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NK Cells in Peripheral Blood and the Endometrium

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Introduction

Investigations form the cornerstone of modern medicine. Without them, clinical practice based on anecdote and experience would be little different from services offered by many complementary practitioners. But, as all clinicians will know, investigations do not always identify a simple cause-and-effect relationship, and treatment based on them is not always certain to cure the problem. Indeed, such clinical interventions are relatively rare. Sometimes investigations provide insight to help patient understanding, or to guide clinical management. Sometimes the very act of doing them provides therapeutic benefit in terms of stress reduction or enhancing the doctor–patient relationship. In other words, while the ultimate purpose of an investigation must be to improve clinical outcome, and studies must strive to prove such a cause-and-effect relationship, clinical benefits may be more subtle.

Natural Killer (NK) cell testing for women with recurrent pregnancy loss is still an evolving science. Benefits are not yet proven, and some academics still argue strongly against it. They claim that there is no causal relationship yet demonstrated between NK test results and miscarriage, that peripheral blood testing can offer no insight into implantation immunology, that any benefit from intervention must be offset by the good prognosis many have without any intervention, and that there is a real danger that "desperate" women will be "taken advantage of" by the promise of illusionary diagnoses and treatments. It is important to understand these issues in order to counsel patients appropriately. And the relevance of NK cell testing must be understood in this context.

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PEARLS TO TAKE HOME

NK cell testing benefits:

1. May identify an immunological cause of recurrent miscarriage (15–25%)
2. May guide supportive therapy for next pregnancy (e.g., Progesterone or prednisolone)
3. Acknowledges patient concern with her immune system
4. Gives patient comfort that "everything" is being tested
5. Provides patient with confidence that her doctor is thinking broadly about her problem

NK cells and reproduction

There is no doubt that NK cells do have an important and perhaps critical role to play in the establishment of early pregnancy. Whilst NK cells are distributed widely in all tissues, they are especially concentrated in the uterus where they are the main immune cells present. Uterine (u)NK cell numbers increase enormously from 10% of stromal cells in the proliferative phase to 20% in the late secretory phase, and >30% in early pregnancy. In the absence of implantation, uNK cells undergo apoptosis that heralds menstruation.

NK cells are part of the innate and evolutionary older branch of the immune system and have a primary function of "immune surveillance." They do not require activation in order to kill cells that are missing "self" markers of major histocompatibility complex (MHC) class I, as in cells affected by intracellular infection or cancer. Since placental cells do not express classic MHC class I proteins except HLA-C (probably to avoid attack by maternal T cells), they are vulnerable to attack by NK cells instead. In theory, therefore, uNK cells may limit (or regulate) trophoblast (placental cell) invasion, by direct cytotoxicity or cytokine production. However, uNK cells have never been shown to be cytotoxic to trophoblast cells *in vivo*, and *in vitro* require coculture with interleukin (IL)-2 to induce cytotoxicity (not surprisingly, IL-2 is not usually present at the maternal–fetal interface).

It is likely that the majority of uNK cells are recruited directly from the peripheral blood pool of NK cells every month, and recent studies have suggested correlation between uNK and blood (b)NK cell numbers. However, the relationship between bNK cells and uNK cells is more complex. All NK cells are, by definition, lymphocytes, which are not T cells ($CD3^-$) and express the surface marker CD56. Two main subtypes exist. $CD56^{+Bright}$ express high-density CD56, are $CD16^-$, and produce cytokines (IFN-γ, TNF-β, IL-10, and GM-CSF). These represent 90% of uNK cells but only 10% of bNK cells. The other subtype, comprising 10% of uNK cells but 90% of bNK cells, are $CD56^{+Dim}$ NK cells, which express low-density CD56, are $CD16^+$, have limited cytokine output, and are primarily responsible for NK cell cytotoxicity. These two subtypes also express different activating receptors (CD69) and inhibiting receptors (killer immunoglobulin-like receptors (KIR) and CD94).

Methods of assessment

Much of the controversy surrounding NK cell analysis is largely the result of poor study design, overinterpretation of results, and little appreciation of the complexities of the laboratory methods used. In most published studies, the "patient" group is very heterogeneous; often including women with both recurrent miscarriage and repeated IVF failure (which themselves can have varied definitions). Controls are difficult to recruit (some studies have had no control group), and even more difficult to define. It is entirely plausible, for example, that a "fertile" woman may have future secondary reproductive failure.

