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Original paper

Natural Killer cells in embryo-implantation

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Abstract Infertility is a global public health problem. Despite progresses in assisted reproductive technologies, failure of treatment exists and poses a difficult challenge to both clinicians and infertile couples. A myriad of factors causes failure of embryo implantation. However, in a significant percentage of the cases, the aetiology remains a topic of study and interpretation. Current data suggest that both peripheral blood and uterine natural killer cells might play a role in successful implantation and subsequent conception. In this paper, we aim to discuss the importance of NK cells in embryo-implantation and the reproductive outcome, as illustrated by current literature.

Keywords Uterine natural killer, *embryo implantation*, infertility.

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Introduction

Local and systemic immune responses involving immunoglobulins, cytokines, hormonal and other endometrial factors influence embryo implantation. A synergism of these factors is critical for successful implantation and subsequent conception. Natural killer cells (NK) are nonspecific innate immune cells critical to antiviral and antitumor defense. In females, two populations of NK cells have been identified: peripheral blood (pbNK) and decidual or uterine natural killer cells (dNK/uNK). Activated NK cells attack their target via exocytosis of perforin – and granzyme-containing granules and modulate the immune response by secreting a wide array of cytokines and chemokines. Nk distribution influences their function. Thus, pbNK are primarily cytotoxic, while uNK are mainly involved in cytokine production. Although recent years have brought new insights into understanding the physiobiology of NK cells, the development and function of uterine NK (uNK) remain a topic of study and interpretation. Current data suggest that peripheral blood and uNK cells might play a role in successful implantation and subsequent conception. In this paper, we aim to discuss the relevance of peripheral blood and uterine NK cells in the reproductive outcome.

NK cells biology

NK cells are non-T, non-B large granular lymphocytes that originate from hematopoietic progenitor cells (HPCs) in the bone marrow and express the surface marker CD56 (M.J. ROBERTSON et al [1]). Conventional NK cells are found in blood and represent 5 to 10 percent of the peripheral blood lymphocytes (G. TRINCHIERI [2]). Tissue-dwelling NK cells have been identified in various areas, such as the lining of the uterus, the uterine decidua during pregnancy, lymph nodes, thymus, liver, and duodenum (D.K. SOJKA et al [3]).

Large intracytoplasmic granules and cell surface markers differentiate NK cells from lymphocytes. Thus, NK cells do not display adaptive lymphocytes' cluster of differentiation markers (CD3, CD4, or CD19) (J.S. ORANGE et al [4]). Instead, most of them express certain surface molecules that function as receptors and recognize other molecules or ligands expressed by target cells (Table 1) (M.J. ROBERTSON et al [1], G. TRINCHIERI [2], J.S. ORANGE et al [4], M.A. COOPER et al [5], R. MARZIO et al [6]). Typically, NK are CD3-negative and CD16/CD56-positive cells. They are derived from HPCs, which are activated through different stages of maturation to produce two patterns of CD56 expression: intermediate affinity (the majority of NK cells) and very high affinity (5% of the peripheral blood NK cells) (R. MARZIO et al [6]). This affinity pattern is linked to different cytotoxicity, secretory cytokine profile and receptor/gene expression. CD56bright NK cells are immature; they exhibit a weak cytotoxic activity, as compared to the powerful lytic effect of CD56dim NK cells. However, CD56bright NK cells exert an important immunostimulative effect, being potent producers of cytokines (J. KWAK-KIM et al [7]).

NK cells also express inhibitory and activation cell-surface receptors (Table 1). Some receptors trigger NK activation after linking their ligands, while other require surpassing a signaling threshold set by the inhibitory receptors in order to activate NK cells cytotoxicity (A. MORETTA et al [8]). In addition, NK cells constitutively express cytokine and chemokine receptors, such as CD128 (a receptor for IL-8 chemokine) (A. CHUNTHARAPAI et al [9]), CD122 (IL-2R) and CD25 (the receptor for IL-2) (D. DAVID et al [10], J. KWAK-KIM et al [11]).

NK cells functions

NK cells are lymphocytes named after their ability to induce natural cytotoxicity during innate immune responses and differ from the lymphocytes belonging to the acquired immune system in that they do not express specific antigen recognition receptors. Instead, NK cells act through invariant, germ-line encoded receptors, which do no attain specificity through genetic recombination (M.J. ROBERTSON et al [1], G. TRINCHIERI [2], R. BIASSONI et al [12]). Interactions between these receptors and their target cell-surface ligands mediate the "natural" or "spontaneous" cytotoxicity function of NK cells. In general, NK cells survey for abnormalities in surrounding cells (M.J. ROBERTSON et al [1], G. TRINCHIERI [2], A. MORETTA et al [8], R. BIASSONI et al [12]).

