Royal College of Obstetricians & Gynaecologists

The Role of Natural Killer Cells in Human Fertility

Scientific Impact Paper No. 53
December 2016

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1. Background

Uterine natural killer (uNK) cells form the major leucocyte population in the endometrium at the time of implantation[1] and have received considerable attention in relation to their role in normal implantation and early placental development. Particular interest has been paid to their potential role in pregnancy pathology; there were more than 70 papers published between 2013 and 2015 on the role of uNK cells in recurrent miscarriage (RM) and recurrent implantation failure (RIF). Although several clinical studies have suggested that peripheral blood (PB) natural killer (NK) cells and/or uNK cells are increased in women with RM[2-6] and RIF,[5,7-9] a meta-analysis[10] and systematic review[11] failed to provide conclusive data because of significant heterogeneity across the studies arising from the use of different methods to quantify NK cells. An understanding of the role of these cells in reproductive failure and their value in clinical practice will not be established until a consensus is reached on how they should be measured.

In this paper, the data relating to NK cell function will be reviewed and recommendations made regarding the measurement of NK cells in women with reproductive failure.

2. NK cells in reproduction

NK cells were originally described as large granular lymphocytes able to lyse cells without priming, and unrestricted by target cell expression of major histocompatibility complex class I molecules. NK cells are now known to have functions other than lysis, such as cytokine secretion. Human NK cells are defined by the expression of various cell surface markers, particularly CD16 and CD56; they do not express CD3, immunoglobulin or T lymphocyte receptors. NK cells are found in PB, and a wide range of lymphoid and nonlymphoid tissues. Two main subsets of PB NK cells have been described: the majority (more than 90%) express CD56 at low density and CD16, and are referred to as $CD56^{dim}$ $CD16^{+}$ cells, which can lyse target cells; while approximately 10% of PB NK cells have high surface expression of CD56, but do not express CD16, and are referred to as $CD56^{bright}$ $CD16^{-}$ cells. PB NK cells have little or no cytotoxic activity and produce abundant cytokines. The relationship between the two main PB NK cell subsets is not clear; and they may have completely distinct roles in the human immune response.

The human endometrium contains a substantial population of NK cells (uNK cells) which vary in number and in proportion to the total number of endometrial stromal cells during the menstrual cycle. Although present in proliferative endometrium, uNK cells increase in number substantially in the mid-secretory phase and are the major endometrial lymphocyte population in the late secretory phase and the first trimester of pregnancy. uNK cells are $CD56^{bright}$ $CD16^{+}$ and also express CD9, which is not expressed by PB NK cells. In contrast to PB $CD56^{bright}$ $CD16^{-}$ NK cells, uNK cells have abundant cytoplasmic granules containing perforin and granzyme.[12]

There is no consensus about the origin of uNK cells. Mature PB NK cells or immature precursors may migrate into the endometrium from the blood possibly in response to chemokines produced by cells within the endometrium at specific stages of the menstrual cycle and pregnancy, and be modified by other factors within the endometrium. For example, production of CXCL-12 by extravillous trophoblast (EVT) cells may attract NK cells into the decidua in pregnancy;[13] interleukin (IL)-15, produced by secretory endometrium and decidua, has a selective chemoattractant effect on PB $CD16^{-}$ NK cells;[14] and transforming growth factor beta 1 (TGF-β1) has been suggested as modifying PB NK cells to uNK cells.[15] An alternative suggestion is that uNK cells are derived from haematopoietic precursor cells within the endometrium.[16]

The increased number of uNK cells in late secretory phase endometrium and early decidua suggests the involvement of progesterone. Although uNK cells do not express progesterone receptor (PR), a

progesterone-mediated response could be facilitated by cytokines and growth factors produced by other endometrial cells, which do express PR. For example, IL-15 production by stromal cells in secretory phase endometrium and early pregnancy decidua is stimulated by progesterone.[14] Macrophage inflammatory protein 1 beta, which is a chemoattractant for PB NK cells, is also upregulated by progesterone in human endometrium.[17] Different chemokines and cytokines within the endometrium may be responsible for the recruitment of PB NK cells and their subsequent modification into uNK cells.

