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Expression of natural cytotoxicity receptor NKp46 on peripheral blood natural killer cells in women with a history of recurrent implantation failures

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Abstract

Aim: The peripheral blood natural killer (NK) cells diversity is highly complex; recent studies described more than a thousand phenotypes sharing NK cell receptors, across the leukocyte lineages. In this study, we investigated the expression of NKp46 in peripheral blood NK cells in women with a history of recurrent implantation failures (RIF) with euploid embryos with pre-implantation genetic diagnosis (PGD) and control group (donors of oocytes and surrogate mothers).

Methods: The expression of NKp46 in peripheral blood lymphocytes and NK cells from women with RIF (n = 57) and control group (n = 50) was analyzed with three-color flow cytometry.

Results: The percentage of NKp46^{+}NK cells was significantly higher in women with RIF compare with the control group and high amount of NKp46^{+}NK cells (>13% of total lymphocytes) was a poor prognostic factor for embryo implantation. Also, women with RIF had a low amount of NKp46^{neg}NK cells, which was a negative prognostic factor for embryo implantation. The analysis of NK subpopulations, on the basis of NKp46 expression, also revealed that NKp46^{neg}NK in low amounts (<20% of NK cells) and NKp46^{dim} in high amounts (>50% of NK cells) are also negative prognostic factors for embryo implantation.

Conclusion: Our results support the clinical significance of the NKp46 expression on NK cells in women with RIF. We suggest that the low level of NKp46^{neg} subset in women with RIF may be a result of an imbalance in the differential development of ILC subsets toward cytotoxic ILC (NK cells), which in turn is a negative condition for successful embryo implantation.

Key words: innate lymphoid cell, natural killer cell, NKp46, recurrent implantation failure.

Introduction

Innate lymphoid cells (ILCs) are functionally heterogeneous and plastic cell populations and are important effector cells in disease and tissue homeostasis. Natural killer (NK) cells are the predominant innate lymphocyte subsets that mediate anti-tumor and antiviral responses.[1]

The role of NK cells in human reproduction has been studied for decades.[2–5] Many of the articles

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Table 1 Age, obstetric, and infertility histories of women with RIF and controls

	Age (average)	IVF failure (average)	Deliveries (average)	IVF cycles (average)
Controls (n = 50)†	29,5 ± 3,6	0	2,8 ± 0,9	4,0 ± 2,5
RIF (n = 57)	34,3 ± 2,0	4,8 ± 1,5	0	4,8 ± 1,5
P	NS	<0.01	<0.01	NS

†The control group consists of oocyte donors and surrogate mothers. and NS, not significant; RIF, recurrent implantation failures.

demonstrate a close relationship between the accentuated parameters of NK cells, both quantitative and functional, and reproductive failures – such as recurrent implantation failures (RIF) and recurrent pregnancy loss. The contradictory nature of the results from all these studies suggests that although abnormal NK cell counts or function may contribute to RIF, there is insufficient evidence from which to draw firm conclusions.[2]

The activity of NK cells is tightly regulated by a combination of cell surface-expressed inhibitory and activating receptors. NKp46 is a major NK cell-activating receptor that is involved in the elimination of target cells.[6,7]

Some studies suggest that regulation of NKp46 expression in various types of NK cells may be one of the key factors in reproductive failure, and analysis of NKp46 expression may be a useful tool in investigating and diagnosing reproductive failures, such as RPL and implantation failures.[5,8]

Previously,[9] we have found that a fraction of NKp46^{+}NK cells has prognostic value for accentuated NK cytotoxicity status, both low and high. Since our previous study was conducted only in healthy persons, the next stage of our work and the aim of this study is to estimate the prognostic value of the NKp46 $^{+}$ cell fraction in a cohort of infertile women, whose IVF failures could be associated with NK abnormalities.

Methods

Study subjects

The expression of NKp46 in peripheral blood NK cells from women with previous implantation failure with PGD embryos (at least three lost in IVF cycle, and at least one lost with PGD embryo) (n = 57) and control group – women who are donors of oocytes (at least one own child) and surrogate mothers (at least one own child and one child as a surrogate mother) (n = 50) – was analyzed with three-color flow cytometry.

