

# Relationship between the cytotoxic activity of peripheral blood natural killer cells and recurrent miscarriage

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## Research

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# Abstract

**Background:** Peripheral natural killer (pNK) cells are granular lymphocytes of the innate immune system that can be recruited by the endometrium to participate in the decidualization and be in contact with the villus in the intervillous space. Moreover, pNK cells were thought to exert cytotoxicity to cytotrophoblast, especially in abnormal pregnancy. However, it is still controversial about the association between pNK cytotoxicity and RM so far. In this study, we aim to compare the percentage, immunophenotype and function of pNK cells between patients with RM and fertile controls.

**Methods:** The peripheral blood was collected from 49 patients with RM and 11 fertile women in their middle luteal phase of the menstrual cycle. pNK cells were co-cultured with K562 cells at three different cell ratios to measure the cytotoxicity. The percentage of CD3<sup>+</sup>CD56<sup>+</sup> pNK was analyzed by flow cytometry and quantified to evaluate the expression of cytotoxic granules (granzyme B, granulysin, and perforin), and the cell surface receptors related to pNK cell cytotoxicity (NKG2D, NKp30, NKp46, CD158a, CD158b) were also detected.

**Results:** The general linear model analysis showed that pNK cell cytotoxicity in patients with RM was significantly lower than that in fertile controls. The RM group possessed a significantly lower level of granzyme B<sup>+</sup> pNK cells and significantly higher level of CD158a<sup>+</sup>, CD158b<sup>+</sup> pNK cells than that in the control group. However, there was no significant difference in the proportion of circulating CD3<sup>+</sup>CD56<sup>+</sup> NK cells expressing the granzyme B, granulysin, perforin, NKG2D, NKp30, NKp46, CD158a, CD158b.

**Conclusions:** Our results suggested that a lower pNK cytotoxicity might be associated with RM.

## Background

Recurrent miscarriage (RM) is defined as two or more spontaneous abortions prior to 20 weeks of gestation (1). It affects approximately 1–3% of women of child-bearing age in worldwide. The etiology of RM is often unknown and the prognosis is always frustrating. Embryo implantation and growth are fundamental for a normal pregnancy, and cytokines, hormones, and endometrial factors are involved in the regulation of both stages (2).

As a large granular lymphocyte in the innate immune system, peripheral natural killer (pNK) cell can be in contact with the villus through the intervillous space. In addition, pNK cells are recruited by the endometrium to participate in the decidualization. Moreover, pNK cells were believed to do harm to pregnancy by exerting cytotoxicity. Some studies showed that the absolute number and ratio of pNK are increased in RM patients, when compared to fertile women (3, 4). However, other studies showed that there was no significant difference in pNK cytotoxicity between healthy controls and women with RM or infertility (5, 6). Therefore, the association between pNK cytotoxicity and RM remains controversial and further research is needed.

The cytotoxic activity of pNK is exerted by the production of cytotoxic granules such as granzymes, granulysin, and perforin. Granulysin with positive charge binds the negatively charged cell membrane, leading to membrane damage (7). Perforin binds to the cell membrane of the target cell to form cylindrical pores that allow the passive diffusion of pro-apoptotic proteases (8) such as the granzymes, which induce cell apoptosis by activating the caspases (9). A higher level of perforin expressed by the uterine NK cells was found in cases of human sporadic miscarriage (10). However, it is still unclear whether the expression of granulysin, perforin, and granzymes are altered in pNK cells of RM patients.

The balance between activating receptors and inhibitory receptors regulates the release of cytotoxic granules from pNK cells (11). The activating receptors NKp30, NKp46 and NKG2D, activate and regulate the secretion of cytotoxic granules (12–14). The inhibitory receptors CD158a and CD158b, transmit inhibitory signals by their immunoreceptor tyrosine-based inhibitory motifs (15). The balance between these two types of receptors determines the cytotoxic activity of NK cells. However, up to now, little is known regarding the expression of these activating and inhibitory receptors in the peripheral blood NK cells in women with RM.