There is also clear evidence that uNK cells are able to proliferate within the endometrium and decidua; several studies[18,19] have demonstrated the expression of the proliferation marker Ki67 by uNK cells, particularly during the secretory phase, which is the time of rapid increase in uNK cell numbers. Whatever the origin of uNK cells, comparison of PB NK and uNK cells within a given woman has shown that uNK cells are distinct from $CD56^{dim}$ $CD16^{+}$ and $CD56^{bright}$ $CD16^{-}$ PB NK cells,[20] and therefore, should be considered as a distinct population.

3. The role of NK cells in normal implantation and pregnancy

Embryo implantation requires the embryo to attach to the luminal epithelium of the endometrium, to penetrate the epithelial layer and to embed within the underlying endometrial stroma. Essential to the process is the invasion of trophoblast cells through the decidualised stromal cells and their differentiation to various cell types.

First, trophoblast cells proliferate to form cytotrophoblast columns, which mushroom outwards to form a cytotrophoblast shell. EVT cells differentiate within these cytotrophoblast columns into highly invasive cells, which invade through the maternal decidua as far as the inner third of the myometrium via two distinct routes. Interstitial EVT invasion occurs through the decidual tissue and is associated with extracellular matrix (ECM) breakdown. In contrast, the endovascular trophoblast migrates within the lumen of the spiral arteries, transiently replacing the endothelium and ultimately remodelling the uterine spiral arteries from thick-walled musculoelastic vessels into dilated tubes.[21] The presence of uNK cells in close proximity to the invading EVT cells suggests that they may play a role in this process.

3.1 Cytotoxicity

Although measurements of uNK cell cytotoxicity in vitro are difficult, there are several reports[22,23] of cytotoxic activity against K562 cells by uNK cells from early pregnancy decidua. However, the consensus is that the cytotoxic activity of uNK cells is reduced compared with PB NK cells. uNK cells isolated from early pregnancy decidua are not cytolytic towards fetal EVT cells, which could be due to the expression of human leukocyte antigen G by EVT cells.[24]

3.2 Cytokines, angiogenic growth factors and protease production

There is considerable interest in the role of cytokines in pregnancy, with the suggestion that a bias towards type 2 cytokines favours successful pregnancy, while type 1 cytokines are considered to be detrimental.[25] uNK cells produce many different cytokines and growth factors (for example, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, tumour necrosis factor alpha, granulocyte-macrophage colony stimulating factor, TGF-β1, leukaemia inhibitory factor and interferon gamma).[26] uNK cells are also an important source of angiogenic growth factors. Production of angiogenin, angiopoietin (Ang)-1, Ang-2, vascular endothelial growth factor (VEGF)-A, VEGF-C, placental growth factor, keratinocyte growth factor, fibroblast growth factor and platelet-derived growth factor-BB by uNK cells from secretory phase endometrium and early pregnancy decidua has been reported.[27,28] There are gestational age differences in the secretory activity of uNK cells: cells from 12–14 weeks of gestation produce more cytokines and less angiogenic factors than those obtained from 8–10 weeks of gestation.[27,29]

Both interstitial trophoblast invasion and spiral artery remodelling require breakdown of the ECM by proteolytic enzymes. uNK cells secrete matrix metalloproteinases (MMP)-1, MMP-2, MMP-7, MMP-9, MMP-10, tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, TIMP-3, urokinase plasminogen activator (uPA) and uPA receptor.[30,31] Immunoreactivity of MMP-7 and MMP-9 by leucocytes surrounding spiral arteries during early pregnancy has also been reported.[32]

