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Review

Implications of uterine NK cells and regulatory T cells in the endometrium of infertile women

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ABSTRACT

A range of studies have shown that the complex process of implantation and an establishment of a pregnancy also involves immune factors. Disturbances in these underlying immune mechanisms might lead to implantation and pregnancy failure and may be involved in the pathogenesis of unexplained infertility. Several studies have reported that imbalances in uterine NK (uNK) cell abundance are associated with infertility; however, controversies exist. An increased amount of $CD56^+$ uNK cells along with a decrease in $CD16^+$ uNK cells have been associated with normal fertility in some studies. Very few studies of $FoxP3^+$ regulatory T cells (Tregs) in the pre-implantation endometrium have been performed. Results are sparse and controversial, studies reporting both increased and decreased numbers of Tregs, respectively, in women suffering from infertility. In conclusion, studies imply that uNK cells, Tregs and HLA-G carry pivotal roles regarding the establishment of a healthy pregnancy, and that abnormal immune mechanisms involving these parameters may be associated with infertility. However, more research in early phases of the reproductive cycle, such as investigating the conditions in the endometrium before implantation, is needed to further clarify the underlying mechanisms.

1. Introduction

The capability for humans to reproduce is limited, not only by age. Some couples face difficulties on their way of obtaining a pregnancy that others do not. Infertility is defined as the inability of a couple to obtain a pregnancy leading to a birth of a live newborn, after one year of unprotected intercourse with regular frequency. Of couples in the reproductive age, 10–15% suffer from infertility [1,2]. Causes can be parental genetic abnormalities, maternal endocrine or metabolic disorders, genital tract infections, abnormal fallopian tubes, ovulation defect or reduced semen count. However, in 8–28% of the cases the cause remains unknown, which hampers the choice of the right assisted reproductive technology (ART) [3]. For patients as well as clinicians, the fact that not being able to identify the underlying reason is frustrating in several aspects: dealing with a condition without knowing the cause, and not at least, an unknown prognosis of ART.

In an immunological context, mammalian pregnancy is a unique phenomenon in which the mother carries a semi-allogenic embryo. To accept the paternal alloantigens during pregnancy, the maternal immune cells must acquire a transient state of tolerance, and unique immune mechanisms are required for obtaining a successful pregnancy. Therefore, altered immunological responses and modulation, and, in line with this, compromised endometrial receptivity, have been thought to play a role in reproductive problems such as infertility [4–6]. Research performed so far suggests an imbalance in endometrial leukocyte population numbers and activity among women suffering from

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; ART, assisted reproductive technology; dNK cells, decidual natural killer cells; CTLA-4, cytotoxic T-lymphocyte antigen-4; EVT, extra-villous trophoblast; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage-colony-stimulating factor; HLA, human leukocyte antigen; HO-1, haem oxygenase isoform 1; IL, interleukin; ILT, immunoglobulin-like transcript; INF-γ, interferon gamma; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; IUGR, intrauterine growth retardation; IVF, in vitro fertilization; KIR, killer cell immunoglobulin-like receptor; LH, luteinizing hormone; LIF, leukaemia inhibitory factor; M-CSF, macrophage-colony-stimulating factor; NCR, natural cytotoxicity receptors; NK cells, natural killer cells; RM, recurrent miscarriage; sHLA-G, soluble human leukocyte antigen-G; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; Tregs, regulatory T cells; uNK cells, uterine natural killer cells; VEGF, vascular endothelial growth factor

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infertility [7]. Much research has been performed to understand the mechanisms at the feto-maternal interface during implantation and during the first trimester. In contrast, the current knowledge of the conditions and immune mechanisms in the endometrium before implantation is not only sparse, but also controversial.

The aim of this review is to evaluate studies reported so far regarding the immune cell subpopulations of natural killer (NK) cells and of regulatory T cells (Tregs), and their possible interplay, in relation to infertility. Our main focus is conditions and mechanisms in the peri-implantation endometrium. Based on existing research already published, hypotheses are: 1) women suffering from infertility have lower amounts of endometrial NK cells, characterized by CD56 and CD16, compared with fertile controls; 2) the number of endometrial Tregs, characterized by FoxP3, is reduced in women with infertility compared with fertile controls.

2. Methods

This review provides an overview of current knowledge on the expression of immune cells as above mentioned. The relevant literature was obtained using the Pubmed database. Both regular advanced free text search and the MeSH search function were used. MeSH terms as 'pregnancy', 'leukocytes/immunology', infertility/pathology, 'pregnancy complications', 'endometrium/pathology', 'biopsy', 'natural killer cells', 'antigens CD56/analysis', 'antigens CD16/analysis', 'regulatory T cells', and 'FoxP3' were used in different combinations. Regarding the search for original studies, main focus was on quantitative measurements of immune cells present in the endometrium. The search was limited to studies investigating the association between expression of CD56, CD16, and FoxP3 in endometrial tissue around the time of implantation and in cases of infertility. According to the method of measuring, CD56 expression may both be referred to as $CD56^{+}$ or $CD56^{-}$, as well as $CD56^{bright}$ or $CD56^{dim}$. As a general rule, $CD56^{bright}$ may be considered as $CD56^{+}$ [8]. Many of the identified studies measured other markers than the above-mentioned but these results were not included. The methods of measuring the expression of especially CD16 and CD56 included immunohistochemistry and flow cytometry. Regarding terminology, studies using both 'endometrial NK cells' and 'uterine NK cells' were included. The identified relevant publications were all written in English. Additionally, reference lists of the identified studies were examined for possible relevant studies not included in the initial search.