Therefore, in this study, the potential correlations between RM and the percentage and cytotoxicity of pNK cells were investigated, together with the expression of cytotoxic granules, surface activating receptors and inhibitory receptors in pNK.

## Materials And Methods

### Study population

Patients who were subjected to a visit at the Fertility Center of Shenzhen Zhongshan Urology Hospital, China, from January 2016 to October 2017 were enrolled in this study. Women who experienced two or more consecutive habitual miscarriage were included in the RM group, while fertile women who experienced at least one successful pregnancy without any abortions were included in the control group. An initial investigation was performed in all women before the recruitment and included the collection of information regarding the medical history, karyotype analysis, systemic and gynecological examination, and endocrine examinations.

The exclusion criteria were the following: patients with (1) abnormal chromosome karyotype; (2) infection with *toxoplasma gondii*, *syphilis*, *HIV*, *hepatitis B virus*, *hepatitis C virus*, *rubella virus*, *cytomegalovirus* or *herpes virus*; (3) genital tract malformations; (4) abnormal endocrine hormone level; (5) a male infertile partner. All the enrolled patients were not pregnant and they did not receive any drug therapy when the peripheral blood was collected. According to the above screening criteria, 49 patients with RM and 11 fertile women were recruited in this study.

This study was performed according to the “Declaration of Helsinki”, and the protocol was approved by the Ethics Committee of Shenzhen Zhongshan Urology Hospital. All patients enrolled in this study provided written informed consent.

# Materials

Human erythroleukemia cell line K562 (China Center for Type Culture Collection, Wuhan, China) were used as target cells to evaluate the cytotoxicity of pNK cells. K562 cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, USA). 3,3'-

Diiodoacetylcarboxycyanine perchlorate (DIO) (1911717; Invitrogen, San Diego, CA, USA) was used as a lipophilic tracer, and it was dissolved in dimethyl sulphoxide (DMSO) (Sigma, St. Louis, MO, USA) at a final concentration of 3 mM. Propidium Iodide (PI) (MKCB0899V; Sigma) was used to detect necrotic cells.

The phenotype and expressions of receptors and cytotoxic granules in NK cells and subpopulations were analyzed by flow cytometry and it is listed below. The following antibodies were used: fluorophore-conjugated monoclonal mouse anti-human antibodies such as PE-cy7-conjugated anti-CD56 (557747; BD Pharmingen, San Jose, CA, USA), PerCP-conjugated anti-CD3 (347344; BD Pharmingen, San Jose, CA, USA), FITC-conjugated anti-NKG2D (11e5878; eBioscience, ThermoFisher, Waltham, MA, USA), PE-conjugated anti-NKp30 (12e3379; eBioscience, ThermoFisher, Waltham, MA, USA), APC-conjugated anti-NKp46 (17e3359; BD Pharmingen, San Jose, CA, USA), FITC-conjugated anti-CD158a (556062; BD Pharmingen, San Jose, CA, USA), PE-conjugated anti-CD158b (559785; BD Pharmingen, San Jose, CA, USA), PE-conjugated anti-granzyme B (561142; BD Pharmingen, San Jose, CA, USA), Alexa Fluor 647-conjugated anti-perforin (563576; BD Pharmingen, San Jose, CA, USA), and Alexa Fluor 488-conjugated anti-granulysin (558254; BD Pharmingen, San Jose, CA, USA). All antibodies have been pre-diluted in aqueous buffered solution containing BSA and  $\leq 0.09\%$  sodium azide.

## Nk Cell Cytotoxicity Assay

NK cell cytotoxicity assay was performed as previously described (16, 17). The peripheral blood mononuclear cells were used as the effector cells and were isolated by density gradient centrifugation using Ficoll-Paque Lymphoprep (Axis-Shield PoC As, Oslo, Norway). K562 leukemia cells were used as the target cell and were stained with DIO after washing in phosphate-buffered saline (PBS). The two types of cells were co-cultured in 96-well Costar plates (Corning Incorporated, Corning, NY, USA) at a effector:target ratio of 12.5:1, 25:1, 50:1, and incubated at 37 °C and 5% CO<sub>2</sub> for 4 h. PI was added into each well, NK cell cytotoxicity was analyzed by flow cytometry and the death rate of K562 cells was calculated.