3.3 Spiral artery development and remodelling

Spiral arteries are key blood vessels in nonpregnant endometrium and pregnant decidua. After menstruation these arteries regenerate from vessel stumps in the remaining endometrial stratum basalis and studies[28,33] have suggested that uNK cells may be involved in this process. uNK cells are frequently aggregated around the spiral arteries and arterioles in early pregnancy and this distribution may reflect their role in mediating vascular changes in pregnancy.[32] In vitro models of vascular remodelling have shown that uNK cell supernatants can modify markers of vascular remodelling.[34]

4. NK cells and reproductive failure

The observation of increased uNK cells in the endometrium of women with reproductive failure (such as infertility, RM and pre-eclampsia) has suggested they may play a role in this pathogenesis. However, the results from numerous studies are varied and despite the emergence of the measurement of the number of cells in clinical practice, there is currently no convincing evidence they are the cause of reproductive failure. For both PB NK and uNK cells, the expression of CD56 is used to define NK cells in most studies. However, some studies using flow cytometry have been able to investigate NK cell subpopulations.

4.1 NK cells and RIF

RIF may often be due to a defective endometrium and functional studies have shown that uNK cells may play a significant role in the implantation process, which suggests that they could be contributing to the failure of an embryo to implant.[35]

A number of studies have shown an increased number of PB NK cells (both the total $CD56^{+}$ and $CD56^{dim}$ populations) in women with RIF[5,9] and in one study, the pregnancy rate of those women with high PB NK cells (approximately 12%) was lower than those with normal NK cells.[5] However, in these studies, a proportion of the women had received immunotherapy prior to their next in vitro fertilisation (IVF) cycle and this will have confounded the results. Other studies[36,37] have failed to find subsequent differences in PB NK cell numbers in women with infertility who failed to get pregnant, in women who became pregnant after IVF, in nonpregnant women with a history of recurrent IVF failure or in women with a history of successful IVF.

One study[7] has clearly shown an increase in the number of uNK cells in prepregnancy endometrium from women with RIF after IVF. The results from other studies are less clear; in one immunohistochemical study[8] some women showed an increased number of uNK cells while others had normal levels, and another study[38] found no difference in uNK cell numbers in young patients with RIF. A systematic review carried out by Tang et al.[11] suggested that abnormal uNK cell numbers could not predict pregnancy outcome in women undergoing IVF. However, the number of uNK cells is likely to be altered by the hormonal regimens used in IVF treatments[39] and this may affect the results obtained.

Measurement of the number of PB NK cells or uNK cells does not address the more significant issue of the biological activity of these cells. A number of studies have attempted to address this, either by measuring the ability of NK cells to lyse K562 cells or the expression of the NK cell activation marker CD69. While studies[40,41] have shown increased cell activity in the PB of women with IVF failure, an

immunohistochemical study[7] reported no difference in the number of $CD69^+$ cells in the peri-implantation endometrium of women with RIF.

The contradictory nature of the results from all these studies suggests that although abnormal PB NK or uNK cell counts may contribute to RIF, there is insufficient evidence from which to draw firm conclusions.

4.2 NK cells and RM

RM is defined as either the loss of three or more consecutive pregnancies (determined by a positive pregnancy test) before 24 weeks of gestation,[42] or the loss of two or more consecutive pregnancies (defined as a nonviable, intrauterine pregnancy with either an empty gestational sac, or an embryo or fetus without fetal heart activity within the first 12 weeks of gestation).[43] The differences in these definitions add considerably to the heterogeneity of the data and makes comparisons of studies difficult. In RM, the initial stages of implantation take place, but the pregnancy is later lost, usually during the first trimester, presumably due to a failure of the later stages of implantation and during placentation. In 50% of women, the cause of the repeated loss is unknown, and in these women in particular, a role for uNK cells has been postulated.