The majority of research published so far has measurements of CD16 and CD56 of interest. Only a few original articles focusing on FoxP3 in pre-implantation endometrium were identified, and the methods used were diverse. One study using immunohistochemistry focused on infertility caused by endometriosis, an exception compared to the other studies.

3. The pregnant woman's immune response to the semi-allogenic fetus

To avoid an aggressive response from the maternal immune system towards the fetus, the maternal immune cells in close contact with fetal tissue may possess functions different from that of peripheral immune cells. A criterion for successful implantation and placentation is trophoblast invasion of the uterine lining. This invasion process has to be well regulated to avoid pregnancy complications and undesirable pregnancy outcomes. Research supports that maternal leukocytes and lymphocytes including T cells, B cells, NK cells, monocytes, and macrophages along with cytokine signalling and growth factor levels play important roles in this process [5,9].

Around the time of implantation, the endometrium is populated by an abundance of maternal leukocytes. The maternal leukocytes may participate in a mechanism for maintenance of pregnancy by protecting the fetus from maternal immunologic rejection. Furthermore, some of the leukocytes are suggested to be directly involved in maternal recognition of the placenta through leukocyte expression of receptors for human leukocyte antigen (HLA)-G and HLA-C antigens, that are present on fetal trophoblast cells [5,10].

Among the proposed mechanisms involved in feto-maternal tolerance are depletion of alloreactive leukocytes and lymphocytes, changes in cell abundances, high levels of leukaemia inhibitory factor, transforming growth factor-β (TGF-β), indoleamine 2,3-dioxygenase, haem oxygenase isoform 1 (HO-1), up-regulation of HLA-G on trophoblast cells, increased apoptosis of activated maternal lymphocytes and reduced complement activity [2,6,11–16].

3.1. Th1/Th2 balance

In order to identify the complex network behind the feto-protective mechanisms, several studies have also focused on cytokine secretion and the Th1/Th2 balance in maintenance of pregnancy. Helper T cells are classified as Th1 or Th2 according to their cytokine secretion profile. Th1 cells produce especially IL-2, INF-γ, and TNF-α. In contrast, Th2 cells produce IL-4, IL-5, and IL-10. Successful pregnancy appears to be characterized by a Th2-dominated response [17,18]. With the onset of pregnancy, the local endometrial immune system seems to switch to an immunity dominated by a Th2 environment. This switch is believed to reduce uNK cell cytotoxicity, but also uNK cells have been reported to facilitate the Th2 immune environment [19]. This might explain one of the mechanisms behind certain reproductive pathologies such as unexplained infertility. Therefore, reasonably, it can be speculated that imbalances in numbers and activity of maternal leukocytes disturb the Th1/Th2 balance by an abnormal cytokine secretion, and promoting a Th1 response instead. A proinflammatory Th1 cytokine response has been associated with lysis of trophoblast cells, and a Th1 shift has been observed in women with unexplained infertility compared to fertile women [17,20,21]. Contrary, an association between excessive Th2 immunity and infertility has been reported [22,23]. However, the concept of Th1/Th2 balance is probably too simplistic, because the two subpopulations have been shown to secrete some common cytokines, and other distinct subpopulations of T helper cells have been defined [2]. Therefore, to explain how tolerance by the maternal immune system is regulated, the Th1/Th2 balance equilibrium is not sufficient as one single explanation and functions as part of a more complex interaction and cooperating immunological network.

4. Common mechanisms for infertility and recurrent miscarriages in reproductive immunopathology

Human endometrial stroma contains endometrial leukocytes as earlier described, and these cells have been associated with reproductive immunopathology such as infertility and recurrent miscarriages (RM) [9]. The majority of current research has had recurrent miscarriage as the focus of interest. However, some studies investigated women with a history of infertility, where some have not necessarily experienced recurrent miscarriage. It has been suggested, that the implantation-related immune abnormality observed for RM patients also exists in infertile patients, and that at least some of these reproductive pathologies may be due to a common underlying mechanism as those in recurrent miscarriages [24–26].

5. $CD56^{+}CD16^{-}$ uterine NK cells – phenotype and function

Natural killer cells are generally known as a minor cell fraction in circulating blood and peripheral organs functioning in innate immune responses. The majority of human NK cells are characterized as $CD56^{dim}$ and are effective killers and mediators of cytotoxicity [4]. However, NK cells play important roles in the female reproductive system too. They infiltrate at different anatomic sites such as the uterine mucosa and the decidual tissue, beyond and during pregnancy. During

pregnancy, especially in the first trimester, decidual NK cells (dNK cells) constitute the cellular majority, > 70%, among leukocytes in the placenta [27]. The uNK cell population has shown to persist throughout normal pregnancies until term, which emphasizes the importance not only initially, but also for maintaining pregnancy [28].

