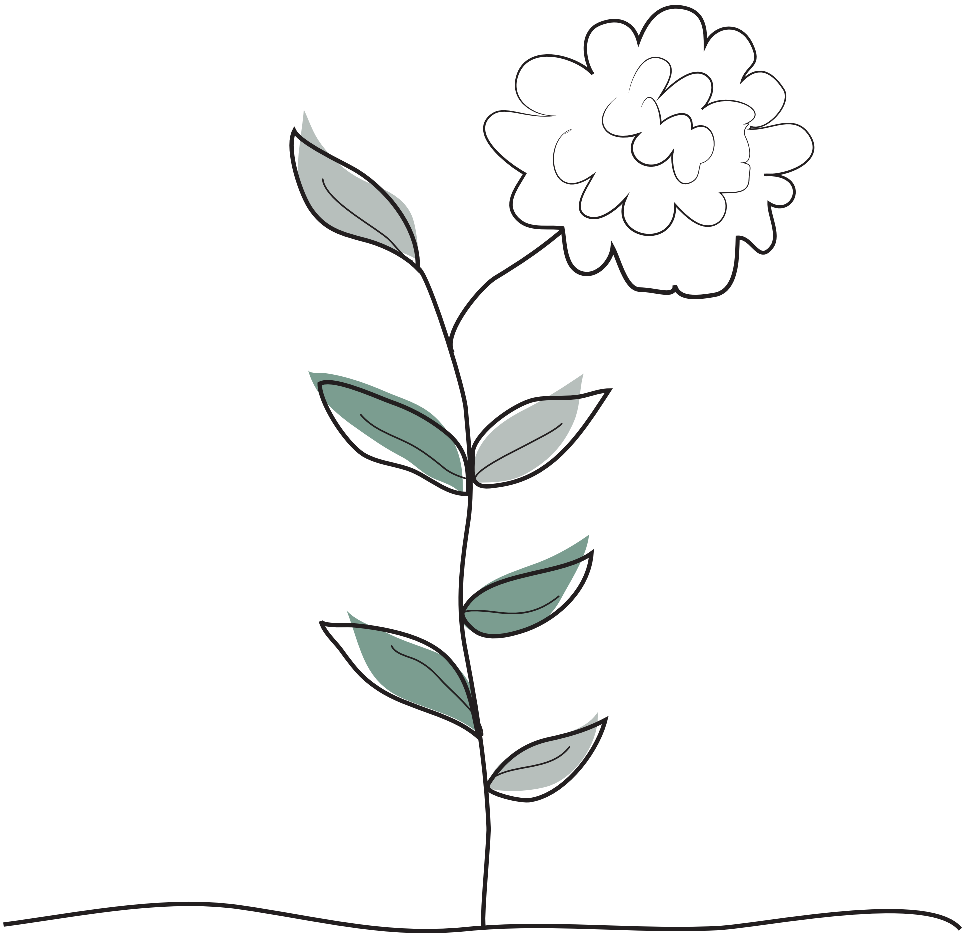

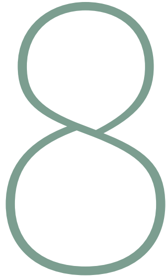

INTRAVENOUS IMMUNOGLOBULINS IMPROVE LIVE BIRTH RATE AMONG WOMEN WITH UNDERLYING IMMUNE CONDITIONS AND RECURRENT PREGNANCY LOSS: A SYSTEMATIC REVIEW AND META-ANALYSIS

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ABSTRACT

Introduction: Intravenous immunoglobulin (IVIG) is increasingly used as a treatment for recurrent pregnancy loss (RPL) despite lack of clear evidence on efficacy. Recent data suggest IVIG might be more effective in a subgroup of women with an aberrant immunological profile.

Methods: Therefore, a systematic review and meta-analysis of studies on the effectiveness of IVIG treatment on pregnancy outcome among women with RPL and underlying immunological conditions (e.g., elevated NK cell percentage, elevated Th1/Th2 ratio, diagnosis with autoimmune disorders) was conducted.

Results: Eight non-randomized controlled trials, including 478 women (intervention: 284; control: 194), met eligibility criteria. Meta-analysis showed that treatment with IVIG was associated with a two-fold increase in live birth rate (RR 1.98, 95% CI 1.44-2.73, P<0.0001). The effect of IVIG was particularly marked in the subgroup of studies including patients based on presence of elevated (>12%) NK-cell percentage (RR 2.32, 95% CI 1.77-3.02, P<0.0001) and when starting intervention prior to or during cycle of conception (RR 4.47, 95% CI 1.53-13.05, P=0.006).

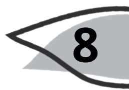
Conclusion: In conclusion, treatment with IVIG may improve live birth rate in women with RPL and underlying immune conditions. However, these results should be interpreted with caution as studies are limited by low number of participants and the non-randomized design, which represent seriously biases. Future randomized controlled trials in women with RPL and underlying immune conditions are needed before using IVIG in a clinical setting.

Keywords: Recurrent Pregnancy Loss, intravenous immunoglobulin.

INTRODUCTION

Recurrent pregnancy loss (RPL), typically defined as two or more pregnancy losses, is both physically and psychologically burdensome for couples trying to conceive[1]. This condition is a frequent reproductive problem worldwide, affecting up to 1% of couples[2]. Despite extensive clinical and laboratory investigations of genetic, hormonal and anatomical factors, the majority of women with RPL have no discernible cause. Currently, there is a prevailing conviction that immunological aberrations may be at fault in women with RPL, as it is evident that the maternal immune system needs regulation to avoid rejection of the semi-allogenic fetus[3].

Among the different immunological aberrations potentially associated with RPL are changes in levels of regulatory T cells and Natural Killer (NK) cells, NK cell cytotoxicity, ratios of T helper cells and the presence of excessive autoimmune reactivity to self-antigens[4]. Auto-antibodies that have been associated with RPL include anti-thyroid, antiphospholipid, lupus anticoagulant, anticardiolipin, antinuclear, anti-ssDNA, anti-dsDNA, and anti-histone[5,6,7]. Furthermore, there is compelling evidence showing that women with RPL have significantly elevated Th1 (proinflammatory) to Th2 (anti-inflammatory) ratios and reduced levels of regulatory T cells compared to normal fertile controls[8,9]. More recently, a study showed that women with RPL have significantly increased activated peripheral blood NK cell levels compared to normal fertile controls[10].



Intravenous immunoglobulin (IVIG) treatment has been broadly applied to suppress excessive immune activation in autoimmune diseases. IVIG has been shown to inhibit the pathological-activity of a large number of disease-associated autoantibodies[11], to downregulate NK cell killing capacity[12], and to inhibit Th1 cytokines[13]. The mechanisms of action of IVIG are complex and a single mechanism might not account for its therapeutic benefit. Although IVIG has been widely used as an immune-modulating agent for more than 30 years, little is known about the factors that predict the success of this therapy[11].

IVIG has frequently been used as a generic treatment strategy for all women with RPL despite lack of clear evidence of improving pregnancy outcomes[14,15]. A recent metanalysis showed that women with unexplained reproductive failure who have abnormal levels of NK cells are more responsive to immunotherapy[16]. However, this study combined the effect of various modalities of immunotherapies on a combined group of spontaneously conceived and IVF pregnancies. Along these lines, IVIG might be more effective in a subgroup of women with spontaneously conceived RPL and underlying immune conditions.

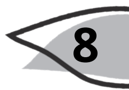
This systematic review and meta-analysis aimed to investigate the effectiveness of IVIG on live birth rate in women with RPL and an underlying immune condition, and to identify the women who might benefit most from IVIG treatment through subgroup analysis.

METHODS

This study was performed in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) reporting guidelines. A standardized Patient, Intervention, Comparison, Outcome (PICO) question was formulated as follows: In women with immunological conditions and recurrent pregnancy loss (P), does the treatment with IVIG (I) increase the live birth rate (O) as compared to no treatment (C)?

Sources and search strategy

A comprehensive literature search was undertaken using PubMed. The literature search was updated up to September 2020. Search terms were combined using 'AND' and included MeSH terms such as 'Recurrent Pregnancy Loss', 'Intravenous Immunoglobulin', 'Cytotoxicity', 'Natural Killer Cells', 'T-Lymphocytes', 'Cytokines', and 'Live birth rate' as well as free text words. The exact search strategy is shown in the appendix. Narrative reviews, systematic reviews, case reports, letters, editorials, and commentaries were excluded, but read to identify other potential studies. Additional strategies to identify studies included manual review of reference lists from key articles that fulfilled the eligibility criteria, use of the "related articles" feature in PubMed, and use of the "cited by" tool in Web of Science and Google scholar.



Eligibility criteria

Studies were selected based on the following criteria: 1) women were included when having three or more pregnancy losses and 2) women were included when studies determined an aberrant immunological profile (defined as elevated number of NK cells or T-lymphocytes, elevated Th1/Th2 ratio or presence of autoimmune diseases). Criteria had to be applicable for both the intervention and the control group. Furthermore, women in the intervention group had to be treated with IVIG. The primary effectiveness outcome was live birth rate. Studies without screening for immunological profile before initial IVIG administration were excluded. Furthermore, studies without a control group or with a control group defined as women with a healthy pregnancy (no immunological aberrations) or non-pregnant women were also excluded. When encountering multiple reports of the same underlying population, the publication with the largest population was included. Lastly, no restriction on age of women was applied, study selection was restricted to English-language and the search was not restricted to a specific publication date.

Study selection and data collection process

The study selection process was performed by one investigator (KP) and critically reviewed by a second investigator (SE). After an initial screening of titles and abstracts, full texts of the potentially eligible studies were retrieved. Full texts were reviewed on their compliance with eligibility criteria and completeness of data by both investigators. Key data were extracted from eligible publications using a data extraction form.

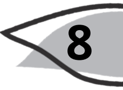
Assessment of risk of bias in included studies

We intended to use the Cochrane Risk of Bias (RoB) Assessment Tool for randomized controlled trials (RCTs)[17]. However, no RCTs were identified (see results). The RoB In Non-randomized Studies of Interventions (ROBINS-I) tool was used to determine the risk of bias per included study[18]. RoB was scored by two independent investigators (KP and DH) on seven domains at three different time points. 1. Pre-intervention (bias due to confounding, bias in selection of participants of the study); 2. At intervention (bias in classification of interventions); 3. Post-intervention (bias due to deviations from intended interventions, bias due to missing data, bias in measurement outcomes, bias in selection of the reported results). Each domain was scored as either low risk, moderate risk, serious risk, critical risk or no information. An overall judgement of RoBs was also performed using the following criteria: 1) Low RoB: studies had low RoB in all domains; 2) Moderate RoB: studies had moderate RoB in at least one domain cut not serious or critical RoB in any domain; 3) Serious RoB: studies had serious RoB in at least one domain, but not critical RoB in any domain; 4) Critical RoB: studies had critical RoB in at least one domain; and 5) No information: when there was no clear indication that the study had serious or critical RoB and there was a lack of information in one or more key domain. Finally, the information on conflict of interest or any funding from commercial agencies was also recorded and evaluated.

Statistical analysis

Included studies were combined and analyzed using comprehensive meta-analysis V3.0 software (Biostat Inc., Englewood, NJ, USA). The risk ratio (RR) with 95% confidence interval (CI) was calculated from the data provided in the studies. Due to anticipated heterogeneity, summary statistics were calculated with a random-effects model. This model accounts for variability between studies as well as within studies. To identify any study that may have exerted a disproportionate influence on the summary effect, we deleted studies one at a time in sensitivity analysis. Statistical heterogeneity was assessed by Cochran's Q statistic and by the I^2 statistic, which is derived from Q and describes the proportion of total variation that is due to heterogeneity beyond chance[19]. I^2 was interpreted based on the following reference points: 25%, 50%, and 75%, representing low, moderate, and high heterogeneity, respectively[20]. We used the Egger's regression test and funnel plots to assess publication bias. A probability value of less than 0.05 (0.10 for heterogeneity) was considered statistically significant.

Potential sources of heterogeneity were assessed through subgroup analysis and subgroups were compared by random effects (method of moments) meta-regression analysis[19]. The sources of heterogeneity analyzed were: 1) use of increased NK cell percentage (>12%) as criterion for inclusion in the study; 2) Initiation of the intervention after pregnancy confirmation; 3) Initiation of the intervention prior to planned conception or during cycle of conception and 4) IVIG dosage/administration scheme.



RESULTS

Study selection

The PRISMA flowchart of the included studies is shown in Figure 1S (Supplementary Information). Out of 243 potentially relevant studies, 8 met eligibility criteria, all were non-randomized controlled trials.

Characteristics of included studies

Characteristics of the included studies are summarized in Table 1. Studies were performed in Iran (2 studies), Spain (2 studies), USA (2 studies), Kuwait (1 study) and Italy (1 study). In total 478 participants were included in these studies: 284 women with an aberrant immunological profile and RPL in the intervention group (IVIG treatment) and 194 women with an aberrant immunological profile and RPL in the control group.