Many studies[5,6,44] have shown increased numbers of $CD56^+$ cells in PB taken from women with a history of RM, either prior to or during pregnancy, compared with PB from healthy fertile nonpregnant or pregnant controls. It has been suggested that such measurements during pregnancy can predict pregnancy outcome.[45] However, there is a lack of consistency in many of these studies. In some studies, women were undergoing treatments with aspirin and heparin,[6] folic acid[44] or immunotherapy,[5] which will have confounded the data. Some studies[44,45] defined RM as a loss of two or more pregnancies, while in others,[6,46] it was defined as loss of three or more pregnancies. Other studies have failed to find a difference in the numbers of PB NK cells in women with RM[47,48] and have suggested that levels of these cells are not predictive of pregnancy outcome.[48] The systematic review by Tang et al.,[11] which excluded studies where patients were treated with immunotherapy, indicated there was no significant correlation between high prepregnancy PB NK cell numbers and pregnancy outcome in women with idiopathic RM. In contrast, the meta-analysis by Seshadri and Sunkara[10] showed increased NK cell numbers in the PB of women with RM compared with the controls. Increased PB NK cell activity has also been shown in nonpregnant women with RM,[45,49] and high levels have been associated with miscarriage in the next pregnancy.[49] However, another study[48] reported that elevated PB NK cell activity was not associated with subsequent miscarriage.

Several immunohistochemical studies[2–4] have shown increased levels of uNK cells in the prepregnancy endometrium of women with RM. However, other studies[50,51] using immunohistochemistry or flow cytometry found no difference in total uNK cells in women with RM, although, one study[50] did find a decrease in $CD16^-$ $CD56^{bright}$ and an increase in the $CD16^+$ $CD56^{dim}$ cell subsets in women with RM. One study,[4] with a small number of patients, reported an increased number of uNK cells in women who subsequently miscarried compared with those who had a live birth, while several other studies[3,50,51] have shown that prepregnancy endometrial uNK cell numbers are not predictive of pregnancy outcome.

There are also a number of contradictory studies on uNK cells in decidual tissue from women with RM reporting further pregnancy loss. One study[52] showed an increased number of NK cells, while another[53] showed a reduction in decidual uNK cells. In both of these studies, comparisons were made between tissue after miscarriage and that from women undergoing elective terminations; differences which could be a result, rather than a cause of the miscarriage.

Again, the inconsistent results from these studies suggest that PB NK or uNK cells may play a role in RM, but that further studies are required for confirmation.

4.3 NK cells and pre-eclampsia

Pre-eclampsia is characterised by inadequate remodelling of the spiral arteries, particularly in the myometrial layer. The evidence that uNK cells play a key role in spiral artery remodelling suggests they may also contribute to pre-eclampsia. Again, there are studies[54,55] showing an increased number of uNK cells in decidual tissue and others[56,57] showing a reduction in decidual uNK cell numbers in women with pre-eclampsia. These studies are of limited value as they measured uNK cell numbers after delivery and it is not clear whether the results reflected cell numbers earlier in the gestation.

5. The role of testing for NK cells

The reported increases in uNK cell numbers in RIF or RM has resulted in increasing demand from women with these conditions for measurements of PB NK and uNK cell counts. The relevance of the results of these tests is currently limited for a number of reasons: 1) a lack of consensus on the methods used for measuring and reporting uNK cell numbers; 2) lack of a clear definition of a 'normal' range or what constitutes a 'high' cell count for either PB NK or uNK cells; and 3) uncertainty that higher levels of NK cells are predictive of an adverse pregnancy outcome.

Measurements of PB NK cells are considerably easier to make than those of uNK cells since they do not require an endometrial biopsy to be performed; an invasive procedure. However, the significance of PB measurements is questionable. In nonpregnant women, the numbers of NK cells in PB do not appear to relate to numbers within the endometrium[58] and it is inappropriate to extrapolate results from PB to events in the endometrium. However, there is some evidence for altered PB NK cell counts in women with RIF or RM, and the measurement of PB NK cells may have a role in the future when more is known about the relationship between uNK and PB NK cells.