The control group – women under 35 years (mean age 29.5). Oocyte donors had an average of four previous cycles of stimulation. Surrogate mothers had an average of 2.8 births. Both egg donors and surrogate mothers did not experience pregnancy or IVF failure.

The RIF[10] patients – women younger than 40 years (mean age – 34, 3). All women in the group had more than three idiopathic implantation failures, (an average – 4, 8) and at least with one euploid PGD tested embryo (an average – 1, 2) (Table 1).

Women with anatomical, endocrine, infectious or autoimmune disorders, including antiphospholipid syndrome and genetic etiologies of RIF, were excluded from the study.

Peripheral blood samples were taken during the implantation window period, on day 16–20.

The local ethical committee's approval in accordance with the Helsinki Declaration of 1975 on human experimentation and patient's informed consent were obtained.

Assessment of NKp46 expression

To determine the NKp46 expression, 100 μL of the whole blood stained by FITC-, PE- and -Cy5-conjugated monoclonal antibodies to CD3, NKp46 and CD56 (BD Bioscience, San Jose, USA) was used. Washed or lysed and washed, the samples were analyzed by FACScan flow cytometer using CellQuest software (BD Bioscience, San Jose, USA). Lymphocyte population was determined using forward versus side scatter (FSC vs SSC) gating. The lymphocyte gate is further analyzed for CD3, CD56, NKp46 expression.

The baseline NKp46 expression on NK cells was assessed by flow cytometry. The CD3^{negative} CD56^{+} NK cells were gated from the total lymphocyte population – %NK. In all the samples, all CD56^{bright} NK cells expressed high levels of NKp46. Three different CD56^{dim} NK CD335(NKp46) phenotypes were identified – NKp46^{high}, NKp46^{dim}, and NKp46^{neg} predominance (Fig. 1).

The percentage of NKp46^{+}NK cells (%NKp46^{+}NK) among all the lymphocytes was determined as both NKp46^{high} and NKp46^{dim} subsets.

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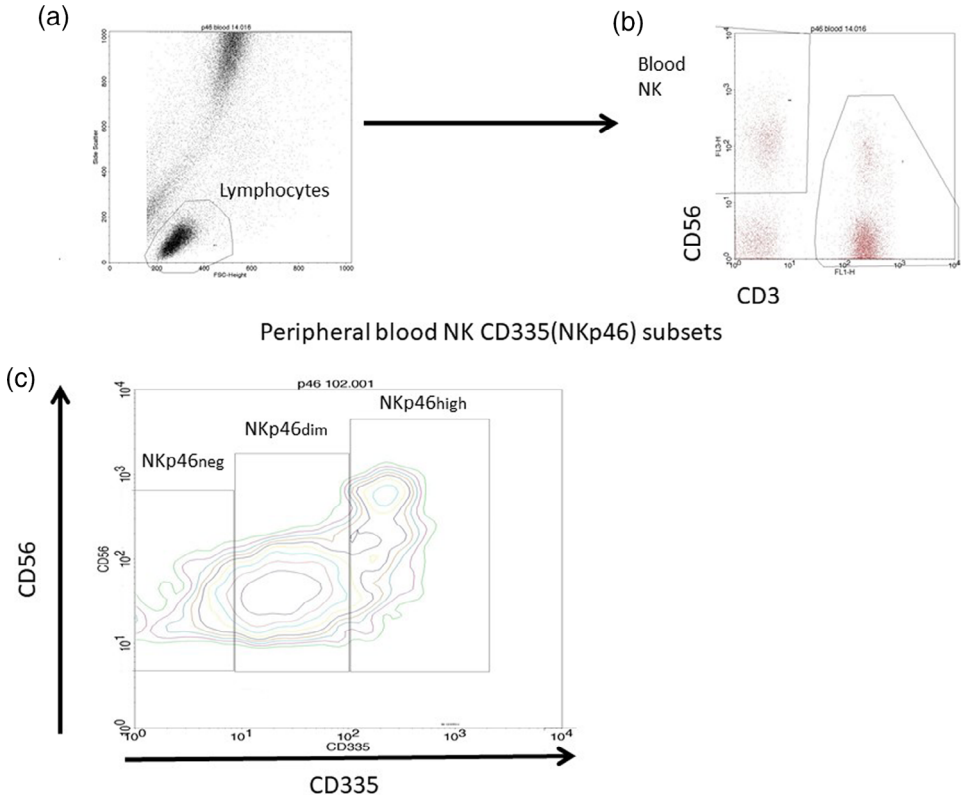