Inclusion and exclusion criteria varied between the included studies. Ahmadi et al.[21], Moraru et al.[24], Ramos-Medina et al.[26] and Winger et al.[28] only included women with an NK cell number above or equal to 12% of total lymphocytes. Perricone et al.[25] only included patients with systemic lupus erythematous and antiphospholipid syndrome and Mahmoud et al.[23] only included patients who were positive for the antiphospholipid syndrome. Stricker et al.[27], Jafarzadeh et al.[22] and Ahmadi et al.[21] reported to exclude women with anatomic, infectious, genetic or endocrine aetiologies of RPL. Moraru et al.[24] reported to exclude women with infectious or lymphoproliferative diseases and Perricone et al.[25], Mahmoud et al.[23] and Winger et al.[28] did not report any exclusion criteria at all. Immunological abnormalities of included patients are further specified in Table 2S.

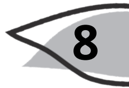
The included studies differed in IVIG dosage regimens and number of cycles. Ahmadi et al.[21] reported to initiate IVIG administration at time of a positive pregnancy test. IVIG administration was continued every 4 weeks during pregnancy until 30-32 weeks of gestation with a dosage of 400mg/kg body weight. Mahmoud et al.[23] and Perricone et al.[25] followed a similar protocol. However, they used a higher dosage (500mg/kg body weight) every 3-4 weeks until a gestational age of 33-34 weeks. Moraru et al.[24] and Ramos-Medina et al.[26] reported administration of 400mg/kg body weight every 3 to 4 weeks until a gestational age of 13 weeks and afterwards patients were given a lower dose of 200mg/kg body weight until 35 weeks of gestation. Stricker et al.[27] reported to initiate IVIG administration 2 weeks prior to planned conception with a dose of 200mg/kg body weight and, after pregnancy was confirmed, IVIG administration was continued every 4 weeks until a gestational age of 26 to 30 weeks using the same dose. Winger et al.[28] reported that 400mg/kg body weight was administered only once during the cycle of conception and/or at least once after a positive pregnancy test.

Risk of bias within studies

Quality assessment of the included studies is shown in Figure 2S (Supplementary Information). Overall, RoB was serious in 1 study and critical in 7 studies. RoB due to confounding and classification of intervention was serious in most studies. Most studies had concerns in RoB due to missing data. Furthermore, RoB during selection of participants (e.g. by screening for NK cell levels before initiation of IVIG treatment) was critical in most studies. Subgroup analysis based on the RoB was unfortunately not feasible due to the low total number of studies.

Meta-analysis

Results of the included studies are calculated as RR for live birth and summarized in Table 3. Meta-analysis showed that treatment with IVIG was associated with a significant increase in 5 out of 8 included studies and an overall significant improvement in live birth rate (8 studies, RR 1.98, 95% CI 1.44–2.73, P<0.0001, Figure 3A), although with a moderate heterogeneity (I^2=58.9%). In sensitivity analyses, excluding one study at a time, the summary RR ranged from 1.81 (95% CI 1.31–2.50), when the study of Ramos-Medina et al.[26] was excluded, to 2.17 (95% CI 1.59–2.96), when the study of Mahmoud et al.[23] was excluded (Figure 3B). Neither visual inspection of the funnel plot (Figure 4S Supplementary Information) nor the regression test of Egger (P=0.362) revealed evidence of publication bias.



We investigated the potential sources of heterogeneity through subgroup analysis. As shown in Table 4, when the 4 studies that used increased NK-cell percentage (≥12%) as criterion for inclusion were pooled[21,24,25,28], meta-analysis showed a stronger association with live birth rate (RR 2.32, 95% CI 1.77–3.02, P<0.0001) and heterogeneity disappeared (I^2=0.0%). In contrast, meta-analysis of the 4 studies that did not use NK-cell percentage as criterion for inclusion[22,23,25,27] showed a RR of 1.73 (95% CI 1.02–2.92) and high heterogeneity (I^2=75.5%). However, meta-regression could not demonstrate a statistically significant difference in effect size between these two subgroups (P=0.266).

Time of initiation of the intervention was also used as subgrouping criterion (Table 4). In 5 studies[21,23-26], treatment with IVIG was initiated after confirmation of pregnancy and the meta-analysis confirmed a moderate heterogeneity in this subgroup (I^2=67.7%). In 2 studies[27,28], IVIG was initiated prior to planned conception or during cycle of conception. Pooling of these two studies yielded a RR of 4.47 (95% CI 1.53–13.05) with a low heterogeneity (I^2=45.2%). Meta-regression could not demonstrate a statistically significant difference (P=0.071) between the effect size of the studies initiating treatment before or after pregnancy confirmation. Finally, although the dose of immunoglobulins had been chosen as a subgroup criterion, the great heterogeneity in this variable did not allow identifying clear patterns for subgrouping.

Table 1 | Characteristics of the included studies

Study	**Country**	**Non-randomized design**	**Definition RPL**	**Inclusion criteria**
Ahmadi et al. 2019	Iran	Prospective	≥ 3 PL before GA of 20 wks	Age 18-40 years NK-cell number > 12%
Jafarzadeh et al. 2019	Iran	Prospective	≥ 3 PL before GA of 20 wks	Age 18-41 years Immune system abnormality
Mahmoud et al. 2004	Kuwait/ USA	Prospective	3 PL between GA of 6 and 22 wks	Positive for APS
Moraru et al. 2012	Spain	Prospective	≥ 3 PL before GA of 20 wks	NK-cell number > 12% NKT-like cells > 10%
Perricone et al. 2008	Italy	Prospective	≥ 3 PL	Systemic lupus erythematosus
Ramos-Medina et al. 2014	Spain	Retrospective	≥ 3 PL before GA of 20 wks	NK-cell number > 12% NK-cell like number > 10%
Stricker et al. 2005	USA	Prospective	≥ 3 PL	Age > 28 years Abnormal immunologic tests including among others T-cell counts and NK-cell levels
Winger et al. 2008	USA	Retrospective	≥ 3 PL	Women with NK-cells > 15% or CD56 number > 12% were offered IVIG

GA: gestational age; IVIG: intravenous immunoglobulin; NK: natural killer; PL: pregnancy loss, RPL: recurrent pregnancy loss, ns: not specified.

N	IVIG (mg/kg)	Cycles	Continued until (weeks of gestation)	Outcomes
78	400 (ns)	Every 4 weeks Initiated after pregnancy confirmation	30-32	Live birth
94	400 (Sandoglobulin)	Every 4 weeks Initiation not further defined	32	Live birth
15	500 (ns)	Every 4 weeks Initiated after pregnancy confirmation	34	Live birth
24	400 (Privigen or Flebogamma)	Every 3-4 weeks Initiated after pregnancy confirmation	13	Live birth
	200 (Privigen or Flebogamma)	Every 4 weeks	35	
24	500 (Flebogamma)	Every 3 weeks Initiated after pregnancy confirmation	33	Live birth
121	400 (Privigen or Flebogamma)	Every 3 weeks Initiated after pregnancy confirmation	1st trimester	Live birth
	200 (Privigen or Flebogamma)	Every 4 weeks	35-36	
64	200 (Venoglobulin-S)	Every 4 weeks Initiated 2 weeks prior to planned conception	26-30	Live birth
58	400 (ns)	At least once during cycle of conception and/or at least once after pregnancy confirmation	-	Live birth

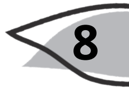


Table 3 | Outcomes of the included studies

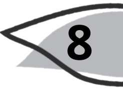
		Mean (SD) pregnancy duration (weeks)	
Study	**N**	**Intervention**	**Control**
Ahmadi et al. 2019	78	39.1 (2.1)	38.3 (2.6)
Jafarzadeh et al. 2019	94	Not reported	Not reported
Mahmoud et al. 2004	15	Not reported	Not reported
Moraru et al. 2012	24	Not reported	Not reported
Perricone et al. 2008	24	37.5 (0.9)	37.2 (2.49)
Ramos-Medina et al. 2014	121	Not reported	Not reported
Stricker et al. 2005	64	Not reported	Not reported
Winger et al. 2008	58	37.2 (3.6)	38.8 (1.0)

NK: natural killer; SD: standard deviation.

Table 4 | Subgroup analysis

Subgrouping criteria	No. of studies	Studies
Use of increased NK-cell percentage (>12%) as criterion for inclusion	4	Ahmadi 2019, Moraru 2012, Ramos-Medina 2014, Winger 2008
No use of increased NK-cell percentage (>12%) as criterion for inclusion	4	Jafarzadeh 2019, Mahmoud 2004, Perricone 2008, Stricker 2005
Initiation of the intervention after pregnancy confirmation	5	Ahmadi 2019, Mahmoud 2004, Moraru 2012, Perricone 2008, Ramos-Medina 2014
Initiation of the intervention prior to planned conception or during cycle of conception	2	Stricker 2005, Winger 2008

Live birth events n (total n)			
Intervention	Control	RR [95% CI]	p-value
33 (38)	18 (40)	1.93 [1.34, 2.78]	<0.001
38 (44)	21 (50)	2.06 [1.45, 2.91]	<0.001
5 (7)	6 (8)	0.95 [0.51, 1.76]	0.877
19 (20)	2 (4)	1.90 [0.71, 5.09]	0.202
12 (12)	9 (12)	1.32 [0.93, 1.86]	0.122
79 (82)	12 (39)	3.13 [1.95, 5.02]	<0.001
38 (44)	2 (20)	8.64 [2.31, 32.33]	0.001
20 (37)	4 (21)	2.84 [1.12, 7.20]	0.028



EFFECT SIZE			HETEROGENEITY	
RR	95% CI	P-VALUE	I^2 (%)	P-VALUE
2.32	[1.77, 3.02]	<0.0001	0.0	0.413
1.73	[1.02, 2.92]	0.041	75.5	0.007
1.71	[1.16, 2.53]	0.007	67.7	0.015
4.47	[1.53, 13.05]	0.006	45.2	0.177

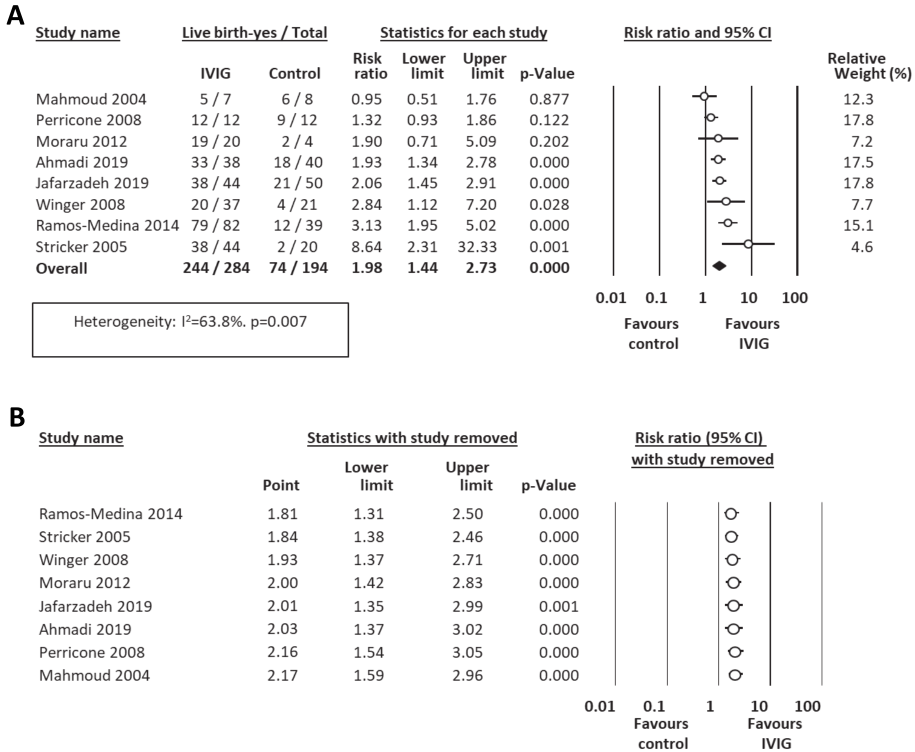


Figure 3 | Meta-analysis on the effect of intravenous immunoglobulin treatment on live birth rate in women with immunological abnormalities and recurrent pregnancy loss or recurrent implantation failure.

A. Forest plot showing the risk ratio (RR) for live birth. A RR>1 means that the event (live birth) is more likely to occur in the intervention than in the control group.

B. Sensitivity analysis showing the effect of removing one study at a time on the pooled RR. Each individual point represents the RR of the meta-analysis if the indicated study is excluded. For example, if the study of Ramos-Medina is excluded, the RR would become 1.81 (95% CI 1.31-2.50) in place of 1.98 (95% CI 1.44-2.73).

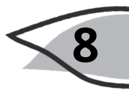
DISCUSSION

Main findings

This systematic review and meta-analysis represent an updated overview of studies on the impact of IVIG treatment in women with RPL who were selected based on an aberrant immunological profile. Five of the eight included studies reported a significant increase in live birth rate (RR ranging between 1.93 and 8.64) among women who received IVIG treatment. The meta-analysis showed that the overall rate of live birth was twice as high in the women treated with IVIG than in the control group (RR 1.98). Although limited by the low number of studies, our data suggest that selection of patients based on the presence of a high percentage of NK cells or initiation of IVIG treatment before pregnancy may further improve pregnancy outcomes.