The number of NK cells in PB is usually determined by measuring the expression of $CD56^+$ by flow cytometry. PB NK cell measurements may be reported as absolute numbers, but more frequently, as a percentage of total leucocytes. There is little consensus as to what constitutes a high PB NK cell number; different studies have used cut-offs of between 12% and 18%.[5,46,59] The numbers of PB NK cells may also be affected by events in the menstrual cycle; NK cell numbers and activity are increased during the periovulatory period and reduced by the use of the contraceptive pill.[47] Thus, blood samples for NK cell measurements should be taken at a defined stage of the cycle and in women who are not taking steroid hormones.

The numbers of NK cells in the endometrium can be determined either by immunostaining tissue sections, or by mincing and digesting the tissue followed by flow cytometry analysis. The advantage of the latter technique is that NK cell subsets can readily be obtained. However, it is sometimes difficult to obtain sufficient endometrial tissue for flow cytometric analysis using a sampler and there is concern that samples may be contaminated by PB. The expression of cell surface markers may also be changed by enzymatic digestion. Immunohistochemical analysis allows measurements of the cells in situ and confirmation that the cells are within the tissue. It also provides an indication of the histology of the endometrium, which might affect uNK cell numbers.

The way in which uNK cell numbers are reported differs among centres; some centres report the absolute numbers of cells, while others express the numbers as a percentage of total stromal cells or total leucocytes. Moreover, there is no clear definition of a normal range of uNK cell numbers and what constitutes a 'high' cell count; two different centres using apparently the same methodology define the cut-off as 5%[60] and 12.9%.[3] In both of these studies, the number of control patients was very small (18 and 10, respectively) and studies with larger numbers of controls are required to determine the normal range. Since uNK cell numbers vary considerably during the menstrual cycle, the timing of sampling is very important. Endometrial tissue sampling is usually carried out during the mid-secretory phase (the

peri-implantation period).The steep increase in uNK cell numbers during this phase of the cycle[61] means that a difference in sampling time as few as 1 to 2 days will make a large difference to the number of uNK cells reported. It is therefore important that the biopsy is timed precisely according to the luteinising hormone surge and preferably taken 7 days post surge.

6. Possible treatment for women with abnormal uNK cell numbers

Intravenous immunoglobulin (IVIg) administered during pregnancy has been used for the treatment of both RM and RIF. Several theories have been proposed for its action, including dampening of NK cell activity and modification of cytokine production.[62] However, a meta-analysis[63] of the use of IVIg for RM showed no significant effect.A subsequent Cochrane review[64] on immunotherapy for RM also reported no overall difference in live birth rates between controls and IVIg groups.

Two observational and case–control studies[65,66] suggested that the use of IVIg may be of benefit to women with elevated PB NK cells, and a further study has suggested that IVIg may be effective in women with high PB NK cells, high PB cell toxicity or increased type 1 T helper/type 2 T helper cell ratios.[67] However, there has been no properly randomised controlled trial (RCT) to establish clinical benefit. Furthermore, there is a limited supply of IVIg, and the Department of Health guideline[68] for the rational prescribing of IVIg does not include IVF and recurrent spontaneous pregnancy loss.

Overall, the evidence suggests that treatment with IVIg is not supported and, since it may have serious adverse effects, should not be used.

6.1 Steroids (glucocorticoids)

Glucocorticoids have been used in a study to treat women with reproductive failure and high numbers of uNK cells;[60] this is based on the observation that uNK cells express the glucocorticoid receptor.[69]

The observational study[60] involved 29 women with a history of three or more RMs and increased numbers of uNK cells on day 21 (±2 days) of the menstrual cycle. It showed that administration of prednisolone (20 mg daily from day 1 to day 21 of the menstrual cycle) led to a significant reduction in uNK cell numbers from a median of 14% before treatment to 9% after treatment.A subsequent feasibility trial[70] randomised subjects with idiopathic RM and a high uNK cell count to prednisolone or placebo. Of the 160 women screened, 72 had uNK cell numbers higher than 5% and 40 of those agreed to randomisation.The live birth rate was 12/20 (60%) with prednisolone and 8/20 (40%) with placebo (RR 1.5, 95% CI 0.8–2.9). This difference was not significant, mainly due to small sample size. The evidence for treatment with glucocorticoids remains inconclusive but merits further investigation.