Uterine NK cells are adjacent to trophoblast cells, and thereby considered as one of the important players behind maternal acceptance of the fetus. Decidual NK cells have generally reduced cytotoxicity, promote vascular formation of spiral arteries and allow trophoblast invasion through various cytokine secretion, including macrophage colony-stimulating factor (M-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) [29]. Additionally, the secretion of growth-promoting factors from NK cells has shown to be essential for fetal growth [30].

5.1. $CD56^{+}CD16^{-}$ uterine NK cells

Natural killer cells are to be found in the non-pregnant endometrial tissue as uNK cells. Here, they constitute over 30% of the total leukocyte population, in contrast to the fraction in peripheral blood, which is only 5–15%; and during pregnancy decidual NK cells comprise 70–80% of the decidual lymphocytes [27,31]. The majority of NK cells in peripheral blood are $CD56^{+}CD16^{+}$. Increased density of the surface antigen CD56 and decreased CD16 expression make $CD56^{+}CD16^{-}$ uterine and decidual NK cells phenotypically distinct from that of peripheral NK cells [32,33]. It is interesting that the uNK cells downregulate CD16 expression; CD16 has shown to trigger NK cell antibody-dependent cell-mediated cytotoxicity (ADCC). A study on peripheral blood NK cells has demonstrated higher cytolytic activity towards NK cell targets among $CD16^{+}$ NK cells than $CD16^{-}$ NK cells [34]. An increased amount of $CD16^{+}$ uNK cells in infertile women is also associated with deficiency of vascular endothelial growth factor (VEGF), normally necessary for vascular formation in the placenta [35]. Consequently, the “normal” lack of CD16 surface expression on uNK cells may be considered as a pro-fertile mediator, and make uNK cells more regulatory than cytotoxic. If the phenotype and function of uterine and endometrial NK cells is to be evaluated, it should primarily be made using sample material from the uterus and not by a peripheral blood sample.

5.2. Functions

The functions of uNK cells are thought to be dependent on unique cell activation, distinguishing them from other NK cells [36]. Several studies have identified interleukin 15 (IL-15) as an important inducer of uNK cell proliferation and function [37]. By specific unfavourable cytokine stimulation, these $CD56^{+}CD16^{-}$ cells have also been found to enhance the release of INF-γ promoting a Th1 response, which might result in an attack on trophoblast cells and maybe pregnancy loss [38]. Therefore, the specific inducer of activation and the activation process per se might be crucial for the subsequent effector functions.

The subset of uNK cells is capable of secreting a wide array of chemokines and cytokines such as leukaemia inhibitory factor (LIF) and angiogenic growth factor not normally produced by peripheral NK cells. As mentioned earlier, this might be important for decidualization and formation of spiral arteries, which is important in early pregnancy establishment [30]. It has been shown that women with unexplained infertility has a reduced expression of LIF [39].

Additionally, uNK cells may be important in controlling the trophoblast invasion [40]. The cytotoxicity of uNK cells is already downregulated before implantation, and therefore uNK cells may be considered a beneficial version of NK cells in relation to fertility and pregnancy [41].

Uterine NK cells have a unique receptor repertoire, expressing killer-cell immunoglobulin-like receptors (KIRs) and immunoglobulin-like transcript-2 (ILT2), and lack NKp30, one of the natural cytotoxicity receptors (NCRs) known to be expressed in peripheral blood NK cells [27,42]. Some of the KIRs are receptors for HLA-G, and especially HLA-C; both are surface antigens on trophoblast cells as outlined above. A decline in the frequency of KIRs specific for HLA-C expressed by uNK cells has been observed among women suffering from recurrent miscarriage [43]. The same picture might emerge for infertile women. However, it illustrates the potential importance of uNK cells in regulation of trophoblast invasion.

The presence of uNK cells seem to coincide with the different phases of the ovarian hormone cycle [44]. One study demonstrated $CD56^{+}$ uNK cell proliferation from day 22 (secretory phase) of a standardized 28-day cycle, with a pre-menstrual maximum, and shedding with the onset of menstruation [31,45]. Whether this mid-secretory rise of uNK cells origins from a recruitment of peripheral NK cells or represents *in utero* proliferation has not been clarified [42]. Some evidence has shown a lack of expression of chemokine receptors such as CXCR3 and CXCR4 indicating that uNK cells do not migrate to the endometrium from other tissues or from the blood, but rather originate from local hematopoietic progenitor cells [33,46]. Other studies have suggested progesterone and estradiol levels to mediate the fluctuations in uNK cell numbers during the cycle [47,48].

If pregnancy occurs, the number of uNK cells increases further, especially in the decidua during the first trimester. Thereafter, the number gradually declines. The abundance at the time of implantation and in early pregnancy suggests that the subset may be important for a successful establishment of pregnancy. The role of uNK cells during pregnancy is still unknown. However, they may carry a pivotal role in angiogenesis as in early pregnancy maintaining the expanding placenta.