Strengths and limitations

The main strength of our systematic review and meta-analysis was the use of strict selection and inclusion criteria. Only studies using IVIG intervention on women with RPL and underlying immune conditions and reporting an effect on live birth were included. This increased the homogeneity of the study population and the clinical applicability, as a trade-off for reducing the total number of included studies, which is an important limitation of our study.



A second limitation of the evidence provided by our meta-analysis is the non-randomized design of included studies, which is more susceptible to selection bias[29]. For example, some studies selected IVIG treatment by patient's preference[28] or based on the number of previous miscarriages[21]. To date, the only available RCTs on the effect of IVIG were in a general group of women with RPL who were not pre-screened on immunological abnormalities and used variable IVIG administration protocols. As argued by Valentine and Thompson, the reason to include non-randomized studies in systematic reviews is the need to synthesize the best available evidence when no or few RCTs are available[29]. As IVIG treatment is already commonly being offered to RPL patients, mainly in private clinics, we believe this review and meta-analysis is of value to both patients and clinicians in refining the indication for treatment. Nevertheless, due to the significant bias in the included studies, the data presented here need to be interpreted with caution.

A third limitation was the moderate statistical heterogeneity across the included studies, which is further addressed using subgroup analyses. Interestingly, pooling the four studies using an elevated percentage of NK cells (>12%) as inclusion criterion led to the disappearance of statistical heterogeneity. In addition, the subgroup analysis including the two studies in which IVIG treatment was initiated before pregnancy, also showed an

increase in the effect size and a reduction in the statistical heterogeneity. Nevertheless, due to the low number of studies, the results of the subgroup analyses should be interpreted with caution and regarded as hypothesis generators for future research. Therefore, it would be necessary to perform RCTs in women with RPL and underlying immune conditions using a standardized protocol for IVIG treatment.

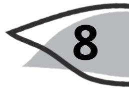
Lastly, substantial clinical heterogeneity was detected in the included studies. The criteria used for defining immunological alterations were variable: including women who were screened with a battery of immunologic tests before inclusion[27] and women with only a diagnosis of autoimmune diseases[23,25]. However, sensitivity analysis by sequential omission revealed that results are robust against omission of these studies. If women with autoimmune diseases were excluded, IVIG treatment still resulted in a significant improvement in live birth rate. Moreover, the laboratory definitions of the immunological alterations were variable. Most studies use the cut-off value of ≥12% of lymphocytes for abnormally raised NK cell levels described by Kwak et al.[30], although peripheral NK cells are considered to have a physiological range between 5-29% of lymphocytes[31].

Future adequately powered studies should define normal and abnormal ranges and determine an aberrant profile, not only for NK cells but also for other immune factors that are clinically relevant for RPL, before they can be used as a diagnostic tool to study IVIG treatment.

Interpretation

Successful pregnancy requires a well-balanced maternal immune system that maintains tolerant toward the foetus while it is still capable of building and adequate immune response against pathogenic microorganisms[32]. Since IVIG can modulate a wide variety of autoimmune and chronic inflammatory diseases and supress excessive and unwanted immune activation[33], it has been proposed to have an immune modulating effect in women with RPL, and especially in those with an aberrant immune profile. The meta-analysis by Christiansen et al.[15] failed to show a significant effect of IVIG on pregnancy outcome in the general group of women with recurrent miscarriage or recurrent implantation failure without pre-screening on immunological conditions. Our results showed that pre-screening women resulted in a two-fold increase in live birth rate, with a particularly beneficial effect in a subgroup of women pre-screened for NK cell percentages (RR=1.99 and 2.32, respectively). These results are in line with a recent study of Woon et al., showing a potential benefit of IVIG in a carefully selected combined population of RIF and RM women with peripheral NK cell dysfunction[16].

Current guidelines, such as the American Society for Reproductive Medicine and the European Society of Human Reproduction and Embryology, do not recommend testing for immune abnormalities in women with RPL due to low level quality of evidence[34,35]. However, our data suggests that pre-screening on immunological biomarkers such as NK cells might be valuable for selecting patients who might benefit from IVIG treatment despite the limited evidence provided here. NK cells are the most abundant leukocytes in early pregnancy decidua and, presumably, they have multiple functions in facilitating healthy pregnancy. First, maternal NK cells in the uterus can directly interact with foetal trophoblasts, allowing trophoblast cells to invade until a certain extent for proper implantation[36]. Furthermore, NK cells secrete an array of cytokines in the uterus that are important for angiogenesis and thus normal placental development. During pregnancy, spiral arterioles are transformed into high capacitance and low-resistance vessels. This vascular adaptation created by the foetal trophoblast is necessary to keep up with the nutritional demands of the growing fetus[37]. When implantation or vascular adjustments are insufficient due to altered uterine NK cell function, it could lead to the early loss of pregnancy[38]. Although to a lesser extent, T cells are also present in early pregnancy decidua and facilitate healthy pregnancy by a predominantly Th2-type immunity[39]. Moreover, large numbers of regulatory T cells are generated during pregnancy and murine studies demonstrated that these are crucial for fetal survival[40,41]. Given these multiple functions, it would be relevant to further explore the potential influence of IVIG on decidual NK and T cells.



In women with reproductive failure, it has become increasingly common to test peripheral blood lymphocytes based on the assumed similarities between lymphocytes in blood and the uterus[10,42]. However, uterine lymphocytes are evidently less cytotoxic[37] and the recent identification of molecularly distinct subgroups of lymphocytes in human decidua[43] suggests that measuring peripheral blood lymphocytes may not provide relevant information on the characteristics of lymphocytes in the uterus. Understanding characteristics of uterine lymphocytes remains a major challenge, as it requires invasive sampling, correlation with menstrual cycle and histological standardization[44,45,46]. Therefore, the data on peripheral blood lymphocytes should be interpreted with caution as their function does not necessarily reflect that of their counterparts in the uterus.

CONCLUSION

Although this systematic review and meta-analysis shows that IVIG improved live birth rate in women with RPL and underlying immune disorders, caution should be taken before offering IVIG as a treatment for reproductive failure. The included studies are potentially biased and limited by the low number and non-randomized design of trials. Our understanding of the immunogenic pathogenesis of RPL is still incomplete and further inquiry into the role of the immune system in RPL is needed to determine a specific biomarker to predict which women with reproductive failure will benefit from IVIG treatment. Even so, these data provide basis for future prospective RCT's in women with RPL and underlying immune conditions using a standardized protocol for IVIG treatment initiated before pregnancy heretofore using IVIG in a clinical setting.

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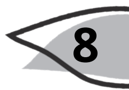

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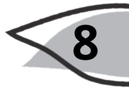

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SUPPLEMENTARY FILES

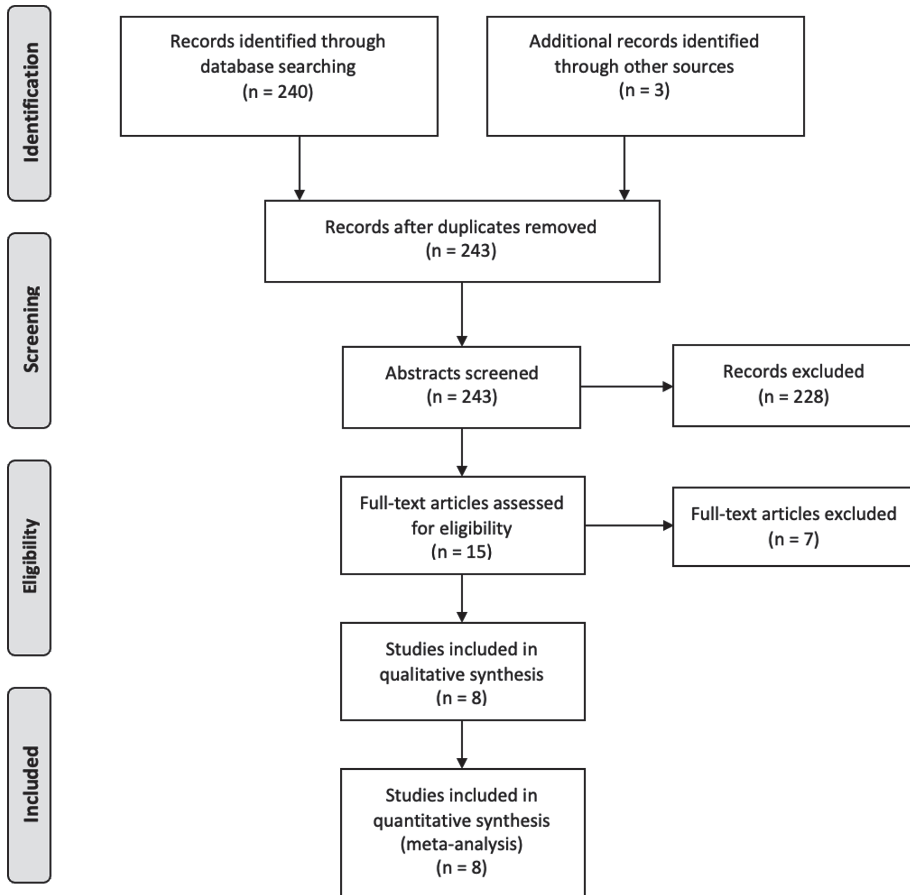


Figure 1S | PRISMA flowchart.

Study	Pre-intervention		At intervention	Post-intervention				Overall risk of bias
First author	Bias due to confounding	Bias in selection of participants into the study	Bias in classification of interventions	Bias due to deviations from intended interventions	Bias due to missing data	Bias in measurement of outcomes	Bias in selection of the reported result	Low/ moderate/ serious/ critical
Jafarzadeh 2019	serious	critical	serious	?	?	moderate	moderate	critical
Ahmadi 2019	serious	critical	serious	?	moderate	moderate	moderate	critical
Mahmoud 2004	moderate	critical	serious	?	?	moderate	moderate	critical
Moraru 2012	serious	critical	serious	?	?	moderate	serious	critical
Perricone 2008	moderate	serious	serious	?	?	moderate	moderate	serious
Ramos-Medina 2014	serious	critical	serious	?	?	moderate	serious	critical
Stricker 2005	serious	serious	critical	?	?	moderate	moderate	critical
Winger 2008	serious	critical	critical	?	?	moderate	moderate	critical

Figure 2S | Assessment of bias using ROBINS-I tool for non-randomized studies.

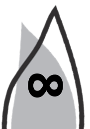
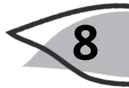


Table 2S | Specification of immunological abnormalities of included patients

Study	Inclusion criteria	Definition of abnormal lymphocyte markers
Ahmadi et al. 2019	Age 18-40 years Elevated NK-cell number	NK-cell number > 12%
Jafarzadeh et al. 2019	Age 18-41 years Immune system abnormality	Not specified
Mahmoud et al. 2004	Positive for APS	Not screened
Moraru et al. 2012	Elevated NK-cell number Elevated NKT-like cells	NK-cell number >12% NKT-like cells > 10%
Perricone et al. 2008	Systemic lupus erythematosus	Not screened
Ramos-Medina et al. 2014	Elevated NK-cell number Elevated NK-cell like number	NK-cell number > 12% NK-cell like number >10%
Stricker et al. 2005	Age > 28 years Abnormal immunologic tests including among others T-cell counts, NK-cell levels and different antibodies	NK-cell levels, CD4 and CD8 T-cell counts
Winger et al. 2008	Elevated NK-cell number Elevated CD56-cell number	NK-cells > 15% CD56-cell number > 12%

Screening for SLE	Screening for APS
Not screened	Not screened
Not screened	Not screened
Not screened	Positive for APS, not further specified
Full fertility screening included determination of antinuclear antibodies; status not further specified for included women	Full fertility screening included determination of lupus anticoagulant and anti-beta-2-glycoprotein (IgG or IgM); status not further specified for included women
Screening included determination of antinuclear antibodies; disease activity was scored using lupus activity index-pregnancy scale (LAI-P; range 0-2.6); status not further specified for included women	Not screened
Screening included determination of antinuclear antibodies; status not further specified for included women	Screening included determination of anticardiolipin, anti-beta-2-glycoprotein I (IgG and IgM) and lupus anticoagulant; status not further specified for included women
Screening included determination of antinuclear antibodies; status not further specified for included women	Screening included determination of antiphospholipid antibody (IgG, IgA, IgM); status not further specified for included women
Not specified	Not specified



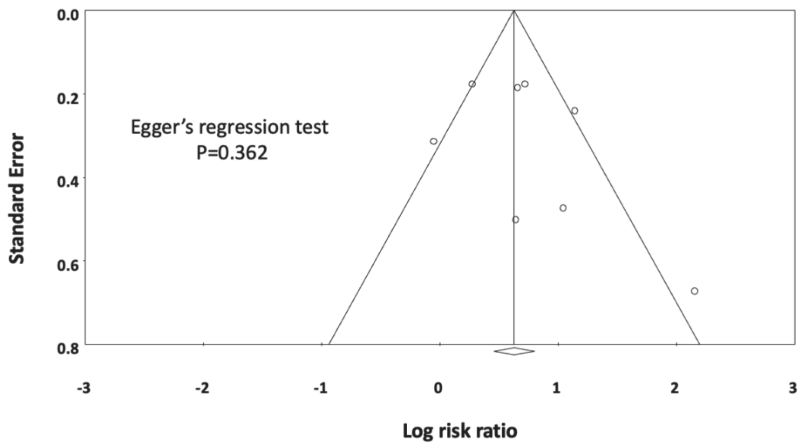


Figure 4S | Funnel plot for publication bias.