Figure 1 Gating strategy used to identify NK cells and NKp46^{+}NK cell subsets. Representative flow cytometry plots from the peripheral blood of one of the study participants. The lymphocytes were live gated during acquisition using the side and forward scatter dot plot display (a). Furthermore, by using the negative gating strategy, CD3^{negative} lymphocyte population was identified (b). The NK cell population was further identified as CD3^{-}CD56^{+} – (%NK) and differentiated into NKp46^{high}, NKp46^{dim} and NKp46^{neg} subsets (c).

Statistical analysis

The statistical analysis of the results was performed using the Fisher's Exact Test (unpaired, non-parametric, two-sided *P*-value) in Stat version 3.0 for Windows Graph Pad Software Inc., San Diego, CA, USA).

Results

The percentage of peripheral blood NK cells (CD3^{-}CD56^{+}) did not differ between the groups (P = 0.353) (Fig. 2). But there was a difference in the percentage of NKp46^{+}NK cells (CD3^{-}CD56^{+}CD335^{+}) between the groups.

The women with RIF had a higher percentage of NKp46^{+}NK compare with a control group (P = 0.008) (Fig. 3). The amount of NKp46^{+}NK (more than 13% of total lymphocytes) was a negative prognostic factor for embryo implantation (OR = 3.512, P = 0.027).

Further analysis revealed that the low amount of NKp46^{neg} NK cells (less than 1,7% of total lymphocytes) also is a negative prognostic factor for embryo implantation (OR = 3.168, P = 0.006) (Fig. 4). We revealed that women with RIF had a high amount of NKp46^{+}NK cells and simultaneously a low amount of NKp46^{neg}NK cells, both of these parameters were negative prognostic factors for embryo implantation.

As described in the Methods section, three different CD56^{dim} NK CD335(NKp46) phenotypes were identified – NKp46^{high}, NKp46^{dim}, and NKp46^{neg} cells (Fig. 1).

We have calculated the percentage composition of each subpopulation in total NK cells in groups and revealed a significant difference, in particular, for NKp46^{neg} and NKp46^{dim} subpopulations (Fig. 5). The women with RIF had a significantly higher amount of

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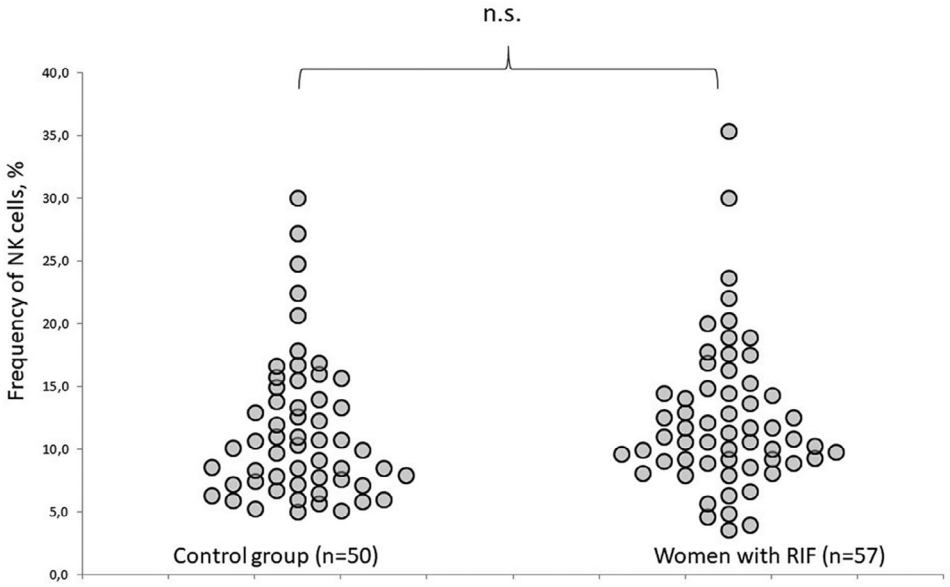