The assessment of uNK cells can be done by flow cytometry but the isolation of the uNK cells for this procedure is technically extremely difficult. Therefore, assessment of uNK cells is normally done by immunohistochemistry, the subjectiveness and limitations of which are rarely appreciated. First of all, it is only possible to count CD56+ cells, without any measurement of subtype or level of activation. Thus, for example, high levels of $CD56^{+Bright}$ may reflect a very different immunological environment to high levels of $CD56^{+Dim}$. Second, the endometrium is a complex glandular histological structure, and counting cells in one area gives wildly different results to counting in another. It takes a considerable effort for a pathologist to develop a reliable and consistent method of counting. Most tests for uNK cells are performed at the time of the "implantation window." But uNK cell numbers vary enormously on a daily basis at that time, and interpretation of cell levels needs to be appropriate for that exact day of the cycle. Few laboratories will have sufficient data to be able to do that.

The main criticism for analysis of bNK cells is that they are mainly of different phenotype to the majority of uNK cells, and therefore cannot bear any useful relationship to uNK cell numbers, and in any case are far from the site of embryo implantation. But endometrial biopsy is an invasive and painful procedure, and the prospect of a blood test assessment of immunological dysfunction has significant appeal. Although the majority (90%) of uNK and bNK cells are of different phenotype, it is simply not known what changes in the ratio of subtypes may have on implantation. Thus, it is hypothesized that higher levels of activated $CD56^{+Dim}$ bNK cells may lead to an altered phenotype ratio in the endometrium due to monthly recruitment. Alternatively, it is also possible that bNK cell activity represents a marker for some other (as yet undefined) immunological disorder. This marker may be nonspecific – in the same way as a raised white blood cell count or C-reactive protein level indicates the likelihood of infection somewhere in the body.

A number of assays have been used for the analysis of bNK cells, including the proportion of bNK cells out of all lymphocytes, concentration, surface markers of activation (flow cytometric analysis) , and *in vitro* assays of biological activity. These methods are not necessarily correlated, and results may be potentially affected by venipuncture conditions and transport to the lab, protocols for preparation and labelling, and the gating of cell populations in flow cytometry analysis. Therefore considerable effort needs to be undertaken to standardise conditions before using an assay for research or clinical purposes, and comparisons with other studies need to take these factors into account. Although population studies demonstrate a wide

reference range for bNK cells (3–31%), the corrected range for females is 5–20% (and this includes women with reproductive failure). There are also numerous other physiological variables that could affect bNK cell levels, including acute stress and exercise (increased), the menstrual cycle and IVF stimulation.

SCIENCE REVISITED

NK cell testing requires complex laboratory methodology that needs to be validated at a local level. Published studies must also be assessed against this background. Endometrial NK cell assessment is invasive and certainly requires a dedicated pathologist to provide reliable results. Blood NK cell assessment can potentially be performed by any hematology lab at a basic level. Our published studies (see Bibliography) indicate that 15–25% of women with RM have high NK cell levels.

NK cell analysis in recurrent miscarriage

Peripheral blood NK cell analysis in reproductive failure was first described in 1996 by Alan Beer's group in Chicago. In a poorly controlled study, it was famously claimed that high levels could be defined by bNK cell numbers >12%, and that no women with bNK levels over 18% had a successful pregnancy outcome, unless treated with immunoglobulin therapy. Other groups have since shown that in women with unexplained reproductive failure (including both those with RM and repeated IVF failure), bNK cells have higher preconceptual activity and cytotoxicity (^{51}Chromium-release assay), higher expression of the surface activation marker CD69, and lower expression of the inhibitory marker CD94. Women with raised NK cell activity have about a fourfold increase risk of miscarriage with karyotypically normal fetuses. In early pregnancy (including after IVF), lower levels of bNK cell cytotoxicity are significantly associated with live birth. One study showed that bNK cell cytotoxicity is higher in women with primary compared to those with secondary recurrent miscarriage.