APPENDIX

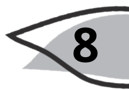
Full electronic search strategy for PubMed
Searched from March 1971 to April 2019

((((((((((((“Abortion, Habitual”[Mesh]) OR Recurrent miscarriage) OR Recurrent pregnancy loss) AND “Killer Cells, Natural”[Mesh]) OR Natural killer cells) OR NK cells) OR NK cell cytotoxicity) OR TH1/TH2 ratio) OR T-lymphocytes) AND “Immunoglobulins, Intravenous”[Mesh]) OR Intravenous immunoglobulin) OR IVIG) AND “Pregnancy Outcome”[Mesh]

240 Hits, 15.12.20

Free terms used for search.

Population	Intervention	Outcome	Design	Setting
Women with Recurrent miscarriage Recurrent pregnancy loss Habitual abortion AND Cytotoxicity Natural killer cells T-lymphocytes Cytokines TNFa	Intravenous immunoglobulin Intravenous IG Intravenous antibodies IVIG	Pregnancy outcome Live birth rate Miscarriage rate	RCT Case-control study Prospective study Retrospective study Cohort study	Clinical setting



9

GENERAL DISCUSSION

GENERAL DISCUSSION

Pregnancy encompasses three trimesters that each have unique inflammatory environments and the ability of the immune system to change and adapt to each specific developmental stage ensures successful outcome[1]. As women with recurrent pregnancy loss (RPL) tend to have successful implantation but lose their pregnancy before 20-24 weeks of gestation[2-5], the switch to an anti-inflammatory environment in the first half of pregnancy seems to be of paramount importance for understanding the immunologic pathogenesis of RPL. In the first half of pregnancy decidual natural killer (dNK) cells play an important role promoting healthy placentation by regulating spiral artery remodeling and trophoblast invasion[6]. These dNK cells interact with invading trophoblast in a fine balance to allow invasion and persistence of semi-allogenic fetal cells while also retaining the ability to inhibit over-invasion and defend against pathogens[7]. How these multiple, and potentially conflicting, effector function of dNK cells are exactly balanced is not yet fully understood. Given the abundant presence, the divergent characteristics in the decidua and the multifactorial regulation of effector function of NK cells, the aim of this thesis was to more integrally profile NK cells in women with RPL. As we anticipate that profiling of NK cells in RPL ultimately could be improved by a more integral and multifactorial approach.

In order to profile NK cells more integrally, we first studied extended phenotypic profiles, including novel immune checkpoint receptors, in peripheral blood and menstrual shed, to see if timing of sampling during the menstrual cycle and location of sampling influenced NK cell phenotypic profiles (Chapter 3). Subsequently, we studied these extended phenotypic profiles in women with RPL and women with a previous successful pregnancy to see if NK cell phenotypic profiles based on the expression of key activating- and inhibitory NK cell receptors in peripheral blood could be valuable in identifying a subgroup of women with RPL with an underlying immune etiology (Chapter 5). We additionally studied the influence of genetic variances in the activating CD16 receptor together with CD16 expression and HLA antibody status as NK cell antibody-dependent cellular cytotoxicity (ADCC) could be an additional pathologic mechanism in RPL (Chapter 6). Moreover, we opted to study associations with abnormal maternal characteristics in cardiovascular or metabolic status that are known to be detrimental for a successful first half of pregnancy (Chapter 7). Finally, we looked at whether there might be a solution that could alter or restore a potential imbalanced immune status in RPL by investigating the effect of intravenous immunoglobulins on live birth rate among women with RPL and underlying immune conditions (Chapter 8).

In **Chapter 3** we showed that the hormonal changes throughout the menstrual cycle did not affect the expression of individual receptors on NK cells in peripheral blood, meaning that, for our type of analysis, it does not matter at what timepoint of the menstrual cycle NK cells are

being sampled in peripheral blood. This information is relevant for studies investigating the association between NK cell phenotypes and reproductive success but also for other disease like cancer, viral diseases and autoimmunity since the relation between NK cell phenotypes is frequently studied in relation to the occurrence and prognosis of these diseases, and because the moment of sampling in the follicular or luteal phase could be an important confounder for studies investigating female immune status.

Moreover, we showed that tissue specific sampling mattered as NK cells derived from early menstrual shed are profoundly different from NK cells in peripheral blood. First, we showed a higher expression of the inhibitory immune checkpoint (IC) receptors TACTILE and TIM3 on NK cells isolated from menstrual shed, underscoring the possible different function of NK cells in the uterus as compared to NK cells in the periphery. Secondly, we showed that NK cells in menstrual shed expressed markers associated with tissue residency that were not expressed by peripheral NK (pNK) cells.

Our first observation on IC receptor expression on menstrual shed NK cells may contribute to unravelling the role of NK cells in reproductive success. Although the critical role of IC receptors in establishing and maintaining immune tolerance is becoming more and more clear, their exact function in reproductive success, and especially their impact on NK cell function in pregnancy, is relatively unexplored (Table 1)[8-16]. IC molecules were originally primarily studied on T cells though and more recently their relevance for controlling NK cell effector function is becoming increasingly clear[17]. Examples of IC receptors are PD1 and CTLA4, well established for their role in inhibition of anti-tumor T cell immunity, as well as less frequently studied IC receptors like TIM3 and LAG3[18] and a group of receptors that interact with ligands of the nectin or nectin-like family including DNAM1, TIGIT, and TACTILE that can act as activating- or inhibitory immune checkpoints[19]. Although IC receptors can be expressed on both T cells and NK cells, it is not yet completely known whether they exert the same effect on the different cell types. It has been shown that interaction of PD1 on T cells with its ligand PDL1 on tumor cells attenuates proliferation, differentiation and survival of T cells[20]. However, elucidating the role of PD1 in NK cells is more challenging and there is still discussion on whether PD1 is actually endogenously expressed on NK cells or that maybe NK cells acquire PD1 exogenously[21]. In chapter 3, we showed expression of the IC receptors TIM3 and TACTILE on NK cells in menstrual shed while these receptors were almost absent on pNK cells. Moreover, two of TACTILE's ligands Nectin-like-5 (usually termed PVR) and Nectin-2 are expressed on endothelial cells of placental blood vessels[22] and TIM3's ligand Galectin 9 is present in decidual tissue[23] providing an incentive for further research into the impact of these receptors on NK cell effector function during pregnancy.

Table 1 | Overview of studies investigating IC receptors on immune cells during pregnancy

IC	Expressed on	Function during pregnancy	Study
PD1	Macrophages	Macrophage polarization M1 to M2	Zhang, 2019[8]
	Cytotoxic T cells	Lower cytotoxicity	Meggyes, 2019[9]
		Th2-type cytokine producing capacity	Wang, 2016[10]
		Decreased cytokine production	Liu, 2020[11]
		Functional exhaustion	Kinder, 2020[12]
	T helper cells	Promote shift to Th2	Wang, 2016[10]
	NK cells	Reduced cytotoxic potential	Meggyes, 2015[13]
	NKT cells	Reduced cytotoxic potential	Meggyes, 2015[13]
	γδ T cells	Reduced cytotoxic potential	Meggyes, 2015[13]
TIM3	NK cells	Reduced lytic activity	Meggyes, 2015[13]
		Increased production of anti-inflammatory cytokines	Li, 2017
		Induction of regulatory T cells	Li, 2017
		Lower perforin production	Li, 2016
		Th2-type cytokine production	Li, 2016
		Suppressing NK cytotoxicity	Sun, 2016
	γδ T cells	Reduced lytic activity	Meggyes, 2015[13]
LAG3	Cytotoxic T cells	Functional exhaustion	Kinder, 2020[12]

9

Our second observation that NK cells in menstrual shed express tissue residency markers, illustrates that NK cells from this source could be used to further characterize their phenotypes as well as their effector functions. As we were able to detect NK cells in first to second day menstrual shed that phenotypically, based on tissue resident markers, resemble the dNK1, dNK2 and dNK3 subsets recently identified in the decidua by Vento-Tormo[23]. Sampling dNK cells during the first half of pregnancy remains a major challenge due to practical and ethical considerations. Moreover, the mechanisms controlling the accumulation of NK cells in the decidua are still being investigated and several possibilities for the origin of dNK cells have been proposed: The first possibility is that NK cells are recruited from the peripheral blood to the decidua, where they undergo further tissue specific differentiation under the influence of the uterine environment[24,25]. This would mean that each menstrual cycle there is a new influx of pNK cells or progenitor cells that can differentiate in dNK cells when arriving in the uterus. Alternatively, it is also possible that dNK cells originate from endometrial (eNK) cells that already reside in the tissue and undergo further differentiation into dNK cells in the new environment that pregnancy creates[26]. At the moment, it is becoming increasingly accepted that dNK cells are likely to be

a heterogeneous population arising from tissue resident progenitors and from homing of pNK cells and/or progenitor cells[6]. By finding tissue residence markers in menstrual shed, which has proven to be an alternative and less invasive method for analysis of eNK cells[27], we provided an opportunity to study specific tissue residency markers identifying 3 novel subsets of NK cells in pregnant decidual tissue, in non-pregnant progenitor endometrial tissue as the endometrium will only transform into decidua if implantation occurs. Without implantation, the endometrial lining will be shed during menstruation[28]. Although we observed exciting differences when sampling menstrual shed, we only sampled on the first to second day of the menstrual cycle. It is unknown if NK cell phenotypic profiles will change between consecutive menstrual cycles, over the course of the menstrual cycle or how expression of menstrual shed NK cells will relate to endometrial NK cells at time of implantation.

Collectively, our data showing differences in thus far relatively unexplored immune checkpoint receptors between NK cells in menstrual shed vs NK cells in peripheral blood and the presence of tissue resident markers when sampling NK cells from menstrual shed provide exciting possibilities for further investigations on the functional relation between IC receptors and NK cells during pregnancy. Moreover, we could envision that screening of NK cell phenotypic profiles in peripheral blood and menstrual shed, could be a valuable tool to study their relation to reproductive success and reproductive failure.

Subsequently, we provided evidence in **Chapter 5**, that pNK cells can be used to detect discriminative phenotypic characteristics between women with RPL and women with previous healthy pregnancies. We observed that not only LILRB1 and TACTILE expression differed, but that expression was also associated with having pregnancy losses even after correction for BMI and was associated with the number of pregnancy losses. However, by doing so we compared differences in expression levels on a per receptor basis while a combination of receptors could also enrich the interpretation of immunological information[29]. Hence in a follow-up study we plan to perform analysis on co-expression of receptors in our dataset e.g., to study NK cell maturation status and the occurrence of so-called memory NK cell subsets in more detail. Unfortunately, we were limited in studying multiple markers simultaneously as we divided our markers over five panels, as the number of markers that could be used per measurement was technologically limited by the presence of 3 fluorescent lasers on the FACS Canto II only allowing us to detect 8 fluorochromes per panel taking into account fluorescent spectral overlap. This approach required every panel to be analyzed separately hereby limiting the simultaneous detection of all the receptors in our study on one cell, as the individual cell that was measured in panel 1 was not the same cell measured in panel 5. Ideally, we would measure all phenotypic characteristics of the cell in 1 panel, to capture the full complexity of the

receptors that control NK function. Through novel techniques (e.g., mass cytometry or spectral analysis) that use for example 30 markers per cell it is becoming increasingly clear that there are many NK cell subsets with different functions; e.g., the dNK1-3[23] but also the innate lymphoid cells (ILC); a heterogeneous family of five subsets including circulating Natural Killer (NK) cells, ILC1, ILC2, ILC3[30] and several kinds of organ-specific NK cells[31]. These subsets probably all have (slightly) different effector functions that allow them to perform diverse roles both pro- and anti-inflammatory and the correct ratio between subsets could then possibly be important for tissue homeostasis. Moreover, a disturbance of this ratio might be pathogenic for example if you have too many cytotoxic NK cells while you need pro-angiogenic NK cells during pregnancy. To be able to measure the nuances in the different NK cell subsets and the ratios between them, simultaneous analysis of more extended sets of markers may be needed. Consequently, we could turn to mass cytometry (CyTOF), which allows for the simultaneous detection of over 40 markers at the single cell level[32].