Figure 2 Frequency of NK cells in peripheral blood in women with recurrent implantation failures (RIF) and control group. $P = 0.353$. n.s, not significant.

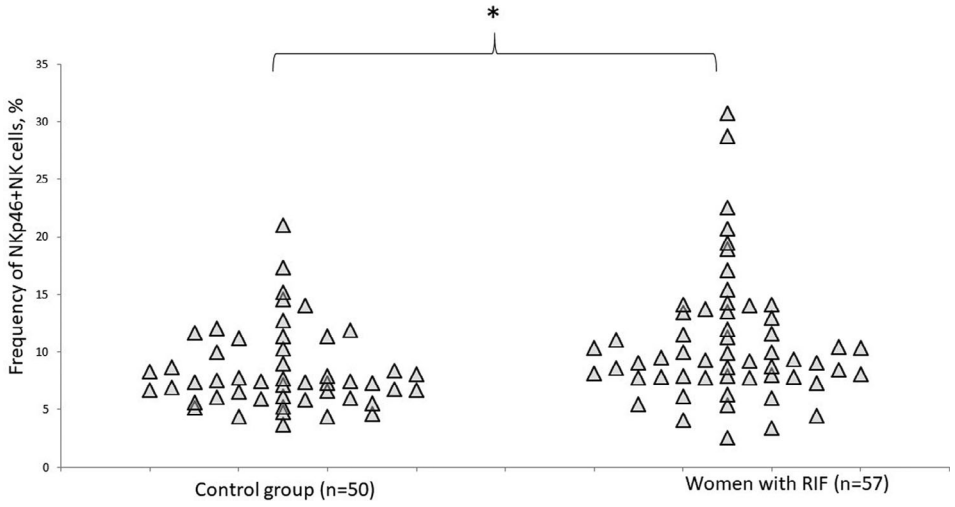


Figure 3 Frequency of $NKp46^{+}NK$ cells in peripheral blood in women with recurrent implantation failures (RIF) and control group. $*P = 0.008$.

$NKp46^{dim}$ and lower of $NKp46^{neg}$ ($P = 0.0001$ and $P = 0.0005$, respectively).

With respect to the different NKp46 phenotypes, we found that the predominance of $NKp46^{dim}$ cells (>50% of NK cells), particularly in women with RIF, is a negative prognostic factor for implantation failure (OR = 4.853, $P = 0.0002$), compared with control group.

Low amount of $NKp46^{neg}$ (<20% of NK cells) in women with RIF also was a negative prognostic factor for implantation failure (OR = 4.714, $P = 0.001$) compared with control group.

Discussion

Natural killer (NK) cells have vital functions in human immunity and reproduction. In the innate and adaptive immune responses to infection, particularly