## Detection Of Pnk Cytotoxic Granules By Flow Cytometry

Peripheral blood was collected before 10:00 AM during the mid-luteal period of the menstrual cycle and placed in heparinized tubes. 100  $\mu$ L peripheral blood was drawn and incubated with antibodies against the cell surface markers CD3 (5  $\mu$ L) and CD56 (5  $\mu$ L) for 15 min. The erythrocytes were lysed by 1x FACS lysis solution (3349202; BD Pharmingen). The cells were permeabilized by 1x Permeabilizing Solution 2 and the cells were incubated for 10 min. Next, the cells were incubated with antibodies against the cytotoxic

granules granzyme B, perforin and granulysin (10  $\mu$ L each) for 30 min. Finally, the cells were analyzed by BD FACSCanto II flow cytometer equipped with BD FACSDiva software.

## Detection of the receptors mediating the cytotoxicity of pNK cells by flow cytometry

In order to evaluate the expression of the receptors on the surface of NK cells, the heparinized blood was incubated with antibodies against CD3 (5  $\mu$ L), CD56 (5  $\mu$ L), NKp30 (5  $\mu$ L), NKp46 (5  $\mu$ L), NKG2D (5  $\mu$ L). In addition, another aliquot of the peripheral blood was incubated with antibodies against CD3 (5  $\mu$ L), CD56 (5  $\mu$ L), CD158a (10  $\mu$ L), CD158b (10  $\mu$ L). Both aliquots were incubated for 15 min in the dark, then 1x FACS lysis solution was used to lyse the whole blood. Cells were analyzed by BD FACSCanto II flow cytometer equipped with BD FACSDiva software.

## Statistical analysis

Statistical analysis was performed using SPSS 20.0 (IBM, Chicago, IL, USA). Kolmogorov-Smirnov test was used to evaluate the normal distribution of the data. Results were expressed as mean  $\pm$  SD when the data were normally distributed, or as median (quartile) when the data were non-normally distributed. Inter-groups differences were examined by independent *t*-test when the data were normally distributed, or by Mann-Whitney U test when the data were non-normally distributed. The difference in NK cytotoxicity between groups was analyzed by a general linear model. The difference was analyzed using Pillai's Trace test when Mauchly's test of sphericity resulted in a value of  $P \leq 0.05$ , or Sphericity Assumed test when the sphericity test resulted in a value of  $P > 0.05$ . A two-tailed  $P < 0.05$  was considered statistically significant.

## Results

### NK cytotoxicity in the fertile control and RM group

The general linear model showed that NK cytotoxicity in the RM group was significantly decreased, when compared to that in the control group ( $P = 0.043$ ). Moreover, NK cytotoxicity was significantly increased along with the increase of the effector:target ratio, regardless of whether this ratio was in the RM group or control group ( $P < 0.001$ , Fig. 1).

### Expression of cytotoxic granules in pNK cells from the fertile control and RM group

The total number of pNK cells and the expression of cytotoxic granules in pNK were measured in the fertile control and RM group. No significant differences were observed in the percentage of circulating CD3<sup>-</sup>CD56<sup>+</sup> NK cells (13.4%  $\pm$  7.6% vs 14.2%  $\pm$  7.1%; Fig. 2A and B), granzyme B<sup>+</sup> (68.2%  $\pm$  11.6% vs