Moreover, we compared differences in expression levels between women with RPL and controls by analysis of the number of positive cells in subpopulations relative to their respective parent population or by analysis of MFI of a marker using a quantitative manual gating strategy. This strategy ensures arranging cells into pre-specified varieties by consecutively setting thresholds on each marker, either alone or in bivariate combinations. This intensive method which is expertise-dependent and likely subjective, impedes unbiased analyses of more than two markers simultaneously and thereby restricts the discovery of novel combinations[33]. The full potential of multicolor flow cytometry also lies in the ability to qualitatively reveal novel subpopulations and correlations by visualizing and dissecting multiparametric datasets which can be done with clustering and dimensionality reduction techniques[34]. In future analyses, it would be exciting to also use these techniques on our multiparametric data per panel, which can be done for example by t-distributed stochastic neighbor embedding (t-SNE)[35] or FlowSom[36]. Another attractive novel method, that is also able to quantify novel populations and correlations, is called DAMACY. DAMACY is based on Principle Component Analysis (PCA); a dimensionality-reduction method that reduces the number of variables of a data set while preserving as much information as possible[37]. DAMACY is unique as it covers information on the multivariate (co)-expression of markers on single cells, on clustering of cells into populations with similar marker expression, on representation of cell populations in a specific individual and on differentiation of this representation in specific phenotypes for multiple individuals[38]. Moreover, it allows for easy projection of samples, as all significant cell clusters are highlighted in the PCA space and the loadings show the marker expression of these cells[38] but can also predict new samples that can easily be cross validated and tested for statistical significance, hereby allowing a quantitate comparison to be made. In a follow-up study, we could use DAMACY

to quantify differences between women with RPL and controls based on the marker variability between single cells all measured in one panel. In this way, multiparametric data from flowcytometric analysis can be used to its full potential when identifying women with RPL with NK cell phenotypic profiles.

When profiling NK cells to discriminate women with RPL and controls, it could be that only looking at receptor level by protein expression underexposes important factors related to NK cell phenotype. As polymorphisms can influence protein expression or protein function, e.g., as it can influence affinity of the protein[39], genotyping might provide additional information. This might be the case when investigating CD16 or FcγRIIIA, one of the most powerful activating receptors on NK cells that enables NK cells to respond to antibody-coated target cells and to exert antibody-dependent cellular cytotoxicity (ADCC)[40]. A FcγRIIIA polymorphism at position 176 encoding for valine has been shown to have a higher affinity for IgG1 and IgG4 than does the one encoding for phenylalanine[41] and homozygosity of the high-affinity p.176val variant has been shown to be associated with the pathogenesis of rheumatoid arthritis[42]. Although, we did not observe an association between the high affinity variant of the FcγRIIIA receptor and RPL with the current set of experiments in **Chapter 6**, it would be relevant to further investigate the possible association of the polymorphism with CD16 expression on eNK or dNK cells and anti-HLA antibody status in RPL pathophysiology. As perhaps the polymorphism does not affect CD16 expression on NK cells in the periphery as much as it does in the uterine environment. An increase of CD16 expression on the abundantly present CD56^{bright} NK cells in the uterus, which under normal circumstances hardly express CD16, might then cause CD56^{bright} NK cells to become more cytotoxic. Such an increase may occur in the presence of a local infection, as especially upon stimulation with cytokines such as IL-2 or IL-12, the cytotoxic activity of CD56^{bright} NK cells dramatically increases as the expression of CD16 increases[43]. Theoretically, the presence of the high-affinity polymorphism in combination with a local stimulus causing increased protein expression of the CD16 receptor on uterine NK cells, may then possibly lead to ADCC in early pregnancy, while under normal conditions no ADCC would occur. This increased CD16 protein expression and function might shift the balance of NK cell effector function in a normal pregnancy, leading to a more pro-inflammatory environment while their predominant role should be anti-inflammatory ensuring implantation and subsequent fetal and placental growth. However, this could only be in the case of the presence of paternal antibodies. Anti-paternal HLA-antibodies are considered a harmless phenomenon during most pregnancies, however their role in RPL is disputed as HLA-antibodies are significantly more frequent in women with secondary RPL with a firstborn boy than in women with primarily RPL and controls and the presence of these antibodies in early pregnancy is associated with a reduced chance of a live birth[44]. Moreover, women experienced higher rejection rates of a transplanted kidney when they received it from

their male partners or their offspring [45]. Suggesting, there might be a higher immunological risk in some women upon re-exposure to paternal antigens on the semi-allogenic fetus. Screening for the presence of anti-HLA antibodies would then possible be relevant for women who previously had a successful pregnancy but subsequently experienced multiple losses, although current guidelines do not recommend doing so. However, it is important to first study this in a larger cohort of women, including both women with primary and secondary RPL in addition to a proper control group. Although we were very fortunate that we were allowed to work with a dataset regarding the *FCGR3A*-p.176 polymorphism, available from a large cohort of healthy women from Sanquin Amsterdam, we do not have information on the obstetric history of these women. If some if these women did have history of pregnancy loss, our results might have been skewed.

Although we realize that the combination of the high-affinity variant, a possible local uterine infection and having anti-HLA antibodies will not be applicable for every woman with unexplained RPL, NK cell phenotypic profiling combined with genomic analysis and with more in-depth analysis of infection status and antibody profiles in a more integral approach, may possibly help to discriminate a subcategory of women with a higher immunological risk related to RPL and subsequently informing them of their prognosis would then be warranted.

Essentially, profiling of NK cells not only helps to discriminate between women with RPL and controls, it also supports to define possible mechanisms of action for reproductive success and provide targets for interventions. Combining our results from **Chapter 3** and **Chapter 5**, TACTILE an immune checkpoint receptor known to be important for immune regulation[46], is higher expressed on NK cells sampled from menstrual shed and is increased on pNK cells in women with RPL. Although the function of TATCILE on NK cells is still largely obscure and not much is known about its role during pregnancy, it might be interesting to investigate if TACTILE controls NK cell effector function, e.g., cytotoxicity and/or cytokine secretion and whether it has a different effect on pNK cells and dNK cells. Therefore, we could envision an experimental setting in which pNK cells and eNK from menstrual shed are being cultured in a functional NK cell CD107a assay (an assay determining the amount of degranulation in NK cells) or NK cell cytotoxicity assay (an assay determining the amount of cell death caused by NK cells) with and without blocking of the immune checkpoint receptor using electroporation of CRISPR/Cas9 plasmids in order to determine its functional relevance[47]. In addition, pNK and eNK cells from women with RPL and controls would then be co-cultured with trophoblast organoids in a functional experimental model, that is transformative for investigating trophoblast interactions with NK cells in RPL. Following implantation, the trophoblast is generated after the trophectoderm of the blastocyst proliferates[48]. Subsequently, the trophoblast differentiates into two main subpopulations:

syncytiotrophoblasts (SCT) that are mainly responsible for nutrient exchange and hormone production, and extravillous trophoblasts (EVT) that secure the developing placenta to the maternal decidua and remodel the maternal spiral arteries[49]. Organoid cultures of trophoblast cells differentiating into SCT and EVT cells have been generated and have proven to organize into villous-like structures that vigorously invade in 3D[50]. By co-culturing trophoblast organoids with isolated pNK or eNK cells, a novel functional experimental model can be developed, in which interactions of NK cells and trophoblasts can be studied but also in which disruptions of the interaction can be studied for example by introducing a pro-inflammatory trigger mimicking infection, as we show in **Chapter 5** that CMV status is related to increased NKG2C expression in women with RPL, or by introducing possible modulators from the cardiovascular system or metabolic system.

Successful NK cell orchestrated spiral artery remodeling might be indirectly influenced by maternal cardiovascular or metabolic status as abnormal maternal characteristics in cardiovascular or metabolic status that are known to be detrimental for a successful placentation during the first half of pregnancy. We hypothesized that shallow cardiovascular reserves[51] and subnormal uterine perfusion[52], related to increased sympathetic tone[53] and dyslipidemia or insulin resistance, variables consistent with the metabolic syndrome[54], might influence NK cell function. In **Chapter 7** we therefore investigated the cardiovascular or metabolic status of women with RPL and controls and observed that there were no major differences in vascular or metabolic parameters between women with RPL and controls. Nonetheless, when analyzing the presence of abnormal constituents, more than 80% of women with RPL had at least one abnormal constituent of a circulatory risk profile and 27% had one or more abnormal constituent of the metabolic syndrome. Moreover, there was a predominance of increased uterine artery PI in the majority of women with RPL, in the absence of overt hypertension, suggesting that subtle changes in uterine perfusion could be linked to inadequate adjustments of the local uterine environment in early pregnancy. Due to different studied cohorts of women with RPL and controls in **Chapters 5 and 7**, we were unable to correlate cardiovascular or metabolic status to NK cell status. However, in the cohort of women observed in **Chapter 5**, we also found an increased BMI in our women with RPL. Possibly women with an increased immunological and cardiometabolic risk may even been more susceptible to RPL. Consequently, an exercise intervention that has favorable effects on weight, BMI, and accumulated visceral fat[55], which is an indicator of cardiometabolic risk, might also have beneficial effects on NK cells, as NK cells have been shown to be the most responsive immune cells to exercise[56]. Moreover, immune modulating effects of metabolic interventions such as the Wim Hof Method (WHM) have been described to causes a shift in metabolism which partly contributes to an anti-inflammatory response as the WHM seems to affect the plasma metabolome by

increasing plasma concentrations of lactate and pyruvate which in part contributed to increases in anti-inflammatory IL-10 and lower pro-inflammatory IL-1b, IL-6 and TNFa[57]. Although a causal link between the immune and the cardiometabolic system has yet to be demonstrated, we can envision a clinical study for which we can hypothesize that an exercise intervention could lead to an improved immune tolerant function of NK cells and eventually improve pregnancy outcome. Subsequently, NK cells can be profiled before an exercise intervention, hereby selecting women with RPL and an aberrant NK cell profile based on for example increased TACTILE expression on pNK cells. In addition to phenotypic profiling of NK cells, it would be relevant to measure metabolic and cardiovascular parameters including uterine perfusion and collecting live birth rates after one year of completion of the intervention, in order to study their association with aberrant NK cell phenotypic profiles. When proven to be effective in improving phenotypic profiles and indirectly also very important manifesting gains in mood and self-reliance in RPL women[58], an exercise intervention could be envisioned as a non-invasive, readily available treatment strategy.

Understanding the immunologic pathogenesis of RPL is urgently needed for providing appropriate care and development of future treatment strategies. In addition to life style adjustment, clinical immune-modulating therapy has been put forward as possible treatment strategy[59] to lower recurrence risks of pregnancy losses and intravenous immunoglobulin (IVIG) is already being offered to RPL patients, mainly in private clinics, despite lack of clear evidence on efficacy. Therefore, in **Chapter 8**, we conducted a systematic review and meta-analysis of studies on the effectiveness of IVIG treatment on pregnancy outcome among women with RPL and underlying immunological conditions, considering this review and meta-analysis could potentially have an impact on the clinician's decision to consider IVIG as a possible treatment strategy for women with RPL. On the one hand, this systematic review and meta-analysis suggest that IVIG improved live birth rate in women with RPL and underlying immune disorders, especially in a subgroup of women with elevated (>12%) NK-cell percentage and when starting IVIG treatment prior to or during cycle of conception. On the other hand, we still should be cautious before offering IVIG as a treatment for reproductive failure, as included studies are biased given their non-randomized design and the still limited number of included participants.

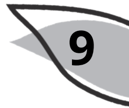
Apart from IVIG, other suggested immunological therapies for RPL include corticosteroids and intralipids**.** The supposed mechanism of immune modulation of corticosteroids in RPL is through improving of trophoblast invasion[60]. However, guidelines do not recommend the use of prednisolone until further placebo-controlled randomized trials are available as adverse events and side effects include a higher risk of diabetes and hypertension in pregnancy and a significantly higher risk of preterm birth[61]. The supposed mechanism of

immune modulation of intralipid; a sterile, non-pyrogenic fat emulsion made up of soybean oil, egg yolk phospholipids, glycerin and water, is through decreasing NK cell cytotoxicity and inhibiting pro-inflammatory mediators such as Th1 cells[62]. Although intralipids are more affordable and do not cause adverse events and detrimental side effects, its impact on life birth rate has not been extensively investigated. Due to the controversialities on corticosteroids, intralipids and IVIG in RPL, guidelines of the Nederlandse Vereniging voor Obstetrie en Gynaecologie[63] (NVOG), the American Society for Reproductive Medicine[64,65] (ASRM) and the European Society of Human Reproduction and Embryology[66] (ESHRE) do not recommend immune modulating treatment strategies in RPL. However, it would be valuable to study immune modulating treatment in a controlled setting of a randomized controlled trial, selecting a subgroup of immune deviant women with RPL based on NK cell profiling. One other immune modulating treatment that we could envision in the future to be investigated in women with RPL is the use of immune checkpoint inhibitors or stimulators. To date, attempts are made to suppress inhibitory immune checkpoint receptors in cancer to increase pro-inflammatory responses, or to stimulate activating immune checkpoint receptors or suppress activating immune checkpoint receptors in autoimmune disease to create a more anti-inflammatory response[67,68]. Similar modulation might also be of great importance for developing new treatment strategies for women with RPL, albeit this first requires a better understanding of the role of IC receptors in pregnancy.

Our understanding of the immunogenic pathogenesis of RPL is still incomplete and further inquiry into the role of the immune system in RPL is needed to determine a specific marker to better predict which women with reproductive failure will benefit from expensive immune modulating treatment. Even so, the data provided in this thesis provide basis for future prospective randomized controlled trials in women with RPL and underlying immune conditions based on NK cell phenotypic profiling using a standardized protocol for treatment before using immune modulators in a clinical setting.