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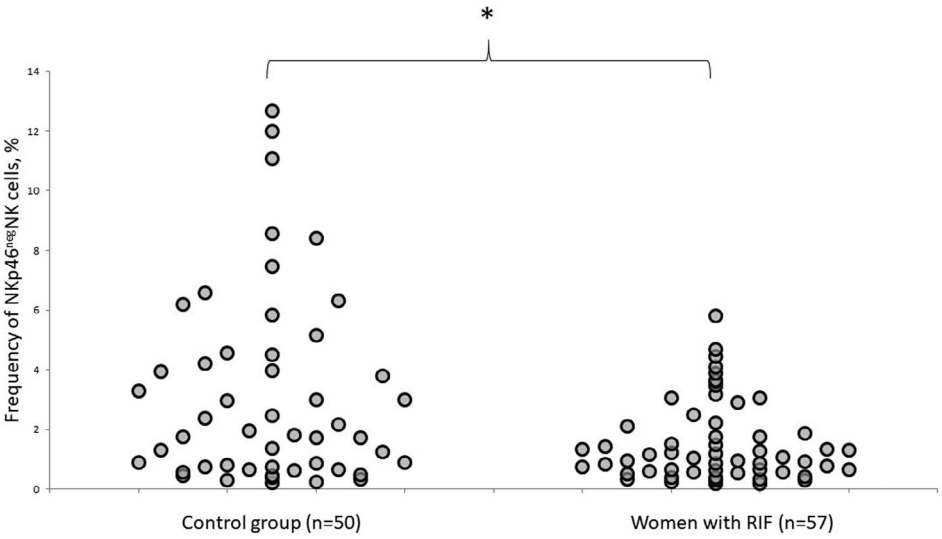


Figure 4 Frequency of NKp46^{neg}NK cells in peripheral blood in women with recurrent implantation failures (RIF) and control group. *P = 0.006.

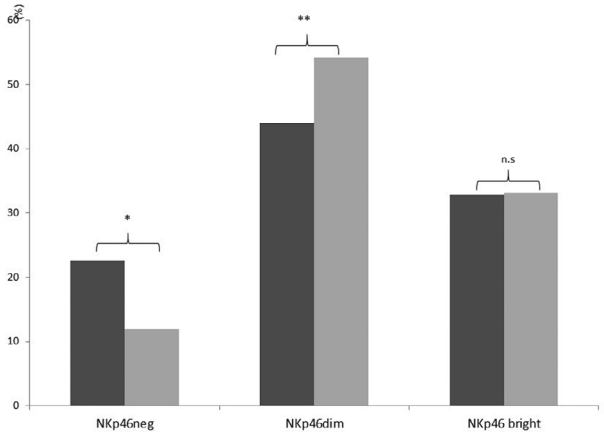


Figure 5 The percentage composition of different subpopulation – NKp46^{high}, NKp46^{dim} and NKp46^{neg} in total NK cells women with recurrent implantation failures (RIF) and control group. (■), Control group (n = 50); (■), women with RIF (n = 57). n.s, not significant; *P = 0.0005; **P = 0.0001.

viruses, NK cells respond by secreting inflammatory cytokines and killing infected cells.[11]

In reproduction, NK cells are critical for the genesis of the placenta, the organ that controls the supply of oxygen and nutrients to the growing fetus. A lot of studies have confirmed the association of NK cells with implantation failures, recurrent miscarriages (RM) or infertility.[2,12,13]

NKp46 is a major NK cell-activating receptor that is involved in the target cell elimination. It was suggested that NKp46 signaling directly regulates the NK lytic immune synapse from early formation to late function. Thus, it is directly involved in cytotoxic activity.[14] Also, cross-linking with anti-NKp46 mAb results in calcium release and the secretion of IFN-g and TNF-a by NK cells and blocking NKp46 signaling with specific mAbs can result in reduced NK cell cytotoxicity of certain tumor cell-lines.[15] The clinical relevance of the NKp46 expression on NK cells has been confirmed in numerous research works.[16–18]

Recently, we have shown that the frequency of the NKp46^{+}NK cells correlates with cytotoxic activity and has significant prognostic value for accentuated NK cytotoxicity status indications, both low and high. Those results showed that the NKp46 expression is a 'link' between an NK cells frequency and their function and afford grounds for using the assessment of the NKp46^{+}NK cells as a responsive, simple, cheap and reliable method for NK cytotoxicity assessment.[9]

In our study, we revealed that percentage of NKp46^{+}NK was significantly higher in women with RIF compared with the control group and was a negative prognostic factor for embryo implantation, which further suggest a tight connection between the NK cells activity and the NKp46 expression and support the clinical significance of the NKp46 expression on NK cells in women with RIF.