Cells that deviate from the self-pattern of expression are detected and destroyed by cytotoxicity, which can be:
1. Direct cytotoxicity (R. BIASSONI et al [12]), or
2. Antibody-dependent cellular cytotoxicity (ADCC) (M.J. ROBERTSON et al [1]).

The latter is achieved with the cooperation of the humoral adaptive immune system. The interaction between NK cells and targets is mediated by the CD16 receptor, a NK cell-surface molecule, which binds the Fc portion of the IgG attached to the targets. Subsequently, the NK cells cytotoxic functions are activated against the cell in check.

NK cells' noncytotoxic functions (T. STROWIG et al [13]) are:
1. Cytokine and chemokine production;
2. Co-stimulation of other immune cells.

Table 1. NK cells phenotype

Surface molecule	Role	Ligand	Discussion
CD2	Receptor	LFA-3*	
CD16	Fc-gamma-RIIIa receptor	Immunoglobulin G	Important in antibody dependent cellular cytotoxicity
CD56		NCAM**	CD56dim and CD56bright
CD335‡	Natural cytotoxicity receptor NKp46	Viral hemagglutinin	Functions as an activating receptor Specific for NK cells
CD336‡	Natural cytotoxicity receptor NKp44	Viral hemagglutinin	Functions as an activating receptor Specific for NK cells
CD337‡	natural cytotoxicity receptor NKp30	HLA-Bassociated transcript3 and H7-B6	Functions as an activating receptor Specific for NK cells
CD69	signal transducing receptor	Specific ligand is unknown	Early activation marker. Induces cytokine release. Positively regulates NKcells activation
CD8	Alpha/alpha homodimer receptor		Expressed by ≈ 50% of NK cells Differs from alpha/beta heterodimers expressed by cytotoxic T cells
2B4 (CD244)	Activation receptor		Non-specific
NKG2D (CD94)	Activation receptor		Non-specific
LFA-1(CD11a/CD18)	Activation receptor		Non-specific
KIR† family	Inhibitory receptors	class I - MHC antigens††	Class I - MHC antigens are expressed by most nucleated cells. Normal expression of class I MHC sends the "healthy self" signal to NK cells, which negatively regulates the activity of NK cells, through KIR signalling. Failure of healthy pattern expression triggers NK cells activation.

* LFA3 - the adhesion molecule for lymphocyte function associated antigen 3; ** NCAM - neural cellular adhesion molecule; ‡NCR (natural cytotoxicity receptor) family includes CD335, CD336 and CD337 molecules
†Killer cell immunoglobulin-like receptor; ††Class I MHC - major histocompatibility complex molecules, also known as the human leukocyte antigen (HLA) system

Although all NK cells are able to produce immunostimulative factors, CD56bright NK cells are highly effective in this role. Interferon (IFN) – gamma production enables NK cells to effectively involve in antiviral immunity. IFN-gamma is an immuno-modulatory cytokine that exerts potent antiviral, antiproliferative properties, activates and facilitates the respiratory burst of macrophages (M.A. COOPER et al [14]). Activated NK cells also secrete TNF-alpha, a pro-inflammatory cytokine with anti-tumor effects, interleukins (IL-2, IL-5, IL-10, IL-13) and TGF-beta, which enables them to play important roles in assisting the development of the adaptive immune response (M.A. COOPER et al [14]).

Recent evidence indicates that, under experimental conditions, NK cells display immunological memory, which is a feature of adaptive immunity (E. VIVIER et al [15], J.C. SUN et al [16]; [17]). However, in humans, evidence for NK cells memory is lacking (A. RÖLLE et al [18]).

Mechanism of target elimination

Cytotoxicity requires the release of the lytic content of cytoplasmatic granules (Table 2). In both NK cell direct and antibody-dependent cellular cytotoxicity, the content of the cytoplasmic granules is released in the interface between NK cells and target cells. Granzymes, which are cell death-inducing enzymes, enter the target cell through perforin-facilitated endocytosis or via perforin pores (J.S. ORANGE [19], M.E. PIPKIN et al [20]). Apoptosis is also induced by interactions between cell-death receptors (Fas expressed on target cell and their ligands, FasL on NK cells) (P. BOLITHO et al [21]).