Nowadays it is well known that the maternal immune system is well aware of an implanting and developing embryo during pregnancy, as it notices and responds to the embryo by establishing an appropriate balance between tolerance, which is crucial for successful implantation and development and growth of the fetus and placenta, and activation, which is needed to respond to pathogens[69]. Nevertheless, the exact mechanisms governing this balanced immune modulation remain poorly understood. In this thesis we described immunological parameters that differ between women with RPL and women with previous uncomplicated pregnancies by integral NK cell profiling. These parameters provide valuable information for developing diagnostic markers, which is highly needed as 50% of RPL cases still cannot be explained by current diagnostic investigations. Next to their diagnostic value of identifying RPL patients with an underlying immune etiology, it

provides opportunities for identifying subgroups of women with an increased risk of RPL because of the presence of a genotypic polymorphism and HLA-antibodies, CMV infection or abnormal cardiometabolic constituents. Moreover, they may provide opportunities for the development of predictive markers (can NK cell profiling predict whether someone will lose a subsequent pregnancy or have a healthy pregnancy), for the development of disease activity biomarkers (can NK cell profiling indicate when an early pregnancy threatens to be detrimental) and for the development of treatment response markers (can NK cell profiling predict which women with RPL will respond to a certain treatment) in the future. In addition to finding clinical associations that can be used to improve current clinical diagnostics, integral NK cell profiles could provide new insights in mechanistic understanding of RPL pathology and may help to guide the development of personalized immune modulation treatment strategies that will hopefully help women with RPL in the future without a one size fits all principle (see schematic representation in Figure 1).



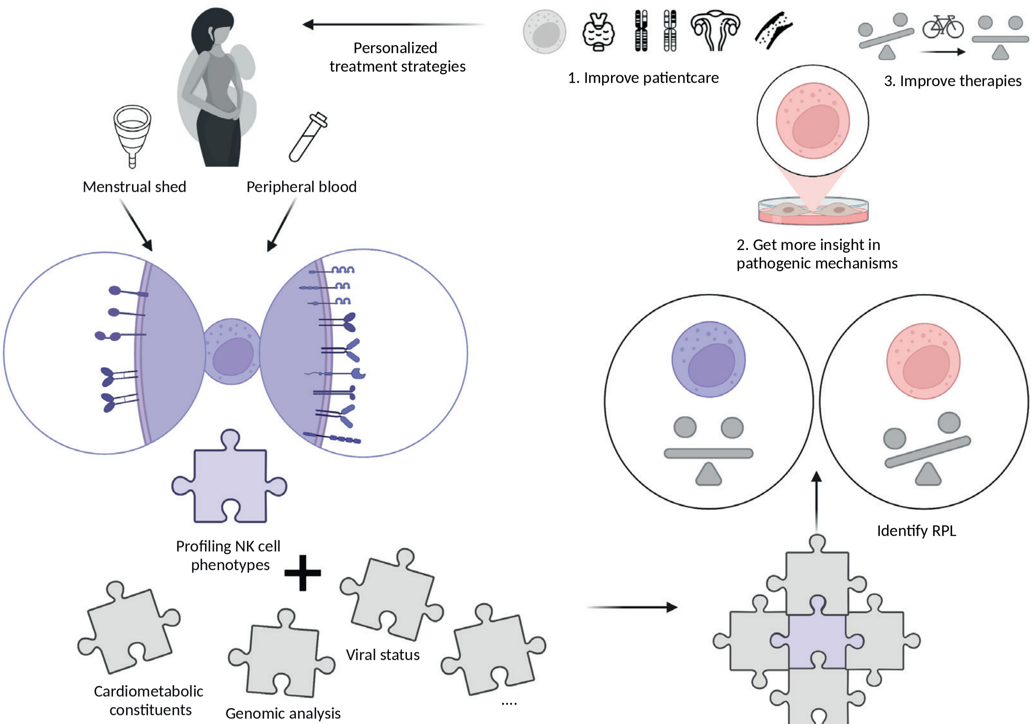


Figure 1 | Preconceptional screening of NK cell profiles in either peripheral blood or menstrual shed, combined with relevant genomic analysis (such as *FCGR3A*-p.176 polymorphism), viral status (such as CMV) and cardiometabolic status (such as presence of constituents of the circulatory risk profile or metabolic syndrome) could provide relevant information on the balance between the different NK cell effector functions relevant for reproductive success (e.g. production of proangiogenic and tissue remodeling factors, attraction of invading trophoblast cells vs controlling over-invasion, protection against pathogens vs contributing to a tolerogenic environment), which consequently could be a valuable tool for stratifying women with RPL hereby improving patient care (new diagnostic markers can be offered), providing new opportunities to study pathological mechanisms (markers can be used for functional in-vitro research) and to initiate new treatment strategies (markers can serve to study the impact of immune modulators), ultimately guiding personalized treatment strategies for future women with RPL.

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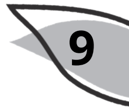

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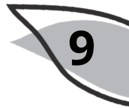

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10

IMPACT

IMPACT

The aim of this thesis was to integrally profile NK cells in women with recurrent pregnancy loss (RPL), an aim for which a unique collaboration was realized between the departments of Obstetrics & Gynecology and Transplantation Immunology in the Maastricht UMC+, for which a specialized outpatient clinic was set up for women with RPL and for which a standardized way of NK cell profiling was developed in the laboratory.

RPL is one of the most complex and challenging scenarios in reproductive medicine. Understanding the mechanisms behind RPL and identifying mediators or effectors and validating targets for prevention or therapy will have a profound impact on the couples' decision making on future family planning. However, identifying immunological factors by NK cell profiling in women with RPL is challenging to say the least. The immune system during early pregnancy is not black and white, it is not on or off, it is driven by a delicate balance of immune tolerance to allow implantation, placentation and growth of the semi-allogenic fetus but also depends on a responsive immune system that can protect both the mother and the fetus against pathogens when necessary.

Given that 50% of the women with RPL do not yet have an explanatory cause for their losses, some cases could perhaps be explained by an immunological etiology. It would be promising if we could uncover this immunological underlying cause, for example to treat these women with immunomodulatory medication. To date, there are no immunological diagnostic or intervention strategies available for women with RPL and current guidelines, such as the NVOG, the ASRM and the ESHRE, do not recommend testing for immune abnormalities in women with RPL due to low level quality of evidence. However, since there is a limited availability of predictors and limited possibilities for diagnosis and treatment for women with RPL, it is very important to investigate novel immunological factors that can offer more perspective for these women. By integrally profiling NK cells we aimed to acquire more insight in whether a poorly balanced regulation of the various effector functions of NK cells may play a role in RPL.

In this thesis we showed that NK cell profiling can be used as a tool to investigate NK cells in peripheral blood and menstrual shed and can be used to identify markers related to RPL that can help patient stratification and prediction of the severity of RPL. Moreover, we showed that genomic analysis, analysis of antibody profiles and viral status analysis, but also overarching analysis between different systems, such as the cardiovascular and metabolic system, could provide more insight in constituents that are important to take into account when profiling NK cells in women with RPL, hereby underlining the importance of complementing NK cell profiles with additional analyses. Although integral NK cell

profiling will not be all encompassing for every woman with RPL, a multidisciplinary approach seems to be the way forward and can certainly be of great value for identifying a subset of women with RPL with an underlying immunological etiology. In the future this could potentially lead to a preconceptional immunological and cardiovascular screening program for the outpatient clinic, unmasking predispositions to disturbances that only become symptomatic later in pregnancy. Such a preconceptional screening program might identify aberrant immunological factors before pregnancy, so that couples at high risk of having a pregnancy loss can make informed reproductive decisions.

However, for the use of integral NK cell profiles as a reliable and predictive biomarker in a preconceptional screening program, our results should first be confirmed in a standardized setting. Furthermore, functional studies are needed to investigate a potential causal relation with pregnancy outcome hereby providing targets for intervention and guiding personalized treatment strategies of a subgroup of women with RPL in the future. Moreover, the added value of NK cell profiling in predicting subsequent loss or healthy pregnancy has not yet been studied well enough. In addition to predictors such as maternal age and the number of previous losses, integral NK cell profiling could be beneficial for predicting pregnancy outcome and is highly needed as prediction is severely limited to date.

Couples who endure RPL are desperate to find an explanation for their repeated losses of pregnancies. The social need for finding diagnosis and treatment strategies for women with RPL is urgent as many general practitioners and also gynecologist often don't have a clue what to offer women with RPL besides care-as-usual. However, there is so much more that can be done, helping these women with a tremendous amount of grief and despair losing pregnancies over and over again. Just because we don't have a validated biomarker or readily available treatment options, doesn't mean we can't try to help these women in the best way possible. Offering appropriate care supported by scientific research can offer these patients a new perspective for the future as many women sadly experience RPL as a traumatic life event.

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Jaarlijks 23 miljoen miskramen, maar nauwelijks aandacht voor het verdriet

Gewoon uithuilen en opnieuw beginnen. Dat lijkt vaak de algemene teneur van de reacties bij een miskraam, valt op te maken uit een analyse in The Lancet. Miskramen worden gezien als onvermijdelijk, maar dat is onterecht en doet geen recht aan het verdriet van vrouwen die het overkomt.

Progress is potentially to be achieved when RPL will gain more attention from patient organizations and gynecologist (in training), who will have to first acknowledge the multifactorial nature of the problem and consequently be comprehensive in their approach looking for novel strategies improving diagnosis and treatment by means

of scientific research and shared discission making. It would therefore be relevant to organize a conference for FREYA, a very important Dutch association for people with fertility problems and professionals, where results of our research could be elucidated. Moreover, this thesis could also serve as a wonderful example for education purposes. The immune system during pregnancy is truly unique and new insights in its role should not only be published in articles but also be implemented in a curriculum for biomedical and medicine studies to make future biomedical scientist and physicians aware of the relevance and the importance of the immune system in reproduction. Losing pregnancies recurrently is not inevitable, the grief of these couples is enormous and should certainly not be underestimated.

Furthermore, these couples are extremely vulnerable, which makes it even more important to provide appropriate care. Private fertility clinics do offer immunology testing and treatment for NK cells, selling couples blood tests despite experts warning that there is no proof that the procedure improves the chances of having a baby. The so-called Chicago test costs €1,000 plus a consultation fee. The blood is sent to Chicago to be examined to see if the woman's immune system is attacking embryos and causing pregnancy losses. If elevated levels of the killer cells are found the woman can be prescribed drugs to suppress her immune system, which can cost thousands of euros per dose. However, there are no guidelines for what normal NK cell numbers or activity are and which imbalance of NK cells will lead to pregnancy loss. Although there is a series of scientific studies that find that NK cells are important for normal pregnancy, it's still not clear what the optimal level of NK cells is and more importantly, what is the best way is to correct any imbalance. Integral NK cell phenotypic profiling can hopefully provide more insight whether a mismatched regulation of the various effector functions of NK cells might play a role in some women with RPL, hereby saving them unnecessary costs and disappointment.

This thesis has provided pieces of a puzzle in order to guide preconceptional screening of NK cells by integrally phenotypic profiling as this might not only be valuable for stratifying women with RPL, but also provides a blueprint for future studies for the development of new clinical strategies and for selecting women whose pregnancy losses may be more likely to respond to specific interventions, subsequently targeting these interventions to pre-selected women when the puzzle is complete.

11

SUMMARY – SAMENVATTING

SUMMARY

Having a positive pregnancy test is for most couples an overwhelming feeling of joy and happiness, yet for some it causes fear and anxiousness when previous pregnancies are lost recurrently. Recurrent Pregnancy Loss, in short RPL, is a distressing pregnancy disorder experienced by 1-3% of couples trying to conceive. Despite extensive clinical investigations of underlying etiologies, still 50% of RPL cases remain unexplained. As we know that the maternal immune system is of great importance for acceptance of a semi-allogenic fetus, after all half of the fetal genes come from the father and the products of those genes could be seen as 'foreign' by the mother's immune system, RPL might be linked to problems with the way the maternal immune system adjusts to early pregnancy. Moreover, there seems to be an important role for a special kind of immune cell, the so-called Natural Killer or NK cell. A NK cell is a type of immune cell loaded with packages containing toxic substances that can kill target cells, for example tumor cells or cells infected with a virus. Surprisingly, during early pregnancy a NK cell in the **decidua** is not a good killer but rather a good nanny that helps to provide a beneficial environment for the developing fetus and placenta.

Decidua: a thick layer of modified mucous membrane which lines the uterus during pregnancy.

Unfortunately, we do not yet know the exact role of NK cells in RPL, but it may be the case that in women with RPL these NK cells do not properly perform their function as a nanny and this leads to the loss of an early pregnancy. NK cell function is regulated by various inhibitory and activating receptors. By means of these receptors a NK cell can distinguish different signals and the combination of these signals ultimately determines the function of the NK cell. If this balance of signals is disturbed, the NK cells can no longer do their job properly. Since we are not yet sure which receptors may play an important role in RPL, it is necessary to profile NK cells in women with RPL in more detail.