The role of uNK cells in the field of infertility is not yet fully understood, but a dysregulation in the pre-implantation endometrium has been associated with infertility (Table 1).

5.3. Uterine NK cell counts in relation to infertility

The studies listed in Table 1 show controversial results. In a recent study, we observed a decrease in the number of $CD56^{+}$ uNK cells in infertile women compared to normal fertile women [49], equal to the observations reported by Klentzeris et al. Conversely, two studies did not find any differences in CD56 [24,25]. As we did not co-stain in our study, the evaluation of CD56 and CD16 expression together was limited. Using flow cytometry, Fukui et al. found a decreased amount of $CD56^{bright}CD16^{-}$ uNK cells in infertile women, who had a spontaneous miscarriage in the following IVF cycle, in some accordance with our result. Additionally, Giuliani et al. observed a higher percentage of $CD16^{+}$ uNK cells in the infertility group compared to fertile women. This support the tendency, we have reported [49].

Given the fact that the uNK cell number rapidly increases in mid-secretory phase from day 22 of a standardised 28-day cycle, the biopsy collection day may be a confounding variable [45]. The confounding effect may be more apparent in studies with biopsies taken during an interval instead of at one well-defined day in the cycle.

Furthermore, it has been suggested that the significantly lower number of $CD56^{+}$ uNK cells among controls, who have previously given birth, could be an effect of this previous birth, as pregnancy and birth involves extensive changes in size and vascularization of the uterus. In addition, some investigators describe an altered composition of endometrial leukocyte subsets in women with reproductive problems, representing a relative difference instead of absolute. It looks like these women tend to express a higher density of CD16 and lesser CD56, the $CD56^{dim}CD16^{+}$ phenotype, compared to controls [26].

Regarding the prognostic value of measuring uNK cells, interpreting the results of Fukui et al., it looks like high density of $CD56^{dim}CD16^{+}$ is unfavourable, when it comes to maintenance of pregnancy (abortion versus delivered group) among infertile women (Fukui et al., 1999). Further support for this is the finding in our own study observing a high percentage of $CD56^{+}$ uNK cells being correlated with a high chance of

Table 1
Studies investigating expression of uNK cells in endometrial tissue related to infertility.

Study	N	Method of analysis	Sample day (in menstrual cycle)	Variables	Purpose of study	Results
Klentzeris et al. [99]	24 infertile 24 control women	Immunohistochemistry and morphometric	LH surge + 4, +7, +10 and +13 days	CD56	Association between uNK and infertility	Infertile women had significantly decreased numbers of $CD56^+$ cells compared to fertile controls
Fukui et al. [26]	76 infertile women of whom 25 obtained a pregnancy (18 women delivered and 7 aborted)	Flow cytrometry	5–10 days after the initial rise of basal body temperature and confirmed by histologic examination	CD56, CD16	Association between uNK and infertility, and subsequent IVF outcome	The abortion group had significantly decreased percentage of the $CD56^{bright}CD16^-$ NK cell subset and an increased percentage of the $CD56^{dim}CD16^+$ NK cell subset compared to those who delivered The percentage of $CD56^{dim}CD16^+$ was significantly higher in the group who implanted successfully, compared to those women who failed to implant[a]
Matteo et al. [25]	10 infertile women 25 control women from the study by Flynn et al. [100]	Flow cytrometry	Day 22–26 assessed by hormone levels	CD56, CD16	Association between uNK and infertility	No differences were found between infertile women compared to fertile controls
Giuliani et al. [24]	30 infertile 10 control women	Immunohistochemistry	LH surge + 7–10 days	CD56, CD16	Association between uNK and infertility	The percentage of $CD16^+$ cells was significantly higher in infertile women compared to fertile controls. No significant differences were found in the expression of CD56
Kofod et al. [49]	41 infertile women 20 control women	Immunohistochemistry	LH surge + 7 days	CD56, CD16	Association between uNK and infertility and subsequent IVF outcome	Infertile women had significantly decreased numbers of $CD56^+$ cells compared to fertile controls Increased number and percentage of $CD56^+$ uNK cells served as predictors for achieving pregnancy. Trend towards decreased numbers of $CD16^+$ cells in women who became pregnant compared to those, who did not

[a] The group who implanted also included the abortion group and this may explain the unexpected result.

achieving pregnancy [49]. Two other studies have failed to predict pregnancy outcome based on CD56 and CD16 expression, a conclusion supported by a meta-analysis by Tang et al. [42].

Due to Table 1, both the absolute count and the percentage of uNK cell markers seem to be important for normal fertility and obtaining pregnancy. In normal endometrium, both the absolute count and the percentage of $CD56^+$ uNK cells have shown to rise dramatically from day 22 in cycle [45]. Whether the absolute count or the percentage is most important in maintaining – and thereby in diagnostic screening of – normal fertility, is yet unknown.