Table 2. NK cells cytoplasmic granules content

Content	Role
Perforins	pore-forming molecules
granzyme serine esterases	cell death-inducing enzymes

Uterine NK cells

Initially known as "granulated uterine metrial gland cells", "endometrial granulocyte", and "K cells", pregnancy-associated uterine NK (uNK) cells are suspected to arise from the trafficking of peripheral NK cells into the uterus (K. KITAYA et al [22], [23], B. ANNE CROY et al [24]), where they differentiate into specialized uterine NK CD56bright cells.

uNK cells are essential components of the innate immuneresponse, being the most abundant immune cells in the endometrium in the middle luteal phase (peri-implantation) and in the basal decidua at the beginning of pregnancy. (I. MANASTER et al [25]). Theyare a single subset of NK cells, strongly stained for CD56 antigens, but not for CD16 antigens.

Uterine NK cells are normally present in the non-pregnant uterus (E.L. PARR et al [26], H. YADI et al [27], T.V. MALLIDI et al [28]) but they expand considerably at the site of embryo implantation, in direct contact with fetal trophoblasts (A. MOFFETT et al [29], K. HATTA et al [30]), accounting for up to 70 percent of local lymphocytes during pregnancy (A. MOFFETT et al [29]). Recruitment of decidual NK cells appears to be controlled hormonally; it is not dependent on the presence of an implanting embryo (J. ORDI et al [31]). However, studies showed that they are the dominant lymphocytes in human and murine embryo implantation sites (A. MOFFETT et al [29], K. HATTA et al [30]). uNK cells are phenotypically different from peripheral NK cells (M.J. MOLLER et al [32]). Unlike NK, most uNK cells are CD56-positive, CD16 (FcγRIII)-negative (J.N. BULMER et al [33]).

Uterine NK cells exhibit different phenotype, distribution, and functions than circulating CD56/CD16 NK cells. In early pregnancy, uNK are numerous around small uterine arteries, endometrial glands and near the extravillous trophoblast. uNK cells were proved to be involved in implantation and early embryo survival. They promote decidual angiogenesis and the remodeling of uterine spiral arteries into dilated vessels invading the trophoblast (J. HANNA et al [34], G.E. LASH et al [35]). Moreover, uNK cells play a role in immune tolerance, via immunoglobulin-like killer cells (KIR) signaling. KIR family consists of inhibitory receptors that regulate NK cells activation by recognizing MHC expression. At uterine level, KIR molecules bind to HLA-C placental antigens, suggesting that NK cells modulate fetal trophoblast recognition (O. CHAZARA et al [36]).

uNK cells exert cytotoxic functions, as they express perforin and granzymes (E.L. PARR et al [37], A.A. ASHKAR et al [38]). In addition, they have an important immunoregulatory potential, being a potent source of IFN-γ (A.A. ASHKAR et al [38]), interleukin-8 and interferon-inducible protein–10 chemokines and proangiogenic factors which promote vascular growth inthe decidua. It is speculated that their major function is to assist fetal development, and to create a tolerant maternal fetal microclimate (J. HANNA et al [34]).

Syncytiotrophoblast and villous cytotrophoblast cells do not express class I human leukocyte or class II alloantigen. It is known that one of the main roles of NK cells is to survey for abnormalities in surrounding cells and detect the "healthy" pattern of expression of class I MHC molecules. Failure of displaying this signature generally triggers NK cells activation and natural cytotoxicity (H.G. LJUNGGREN [39]). As decidual NK are cytotoxic, it can be speculated that lack of expression of class I MHC may render villous trophoblasts vulnerable to their attack. However, under normal conditions, the immune system is tolerant towards fetal tissue development. Thus, regulation of NK activity is a crucial element in pregnancy outcome. The role of decidual NK cells, as protective factors or negative regulators on reproductive outcome, remains largely unknown. The activation and inhibitory receptor repertoire of uNK is similar to that of peripheral blood NK cells (J. KWAK-KIM et al [7]).

Increasing evidence pleads for their role in female reproductive performance (A.E. BEER et al [40], S. QUENBY et al [41]). Uterine NK cells regulate placental vascularization (J. HANNA et al [34], A. MOFFETT et al [42]; [43], S.E. HIBY et al [44]), and are responsible for trophoblast invasion (J.N. BULMER et al [33]). Also, uNK cells appear to be associated with implantation failures, recurrent spontaneous abortions, infertility (J. KWAK-KIM et al [7]), or pre-eclampsia (S.E. HIBY et al [44]).

Methods of Assessment

In women with reproductive failures, NK cells were analyzed from numerical, phenotypical, and functional perspectives. NK cells can be assessed by immuno-histocytochemical staining of tissue samples or flow-cytometry studies of peripheral blood, endometrial biopsy samples and uterine flushing samples. Samples collection can be performed using a Pipelle catheter for biopsy or flushing the uterine cavity with saline solution.