This thesis is dedicated to identifying NK cells in women with RPL by means of NK cell profiles, hereby determining what the NK cell looks like based on subgroups and receptors. Understanding the immunologic pathogenesis of RPL is very much needed for providing appropriate care and knowing what is the most beneficial way of profiling NK cells, could benefit diagnosis of these women.

We first give a general introduction in **chapter 1**, for providing more insight into the clinical problem of RPL. In addition, we describe the physiology of the immune system during pregnancy, in particular how the immune system should adapt to the different trimesters of pregnancy, and we explain the importance of NK cells in early pregnancy. NK cells have multiple functions during early pregnancy and should be both tolerant acting as a nanny but still able to respond to pathogens as killers. They also play an important role in facilitating changes to the blood vessels in the uterine wall so that nutrients and oxygen can be exchanged efficiently between the mother and the growing fetus. We end with a description of the possible pathology of NK cells in RPL.

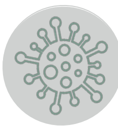
To get a better insight into what is known and also not known yet, we provide in **chapter 2** a concise overview of studies investigating NK cells profiles, measured in different tissues by different laboratory techniques in women with RPL versus controls. We found that especially NK receptors belonging to the inhibitory killer cell receptor family, and to some extent also the activating natural cytotoxicity receptor family, have been studied in the context of RPL. Studies investigating **immune checkpoint** receptors are almost completely lacking, while these receptors are known to play an important role in maintaining immunological tolerance in cancer.

Immune checkpoint: a regulator of the immune response; inhibitory immune checkpoint molecules contribute to immune tolerance and prevent the immune system from overreacting to the body's own or harmless cells, activating immune checkpoint molecules provide immune activation and are important for clearing diseased and foreign cells.

We first wondered whether timing of sampling and location of sampling are important for what the NK cell looks like, because hormones may have an influence on this and we know that NK cells in different tissues (e.g., the liver) have a different function and therefore they may also look different and be regulated differently. That is why, **chapter 3** examines the influence of the menstrual cycle on NK cell profiles in peripheral blood and investigates whether profiles are different when investigated in menstrual blood. We found that NK cell profiles based on receptor expression were comparable on the different days of the menstrual cycle, meaning that timing of sampling is not critical for analysis of individual NK receptors. Compared to NK cell profiles in peripheral blood, NK cell profiles in menstrual blood showed higher expression of some inhibitory receptors and some immune checkpoint receptors and additionally showed high levels of tissue-residency markers, hereby empowering future studies on the role of peripheral blood and menstrual blood NK cell profiles in reproductive success.

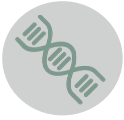
To compare NK cell profiles in women with RPL and women with a previous uncomplicated pregnancy and see if specific subgroups or receptors are associated with RPL, we set up a study of which **chapter 4** gives a description of the protocol. The so-called OVIDE study was conducted in Maastricht combining expertise from both the department of Gynecology, by setting up a specialized outpatient clinic for women with RPL, as well as the department of Transplantation Immunology, by setting up standardized operating procedures for isolation, measurement and analysis of NK cell profiles.

Results of the OVIDE study, investigating whether NK cell profiles in peripheral blood of women with RPL are different from women with a previous uncomplicated pregnancy and studying the association with the severity of RPL, are presented in **chapter 5**. We found that NK cell profiles from women with RPL showed a higher expression of the inhibitory LILRB1 receptor and the immune checkpoint receptor TACTILE and that higher expression of both was associated with the number of losses, meaning the higher the expression of these receptors the more losses the women endured, hereby identifying LILRB1 and TACTILE as NK cell receptors associated with RPL. In addition, we found a large variation in expression of the NKG2C receptor in women with RPL whose higher levels could be explained by going through an infection with the **cytomegalovirus**, hereby providing first support for the potential role of the cytomegalovirus in RPL via its impact on NK cells.



Cytomegalovirus: a common virus for people of all ages, however a healthy person's immune system usually keeps the virus from causing illness.

As it is known that for some receptors, their function is influenced by the presence of a **polymorphism** in the DNA, we studied the so-called p.V176F polymorphism and its relation to the activating CD16 receptor expression and Human Leukocyte Antigen or HLA antibody status in **chapter 6**. As this could have a possible influence on NK cell antibody-dependent cellular cytotoxicity, in short ADCC in women with RPL in this way allowing the NK cell to assume more of a killer function. First, we found similar frequencies of the polymorphism in women with RPL and in healthy controls. Second, we only found a higher intensity of the CD16 receptor but no differences in expression in the presence of the polymorphism. Third, no differences in frequencies of the polymorphism were detected when comparing women with or without antibodies against HLA, therefore we could not provide strong evidence for an association between the polymorphism and RPL.



Polymorphism: changes in the genetic code that cause multiple variants (alleles) of a gene to coexist in a population and create genetic differences between individuals.

In addition to studying a particular polymorphism that could be related to NK cell function, it could also be that other factors are valuable when profiling NK cells. As NK cell orchestrated **spiral artery** remodeling during early pregnancy might indirectly be influenced by maternal cardiovascular or metabolic status, we studied the additional influence of the metabolic and cardiovascular system in RPL in **chapter 7.** Although we found no major differences in preconceptional vascular or metabolic parameters between women with RPL and controls, we found in our study where we compared deviating parameters that more than 80% of women with RPL had at least one abnormal constituent of a circulatory risk profile and that 27% had at least one abnormal constituent of the metabolic syndrome. Meaning that the presence of abnormal circulatory factors prior to pregnancy, and to lesser extent of the metabolic syndrome, may predispose to RPL.

Spiral artery: small arteries whose role is to supply blood to the upper functional layer of the uterus, and play a vital role in supplying nutrients to the placenta and fetus during pregnancy.

As immune interventions which may also affect NK cells are already being offered to women with RPL even though we don't quite know how these exactly work yet, we studied the effectiveness of intravenous immune globulin, in short **IVIG** treatment on live birth rate in women with RPL and an underlying immune condition by reviewing available literature in **chapter 8**. Although we showed that treatment with IVIG was associated with a two-fold increase in live birth rate and that the effect of IVIG was particularly marked in the subgroup of studies including patients based on presence of elevated NK-cell percentage, results should be interpreted with caution. We concluded that future trials in women with RPL and underlying immune conditions are needed before using IVIG can be safely used in a clinical setting.

IVIG: a therapy made up of a large amount of antibodies (IgG) from healthy individuals which help fight infection and disease which is given intravenously and is for example used to treat various autoimmune diseases.

To address findings of this thesis within the context of recent literature and to provide suggestions for future research, we gave a general discussion in **chapter 9.** By profiling NK cells, we have described parameters associated with RPL. These parameters are very valuable for developing diagnostic markers to better identify women with RPL and an underlying immune etiology and for developing predictive markers, to see when early pregnancy is heading in the wrong direction or to see which women will respond best to a particular treatment. In addition, NK cell profiles can help us to better understand the pathology of RPL in order to develop new immune modulating strategies.

Finally, we elaborated on wider implications and the impact of this thesis in **chapter 10** and we conclude that it is important to keep looking for new causes of RPL, as 50% of all causes cannot be explained yet. This allows us to have a major influence through our immunological research on future family planning of couples dealing with RPL.

SAMENVATTING

Het hebben van een positieve zwangerschapstest is voor de meeste stellen een overweldigend gevoel van vreugde en geluk, maar voor sommigen veroorzaakt het angst en spanning wanneer eerdere zwangerschappen herhaaldelijk verloren zijn gegaan. Herhaalde miskramen, kortweg HM, is een verdrietige zwangerschapscomplicatie die wordt ervaren door 1-3% van de stellen die proberen zwanger te worden. Ondanks uitgebreid klinisch onderzoek naar onderliggende oorzaken, blijft nog steeds 50% onverklaard. Omdat we weten dat het immuunsysteem van de moeder van groot belang is voor de acceptatie van een semi-allogene foetus, immers de helft van de foetale genen komt van de vader en de producten van die genen zouden door het immuunsysteem van de moeder als ‘lichaamsvreemd’ gezien kunnen worden, kan HM in verband worden gebracht met problemen met de manier waarop het immuunsysteem van de moeder zich aanpast aan de vroege zwangerschap. Bovendien lijkt er een belangrijke rol te zijn weggelegd voor een speciaal soort afweercel; de zogenaamde Natural Killer of NK cel. Een NK cel is een type afweercel beladen met pakketjes met daarin toxische stofjes die andere cellen kunnen doden, bijvoorbeeld tumorcellen of cellen die zijn geïnfecteerd met een virus. Verrassend genoeg is een NK cel in de **decidua** tijdens de vroege zwangerschap geen goede moordenaar, maar eerder een goede oppas die helpt om een bevorderende omgeving te creëren voor de ontwikkelende foetus en placenta.

11

Decidua: een dikke laag hervormd slijmvlies dat de baarmoeder bekleedt tijdens de zwangerschap.

We weten jammer genoeg nog niet de exacte rol van NK cellen in HM, maar mogelijk kan het zo zijn dat in vrouwen met HM deze NK cellen niet goed hun functie als oppas uitvoeren en dit ertoe leidt dat een vroege zwangerschap verloren gaat. Het reguleren van NK cel functie gebeurt door verschillende remmende en activerende receptoren. Middels deze receptoren kan een NK cel verschillende signalen onderscheiden en de combinatie van deze signalen bepaald uiteindelijk wat de functie van de NK cel is. Mocht deze balans van signalen verstoord worden, dan kunnen de NK cellen hun werk niet meer goed doen. Gezien we nog niet zeker weten welke receptoren mogelijk een belangrijke rol spelen in HM, is het nodig om NK cellen in vrouwen met HM in meer detail in kaart te brengen.

Dit proefschrift is gewijd aan het karakteriseren van NK cellen in vrouwen met HM middels NK cel profielen, waarbij gekeken wordt hoe de NK cel eruit ziet op basis van subgroepen en receptoren. Het begrijpen van de immunologische pathogenese van HM is hard nodig om de juiste zorg te bieden en als we weten wat de meest gunstige manier is om NK cellen te karakteriseren, zou dit de diagnose van deze vrouwen ten goede kunnen komen.

In **hoofdstuk 1** geven we eerst een algemene introductie om meer inzicht te krijgen in de klinische problematiek van HM. Daarnaast beschrijven we de fysiologie van het immuunsysteem tijdens de zwangerschap, met name hoe het immuunsysteem zich dient aan te passen aan de verschillende trimesters van de zwangerschap en lichten we het belang van NK cellen in de vroege zwangerschap toe. NK cellen hebben meerdere functies tijdens de vroege zwangerschap en behoren zowel verdraagzaam te zijn als oppas, maar ook nog steeds kunnen te kunnen reageren op ziekteverwekkers als moordenaars. Tevens vervullen ze een belangrijke rol bij het faciliteren van veranderingen aan de bloedvaten in de baarmoederwand waardoor voedingstoffen en zuurstof efficiënt uitgewisseld kunnen worden tussen de moeder en de groeiende foetus. We eindigen met een omschrijving over de mogelijke pathologie van NK cellen in HM.

Om een beter inzicht te krijgen in wat er wel en ook nog niet bekend is, geven we in **hoofdstuk 2** een beknopt overzicht van studies naar NK cel profielen, gemeten in verschillende weefsels door verschillende laboratoriumtechnieken bij vrouwen met HM versus controles. We ontdekten dat vooral receptoren die behoren tot de familie van de remmende killer cel receptoren, en tot op zekere hoogte ook de familie van activerende natuurlijke cytotoxiciteit receptoren, zijn bestudeerd in de context van HM. Studies naar **immune checkpoint** receptoren ontbreken bijna volledig, terwijl bekend is dat deze een belangrijke rol spelen bij het handhaven van immunologische tolerantie in kanker.

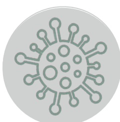
Immune checkpoint: een regulator van de immuunrespons; remmende immune checkpoint moleculen dragen bij aan immunologische tolerantie en voorkomen dat het immuunsysteem te sterk reageert op lichaamseigen of onschadelijke cellen, activerende immune checkpoint moleculen zorgen voor immuun activatie en zijn belangrijk voor het opruimen van zieke en ongewenste cellen.

Bij het bestuderen van NK profielen vroegen we ons eerst af of timing van afname en locatie van afname belangrijk zijn voor hoe de NK cel eruit ziet, omdat hormonen wellicht een invloed hierop kunnen hebben en we weten dat NK cellen in diverse weefsels (bijvoorbeeld de lever) een andere functie hebben en ze er daarom mogelijk ook anders uitzien en anders gereguleerd worden. Daarom wordt in **hoofdstuk 3** de invloed van de

menstruatiecyclus op NK cel profielen in perifeer bloed onderzocht en wordt onderzocht of profielen anders zijn wanneer onderzocht in menstrueel bloed. We ontdekten dat NK cel profielen op basis van receptor expressie vergelijkbaar waren op de verschillende dagen van de menstruatiecyclus, wat betekent dat timing van afname niet cruciaal is voor analyse van individuele NK cel receptoren. Vergeleken met NK cel profielen in perifeer bloed, vertoonden NK cel profielen in menstrueel bloed een hogere expressie van sommige remmende receptoren en sommige immune checkpoint receptoren en vertoonden deze bovendien hoge niveaus van weefselspecifieke markers, wat toekomst biedt voor toekomstige studies naar de rol van perifeer bloed en menstrueel bloed NK cel profielen in reproductief succes.