68.8% ± 14.5%, Fig. 2C and D), granulysin<sup>+</sup> (66.1% ± 11.4% vs 68.2% ± 14.4%, Fig. 2C and E) and perforin<sup>+</sup> NK cells (62.3% ± 13.8% vs 64.3% ± 15.3%, Fig. 2C and F) between the fertile control and RM group. The mean fluorescence intensity (MFI) of granzyme B reflecting its expression level in the pNK cells in the control group was significantly higher than that in RM group (27318.6 ± 3643.8 vs 23774.4 ± 8991.2, *P* = 0.04, Fig. 2C and G). However, the MFIs of granulysin (4756.5 ± 1370.7 vs 5043.5 ± 1578.0, *P* = 0.54, Fig. 2C and H) and perforin (4454.8 ± 1243.9 vs 4972.4 ± 1426.4, *P* = 0.27, Fig. 2C and I) in the pNK cells was not significantly different between the two groups.

## Expression of receptors in the pNK cells of both the fertile control and RM group

The percentage of NKG2D<sup>+</sup> (67.9% ± 12.6% vs 68.4% ± 12.6%, Fig. 3A and B), NKp30<sup>+</sup> (45.3% ± 16.5% vs 52.1% ± 21.9%, Fig. 3A and C), NKp46<sup>+</sup> (53.6% ± 16.4% vs 55.2% ± 19.6%, Fig. 3A and D), CD158a<sup>+</sup> (42.8% ± 21.2% vs 45.0% ± 18.3%, Fig. 4A and B), CD158b<sup>+</sup> (36.8% ± 18.9% vs 40.1% ± 18.4%, Fig. 4A and D) pNK cells were not significantly different between the fertile control group and RM group. Furthermore, the MFI of NKG2D (685.1 ± 154.4 vs 641.3 ± 95.0, Fig. 3A and E), NKp30 (1966.3 ± 374.6 vs 2052.3 ± 499.8, Fig. 3A and F), and NKp46 (1795.1 ± 258.3 vs 1809.2 ± 663.2, Fig. 3A and G) expression in the pNK cells between these two groups were also not significantly different. However, the MFI of CD158a (1509.7 ± 644.0 vs 2060.7 ± 525.0, *P* = 0.004, Fig. 4A and C) and CD158b (4850.4 ± 333.1 vs 5584.9 ± 1580.1, *P* = 0.004, Fig. 4A and E) expression in the pNK cells were higher in RM group than that in the control group.

## Discussion

Although a bunch of evidence is available regarding the association between NK cell cytotoxicity and female fertility (18), distinct opinions were formulated regarding the relationship between the cytotoxic activity of pNK and RM (19–21). In this study, the cytotoxic-related parameters, such as the percentage, immunophenotyping, and cytotoxicity of pNK cells in RM patients were evaluated. Interestingly, our results showed that NK cytotoxicity in the RM group was significantly lower than that in the control group. Moreover, the RM group showed a lower MFI of granzyme B and higher MFI of inhibitory receptors of pNK compared to that in the control group. These opposite results questioned the role of the cytotoxicity-related function of pNK cells in the occurrence of RM.

During implantation, the first step is the attachment of the embryo to the surface of the endometrium, followed by its migration and invasion until it is immersed in the depth of the uterine tissue (22). Uterine NK cells are a key factor in regulating the invasion of trophoblasts by secreting proteolytic enzymes such as matrix metalloproteinase-2 (MMP2) and MMP9 (23), and reducing extravillous trophoblast cell apoptosis (24). Nevertheless, it is still not clear whether the recruited pNK cells can regulate the pregnancy outcome or reflect what is happening at the maternal-fetal interface (19, 25, 26). Some scientists reported that patients with RM are accompanied with high pNK cells ratio (3, 20) or cytotoxicity (4). However, other scientists reported contradictory results indicating the same NK cell ratio (27) or lower

pNK cytotoxicity (28) in RM patients compared with the fertile control, and our results are actually consistent with these results. A large cohort study suggested that the relationship between the subsequent pregnancy outcome and pNK cytotoxicity is not linear, and both patients with low and high pNK cytotoxicity tend to have high miscarriage rate (6). Although pNK cells contribute in creating an immune tolerant environment for placentation, they should still be equipped with a certain cytotoxic potential to fight infections during normal conditions (29). Therefore, both RM patients with higher pNK cytotoxicity and that with lower pNK cytotoxicity compared to fertile control should be taken into serious consideration.