Most of the studies in Table 1 indicate that the status of uNK cells may influence the ability to reproduce. However, to determine whether the abnormality of leukocyte subpopulations is the cause or the result of abnormal pregnancies can be very difficult. Especially because an imbalance in leukocyte expression also might be due to a post-miscarriage inflammatory response, challenging studies that investigate women with infertility.

In mice, restraint stress has shown to decrease the density of uNK cells up to almost 50% and alter the composition of T lymphocytes [50]. In humans, peripheral NK cell numbers and activity has shown to fluctuate under influence of several factors, such as progesterone and estradiol levels, exercise, time of day, and sympathetic response to stressors [47,51]. These are all potential confounders, important to be aware of.

The studies use different methods of analysis; some employing flow cytometry, others immunohistochemistry. Analyses based on flow cytometry may not be an accurate estimation of the factual *in vivo* counts because of the procedure of tissue digestion in the preparation process. On the other hand, assessing staining results of immunohistochemistry might be biased by intra- and interobserver variability. The different methods of analysis and the variation in study populations influence the comparability. Also, a lack of consensus concerning counting of immune cells in endometrial biopsies exists, and relevant reference ranges for the respective immune cell counts need to be established. A recent study has established such a protocol for immunohistochemical analysis, especially highlighting the importance of equal timing of the endometrial biopsy and suggesting how to select the most appropriate fields for counting [31,45]. The protocol recommends to count only cells with an identified nuclei, to exclude perivascular and periglandular aggregates and to count five fields with $\times 40$ objective. For future research, this approach may be very useful.

Characteristic for the studies discussed above is their relatively small sample size. By increasing the study population, the probability of detecting a possible difference might increase as well. Despite the lack of consensus in current clinical evidence, there is a biological plausibility for a role for uNK cells in infertility. Uterine NK cells are most numerous in the implantation window and in early pregnancy, and not at least, they are adjacent to and interact with extravillous trophoblast cells making them an important player in feto-maternal tolerance.

6. $FoxP3^+$ regulatory T cells

The T cell population in the endometrium consists of different subsets, including regulatory $CD4^+CD25^+$ T cells (Tregs) [52]. The Treg subset develops in the thymus and peripheral tissue, and functions by e.g. secreting immunosuppressive cytokines to prevent the immune system from reacting against auto-antigens. Besides avoiding destructive autoimmunity, Tregs are also involved in graft tolerance [53].

6.1. *Generation of $FoxP3^+$ regulatory T cells*

In order to differentiate from naive $CD4^+$ T cell precursors, TGF-β is the key cytokine driving this process. In the presence of TGF-β, T cells differentiate into a regulatory T cell phenotype expressing FoxP3 [52]. In continuation, IL-2 and IL-15 have also been shown to stimulate FoxP3 expression in Tregs [54]. FoxP3 was first identified in relation to

the X-linked disease, the IPEX syndrome (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked), where FoxP3 is mutated. IPEX is a severe systemic autoimmune disease [52]. This helped investigators to define FoxP3 as a 'fate-determining' transcription factor, and a specific marker for Tregs, a unique phenotype of T cells with immunosuppressive properties [52]. Therefore, measuring the expression of FoxP3 may evaluate the differentiation capability, but can also be used as a surrogate measure of the relative abundance of Tregs in different tissues [55].

6.2. Proliferation and differentiation of Tregs in relation to pregnancy

Tregs are essential for successful pregnancy, protecting the semi-allogenic fetus from immune rejection [56]. Interestingly, tumor grafts expressing paternal antigens have shown to be rejected after normal delivery, suggesting that the immunosuppressive mechanism led by Tregs is transient, limited to the period of pregnancy [57]. To elicit activation and differentiation of Tregs before pregnancy and at the time of conception, seminal fluid from the male partner may be important [58]. Seminal fluid contains many of the same paternal antigens later expressed by the fetus. Therefore, a possible role for seminal fluid in driving Treg activation and proliferation is interesting, because it may stimulate initial priming of Tregs and promote tolerance to paternal alloantigens prior to embryo implantation [59]. Seminal fluid may be considered a key regulator of the uterine Treg abundance. In addition, HLA-G expressed by trophoblast cells is also a possible regulator of Treg proliferation [52]. One study reported that soluble HLA-G (HLA-G5), secreted by human mesenchymal stem cells, induced $CD4^+CD25^+FoxP3^+$ regulatory T cells [60]. The observations suggest a local as well as a peripheral inducer of Treg proliferation and differentiation in relation to pregnancy.

6.3. Functions of Tregs

Compared to peripheral Tregs, the Tregs localized in the decidua have shown to display a more frequent expression of FoxP3 in normal pregnancy enhancing a suppressive phenotype [61]. The subset is less frequent compared to decidual NK cells in first trimester tissue, but not less important [62]. An increase is seen throughout the pregnancy and a decrease in the late puerperium [6,12].

One of the main functions of Tregs is to regulate allo-reactive Th1 cell subsets mentioned earlier [6,13]. Secondly, Tregs have been shown to increase placental HO-1, leading to up-regulation of IL-10, TGF-β and cytotoxic T-lymphocyt antigen-4 (CTLA-4), which all in different ways suppress immune responses facilitating a protective microenvironment in the uterus [6,63,64]. Tregs may directly block maternal effector T cells, either by expression of CTLA-4 or by direct cell-to-cell contact, preventing a harmful immunologic reaction towards paternal antigens.