Om NK cel profielen te vergelijken in vrouwen met HM en vrouwen met een eerdere ongecompliceerde zwangerschap en te zien of specifieke subgroepen of receptoren geassocieerd zijn met HM, hebben we een studie opgezet waarvan **hoofdstuk 4** een beschrijving van het protocol geeft. Het zogenaamde OVIDE-onderzoek is uitgevoerd in Maastricht en combineert expertise van zowel de afdeling Gynaecologie, door het opzetten van een gespecialiseerde polikliniek voor vrouwen met HM, als de afdeling Transplantatie Immunologie, door het opzetten van gestandaardiseerde werkwijzen voor isolatie, meting en analyse van NK cel profielen.

Resultaten van de OVIDE studie, waarin wordt onderzocht of NK cel profielen in perifeer bloed van vrouwen met HM anders zijn dan die van vrouwen met een eerdere ongecompliceerde zwangerschap en waarin de associatie met de ernst van HM wordt bestudeerd, worden gepresenteerd in **hoofdstuk 5**. We vonden dat NK cel profielen van vrouwen met HM een hogere expressie van de remmende LILRB1 receptor en de immune checkpoint receptor TACTILE vertoonden en dat hogere expressie van beide markers geassocieerd was met het aantal verliezen, wat betekent dat hoe hoger de expressie van deze receptoren, hoe meer miskramen de vrouwen in onze studie leden, waardoor LILRB1 en TACTILE werden geïdentificeerd als NK cel receptoren geassocieerd met HM. Daarnaast vonden we een grote variatie in expressie van de NKG2C receptor in vrouwen met HM waarvan de hogere niveaus verklaard konden worden door het doormaken van een infectie met het **cytomegalovirus**, waarmee we de eerste ondersteuning bieden voor de mogelijke rol van het cytomegalovirus in HM via impact op NK cellen.



Cytomegalovirus: een veelvoorkomend virus in mensen van alle leeftijden; het immuunsysteem van een gezond persoon zorgt er echter meestal voor dat het virus geen ziekte veroorzaakt.

Omdat het bekend is dat voor sommige receptoren diens functie wordt beïnvloed door de aanwezigheid van een **polymorfisme** in het DNA, hebben we in **hoofdstuk 6** het zogenaamde p.V176F-polymorfisme en de relatie met de activerende CD16 receptor expressie en humaan leukocytenantigeen of HLA antilichaam status bestudeerd. Mogelijk zou dit p.V176F-polymorfisme namelijk een invloed kunnen hebben op NK cel antilichaam-afhankelijke-cellulaire-cytotoxiciteit, kortweg ADCC bij vrouwen met HM waardoor deze NK cel meer de functie van moordenaar zou kunnen aannemen. Ten eerste vonden we vergelijkbare frequenties van het polymorfisme bij vrouwen met HM en bij gezonde controles. Ten tweede vonden we alleen een hogere intensiteit van de CD16 receptor, maar geen verschillen in expressie, in de aanwezigheid van het polymorfisme. Ten derde werden er geen verschillen in frequenties van het polymorfisme gevonden bij het vergelijken van vrouwen met of zonder antilichamen tegen HLA, waardoor we uiteindelijk geen sterk bewijs hebben kunnen leveren voor een verband tussen het polymorfisme en HM.

Polymorfisme: veranderingen in de genetische code waardoor meerdere varianten (allelen) van een gen naast elkaar voorkomen in een populatie en genetische verschillen tussen individuen ontstaan.

Naast het bestuderen van een polymorfisme dat verband zou kunnen houden met de functie van NK cellen, kan het ook zijn dat andere factoren waardevol zijn voor het karakteriseren van NK cellen. Omdat remodellering van **spiraal arteriën** gereguleerd wordt door NK cellen tijdens de vroege zwangerschap, kan maternale cardiovasculaire of metabole status mogelijk ook een vroege zwangerschap beïnvloeden en daarom hebben we de invloed van het metabole en cardiovasculaire systeem in HM in **hoofdstuk 7** bestudeerd. Hoewel we geen grote verschillen vonden in preconceptionele vasculaire of metabole parameters tussen vrouwen met HM en controles, vonden we in onze studie waarbij we afwijkende parameters vergeleken dat meer dan 80% van de vrouwen met HM ten minste één abnormale parameter van een circulatoir risicoprofiel had en dat 27% ten minste één abnormale parameter had van het metabool syndroom. Dit betekent dat de aanwezigheid van abnormale circulatoire factoren voorafgaand aan de zwangerschap, en in mindere mate van het metabool syndroom, van invloed kunnen zijn voor HM.

Spiraal arterie: kleine slagaders die de bovenste functionele laag van de baarmoeder van bloed voorzien, en die een vitale rol spelen bij het leveren van voedingsstoffen aan de placenta en de foetus tijdens de zwangerschap.

Omdat immuun interventies die mogelijk ook NK cellen beïnvloeden reeds worden aangeboden aan vrouwen met HM ondanks dat we nog niet helemaal weten hoe deze werken, hebben we de effectiviteit van intraveneuze immunoglobuline, kortweg IVIG behandeling op levendgeborenen bij vrouwen met HM en een onderliggende immuun aandoening bestudeerd in **hoofdstuk 8** door beschikbare literatuur te bestuderen. Hoewel we aantoonden dat behandeling met **IVIG** geassocieerd was met een tweevoudige toename van het aantal levendgeborenen en dat het effect van IVIG bijzonder uitgesproken was in de subgroep van onderzoeken met patiënten op basis van de aanwezigheid van een verhoogd NK cel percentage, moeten de resultaten met voorzichtigheid worden geïnterpreteerd. We concludeerden dat toekomstige studies bij vrouwen met HM en onderliggende immuun aandoeningen nodig zijn voordat IVIG veilig kan worden gebruikt in een klinische setting.

IVIG: een therapie die bestaat uit een grote hoeveelheid antilichamen (IgG) van gezonde personen die infecties en ziekten helpen bestrijden, die intraveneus wordt toegediend en wordt gebruikt om bijvoorbeeld verschillende auto-immuunziekten te behandelen.

Om de bevindingen van dit proefschrift binnen de context van recente literatuur te bekijken en suggesties te doen voor toekomstig onderzoek, hebben we een algemene discussie in **hoofdstuk 9** gegeven. Door middel van het karakteriseren van NK cellen middels NK cel profielen, hebben we parameters beschreven die geassocieerd zijn met HM. Deze parameters zijn zeer waardevol voor het ontwikkelen van diagnostische markers om vrouwen met HM en een onderliggende immuun etiologie beter te karakteriseren en voor het ontwikkelen van voorspellende markers bijvoorbeeld om te zien wanneer een vroege zwangerschap de verkeerde kant op dreigt gaat of om te zien welke vrouwen het beste op een bepaalde behandeling zullen reageren. Daarnaast kunnen NK cel profielen ons helpen om de pathologie van HM beter te begrijpen om zo nieuwe immuun modulerende strategieën te ontwikkelen.

Tenslotte zijn we dieper ingegaan op bredere implicaties en de impact van dit proefschrift in **hoofdstuk 10** en concluderen we dat het belangrijk is te blijven zoeken naar nieuwe oorzaken in HM, omdat 50% van alle oorzaken nog niet verklaard kan worden. Hierdoor kunnen we middels ons immunologisch onderzoek een grote invloed hebben op toekomstige gezinsplanning van koppels die te maken hebben met HM.

12

DANKWOORD

DANKWOORD

Met een goed glas wijn schrijf ik dan mijn laatste hoofdstuk en dat voelt best gek. Dat er nu een einde is gekomen aan een gigantisch leuke tijd, aan mijn reis als promovenda, aan de meest bijzondere jaren van mijn leven. Niet alleen door mijn waanzinnig leuke project, maar zeker ook door de fantastische mensen die ik gedurende deze jaren heb leren kennen. Mensen die mij ieder op hun eigen manier hebben geholpen om dit mooie proefschrift uiteindelijk vorm te kunnen geven en daar wijd ik dan ook met heel veel liefde en trots mijn laatste hoofdstuk aan.

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Professor Spaanderman, beste **Marc**. Als ik een ding zeker weet, dan is het dat ik enorm geboft heb met een promotor als jij. Als ik je in een woord zou moeten omschrijven dan hoef ik niet lang na te denken: uitzonderlijk! Op de best mogelijke manier. Je was er voor mij tijdens alle moeilijke momentjes, maar je was er ook om alle succesjes te vieren. Duimpjes omhoog na het submitten van een artikel of het behalen van een finishlijn. En wat jij weet als geen ander, is dat een promotietraject niet alleen een traject is om wetenschappelijk te groeien. Persoonlijk ben ik ook enorm gegroeid van gymnastiek docent naar doctor en dat heb ik samen met jou gedaan. Je maakte altijd tijd voor me in je gigantisch drukke agenda en leerde mij als geen ander om op mezelf te vertrouwen. Dat ik in mijn eigen ontwikkeling en kracht mag geloven en er trots op mag zijn. En één ding in het bijzonder zal ik nooit meer vergeten; je aanbevelingsbrief! Ik heb een traantje gelaten van geluk, geluk dat ik zo een mooi mens als jou heb mogen leren kennen. Lieve Marc, enorm bedankt voor alles!

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A

ABOUT DENISE

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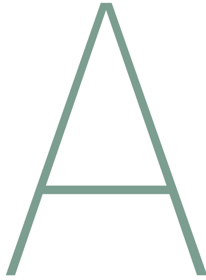
Denise Habets was born on May 3th 1987 in Valkenburg, the Netherlands. She attended secondary school at the Stella Maris College in Meerssen and combined this with top level sport. After graduating from secondary school with a gymnasium diploma she studied at the Fontys Sports Academy in Tilburg and obtained her first-degree teaching qualification in physical education in 2009. After working for two years as a secondary school PE teacher and mentor of gifted students, she took on a new challenge in Maastricht to study BioMedical Sciences (specialization Molecular Life Sciences) after which she followed a selective research master in Cognitive and Clinical Neuroscience (specialization Fundamental Neuroscience) chaperoned by Jos Prickaerts.

After completion of her senior internship at the department of Pediatrics supervised by Tim Wolfs, she applied for a full-time position as promovenda on a novel project in the field of Reproductive Immunology under the supervision of Marc Spaanderman, Lotte Wieten and Salwan Al-Nasiry. The results of her research are published in this thesis.

During her PhD Denise combined her work in the clinic and the laboratory with sports, finishing the New York marathon in 2017 and the Ironman 70.3 Hawaii in 2019. In addition, she obtained her university teaching qualification (BKO) and is in preparation for the SMBWO recognition as an immunologist. She currently works as a postdoc in the group of Lotte Wieten at the department of Transplantation Immunology in the Maastricht UMC+.



PUBLICATIONS - BURSARY

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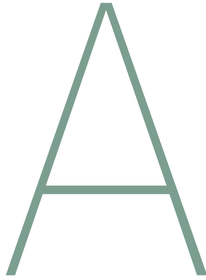
BURSARY

Personal bursary for the 35st European Immunogenetics and Histocompatibility Conference in Amsterdam, the Netherlands 2022.



STELLINGEN

Stellingen behorende bij het proefschrift

NATURAL KILLER CELL PROFILING IN WOMEN WITH RECURRENT PREGNANCY LOSS

1. Het karakteriseren van natural killer cellen vraagt een allesomvattende benadering vanwege hun diverse en soms zelfs tegengestelde functies tijdens de zwangerschap (dit proefschrift).
2. Menstrueel bloed kan gebruikt worden om immunologische veranderingen van het baarmoederslijmvlies non-invasief te onderzoeken in vrouwen met herhaalde miskramen (dit proefschrift).
3. Het identificeren van een subgroep vrouwen met een immunologisch probleem kan een eerste stap zijn naar gepersonaliseerde zorg voor vrouwen met herhaalde miskramen (dit proefschrift).
4. Het beschikbaar zijn van een medische interventie rechtvaardigt niet het gebruik ervan (dit proefschrift).
5. Gegeven de psychologische impact verdienen vrouwen met herhaalde miskramen een zorgvuldige preconceptionele counseling.
6. Het op een begrijpelijke manier verstrekken van informatie over het fascinerende immuunsysteem dat ons beschermt, verbetert de zelfregie van de patiënt.
7. De kennis en kunde van een medisch immunoloog biedt clinici onmisbare ondersteuning bij diagnostiek en behandeling van een patiënt met een immunologisch ziektebeeld.
8. Een menstruatie cup is een innovatief, veilig en milieuvriendelijk alternatief voor tampons en maandverband en zou daarom gratis verkrijgbaar moeten zijn voor iedere vrouw in Nederland.
9. Wie te vroeg juicht, heeft in ieder geval plezier gehad. (Eva Mulder)
10. Moeite is moeiteloos voor wie echt wil. (Roald van der Vliet)