NK cytotoxicity is controlled by the complex interaction between inhibitory and activating receptors (11, 30). Indeed, NK cells exert their cytotoxicity by secreting cytotoxic granules when the activating receptor signals are predominant (31). In order to explore the potential mechanism for low pNK cytotoxicity in RM patients, the expressions of receptors and cytotoxic granules in the pNK cells were also evaluated. However, our results showed no significant difference about their percentage between the RM and control groups, while the significant differences were observed in the MFI of granzyme B, CD158a, and CD158b in the pNK cells between the RM and control groups, which was consistent with the tendency for the cytotoxicity of pNK in the RM group. Thus, the predominant inhibitory signal might result in a low cytotoxicity in the RM group. It is also worth noting that some other pathways can actually be used by NK cells to kill infected or transformed cells. For instance, they can kill cells by the activation of the death receptor pathway, such as FasL-Fas signaling pathway and TNF-related apoptosis-inducing ligand (TRAIL) (32, 33). However, this was not the aim of our present work, and further studies should consider these different pathways used by the pNK cells to kill the target cells.

Our present work contains the following limitations that should not be underestimated. Firstly, this is a retrospective study. Secondly, several factors that affect the level and function of peripheral blood NK cells were not considered, including mental stress (34, 35), exercise (36, 37) and age. Although the patients with diseases that can influence peripheral blood NK cells were excluded, it is possible that some undetected autoimmune diseases or infections affected the results.

## Conclusions

In conclusion, this study found that a lower pNK cytotoxicity could also be associated with RM and it is potentially related to a receptor-mediated pathway. Therefore, clinicians should evaluate the range of pNK cytotoxicity cautiously. Larger prospective studies and the exploration of the underlying mechanisms are needed to confirm the relationship between pNK cytotoxicity and RM.

## Abbreviations

RM: Recurrent miscarriage; pNK: Peripheral blood natural killer cell; DIO: 3,3'-Diocetadecyloxycarbocyanine perchlorate; DMSO: Dimethyl sulfoxide; PI: Propidium Iodide; PBS: phosphate-buffered saline; MFI:

Mean fluorescence intensity; MMP2: Matrix metalloproteinase-2; MMP9: metalloproteinase-9; TRAIL: TNF-related apoptosis-inducing ligand.

## Declarations

### Acknowledgement

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### Authors' contributions

Y-NZ, JX, Y-FF performed the experiments. Y-NZ, C-YH analyzed the data. R-CL recruited the patients. Y-NZ wrote the manuscript. C-YH, W-WT reviewed the manuscript. C-YH, W-WT, YZ designed the study. All authors read and approved the final manuscript.

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### Availability of data and materials

The datasets supporting the findings of this study are included in this published article.

### Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shenzhen Zhongshan Urology Hospital. All patients enrolled in this study provided written informed consent.

