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Research paper

Enumeration of peripheral blood NKp46 positive NK lymphocytes reflects NK cytotoxic activity *in vitro*

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ABSTRACT

Natural killer (NK) cells are the predominant innate lymphocyte subsets that mediate anti-tumor and anti-viral responses. The monitoring of NK cells function is important in various physiological and pathological conditions. Different approaches have been used to directly or indirectly evaluate NK cells activities.

The purpose of this study was to investigate the correlation between the number of NK cells and cytotoxic activity of NK cells and to determine whether NKp46$^+$NK cells reflect NK cytotoxicity status.

In our study, we retrospectively analyzed laboratory data on NK cytotoxicity and NK lymphocyte levels of 4896 infertile women which underwent routine immunology investigation after IVF failures. In healthy women, NKp46 expression was assessed on NK cells (n = 214) and cytotoxicity activity was evaluated with regard to NKp46 expression. We found that despite a significant correlation coefficient (n = 4689, r = 0.447), the correlation with cytotoxicity is maintained only within the zones with a low or high NK cells frequency. NK cells frequency has no significant prognostic value for their cytotoxicity – within the medium NK frequency zone the samples may have any cytotoxicity, both reduced and elevated.

However, our data demonstrate that NKp46$^+$NK cells frequency correlates with cytotoxicity activity even more significantly than the NK cells frequency (n = 214, r = 0.67 and r = 0.62, respectively) and has significant prognostic value for the abnormal NK cytotoxicity status indications, both low and increased.

Our results further support an important role of NKp46 in NK cells killing and afford grounds for using the measurement of the NKp46$^+$NK cells frequency as an alternative method for abnormal NK cytotoxicity status indication, which is responsive, simple and reliable.

1. Introduction

Natural killer (NK) cells are large granular lymphocytes endowed with the inherent capacities to recognize and kill foreign, infected, and malignant cells and also to modulate other aspects of the immune system through their rapid production of numerous cytokines and chemokines (Caligiuri, 2008; Orr and Lanier, 2010).

The accurate measure of the cytotoxic function is critical for investigation of a number of immunodeficiency states (Ham and Billadeau, 2014; Orange, 2013), reproductive failures (Chernyshov et al., 2010) and utilization of these cells as immunotherapeutic agents (Mandal and Viswanathan, 2015).

Different approaches have been used to directly or indirectly evaluate NK cells and analyze various parameters (Claus et al., 2009; Dons'koi et al., 2011).

The most common parameters in NK cells evaluation are their number and functional characteristics, *e.g.* cytotoxicity, degranulation activity and cytokines production (Queirós, 2014). Some techniques have been developed for measuring NK cytotoxic activity by flow cytometry (FCA – flow cytometry assay), offering an alternative method to the standard and widespread chromium (51Cr) release assay (Queirós, 2014; Dons'koi et al., 2011).

However, the FCA with K562 cells method, which is considered a gold standard, is not infallible. Lysis of sensitive target cells depends on the cells cycle, growth and medium condition, labeling procedure, as well as on the correct, effector-comfortable test design (incubation time, volume, real NK/K562 and PBMC/NK ratio) that can affect the probability of effector-target contact. Given the above and the fresh

Abbreviations: NK%, natural killer cells frequency; FCA, flow cytometry assay; PBLs, peripheral blood lymphocytes; PI, propidium iodide; E/T ratio, effector/target ratio; IVF, *in vitro* fertilization; NKRs, NK cell receptors

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peripheral blood as a prerequisite, it is almost impossible for any laboratory to perform the tests routinely.

The assessment of the number of NK cells is a technically simple procedure; however, this parameter alone does not represent the functional potential of NK population.

Many phenotypically distinct subpopulations of NK cells have been discovered, usually by dividing cells using cell-surface markers. These subpopulations are typically described as related to activation or developmental status of the cells (Dons'koi et al., 2011; Forslund et al., 2012). However, how these distinct phenotypes correlate with behavior of, *e.g.*, NK-target interactions is less widely understood (Vanherberghen et al., 2015).

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. NKp46 is uniquely expressed on all NK cell subsets and has been suggested as a possible target for NK cell ablation and as a pan NK cell marker (Narni-Mancinelli et al., 2011; Walzer et al., 2007). To date, few pathogen-associated ligands and cellular co-ligands for NKp46 have been documented; it has been shown that the latter plays a role in regulating the NK lytic immune synapse from early formation to late function (Hadad et al., 2015). The data also suggest that NKp46 signaling directly regulates the NK lytic immune synapse from early formation to late function. NKp46 was considered to be a predictive biomarker for clinical outcome after allogeneic transplantation, in patients with AML (Chretien et al., 2017). The purpose of this study was to determine whether the frequency of NKp46$^+$NK cells can be indicative of abnormal NK cytotoxicity.