There is no consensus about the timing of testing. Levels of peripheral NK cells vary during the different phases of the menstrual cycle (A.N. SULKE et al [45]). Similarly, a cycle-to-cycle variation in the number of uterine NK cells was observed (N. MARIEE et al [46]).

The uNK cell density was determined as the percentage of uNK cells in the stromal cell population. Because uNK cell density varies with endometrium depth, the measurement was limited to the luminal endothelium. The normal uNK cell density was defined as 5% or less of the total number of cells in the luminal endothelial layer. Lately, an improved uNK numbering methodology has been adopted. In order for there to be no major difference between the results of measurements in different laboratories, it is recommended to observe some rules in the counting of uNK:

- Using the same control samples in each batch to ensure staining consistency;
- Excluding glands and vessels from the count;

- Counting only CD56 + cell near the epithelium because uNK cell density varies depending on endometrium depth;
- Counting at least eight randomly selected high-power fields and 5000 stromal cells per patient;
- Defining a CD56-positive cell as one having a nucleus (CD56 stains the cell surface).

Reportedly, high levels of CD 56-positive NK cells in peripheral blood are associated with poor reproductive outcomes (V.I. MICHOU et al [47], M.Y. THUM et al [48]). A percentage of NK cells over 18% in peripheral blood is highly associated with recurrent spontaneous miscarriages and recurrent implantation failure (K. KING et al [49]).

Inter-relation between peripheral NK cells and uNK cells in infertile women and in those with recurrent miscarriages is difficult to interpret. Also, pbNK cells do not correlate with uNK in terms of number and function. Moreover, studies failed to find differences in pre- and post-conception pbNK levels in both women with recurrent miscarriages and healthy controls (A.E. BEER et al [40], C. PERRICONE et al [50]). Some recent studies found no correlations between the levels of uNK during luteal phase and pregnancy outcome (E. TUCKERMAN et al [51], K. KURODA et al [52]). Assessment of the uNK number is limited by their expression that significantly varies over the menstrual cycle. Thus, the uNK number follows a rapid upward trend after ovulation, being positively regulated by IL-5 synthesis in stromal cells, under progesterone overexpression (J. WILKENS et al [53]). Other factors influencing access to uNK are local edema, site in the uterus, and depth from the epithelium surface. Given the difficulties in standardizing this evaluation and its invasive character, counting uNK is not routinely recommended and is currently used solely for research purposes.

NK cells relation with infertility

Despite recent advances in reproductive immunology, areas of controversy remain. Currently, the relationship between uNK and reproductive outcome is insufficiently elucidated.

Several studies have reported an association between increasing the number of uNK cells in the midluteal endometrium and reproductive failure (O. CHAZARA et al [36], S. QUENBY et al [54], K. CLIFFORD et al [55]). In particular, there is convincing evidence in relation to the increase in endometrial uNK density in the case of repeated pregnancy losses, defined here as three or more consecutive spontaneous abortions. RPL is a predominant disorder affecting 1-2% of couples and a major cause of physical and psychological morbidity (R. RAI et al [56]). Furthermore, RPL is associated with an increased probability of obstetric and perinatal complications in a subsequent pregnancy (E. JAUNIAUX et al [57]).

A meta-analysis of 22 studies investigating the relevance of NK cells in embryo-implantation, recurrent miscarriages and female infertility documented the lack of correlations between the percentage of uNK in infertile women and healthy controls. A similar aspect was observed with regard to the expression of uNK in women with recurrent miscarriages versus fertile controls. However, the same study found a significantly higher expression of peripheral NK, both numerical and as a percentage, in infertile women, and in those with recurrent miscarriages, compared to healthy controls (S. SESHADRI et al [58]). In addition to the increase in number of uNK cells in infertile women, studies demonstrated an increase in their cytotoxic activity (P.M. EMMER et al [59], K. SHAKHAR et al [60]). Also, a favorable reproductive outcome appears to be paralleled by a decrease of NK cell activity during pregnancy. Similarly, an intense cytotoxic activity is positively correlated with abortion and an unfavorable outcome (H. YAMADA et al [61]).