6.4. Tregs in reproductive immunopathology

Several studies have reported an association between Tregs and reproductive pathologies in different biological contexts including peripheral and local Tregs. In this review, local Tregs are defined as located in the endometrium and decidua.

6.4.1. Peripheral Tregs

An increase in Tregs in peripheral blood is observed prior to embryo implantation and in decidual tissue of normal early pregnancy [52]. Arruvito et al. have examined $FoxP3^+$ Tregs in peripheral blood in women experiencing recurrent miscarriages, and observed a reduced number of $CD4^+CD25^+FoxP3^+$ Tregs compared with fertile controls [65].

6.4.2. Local Tregs counts

In line with the observations mentioned above, a decreased proportion of Tregs has been demonstrated in decidual tissue of women with spontaneous abortion compared with cases of elective abortions [66,67]. Other investigators comparing women with recurrent miscarriage with fertile controls confirmed the significantly decreased expression of $CD4^+CD25^+$ Tregs in the decidua [68].

Also, the function of these cells showed to be deficient in women with recurrent miscarriages. Data showed a correlation between $FoxP3^+$ Treg counts and the menstrual cycle, showing a Treg expansion in late follicular phase [65].

6.5. Experiments in mice

Previous experiments in mice have shown an expansion of Tregs around the time of implantation, and a depletion of Tregs terminates pregnancy [69]. Another study demonstrated that transferring exogenous Tregs in the peri-implantation period in depleted mice resulted in protection from fetal loss [11]. Transferring Tregs after 4–5 days of pregnancy was unsuccessful, suggesting that Tregs play an important role in the time just around implantation. Studies by Shima et al. support the immunosuppressive role of Tregs in the pregnancy peri-implantation phase, and also in early pregnancy, with data from mouse experiments [70]. Depletion of Tregs in late pregnancy did not terminate pregnancy, nor induce abnormal pregnancy outcome, such as intrauterine growth retardation (IUGR), hypertension or proteinuria; thereby supporting the role of Tregs around implantation and in early pregnancy.

6.6. Endometrial FoxP3 expression in relation to infertility

To date, very few human studies of FoxP3 expression in peri-implantation endometrium have been reported using different methods

Table 2
Studies investigating expression of regulatory T cells (Tregs) in endometrial tissue related to infertility.

Study	N	Method of analysis	Sample day (in menstrual cycle)	Variables	Purpose of study	Results
Jasper et al. [55]	10 infertile women 12 control women	RT-PCR	5–9 days after ovulation, timed according to the last menstrual period along with histological dating	FoxP3	Association between Tregs and infertility	Infertile women have significantly reduced endometrial expression of FoxP3 mRNA compared to fertile controls
Chen et al. [71]	7 infertile women with mild endometriosis (EM) 20 infertile women with advanced EM 20 control women	Immunohistochemistry and RT-PCR	Day 19–23 of menstrual cycle, based on last menstrual period along with histological dating	FoxP3	Association between Tregs and infertility caused by endometriosis	Immunohistochemistry: No significant difference RT-PCR: Significantly higher FoxP3 mRNA expression in infertile patients compared to the control group
Kofod et al. [49]	41 infertile women 20 control women	Immunohistochemistry	LH surge + 7 days	FoxP3	Association between Tregs and infertility	No significant differences

assessing the FoxP3 expression (Table 2). In general, few patients were included in these few studies.

Jasper et al. used RT-PCR to quantify possible differences in FoxP3 mRNA expression, and found significantly reduced FoxP3 mRNA in endometrial tissue of infertile women compared with fertile controls [55]. These results are in conflict with the findings in the study by Chen et al. [71]. Chen et al. observed a marginally higher number of FoxP3$^+$ cells in the endometrium of infertile women assessed by immunohistochemistry, not statistically significant though [71]. They supplemented with RT-PCR analyses, revealing a significantly higher FoxP3 mRNA expression in infertile patients than in the control group. However, the studies were based on infertility with a known cause: endometriosis. It can be considered that other mechanisms are important in relation to endometriosis compared to unexplained infertility, leading to the contrary results. Investigating the presence of FoxP3$^+$ cells by immunohistochemistry, we have recently failed to detect any significant differences between infertile and normal fertile women in the numbers per area [49]. Generally, studies agree that numerically fewer Tregs may result in reduced immunosuppressive capability, in the end possibly causing infertility or miscarriage.

The exact way of immunosuppression carried out by Tregs is not yet fully defined. A systematic review by Guerin et al. confirms Tregs as important, and not at least potent immunosuppressors in pregnancy, and that a Treg numerical and functional deficiency is associated with reproductive problems [52]. Future research may help to acquire knowledge of the antigens, cytokines and hormones regulating these cells. This knowledge may be the groundwork for developing therapeutic options for treating reproductive pathologies. However, it seems reasonable to boost the number or activity of Tregs targeting conceptual antigens, which, in theory, will improve maternal immune tolerance in women suffering from infertility or recurrent miscarriages. With regard to the current knowledge of Tregs enrichment at the feto-maternal interface, it still appears plausible that the suppressive actions of Tregs are also exerted prior to embryo implantation.