2. Methods

2.1. Blood specimens

Venous blood was obtained from healthy individuals – Caucasian women (local Ukrainian population) under 35 years. None had an active infection, allergy, immunodeficiency or a history of autoimmune disease. Also, they had no history of reproductive and IVF failures and had at least 1 healthy child (n = 214). All the study subjects have signed informed consent before they entered the study. The cell isolation and all the study procedures were started at the blood sampling date (4 h at the latest). During the retrospective study, we analyzed our laboratory data on NK cytotoxicity and the NK frequency of 4896 somatically healthy Caucasian women under 42 years (the average age was 36 years) with normal BMI and without any chronic diseases, which could be contraindications to IVF. All of them had at least one reproductive failure or IVF failure (on average, 3.2 IVF failures).

2.2. Flow cytometry NK cytotoxicity assay (FCA)

The target K562 cells were labeled with 5 μM CellTracker™Green CMFDA (5-chloromethylfluorescein diacetate) (Molecular Probes, Eugene, Oreg. USA) for 20 min at 37 °C in a humidified 5% CO_2 incubator. The labeled cells were washed in PBS twice, resuspended in RPMI1640 with 10% NBCS and counted using Flow-Count™ Fluorospheres (Beckman Coulter, USA). The effector cells were co-incubated at the E:T ratios of 25, 12.5 and 7.5 for 2.5 h at 37 °C in the atmosphere of 5% CO_2 (air mixture). After the incubation period, the cells were mixed with 10 μL of 2 mg/mL PI solution (SIGMA) in PBS to stain dead cells. For each E/T ratio, the NK cytotoxicity was measured by analyzing 10,000 target cells per sample using a FACScan flow cytometer (BD Bioscience, San Jose, USA) equipped with CellQuest software. The background target cells death was identified based on the cells incubated in the absence of effector cells.

For each E/T ratio specific NK cytotoxicity was calculated as a percentage of PI positive (dead cells) minus the spontaneous lysis level. After that, we construct a calibration curve and calculated virtual cytotoxicity under the E/T ratio condition (E/T ratio - 15/1, 10/1 or 20/1).

The level of spontaneous lysis was determined as the percentage of dead cells under the same conditions of incubation, but in the absence of effectors cells. Less than 5% of the target cells spontaneous lysis was observed in these experiments.

The concept of “normal cytotoxicity” was based on the reference values of NK cytotoxicity favorable for reproductive prognosis in accordance with (Chernyshov et al., 2014).

The cytotoxicity activity levels of > 30% (E/T ratio 10/1) and < 10% (E/T ratio 10/1) were considered as high and low, respectively.

To measure the E/T ratio correctly, we have labeled the PBLs permeabilized by the 0.25% Triton and 1% paraformaldehyde solution with PI; after that, we added labeled K562 cells. The ratio of E/T in each sample was measured by flow cytometry (PBLs – FL3, K562 cells – FL1 Supplementary Fig. Ap.1)

For the target-gate correction (FL1 lost) we used (50% permeabilized K562 culture) 100 μL of the target cells permeabilized by 50 μL ethanol for 10 s. After vortexing, 100 μL of PBS and 100 μL of unaffected targets were added. The obtained suspension consisted of 50% live and 50% permeable cells (Supplementary Fig. Ap.2).

2.3. 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).

2.4. Gating strategy for NKp46$^+$NK cells

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^{medium}, and NKp46^{neg} predominance.

The percentage of NKp46$^+$NK cells (%NKp46$^+$NK) among all the lymphocytes was determined as both NKp46^{high} and NKp46^{medium} subsets (Supplementary Fig. Ap.3.)

2.5. Statistical analysis

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

3. Results

3.1. Correlation between cytotoxicity and frequency of NK cells in PBMCs (%NK)

The specific lysis value measured by FCA (n = 4869) significantly correlated with %NK (r = 0.447) (Fig. 1). We have divided them into subgroups according to %NK and analyzed the correlation ratio in each of the subgroups. Our results have shown that the correlation ratio continues to persist only in subgroups with high (17–40%, n = 690, r = 0.167, 14–17%, n = 712, r = 0.121) and low (< 6%, n = 650, r = 0.269) %NK. Whereas within different subgroups with the medium %NK of 12–14% (n = 645, r = 0.020), 10–12% (n = 694, r = 0.08), 8.3–10% (n = 704, r = 0.016), 6–8% (n = 703, r = 0.112) there is no correlation with NK cytotoxicity (Fig. 2).