In women with unexplained infertility, high bNK cell activity is associated with significantly lower conception rates over a 2-year follow-up. In the IVF setting, it has been claimed that lower bNK cell cytotoxicity on the day of embryo transfer is significantly associated with live birth. Another study used a receiver operating characteristic analysis to show that women undergoing IVF with raised CD69 expression on bNK cells had a significantly reduced implantation rate (13.1 vs. 28.2%), pregnancy rate (23.1 vs. 48.3%) and live birth rate (7.7 vs. 40.2%), and manifested a higher miscarriage rate (66.7 vs. 16.7%).

Given the invasive nature of uNK cell testing, there are fewer such studies in women with reproductive failure. However, numerous studies have similarly demonstrated that women with unexplained RM or repeated IVF failure have "high" uNK cell levels. Perhaps most significantly, it has been shown that preconceptual numbers are increased in women who subsequently have karyotypically normal miscarriages. Furthermore, a critical study using flow cytometry rather than immunohistochemistry showed that women with unexplained RM have increased uNK cells of the $CD56^{+Dim}$ subtype. This supports the hypothesis that increased or activated bNK

cells (primarily $CD56^{+Dim}$ cells) alter the uNK cell subtype population, which may be in turn detrimental to successful implantation.

NK cell testing in practice

In one of the largest studies to date, work in Sydney has attempted to assess the reliability and establish reference levels for NK cell testing in women with RM. Endometrial biopsies were taken in the luteal phase (the time of implantation) and assessed by immunohistochemistry. It was immediately apparent that (i) the complexity of the tissue led to significant variability in cells counted (e.g., due to the presence of glands or cell clusters), and (ii) uNK cell numbers increased rapidly each day of the mid to late luteal phase. This new data challenges previous assumptions defining fixed reference ranges and illustrates two critical principals for uNK cell testing: (i) It requires a dedicated pathologist with an interest in this area in order to report reliable cell counts, and (ii) it requires a large database to provide daily reference ranges for a given population. It is clear that simply sending a sample to a general pathology laboratory is unlikely to produce any useful information on NK cell levels. Testing for uNK cells is still a highly specialized investigation that is only available in certain tertiary centers with a research interest in the topic.

Testing for bNK cells, on the other hand, has potential advantages and may be more widely available. In terms of a diagnostic test, a simple blood test is obviously far preferable to a uterine biopsy for the patient. The criticism that blood testing is irrelevant for assessment of events at implantation in the uterus ignores the facts that (i) in general medicine many blood tests gain information about distant organs, and (ii) numerous studies have shown high bNK cell levels in women with RM. Although our work in Sydney (and others elsewhere) has showed that high levels of bNK cells are strongly correlated with high levels of uNK cells, such a demonstration is more interesting from a research pathophysiological perspective than a clinical one. In reality, in comparison with uNK cell testing, bNK cell testing is an independent test, which uses different methodology (flow cytometry) on different cell types. In one of the largest and most detailed studies to date, we have shown that for bNK cells, the strongest discriminating factors (for nonpregnant women with RM vs. controls) are (i) the number of bNK cells expressed as a percentage of lymphocytes (normal <18%), and (ii) the concentration of activated $CD56^{+Dim}$ bNK cells (determined with the CD69 marker; normal $<12 \times 10^6$/l). Flow cytometry is performed in all hospitals to varying degrees, and simple enumeration of bNK cell numbers (as a percentage of lymphocytes) should be possible in most large hematology labs. The CD69 marker test of bNK cell activation requires a dedicated flow cytometrist to perform labelling and gating. There are also important issues to take into account such as transport of specimens to the lab (time and shaking could affect bNK cell function), and protocols for fixation and labelling. In other words, just as is the case for uNK cell testing, bNK cell testing also requires the setting up of local systems and controls to obtain reliable results.

Using methods recently published, we have defined a population of 15–25% of women with unexplained RM as having "high" uNK cell levels, "high" bNK cell levels or activation, or both. It is not known why some women have higher levels than others, how or when their NK cell levels became raised. No longitudinal study has yet been

done. Such a diagnosis is useful because of the association with RM, although it is certainly not proven that high NK cell levels are in themselves a cause of miscarriage. In normal pregnancy, it is well recognized that bNK cell levels and functional activity are suppressed, and this provides the rationale to attempt to suppress NK cell levels in women with known high levels and a clinical diagnosis of RM.

