Natural Killer cell analysis

Gavin Sacks
MA BM BCh DPhil MRCOG FRANZCOG

Fertility Specialist
IVFAustralia
Sydney, Australia

gsacks@ivf.com.au

Natural killer cell analysis

In reproductive medicine, Natural Killer (NK) cell analysis is at once a marketing dream and an academic nightmare. This chapter aims to help clinicians counsel patients effectively and contemplate a progressive approach to this new and still unproven topic. The lack of large trials and proven pathological mechanisms should not deter clinicians from trying to understand what is actually being tested, what it may mean, and how it may guide adjuvant therapy to improve IVF success rates.

Natural killer cell biology

NK cells 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. Hence NK cells, part of the innate and evolutionary older branch of the immune system, have a primary function of 'immune surveillance'. Since placental cells do not express classic MHC class I proteins (probably to avoid attack by maternal T cells) they are vulnerable to attack by NK cells instead.

NK cells are lymphocytes with a $CD3^{-}CD56^{+}$ phenotypic profile. Two main subtypes exist. $CD56^{+Bright}$ express high density CD56, are $CD16^{-}$, and produce cytokines (IFN-γ, TNF-β, IL-10, and GM-CSF). $CD56^{+Dim}$ 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).

NK cells are distributed widely in all tissues, but are especially concentrated in the uterus. 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. Thus uNK cells appear to regulate trophoblast (placental cell) invasion, although their precise function in reproduction is still not known. 90% of uNK cells are the $CD56^{+Bright}$ phenotype. They have never been shown to be cytotoxic to trophoblast cells in vivo, and in vitro require co-culture with interleukin (IL)-2 to induce cytotoxicity (not surprisingly, IL-2 is not usually present at the maternal-fetal interface). In

the absence of implantation, uNK cells undergo apoptosis that heralds menstruation. 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 unclear, especially since over 90% of bNK cells are $CD56^{+Dim}$. The overwhelming majority of $CD56^{+Bright}$ uNK cells represents <10% of the bNK cell population.

Methods of assessment

Much of the controversy surrounding NK cell analysis is largely the result of poor study design, over-interpretation of results, and little appreciation of the complexities of the laboratory methods used. In most published studies the 'patient' group is very heterogenous, 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 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 $CD56^{+Dim}$. Secondly, 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 hypothesised 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 non-specific - 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, and in vitro assays of biological activity. These methods are not necessarily correlated, and results may be potentially affected by venepuncture conditions and transport to the lab, protocols for preparation and labelling, and the gating of cell populations in flow cytometry analysis. 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 (differential effects on different tests performed).

NK cell analysis in reproductive failure

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, 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 four-fold increase risk of miscarriage with karyotypically normal fetuses. In early pregnancy (including after IVF), lower levels of bNK cell cytotoxicity are significantly associated with livebirth. 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 are 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 livebirth. And another study used a receiver operating characteristic (ROC) analysis to show that women with raised CD69 expression on bNK cells had a significantly reduced implantation rate (13.1 versus 28.2%), pregnancy rate (23.1 versus 48.3%) and livebirth rate (7.7 versus 40.2%) and manifested a higher miscarriage rate (66.7 versus 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 recurrent miscarriage 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. And a critical study using flow cytometry rather than immunohistochemistry showed that women with unexplained recurrent miscarriage 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.

In Sydney, we have showed that high levels of bNK cells are strongly correlated with high levels of uNK cells, and for bNK cells the strongest discriminating factors (for women with reproductive failure versus controls) are (1) the number of bNK cells expressed as a percentage of lymphocytes (normal <18%), and (2) the concentration of activated $CD56^{+Dim}$ bNK cells (determined with the CD69 marker; normal <12x106/l). The assessment of uNK cells is obviously more invasive and is potentially useful if (1) further confirmation is wanted, or (2) bNK cell levels are low or borderline.

Ultimately though, it boils down to whether it is worth measuring NK cells anyway. Is there effective treatment? And does that treatment improve IVF success rates.

Targeted immune therapy

Immune therapy to try to improve IVF success rates (and 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. More recent work has significantly refined that hypothesis, with some elements of the maternal immune system suppressed and others activated. 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 hypothesised that the absence of inflammation can be just as detrimental as an excessive inflammatory state.

Most women having IVF do not require additional immune suppressive therapy (trials from 15-20 years ago did not show benefit). Some more recent trials have shown that immune therapy improves IVF success rates in women with repeated IVF failure, suggesting that there may be a subgroup of women who do benefit from immune therapy. So, can NK cell testing identify that subgroup who have excessive immunological activity leading to a poor endometrial environment (eg. abnormal local cytokine profile) and lower success rates? Can such women be targeted for immune therapy?

A randomised controlled trial to assess the effectiveness of immune therapy in women with high NK cell activity is urgently needed. A number of observational studies since Beer's 1996 paper have suggested benefit, although their interpretation is prone to possible bias. They have also all involved women with unexplained repeated reproductive failure (rather than first time IVF couples for example), and tended to include a mix of women with repeated IVF failure and miscarriage. Considerable care is essential to assess the methods of NK analysis, and treatment protocols are highly variable, often including multiple therapy with intravenous immunoglobulin (IVIG), aspirin, heparin and dexamethasone. Given these constraints though, it has been shown that both uNK and bNK cell numbers and activity can be suppressed by IVIG and by prednisolone. And in women with repeated IVF failure with high NK cell activity, treatment produces significantly better pregnancy rates.

Immune therapy is currently crude and nonspecific. Options 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.

Conclusions

Interest in NK cell analysis has so far primarily been for patients with otherwise *unexplained* reproductive failure. It has been used as a means of exploring possibilities that are, by definition, at the frontiers of knowledge. In the absence of a randomised controlled trial on the effectiveness of NK cell testing and treatment, we simply do not yet know who (if anyone) benefits.

We must be cautious in assuming that everyone with 'unexplained infertility' 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 place of NK cell testing in women about to start IVF?

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. And 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 individualising their problem rather than simply booking IVF cycles. 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 preceeds 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.

References

King K, Smith S, Chapman M, Sacks G. Detailed analysis of peripheral blood natural killer cells in women with recurrent miscarriage. Hum Reprod 2010. 25:52-8

Tuckerman E, Mariee N, Prakash A. Li TC, Laird S. Uterine natural killer cells in peri-implantation endometrium from women with repeated implantation failure after IVF. J Reprod Immunol 2010. (Epub ahead of print)

Heilmann L, Schorsch M, Hahn T. CD3-CD56+CD16+ natural killer cells and improvement of pregnancy outcome in IVF/ICSI failure after additional IVIG-treatkment. Am J Reprod Immunol 2010. 63:263-5.

Clark DA, Coulam CB, Stricker RB. Is intravenous immunoglobulins (IVIG) efficacious in early pregnancy failure? A critical review and meta-analysis for patients who fail in vitro fertilization and embryo transfer (IVF). J Assist Reprod Genet. 2006;23:1-13.

Lachapelle MH, Miron P, Hemmings R, Roy DC. Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome. J Immunol. 1996;156:4027-34.