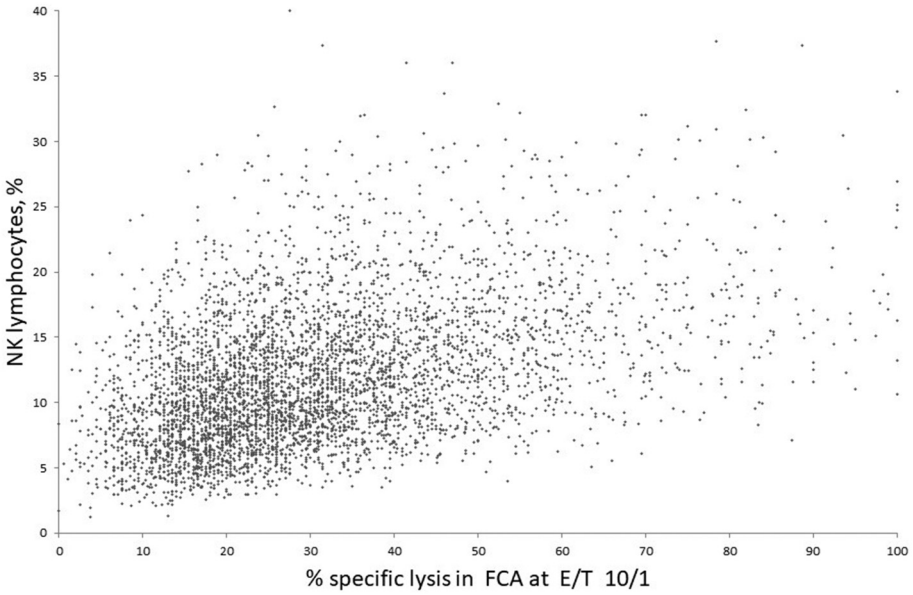

Fig. 1. Correlation of specific lysis in FCA at E/T ratio 10/1 with NK frequency in group of infertile women which underwent routine immunology investigation after IVF failures. (n = 4869, r = 0.447.)
The NK frequency was defined as % $CD3^{negative}$ $CD56^{+}$ NK cells gated from the total lymphocyte population.

3.2. Prognostic value of %NK and %NKp46^{+}NK for cytotoxicity status

Next, we investigated whether %NK or %NKp46^{+}NK have prognostic values for cytotoxicity status (n = 214). In this group, %NKs correlates with NK cytotoxicity (r = 0.62, Fig. 3a), but %NKp46^{+}NK has higher correlation coefficient as far as NK cytotoxicity is concerned (r = 0.67, Fig. 3b). Also, we analyzed the correlation between cytotoxicity and a separated $NKp46^{high}$ or $NKp46^{medium}$ subset, but both correlation coefficients weren't higher than the correlation coefficient between the NK frequency and cytotoxicity. Further, NKp46^{+}NK cells were determined as both $NKp46^{high}$ and $NKp46^{dim}$ subsets.

To investigate whether the %NK is a prognostic marker for cytotoxicity status, patients were divided into subgroups according to %NK, and rate of the subjects with normal cytotoxicity (10–30%, E/T - 10/1) was calculated for each of the subgroups (Fig. 4).

Our data indicate that the high %NK (such as > 17% or > 14.5%) is not a significant prognostic marker for an increased (> 30%, E/T – 10/1) cytotoxicity (RR = 1470, p = .07 and RR = 1.125, p = .82, respectively) compared to the medium (7,3–14,5%) %NK. Also, low %NK (such as < 5.5% or 7.3%) was not prognostically significant for a reduced (< 10%, E/T – 10/1) cytotoxicity level (RR = 1.657, p = .31 and RR = 1.252, p = .79, respectively) (Fig. 4), compared to samples with medium %NK.

Next, we investigated whether %NKp46^{+} NK has a prognostic value for cytotoxicity, in the same way described above; this time, patients were divided according to %NKp46^{+}NK, and the rate of subjects with normal cytotoxicity (10–30%, E/T 10/1) was calculated for each of the subgroups (Fig. 5).

Subgroup analysis revealed that the presence of %NKp46^{+}NK cells in the amount of > 12.5% and > 11.2% has significant prognostic value for high (> 30%, E/T – 10/1) NK cells cytotoxicity in these subgroups (RR = 2.792, p < .0001 and RR = 2.489, p < .0272, respectively), compared to the samples with medium %NKp46^{+}NK (5.3–11.2%).

Importantly, low %NKp46^{+}NK (< 4%) has even more significant prognostic value for NK with low (< 10%, E/T – 10/1) cytotoxicity state (RR = 4.025, p < .0021).