Targeted immune therapy

Immune therapy to try to reduce miscarriage rates has a long and chequered history. This is partly due to the legacy of Peter Medawar's classic 1950s paper in which pregnancy immunology was compared with a tissue transplant, and hence the need for maternal immune suppression. While more recent work has significantly refined that hypothesis, it is clear that NK cells are a critical part in the maternal recognition of a conceptus and establishment of the maternal–fetal interface. Animals with depleted NK cells do not have successful pregnancies. Implantation and pregnancy in general are inflammatory states, and it is hypothesized that the absence of inflammation can be just as detrimental as an excessive inflammatory state. So, can NK cell testing identify that subgroup of women with "excessive" immunological activity leading to a poor endometrial environment (e.g., abnormal local cytokine profile) and higher risk of miscarriage? Can such women be targeted for immune therapy?

A RCT to assess the effectiveness of immune therapy in women with high NK cell activity is urgently needed, and trials in the United Kingdom and Australia are currently being undertaken. At present, clinical guidance relies on less rigorous evidence. There have been a number of case reports, one of which described a woman with 19 successive miscarriages and high uNK cells who was successfully treated with prednisolone (at a dose that was shown to suppress uNK cell levels). A number of other observational studies have suggested benefit, although their interpretation is prone to possible bias. Options for immune therapy include prednisolone, dexamethasone, IVIG, intralipid, and anti-TNF-alpha. Heparin and even progesterone provide milder immune-suppressive effects and should be considered given their safety and cost. There is no NK specific drug and, given our current understanding of pregnancy immunology, no particular therapy is obviously preferable (immune therapies have never been compared in a single trial). Therapies should be regarded as experimental, with determining factors including cost, potential harm to mother and fetus, and availability.

In Sydney, having defined a population of women with RM who have high NK cell levels (by blood test or endometrial biopsy), empirical treatment is given in the form of prednisolone 20 mg daily. This is commenced as soon as the woman has a positive pregnancy test, and continued until 12 weeks gestation. In a population of women (n = 87) with a very poor prognosis (median age = 39, median number of miscarriages = 6 over 4 years of trying to conceive), in those treated with prednisolone for high NK cell levels, 52% had a live birth following treatment, 41% within a year, and the majority (66%) succeeded in their first pregnancy with immune therapy. Those who did not succeed were noted to have multiple miscarriage factors compared to the successful cases that were more likely to have "unexplained" RM (i.e., high NK cell levels only).

PATIENT ADVICE

On current evidence, it is reasonable to provide empirical therapy with progesterone and/or heparin first line. Both have mild NK-suppressive effects. A more powerful agent is prednisolone, which is commonly available and as effective at suppressing NK cells as other more expensive regimes (e.g., IVIG). Common side effects include insomnia and fluid retention. More severe potential maternal side effects such as diabetes, osteoporosis, and premature labor are extremely rare (never been reported) when given at moderate doses (20 mg daily) for the first trimester only. The only reported possible fetal side effect is cleft palate, although the incidence is also extremely rare.

Immune therapy caution

A key issue to take into account is the potential that immune therapy could do more harm than good. In the absence of randomized trials, it is of course impossible to be sure that immune therapy doesn't *reduce* overall success rates. That is a major reason to be particularly cautious in using immune therapy in women without a clinical diagnosis (e.g., in women who might have high NK cell levels but no history of miscarriage). Furthermore, therapy in early pregnancy has the potential to affect the fetus, and some effects may only be discovered some years later. The side effect profile and risks of immune therapy (for mother and fetus) need to be carefully weighed up and managed in any program offering NK cell testing. Ongoing studies and long-term follow-up are essential.

CAUTION

NK cell testing inevitably leads to the potential for immune therapy. Options in increasing order of strength are:

- progesterone
- heparin
- prednisolone/IVIG/intralipid/anti-TNF-alpha

Potential risks and side effects on both the fetus and mother need to be discussed with the patient. Therapy is unproven in this context, and no therapy is known to be superior. Therefore, use the safest, simplest, and cheapest first.