In a RCT[71] involving 112 women undergoing intracytoplasmic sperm injection who had recorded high levels of PB NK cells, those treated with prednisolone achieved a significantly higher ($P < 0.05$) clinical pregnancy rate than in the placebo group (48% versus 30%).

Polanski et al.[72] reviewed the literature regarding interventions to improve reproductive outcome in women with elevated NK cell numbers undergoing assisted conception and highlighted the deficiency of high-quality RCTs in this particular area.

7. Opinion

Despite intensive research the role of uNK cells in pregnancy remains uncertain, and whether the increased uNK cell numbers reported in association with abnormal pregnancy pathology (RM, RIF or pre-eclampsia) are directly causal or reflect more fundamental problems with the endometrium is not known. Despite this, a number of women are requesting and being offered analysis of either PB NK or

uNK cells and the value of these measurements remains controversial.[73,74] In response to patients who wish to discuss or request NK cell testing, clinicians should be aware that:

- uNK cells are different from PB NK cells, and that measurements of the latter are of limited value in aiding our understanding of the role of uNK cells in reproductive failure.
- There is no indication to offer routine uNK cell testing in women presenting with infertility or seeking IVF treatment; uNK cell testing in women with RM and RIF is still a matter for debate pending further evidence and should be regarded, for the time being, as within the realm of experimental medicine.
- The measurement of uNK cells must be standardised and the definition of 'normal' and 'high' levels based on established reference ranges derived from standardised methodology.
- Women undergoing uNK cell testing should understand that there is, as yet, no proven effective treatment for those with what may be considered abnormal results, although preliminary data suggest a possible positive effect of prednisolone.

In planning RCTs, the need to standardise uNK cell measurement cannot be overemphasised. Resolution of this issue should be made a priority in order to provide answers to the points above and to give clarity to both clinicians and patients.

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This Scientific Impact Paper was produced on behalf of the Royal College of Obstetricians and Gynaecologists by:
Professor SM Laird, Sheffield Hallam University; Dr GE Lash, Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou, China, and the Institute of Cellular Medicine, Newcastle University; Professor TC Li FRCOG, Hong Kong; and Dr JN Bulmer, Royal Victoria Infirmary, Newcastle upon Tyne

and peer reviewed by:
Professor OB Christiansen, Aalborg University Hospital and the Juliane Marie Centre, Rigshospitalet, Copenhagen University Hospital, Denmark; Professor HOD Critchley FRCOG, Edinburgh; Mr DI Fraser FRCOG, Norwich; Dr J Kwak-Kim, Chicago Medical School at Rosalind Franklin University of Medicine and Science, IL, USA; Professor N McClure FRCOG, Belfast; Dr LT Polanski, University of Nottingham; RCOG Women's Network; Dr GP Sacks FRCOG, Sydney, Australia; Miss S Seshadri MRCOG, London; Mrs EM Tuckerman, Sheffield; and Dr RG van der Molen, Radboud University Medical Center, Nijmegen, The Netherlands.

The Scientific Advisory Committee lead reviewer was: Mr M Metwally FRCOG, Sheffield.

The chair of the Scientific Advisory Committee was: Dr S Ghaem-Maghami MRCOG, London.

All RCOG guidance developers are asked to declare any conflicts of interest. A statement summarising any conflicts of interest for this Scientific Impact Paper is available from:
https://www.rcog.org.uk/en/guidelines-research-services/guidelines/sip53/.

The final version is the responsibility of the Scientific Advisory Committee of the RCOG.

The review process will commence in 2019, unless otherwise indicated.