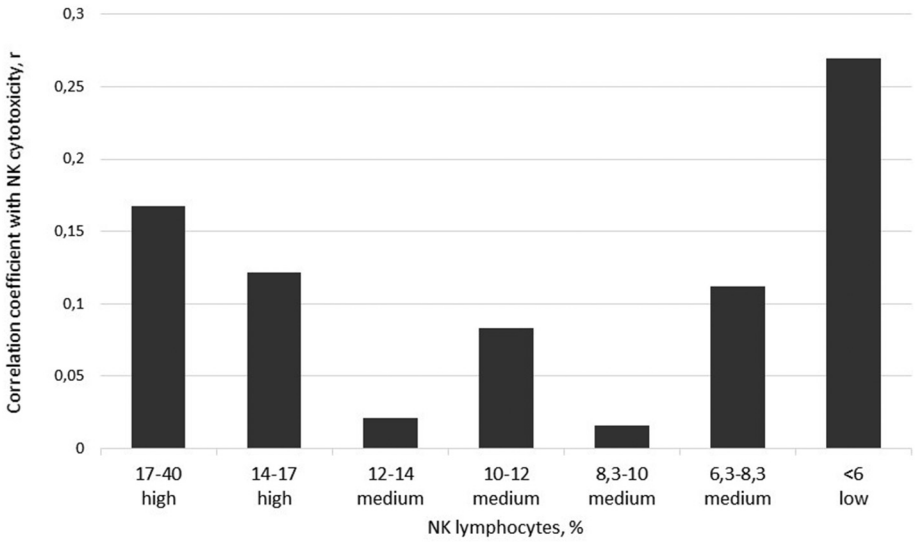

Fig. 2. Correlation of specific lysis in FCA at E/T ratio 10/1 with NK frequency. Samples were divided into subgroups according to NK frequency (high - 14–17%, 17–40%, low - < 6% and medium - 6–8%, 8.3–10%, 10–12%, 12–14% subgroups) and correlation relationship with NK cytotoxicity was analyzed in each subgroup.
High (17–40%, n = 690, r = 0.167, 14–17%, n = 712, r = 0.121) and low frequency of NK (< 6%, n = 650, r = 0.269) correlates with NK cytotoxicity. Medium frequency of NK within different subgroups 12–14% (n = 645, r = 0.020), 10–12% (n = 694, r = 0.08), 8.3–10% (n = 704, r = 0.016), 6–8.3% (n = 703, r = 0.112) doesn't correlate with NK cytotoxicity.

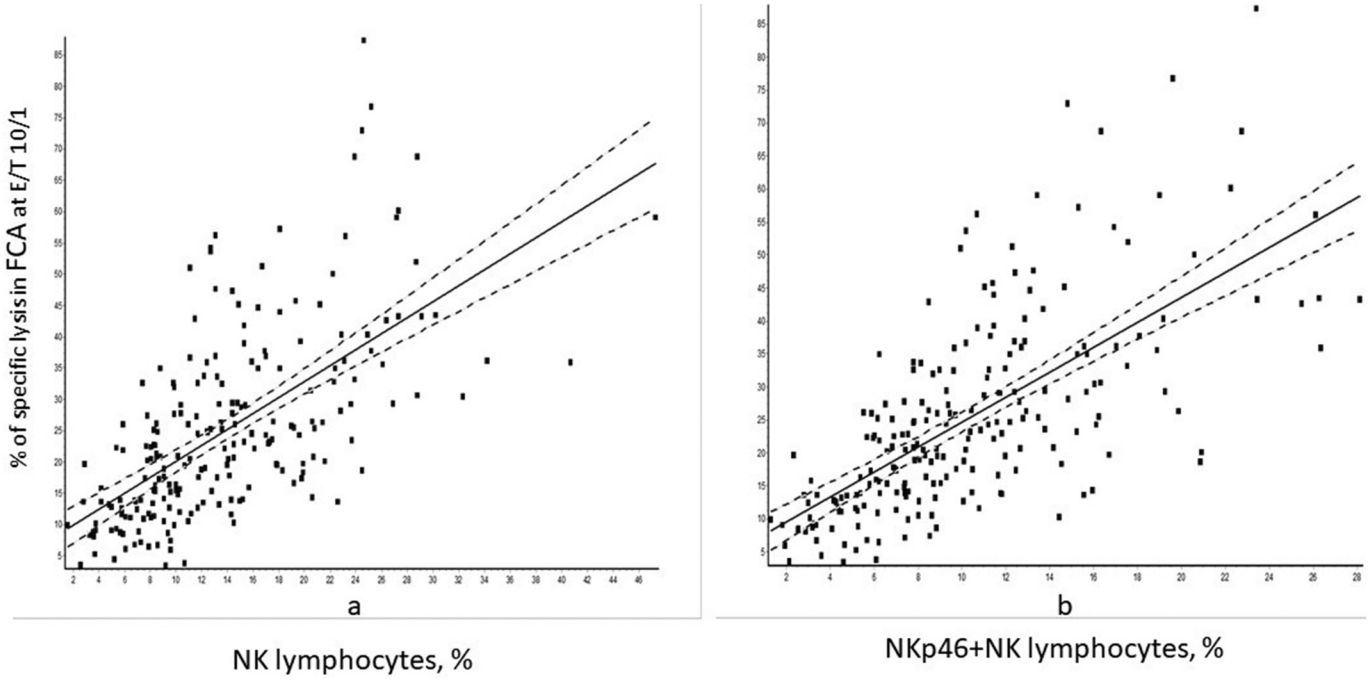

Fig. 3. Correlation of specific lysis in FCA at E/T ratio 10/1 with frequency of NK($r = 0.62$) and NKp46$^+$ NK cells ($r = 0.67$) in group with healthy women ($n = 214$).