### Consent for publication

Not applicable

### Competing interests

Not applicable

### Author details

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## Figures

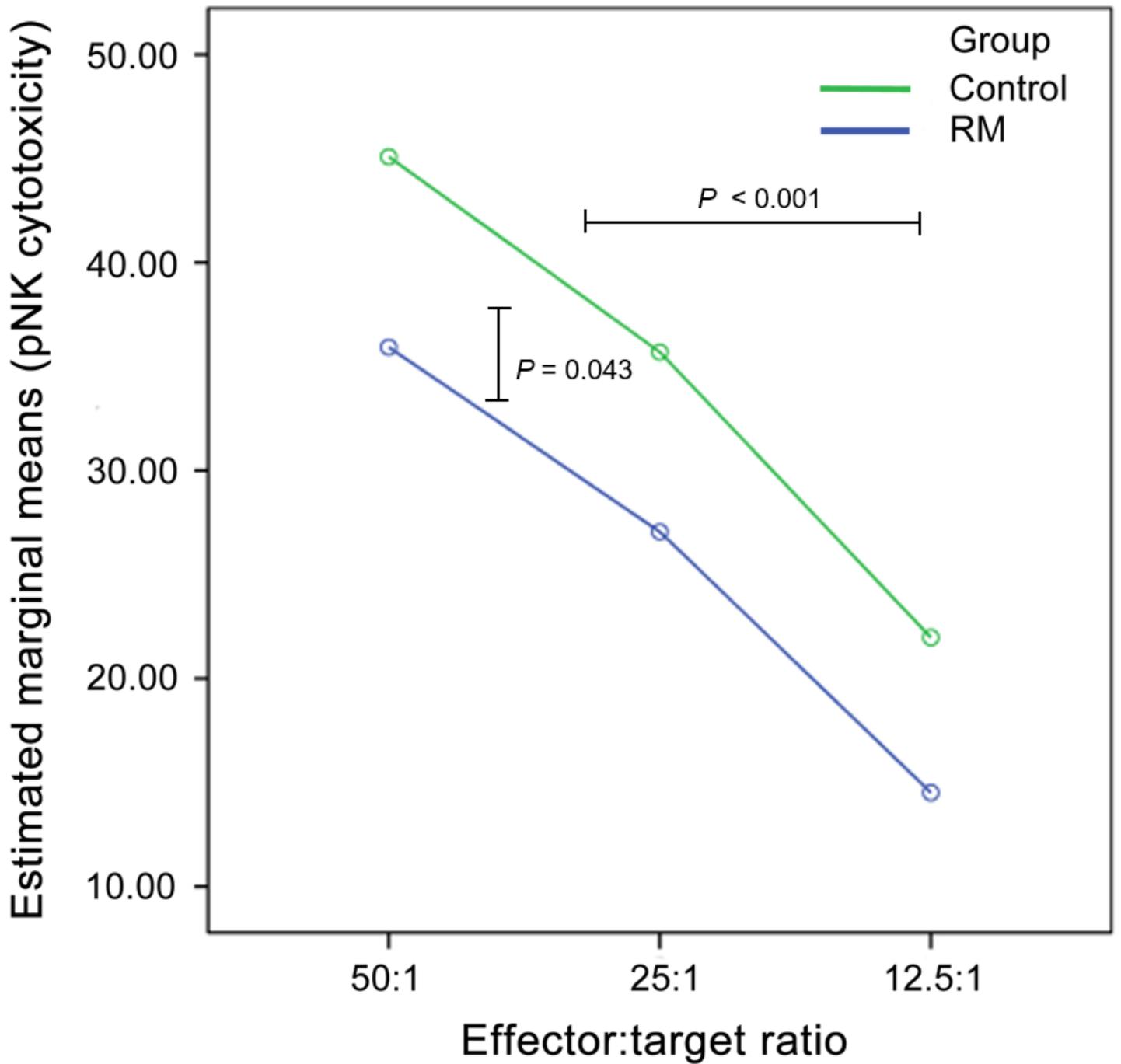
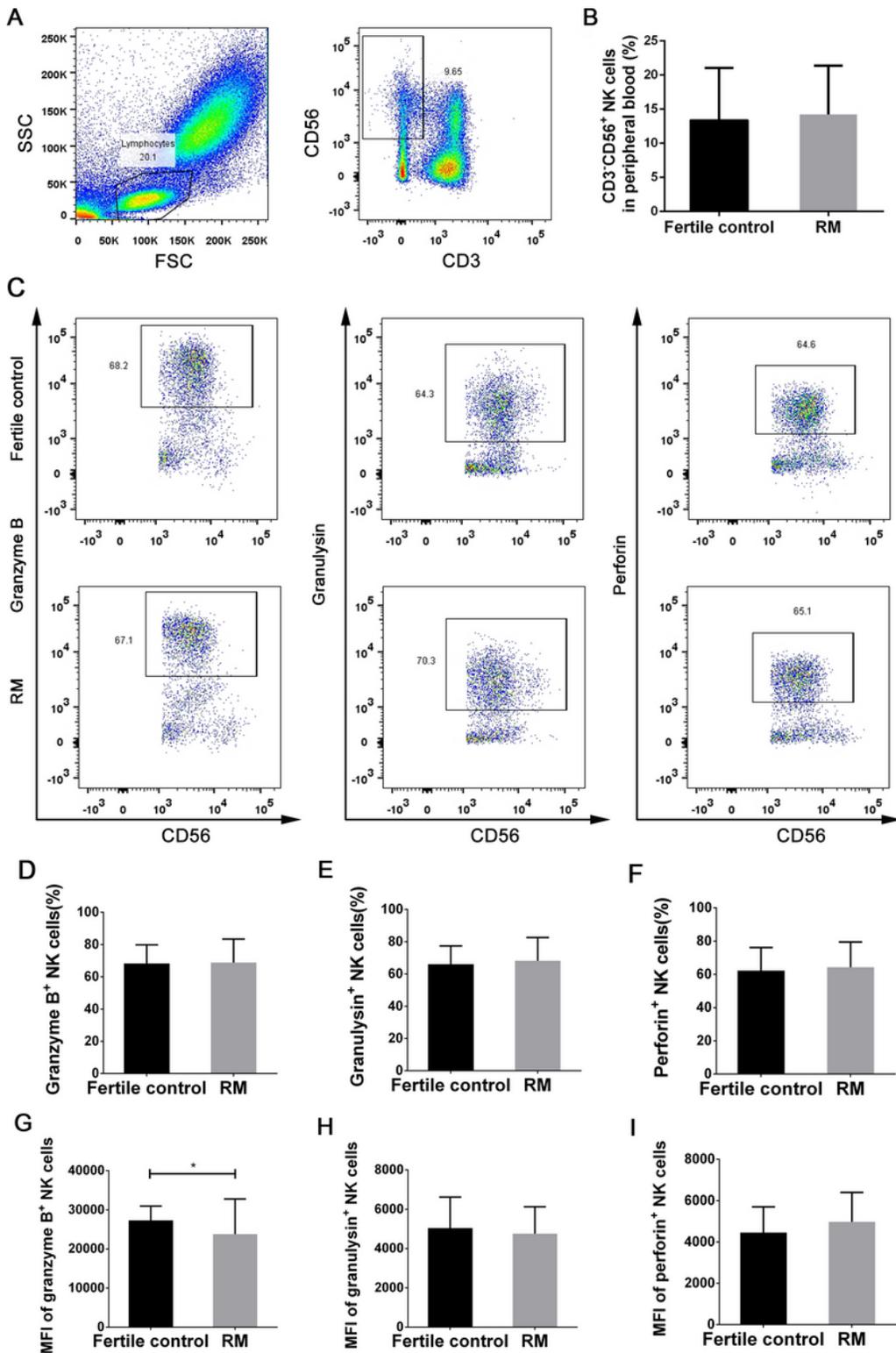