Fukui *et al*. have demonstrated the importance of p46 expression on CD56^{+} lymphocytes in reproduction. They reported decreased expression of NKp46 in peripheral blood and uterine endometrial NK cells in women with previous reproductive failures, such as recurrent pregnancy loss (RPL) and implantation failure, as well as lower production of IFN-γ and TNF-α

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by uterine NK cells from women with RPL compare to control.[5,19,20]

Endometrial immune profiles are registered as deregulated in most of the patients with RIF compared to controls.[21] Some authors confirmed the association of uterine NK cells and infertlity,[22,23]while others found no association.[23,24]

Our study confirmed that there is no association between the main subsets of T and NK cells of peripheral blood and endometrium in healthy fertile women. However, there are associations that exist for some separate subsets, particularly HLA DR^+ cytotoxic T lymphocytes (HLA DR^+ [%] in $CD3^+CD8^+$) and CD8+ NK cells ($CD8^+$ [%] in $CD56^+CD3^-$), which may reflect some regulatory mechanisms.[25] More, in egg donors we found significant correlation in NKp46 expression on NK cells between blood and endometrial lymphocytes,[26] indicating that although the endometrium is a fairly autonomous, but not completely isolated structure.

These studies suggest that regulation of NKp46 expression in various types of NK cells may be one of the key factors in a reproductive failure, and analysis of NKp46 expression may be a useful tool in investigating and diagnosing reproductive failures, such as RPL and implantation failures.[5]

Interestingly, a low amount of $NKp46^{neg}$NK cells also associated with poor prognosis for embryo implantation.

The density of NKp46 surface expression clearly segregated $NKp46^{neg}$, $NKp46^{dim}$ and $NKp46^{high}$ subsets.[27] We found that there is a difference in the percentage composition of different subsets between the groups, in particular, women with RIF have a decreased amount of $NKp46^{neg}$ subset and increased amount of $NKp46^{dim}$ subset compared with the control group. Both of these parameters were negative prognostic factors for embryo implantation. Our previous studies of NK cells showed that accentuated parameters in this population are often adverse.[28–30]

NK cells (subsets pool) are a subset of ILCs, that mirror CD8+T cells, thus, they may be termed 'cytotoxic ILCs'. Innate lymphoid cells (ILCs) are a growing family of immune cells that mirror the phenotypes and functions of T cells. ILCs develop from hematopoietic precursors that may migrate from their primary site of production into infected and injured tissues, where they complete their maturation, similar to the differentiation of naïve T cells into TH effectors. Cytokines produced by local cells as well as stress ligands and bacterial and dietary compounds regulate the maturation and activation of ILCs into effectors that play a major role in early immune responses to pathogens and symbionts, helminths and allergen.[1,31,32]

Taking into account our results, we can speculate that the low level of $NKp46^{neg}$ subset in women with RIF may be a result of an imbalance in the differential development of ILCs subsets toward cytotoxic ILCs (NK cells), which in turn is a negative condition for successful embryo implantation. Further research with a more accurate analysis of ILCs subpopulations is needed to assess their impact on the reproductive process.

We showed the NK-associated difference between a group of women with RIF and a healthy control group. But we do not know if the reduced amount of $NKp46^{neg}$NK cells and increased amount of $NKp46^{dim}$NK cells, founded in women with RIF, is the cause or consequence of RIF, and whether these deviations will have a negative prognostic value for the implantation. We are now conducting a double blind study with randomized groups to determine more accurately if such differences are the cause of RIF.

Here, we further suggest a tight connection between the NK cells activity and the NKp46 expression and support the clinical significance of the NKp46 expression on NK cells in women with RIF. Further, we hypothesize that women with a history of RIF have an imbalance in the differential development of ILCs subsets toward cytotoxic ILC (NK cells).

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Disclosure

None declared.

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