4. Discussion

In a clinical laboratory, assessment of NK cells cytotoxicity is increasingly important for the diagnosis and monitoring of the disease progression. Cytotoxic assays allow *in vitro* evaluation of the lytic NK cells activity against tumors or transformed target cells. Since the earliest cytotoxic tests based on the direct visualization of effector/target cell conjugates and the use of trypan blue to exclude nonviable target cells using a light microscope, a variety of cytotoxic assays have been developed (Queirós, 2014).

Despite that, there are still a lot of points to be taken into account when we want to evaluate NK cells activity response. The widely used FCA assay, like the most functional tests, highly depends on the smallest variations in the components and test conditions (incubation time, volume, real NK/K562, PBMC/NK ratio *etc.*); furthermore, NK cytotoxicity assay requires fresh peripheral blood; all this makes it a very complicated method – not only from technical, but also from a logistical point of view.

We have shown that the frequency of NK cells strongly correlates with NK cytotoxicity. Nevertheless, these parameters are not equal. Our data demonstrate that they are characterized by zone dependent linkage – the correlation with cytotoxicity is maintained only within the zones with a low or high NK cells frequency. In other words, we can say that in those zones the functional activity of the NK population is determined mainly by their frequency, but the NK frequency still has no significant prognostic value for their cytotoxicity, and the main reason for that is that samples within the medium NK frequency zone may have any cytotoxicity levels, both reduced and increased.

Taking into account the complexity of NK cells activity regulation by a combination of cell surface expressed inhibitory and activating receptors, it seems logical to assume that a single quantitative parameter couldn't completely define the functional potential of the population. In our previous study, we demonstrated that only a subset of the peripheral blood NK lymphocytes is able to respond to K562, and that only a fraction of this subset determines the entire population NK cytotoxicity levels (Dons'koi et al., 2011).

The peripheral blood NK cells diversity is highly complex; recent studies have described more than a thousand phenotypes sharing NK

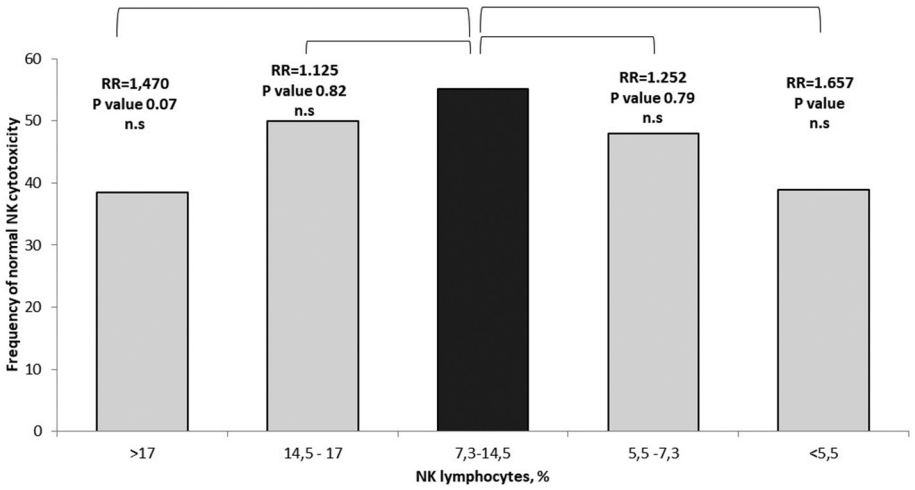

Fig. 4. The NK frequency as a prognostic marker for cytotoxicity status.
Patients were divided into subgroups according to NK cell frequency, and rate of subjects with normal cytotoxicity (10–30%, E/T - 10/1) was calculated for each subgroup.
Relative risk for low (< 10%, E/T - 10/1) or increased (> 30%, E/T - 10/1) cytotoxicity was calculated in subgroups with low (< 5.5%, $n = 18$, 5.5–7.3%, $n = 17$) an respectively high (14.5–17%, $n = 22$, > 17%, $n = 62$) NK cells frequency compared to subgroup with medium (7.3–14.5%, $n = 95$) NK frequency.