To further define the role of human FoxP3$^+$ Tregs, it will be relevant to examine the expression in peri-implantation endometrial tissue among women with infertility and compare them to fertile controls.

7. Interactions of dNK cells, Tregs and trophoblast – and a role for HLA-G

Understanding the complex mechanisms by which immune cell subsets interact at the feto-maternal interface during implantation is very important. The inability of extravillous trophoblast (EVT) to invade the uterine lining may be considered as one of the primary defects in women with reproductive dysfunctions including infertility. One mechanism is regulated by interaction between dNK cells and EVT (Fig. 1). Supported by several studies, one of the key players regarding this interaction appears to be HLA-G expressed on EVT [72–74]. The specific interaction between KIR2DL4 and HLA-G has been a focus of interest; the KIR2DL4 receptor is markedly present in the decidua [75]. Generally, KIRs are highly polymorphic, but a limited repertoire along with a lack of inhibitory KIRs specific for fetal HLA-C, and KIR2DL4 specific for HLA-G antigens, have been reported in dNK cells from women experiencing recurrent miscarriages [72,76]. Together, this might lower the threshold for uNK cell activation and cytotoxicity, probably contributing to the pathogenesis of infertility. In line with this, HLA-G has been shown to trigger secretion of pro-angiogenic factors as VEGF from dNK cells in vitro via KIR2DL4 (Fig. 1) [77,78]. Human leucocyte antigen-G has been shown with high affinity to bind to the receptors ILT2 and ILT4 [79]. *In vitro* studies have shown that the effect of binding is prevention of lysis carried out by dNK cells accompanied by impairment of IFN-γ release [80,81]. The ILT4 receptor is expressed on monocytes, macrophages and dendritic cells [82]. As a result of the interaction between HLA-G and ILT4, decreased activation of NK cells has been observed (Fig. 1) [83].

Whether the primary role of dNK cells *in vivo* is cytotoxicity and/or cytokine-secretion has been difficult to evaluate. However, a study by Chen et al. showed considerable NK cell cytotoxicity towards HLA-G knockdown cells compared to the non-knockdowned cells, demonstrating the importance of HLA-G interactions with the receptors ILT2, ILT4 and KIR2DL4 [84]. In addition, van der Meer et al. reported that HLA-G stimulation induced dNK cell proliferation [78]. This illustrates another essential pro-fertile function of the dNK cell/HLA-G interaction.

The uterine mucosa also contains Tregs, and the local abundance has been suggested to be influenced by dNK cells. Upon dNK cell activation, IFN-γ and galectin-1 is secreted [5]. This secretion induces monocyte-derived indoleamine-pyrrole 2,3-dioxygenase (IDO) expression and tolerogenic dendritic cell up-regulation, which promote generation of Tregs, thereby enhancing fetal survival [85]. Experimental studies in mice have reported that uNK cell-deficient mice secrete lower amounts of IFN-γ, resulting in increased incidence of fetal loss because of intrauterine fetal growth restriction, aberrant placental vascularity and hypocellular decidua. This exemplifies an essential role of uNK cells in normal pregnancy and thereby a possible quantitative importance of IFN-γ [86]. Furthermore, the absence or reduced expression of decidual galectin-1 has been associated with spontaneous fetal loss in mice, probably due to low numbers of Tregs [87].

A newly published study observed an interaction of EVT with CD4$^+$ T cells. A co-culture of EVT and CD4$^+$ T cells resulted in increased numbers of FoxP3$^+$ Tregs, and increased the relative expression level of FoxP3 in these cells [88]. Although the mechanisms responsible remain unknown, it might be plausible that HLA-G expressed on EVT could be involved (Fig. 1). However, we were not able to demonstrate the involvement of HLA-G in changes in Tregs using RNA-interference to downregulate HLA-G on the choriocarcinoma cell line JEG-3 derived from trophoblast [89].

Soluble HLA-G (sHLA-G) has been shown to interfere with especially the killing function of dNK cell at the feto-maternal interface and may, therefore, be a critical modulator of immunological tolerance during pregnancy [90,91]. Studies have reported that circulating sHLA-G, either transported from the serum or locally synthesized, is capable of eliminating both maternal T lymphocytes and dNK cells programmed to kill HLA-G$^+$ fetal cells by inducing the killer cells to undergo apoptosis [92–94]. However, the basis for the interaction is not yet clarified.

8. Immunomodulatory treatment for infertile women

To facilitate the implantation process in women suffering from infertility, several studies have investigated the efficacy of immunosuppressant drugs such as glucocorticoids, intravenous immunoglobulin and TNF-α blockers without any promising result [95]. The results are conflicting.

A systematic review and meta-analysis by Li et al. found a significantly higher implantation rate, clinical pregnancy rate and live birth rate compared to placebo in women with unexplained infertility or repeated IVF/ICSI failure receiving intravenous immunoglobulin [96].