Low levels of NK activity predict the success rate of immunotherapy for recurrent spontaneous activity (C. PERRICONE et al [50], K. KATANO et al [62]). A similar pattern of increased NK cells cytotoxicity was observed in infertile women (H. MATSUBAYASHI et al [63]). Moreover, an elevated activity of peripheral blood NK cells proved to be a risk factor for infertility (H. MATSUBAYASHI et al [64]). Overexpression of IL-2 is positively correlated with high NK cell activity and it appears to have prognostic value in pregnancy outcome (H. HADINEDOUSHAN et al [65]). Up-regulation of specific NK cell activation markers (NKp46, NKp44, and NKp30) is noted in women with recurrent spontaneous abortion and implantation failure, suggesting that dysregulation of NK cytotoxicity is relevant for reproductive outcome. Elevated systemic levels of IFN-gamma and TNF-alphaare associated with high levels of activated NK cells, butdo not correlate with implantation success (M.Y. THUM et al [66]). An imbalance between inhibitory and activating receptor expression was also found in women with implantation failures. Infertile women have a higher level of expression of NK activation markers than controls (E.I. NTRIVALAS et al [67]). Unsurprisingly, p-regulation of CD94, an inhibitory receptor for NK cells, is negatively correlated with cytotoxic function in implantation failure (C.B. COULAM et al [68]). Unbalanced activating and inhibitory receptors may explain the poor reproductive outcome (A.M. IONESCU et al [69]).

Peripheral blood NK cells cytotoxic function does not correlate with percentages and absolute counts of different subsets (J. KWAK-KIM et al [7]). Therefore, an increased level of peripheral NK cells does not imply their increased activity.

Evaluation of peripheral blood NK cells is a promising diagnostic tool for infertility and recurrent spontaneous abortion, and a potential target of immunotherapy. However, it is important to understand that NK cell indices are not static parameters but vary considerably in different contexts (K. SHAKHAR et al [70], K. TERAO et al [71]).

It has been suggested that uNK cells play an important role in embryo-implantation and that an increased cytotoxic activity of peripheral and uNK cells can affect IVF outcome

(A. FUKUI et al [72]). However, studies regarding NK cells and in vitro fertilization outcome bring contradictory results (A.E. BEER et al [40], M.Y. THUM et al [48], T. BACZKOWSKI et al [73]). Recent studies showed no significant correlation between the number of activated peripheral NK cells and the success rate of embryo implantation in IVF treatment (M.Y. THUM et al [48]; [66]). These differences may reflect heterogeneity in infertility population and multifactorial etiologies for infertility. Also, the number of uNK cells does not appear to predict pregnancy outcome. No significant difference in uNK numbers was observed in women who miscarried versus women who had a live birth in a subsequent pregnancy (E. TUCKERMAN et al [51]).

In terms of cytotoxic activity, significantly increased perforin expression of NK cells was observed in women with miscarriages with abnormal karyotype, compared with those with normal karyotype (H. YAMADA et al [74]). Along with an increased cytotoxic activity of NK cells, a significant activation of leucocytes was also detected in the decidua of women with unexplained recurrent spontaneous abortion (K.C. QUACK et al [75]). In terms of uNK cytotoxic function, as revealed by the activation/inhibitory repertoire, activation receptors such as CD94 (the early activation receptor) are down-regulated in women with miscarriage and normal karyotype compared to women with miscarriage and abnormal karyotype, which was interpreted in the etiological context of sporadic miscarriage with normal karyotype (H. YAMADA et al [74]).

Systemic corticosteroid therapy proved to reduce the number of uNK in women with recurrent miscarriage (S. QUENBY et al [76]). However, immunosuppressive agents create an environment for potential severe and irreversible side effects that do not justify their usage during pregnancy.

Conclusions

Uterine NK cells were proved to be involved in implantation and early embryo survival. They promote decidual angiogenesis and the remodeling of uterine spiral and trophoblast invasion. It is speculated that their major function is to assist fetal development, and to create a tolerant maternal fetal microclimate. An increase uNK cell density of more than 5% of the total number of cells in the luminal endothelial layer is highly associated with implantation failures, recurrent spontaneous abortions, infertility and pre-eclampsia. Also, a percentage of NK cells over 18% in peripheral blood is associated with recurrent spontaneous miscarriages and recurrent implantation failure. However, many studies still produce contradictory results and have not yet reached a consensus about normal levels of uNK and pbNK.

Immune regulation is of undeniable importance in successful reproductive outcome. Results regarding the clinical relevance of peripheral and uterine NK cells counts and cytotoxic activity bring inconclusive data. Further studies on the predictive value of these cells on the obstetrical outcome are needed. The evaluation of peripheral blood NK cells is a promising diagnostic tool for infertility and for recurrent spontaneous abortion, and a potential target of immunotherapy.

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