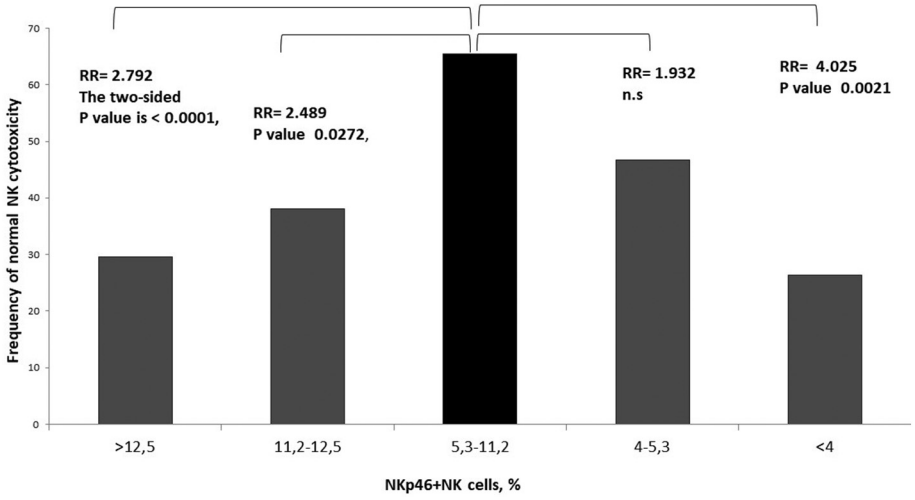

Fig. 5. The NKp46$^+$NK frequency as a prognostic marker for cytotoxicity status.
Patients were divided into subgroups according to NKp46$^+$NK cell frequency, and rate of subjects with normal cytotoxicity (10–30%, E/T - 10/1) was calculated for each subgroup.
Relative risk for low (< 10%, E/T – 10/1) or increased (> 30%, E/T - 10/1) cytotoxicity was calculated in subgroups with low (< 4%, $n = 19$, 4–5.3%, $n = 16$) and respectively high (11.2–12.5%, $n = 22$, > 12.5%, $n = 60$) NKp46 + NK cells frequency compared to subgroup with medium (5.3–11.2%, $n = 97$) NKp46 + NK cells frequency.

cell receptors (NKRs), across the leucocyte lineages (Ansari et al., 2015). NCRs are germ-line-encoded major NK cell-activating receptors comprising NKp30, NKp44 and NKp46. The binding of these receptors to their putative ligands is mostly observed on stressed, tumor and virus-infected cells and triggers lysis of these cells (Horton and Mathew, 2015). 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 (Hadad et al., 2015).

Furthermore, the clinical relevance of the NKp46 expression on NK cells has been confirmed in numerous research works (Han et al., 2018; Chretien et al., 2017; Garcia-Iglesias et al., 2009).

In particular, a group of researchers (Fauriat et al., 2007) previously reported that low NKp46 expression on NK cells was significantly associated with a reduced overall survival (OS) in AML and validated the prognostic value of NKp46 expression at diagnosis in AMLs treated with allo-stem cells transplantation (Chretien et al., 2017). NK cells are associated with implantation failures, recurrent miscarriages (RM) or infertility due to either NK cell cytotoxicity or receptor/gene expression. (Kwak-kim and Gilman-Sachs, 2008). The natural killer cell testing is currently practiced widely in the field of reproductive immunology (Chernyshov et al., 2010, 2014). Fukui et al. have demonstrated the importance of p46 expression on CD56$^+$ lymphocytes in reproduction. They reported decreased expression of NKp46 in peripheral blood in women with previous reproductive failures, such as recurrent pregnancy loss (RPL) and implantation failure (Fukui et al., 2006, 2009).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 (Fukui et al., 2017).

These findings emphasize the clinical significance of this parameter and further suggest a tight connection between the NK cells activity and the NKp46 expression.

Kramer et al. and Pembroke et al. have shown that NKp46 surface density defines, phenotypically and functionally, the different human NK cell subsets. The NKp46^{high} cell subset displayed a significantly higher cytolytic activity than the NKp46^{Dim} subpopulation against K562 cells, as well as IFN-gamma production upon stimulation (Pembroke et al., 2013, 2014; Krämer et al., 2012). We have analyzed the correlation between the cytotoxicity (FCA) and the frequency of NKp46^{high} and NKp46^{medium} subsets, separately; but both correlation coefficients weren't higher than the correlation coefficient between the frequency of NK and the cytotoxicity. Further, NKp46$^+$NK cells were identified as both NKp46^{high} and NKp46^{dim} subsets.

Our data demonstrate that the NKp46$^+$NK cells frequency correlates with cytotoxic activity and has significant prognostic value for abnormal NK cytotoxicity status indications, both low and high.

Similarly, we have shown previously that the accentuated (both high and low) frequency of activated NK was a significant negative predictive factor for embryo implantation while simple NK lymphocytes enumeration does not have clinical significance (Dons'koi et al., 2014). Also, we have observed that the expression of NKp46 on NK surface remains stable for several days after the blood intake; this makes it possible to transport and store the samples [unpublished data]. Our results afford grounds for using the NKp46$^+$NK cells assessment as an alternative to the sophisticated FCA K562 cells assay.

5. Conclusions

We have shown in our work that the frequency of NK cells is strongly correlated with NK cytotoxicity only within the zones with a low or high NK cells frequency. Moreover, NK cells frequency has no significant prognostic value for NK cytotoxicity status, both low and high. We have found that a fraction of NKp46$^+$NK cells has prognostic value for abnormal NK cytotoxicity status, both low and high.

Our results show that the NKp46 expression is a “link” between a NK cells frequency and their function and afford grounds for using the NKp46$^+$NK cells assessment as a responsive, simple, cheap and reliable method for NK cytotoxicity assessment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.112639.

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Declarations of Competing Interest

None.

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References

Ansari, A.W., Ahmad, F., Meyer-Olson, D., Kamarulzaman, A., Jacobs, R., Schmidt, R.E., 2015. Natural killer cell heterogeneity: cellular dysfunction and significance in HIV-1 immuno-pathogenesis. Cell. Mol. Life Sci. 72, 3037–3049. https://doi.org/10.1007/s00018-015-1911-5.