Other biologic therapy options have recently been described as a new alternative way to manage reproductive pathological conditions. Granulocyte colony-stimulating factor (G-CSF) has shown to influence Tregs generation and selection via dendritic cell maturation. Unfortunately, administration of G-CSF has failed to improve implantations rates, clinical pregnancy rates, ongoing pregnancy rates and miscarriage rates in infertile women in randomized controlled clinical trials [97,98].

In general, the majority of studies within this field investigate RM and recurrent implantation failure (RIF). To our knowledge, tacrolimus, cyclosporine and intralipid treatment has only been investigated in women with RM.

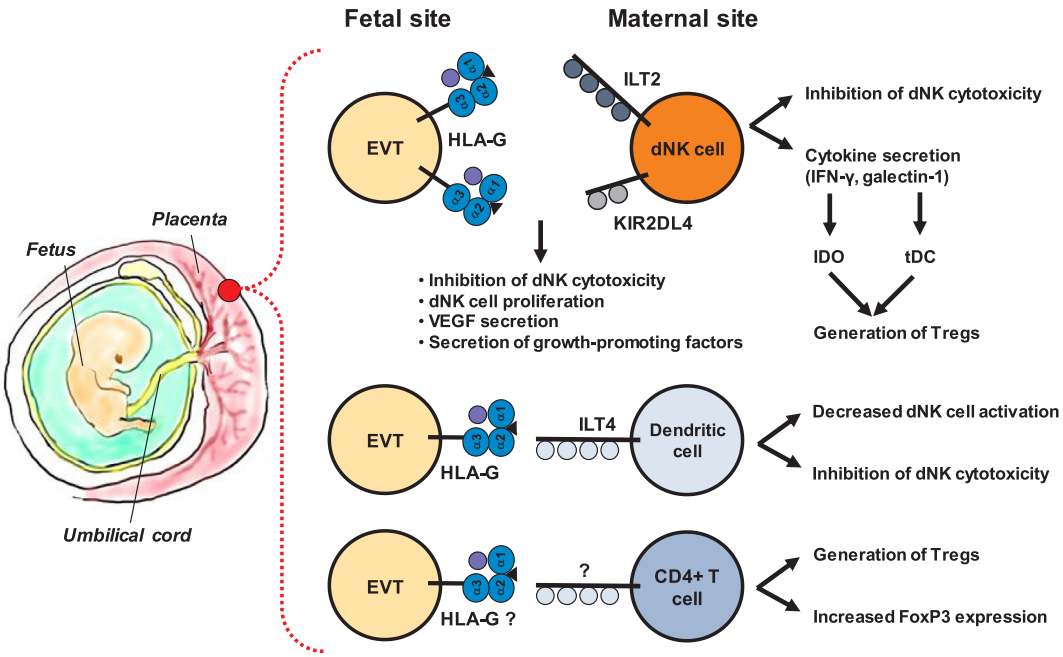

Fig. 1. Key interactions at the feto-maternal interface between dNK cells, T cells, dendritic cells and HLA-G involved in maternal immune tolerance towards the semi-allogenic fetus. The extravillous trophoblast (EVT) is one of the trophoblast subpopulations known to express HLA-G. *ILT4 is expressed on dendritic cells, monocytes and macrophages. See text for details.

In conclusion, the value of immunotherapy as a treatment for infertility is still not clear. Although some evidence exists, we still need strong evidence to support or refute the role of immuno-modulatory treatment in cases of infertility.

9. Conclusion

For decades, several studies have tried to correlate reproductive pathologies including infertility with an immunological origin. Immune cell density as well as receptor expression and functioning in endometrial tissue have been general focuses of interest.

Several studies report imbalances in the uNK cell profile among women with a history of infertility, while other studies are inconclusive. Therefore, some degree of controversy still exists. If a strong association can be evidenced regarding immune cell imbalances in endometrial tissue of infertile women, there might be an option for developing treatments based upon immunotherapy.

Future research may strive to use a standardised protocol for the assessment of immune cells in endometrial tissue of larger cohorts compared to current studies. Internationally accepted reference ranges need to be established promoting inter-study comparability and for the purpose of defining what is a normal and an abnormal count of uNK cells.

Regulatory T cells are reported to increase due to pregnancy, and might function to suppress immune responses to paternal alloantigens. This immunosuppressing mechanism is thought only to be active during pregnancy, especially around the time of implantation and in early pregnancy [70]. Among women suffering from infertility a reduced number of Tregs has been demonstrated, while studies on FoxP3 are contrary.

Women with reproductive problems are a heterogenic group, which is an important fact to take into account for studies within the field of reproductive immunology. Furthermore, when focusing on reproductive immunology, one single pathological mechanism may not necessarily be a sufficient explanation, and only in a subgroup is immunological dysfunction probably involved. Perhaps further investigations should search for an impaired interaction between several immunological mechanisms involving both changes in the distribution of leukocytes, atypical cytokine secretion, and changes in receptor expression and function, perceiving reproductive pathologies as multifactorial.

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