When to offer NK cell testing

As information has become so widely available, NK cell testing is almost impossible to ignore. Patients frequently discover the possibility of such testing on simple Internet searches or via patient forums. There is little point in doctors denying access to such testing on the basis that it is not yet proven to be effective. It may turn out to be impossible to actually prove that it is either effective or ineffective. Doctors would be best advised to understand the background (as discussed earlier), and take a progressive empirical approach with individualized care. What does this mean?

First of all, we must all be aware that many women are particularly concerned that their immune system is a problem. Women are prone to autoimmune conditions such as thyroid disease, and have an innate concern of their ability to carry a pregnancy. A survey in Sydney has found that nearly two-thirds of patients admitted using alternative therapies such as acupuncture and herbs. Such alternative practitioners have been successful as they acknowledge concern that a successful pregnancy requires complex whole body interactions, and this is barely assessed by conventional highly specific RM investigations such as karyotype and uterine morphology. For better or worse, NK cell testing is currently the most widely known and available test of the immune system, and as such offers a potential insight into the broader assessment of a woman's reproductive health. Some women request such testing at an early stage, and there is no reason to deny them if reliable NK testing is available. In Sydney, a blood test for NK cell analysis costs less than $100.

On current evidence, NK cell testing should not be offered as part of "routine" investigation for RM (defined as three or more miscarriages). However, if a reliable test is available, it should be done on request, or in more significant reproductive difficulty (e.g., five or more unexplained miscarriages, age over 36, IVF). Clearly, a blood test would be preferable, although on current evidence, optimal information is achieved with testing for both bNK and uNK cells. I do not believe that uNK cell testing by immunohistochemistry is a "better" test even though it samples the site of the problem (implantation). Flow cytometry offers significantly more detailed information on NK cell subtypes and activation status that may be more relevant. Also, flow cytometry for uNK cells is technically extremely difficult. Not many centers are able to offer both uterine and blood testing.

Interest in NK cell analysis has so far primarily been for patients with otherwise *unexplained* RM. It has been used as a means of exploring possibilities that are, by definition, at the frontiers of knowledge. We must be cautious in assuming that everyone with "unexplained RM" must have an "overactive" immune system. In Sydney, 15–25% of women with unexplained repeated reproductive failure have high NK cell levels (although a normal NK result does not exclude the possibility of an immune disorder). We must also remember that high NK cell levels may not be the cause of the problem – they may simply be associated with it. On the other hand, treatment with immune therapy (on an empirical basis) is not necessarily confined to women with high NK cells. So, what is the relevance of NK cell testing in women with RM?

NK cell testing offers the *potential* to target immune therapy to women who are more likely to benefit from it, and so may improve success rates. NK testing may be beneficial in other ways too. Many women appreciate the concept of looking for a cause of their infertility. It gives them confidence that their doctor is thinking and individualizing their problem. By acknowledging the importance of the immune system, it may reduce stress, and can give some patients the hope they need to keep trying.

Methodology is critical. Any test must be thoroughly validated and, in the absence of better-quality evidence, NK testing for targeted immune therapy should be done in the context of a trial. Patients should be advised of the experimental nature of the approach, and considerable caution should be undertaken to avoid the situation where marketing precedes the evidence. In that way, it is incumbent on us to push this frontier of reproductive medicine, rather than simply turn our back on it. Our patients expect nothing less.

☆ TIPS AND TRICKS

In view of the lack of RCTs, an empirical, individualized approach to NK cell testing is recommended:

Offer testing (i) on request, (ii) after conventional miscarriage investigations have been found normal, (iii) after failure of conservative management or treatment of other miscarriage causes, (iv) after five or more unexplained miscarriages, (v) women over age 36, (vi) women with miscarriage after IVF.

Management of high NK cell levels must account for (i) strength of evidence for immune dysfunction (e.g., exact NK cell levels, whether information from just blood or uterine testing, reliability of labs, other autoimmune abnormalities), (ii) severity of clinical problem (e.g., number of miscarriages, requirement of IVF), (iii) immune therapy available, tolerable, and affordable.

The choice of immune therapy depends on how important the problem (high NK cells) is considered to be in relation to the risks, side effect, and cost of therapy

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