Caligiuri, M.A., 2008. Human natural killer cells. Blood 112, 461–469. https://doi.org/10.1182/blood-2007-09-077438.

Chernyshov, V.P., Sudoma, I.O., Dons'koi, B.V., Kostyuchyk, A.A., Masliy, Y.V., 2010. Elevated NK cell cytotoxicity, CD158a expression in NK cells and activated T lymphocytes in peripheral blood of women with IVF failures. Am. J. Reprod. Immunol. 64, 58–67. https://doi.org/10.1111/j.1600-0897.2010.00825.x.

Chernyshov, V.P., Dons'koi, B.V., Sudoma, I.O., Goncharova, Y.O., 2014. Favorable immune phenotype predicts successful implantation and pregnancy. Immunol. Lett. 162, 217–221. https://doi.org/10.1016/j.imlet.2014.10.022.

Chretien, A.S., Devillier, R., Fauriat, C., Orlanducci, F., Harbi, S., Le Roy, A., Rey, J., Bouvier Borg, G., Gautherot, E., Hamel, J.F., Ifrah, N., Lacombe, C., Cornillet-Lefebvre, P., Delaunay, J., Toubert, A., Arnoulet, C., Vey, N., Blaise, D., Olive, D., 2017. NKp46 expression on NK cells as a prognostic and predictive biomarker for response to Allo-SCT in patients with AML. Oncoimmunology 6. https://doi.org/10.1080/2162402X.2017.1307491.

Claus, M., Greil, J., Watzl, C., 2009. Comprehensive analysis of NK cell function in whole blood samples. J. Immunol. Methods 341, 154–164. https://doi.org/10.1016/j.jim.2008.11.006.

Dons'koi, B.V., Chernyshov, V.P., Osypchuk, D.V., 2011. Measurement of NK activity in whole blood by the CD69 up-regulation after co-incubation with K562, comparison with NK cytotoxicity assays and CD107a degranulation assay. J. Immunol. Methods 372, 187–195. https://doi.org/10.1016/j.jim.2011.07.016.

Dons'koi, B.V., Chernyshov, V.P., Sirenko, V.Y., Strelko, G.V., Osypchuk, D.V., 2014. Peripheral blood natural killer cells activation status determined by CD69 upregulation predicts implantation outcome in IVF. Immunobiology 219, 167–171. https://doi.org/10.1016/j.imbio.2013.09.002.

Fauriat, C., Just-landi, S., Arnoulet, C., Sainty, D., Olive, D., Costello, R.T., 2007. Deficient expression of NCR in NK cells from acute myeloid leukemia: evolution during leukemia treatment and impact of leukemia cells in NCR. Hematology 109, 323–330. https://doi.org/10.1182/blood-2005-08-027979.The.

Forslund, E., Guldevall, K., Olofsson, P.E., Frisk, T., Christakou, A.E., Wiklund, M., Önfelt, B., 2012. Novel microchip-based tools facilitating live cell imaging and assessment of functional heterogeneity within NK cell populations. Front. Immunol. 3, 1–7. https://doi.org/10.3389/fimmu.2012.00300.

Fukui, A., Ntrivalas, E., Gilman-Sachs, A., Kwak-Kim, J., Lee, S.K., Levine, R., Beaman, K., 2006. Expression of natural cytotoxicity receptors and a2V-ATPase on peripheral blood NK cell subsets in women with recurrent spontaneous abortions and implantation failures. Am. J. Reprod. Immunol. 56, 312–320. https://doi.org/10.1111/j.1600-0897.2006.00431.x.

Fukui, A., Ntrivalas, E., Fukuhara, R., Fujii, S., Mizunuma, H., Gilman-Sachs, A., Beaman, K., Kwak-Kim, J., 2009. Correlation between natural cytotoxicity receptors and intracellular cytokine expression of peripheral blood NK cells in women with recurrent pregnancy losses and implantation failures. Am. J. Reprod. Immunol. 62, 371–380. https://doi.org/10.1111/j.1600-0897.2009.00750.x.

Fukui, A., Funamizu, A., Fukuhara, R., Shibahara, H., 2017. Expression of natural cytotoxicity receptors and cytokine production on endometrial natural killer cells in women with recurrent pregnancy loss or implantation failure, and the expression of natural cytotoxicity receptors on peripheral blood natural kille. J. Obstet. Gynaecol. Res. 43, 1678–1686. https://doi.org/10.1111/jog.13448.

Garcia-Iglesias, T., del Toro-Arreola, A., Albarran-Somoza, B., del Toro-Arreola, S., Sanchez-Hernandez, P.E., Ramirez-Dueñas, M., Balderas-Peña, L.M.A., Bravo-Cuellar, A., Ortiz-Lazareno, P.C., Daneri-Navarro, A., 2009. Low NKp30, NKp46 and NKG2D expression and reduced cytotoxic activity on NK cells in cervical cancer and precursor lesions. BMC Cancer 9, 1–8. https://doi.org/10.1186/1471-2407-9-186.

Hadad, U., Thauland, T.J., Martinez, O.M., Butte, M.J., Porgador, A., Krams, S.M., 2015. NKp46 clusters at the immune synapse and regulates NK cell polarization. Front. Immunol. 6, 1–11. https://doi.org/10.3389/fimmu.2015.00495.

Ham, H., Billadeau, D.D., 2014. Human immunodeficiency syndromes affecting human natural killer cell cytolytic activity. Front. Immunol. 5, 1–17. https://doi.org/10.3389/fimmu.2014.00002.

Han, B., Mao, F.Y., Zhao, Y.L., Lv, Y.P., Teng, Y.S., Duan, M., Chen, W., Cheng, P., Wang, T.T., Liang, Z.Y., Zhang, J.Y., Liu, Y.G., Guo, G., Zou, Q.M., Zhuang, Y., Peng, L.S., 2018. Altered NKp30, NKp46, NKG2D, and DNAM-1 expression on circulating NK cells is associated with tumor progression in human gastric cancer. J Immunol Res 2018. https://doi.org/10.1155/2018/6248590.

Horton, N.C., Mathew, P.A., 2015. NKp44 and natural cytotoxicity receptors as damage-associated molecular pattern recognition receptors. Front. Immunol. 6, 1–6. https://doi.org/10.3389/fimmu.2015.00031.

Krämer, B., Körner, C., Kebschull, M., Glässner, A., Eisenhardt, M., Nischalke, H.D., Alexander, M., Sauerbruch, T., Spengler, U., Nattermann, J., 2012. Natural killer p46 High expression defines a natural killer cell subset that is potentially involved in control of hepatitis C virus replication and modulation of liver fibrosis. Hepatology 56, 1201–1213. https://doi.org/10.1002/hep.25804.

Kwak-kim, J., Gilman-Sachs, A., 2008. Clinical implication of natural killer cells and reproduction. Am. J. Reprod. Immunol. 59, 388–400. https://doi.org/10.1111/j.1600-0897.2008.00596.x.

Mandal, A., Viswanathan, C., 2015. Natural killer cells: in health and disease. Hematol. Oncol. Stem Cell Ther. 8, 47–55. https://doi.org/10.1016/j.hemonc.2014.11.006.

Narni-Mancinelli, E., Chaix, J., Fenis, A., Kerdiles, Y.M., Yessaad, N., Reynders, A., Gregoire, C., Luche, H., Ugolini, S., Tomasello, E., Walzer, T., Vivier, E., 2011. Fate mapping analysis of lymphoid cells expressing the NKp46 cell surface receptor. Proc. Natl. Acad. Sci. 108, 18324–18329. https://doi.org/10.1073/pnas.1112064108.

Pembroke, T., Gallimore, A., Godkin, A., 2013. Intrahepatic natural killer cell NKp46 expression drives HCV-associated liver inflammation and viral resistance to treatment with interferon alpha. Lancet 381, S85. https://doi.org/10.1016/s0140-6736(13)60525-5.

Orange, J.S., 2013. Natural killer cell deficiency. J. Allergy. Clin. Immunol. 132 (3), 515–526. https://doi.org/10.1016/j.jaci.2013.07.020.

Orr, M.T., Lanier, L.L., 2010. Natural Killer Cell Education and Tolerance. Cell 142, 847–856. https://doi.org/10.1016/j.cell.2010.08.031.

Pembroke, T., Christian, A., Jones, E., Hills, R.K., Wang, E.C.Y., Gallimore, A.M., Godkin, A., 2014. The paradox of NKp46+ natural killer cells: drivers of severe hepatitis C virus-induced pathology but in-vivo resistance to interferon α treatment. Gut 63, 515–524. https://doi.org/10.1136/gutjnl-2013-304472.

Queirós, M., 2014. When and how to evaluate the natural killer cell function. Annu. Res. Rev. Biol. 4, 3586–3614. https://doi.org/10.9734/arrb/2014/10968.

Vanherberghen, B., Vanherberghen, B., Olofsson, P.E., Forslund, E., Sternberg-simon, M., Khorshidi, M.A., 2015. Classification of human natural killer cells based on migration behavior and cytotoxic response classification of human natural killer cells based on migration behavior and cytotoxic response. 121, 1326–1335. https://doi.org/10.1182/blood-2012-06-439851.

Walzer, T., Blery, M., Chaix, J., Fuseri, N., Chasson, L., Robbins, S.H., Jaeger, S., Andre, P., Gauthier, L., Daniel, L., Chemin, K., Morel, Y., Dalod, M., Imbert, J., Pierres, M., Moretta, A., Romagne, F., Vivier, E., 2007. Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. Proc. Natl. Acad. Sci. 104, 3384–3389. https://doi.org/10.1073/pnas.0609692104.