Figure 1

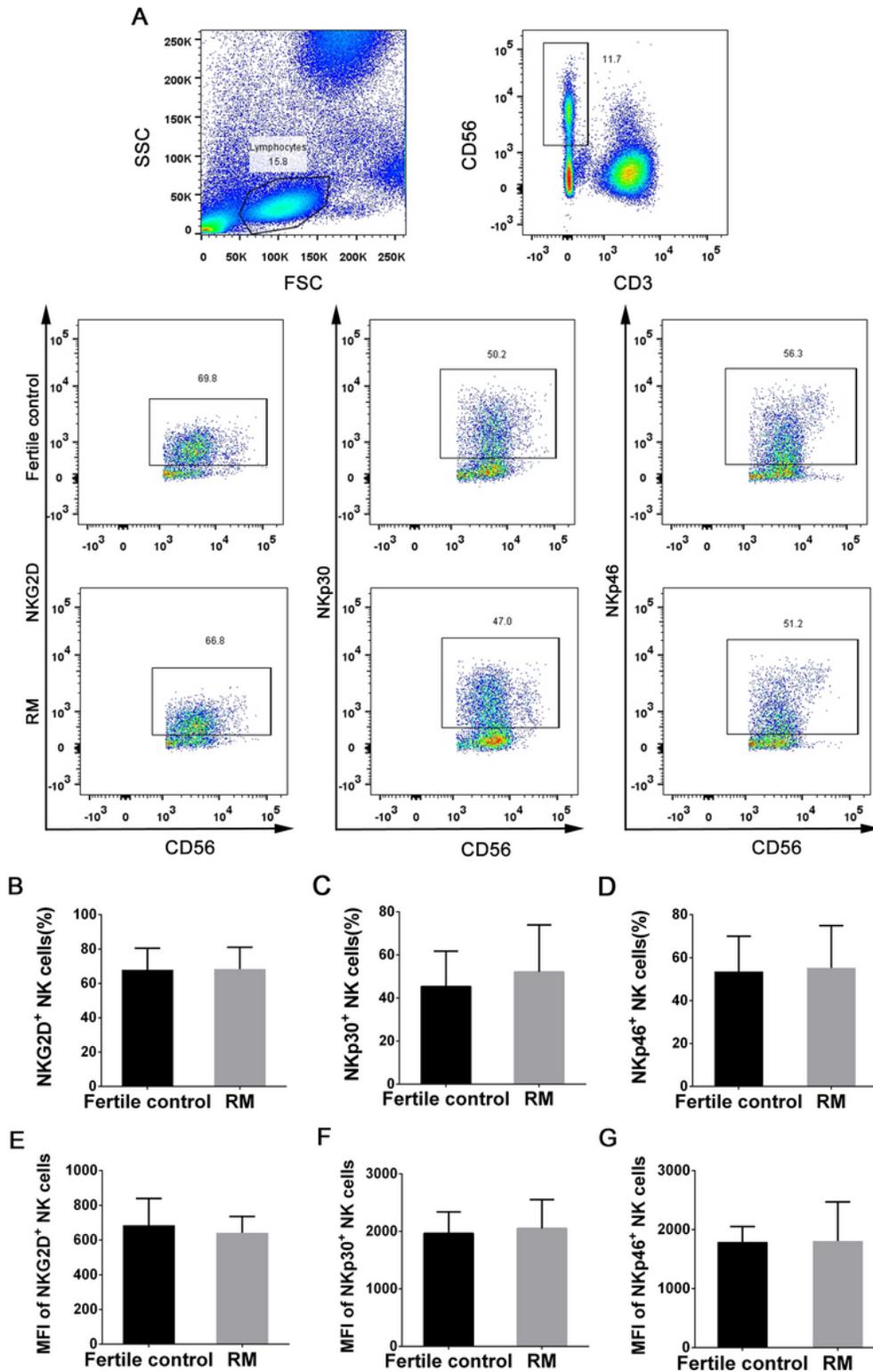
The general linear model analysis evaluating the correlation between pNK cytotoxicity and RM. The estimated marginal means of pNK cytotoxicity was compared between the fertile control (green) and RM patients (blue).



**Figure 2**

Expression of cytotoxic molecules on the peripheral blood NK cells. (A) Representative flow cytometry images of NK cells. (B) The percentage of pNK cells in the fertile control and RM patients. (C) Representative flow cytometry images of granzyme B<sup>+</sup>, granulysin<sup>+</sup>, and perforin<sup>+</sup> pNK cells. The percentage and MFI of granzyme B<sup>+</sup> (D, G), granulysin<sup>+</sup> (E, H), and perforin<sup>+</sup> (F, I) pNK cells in the fertile

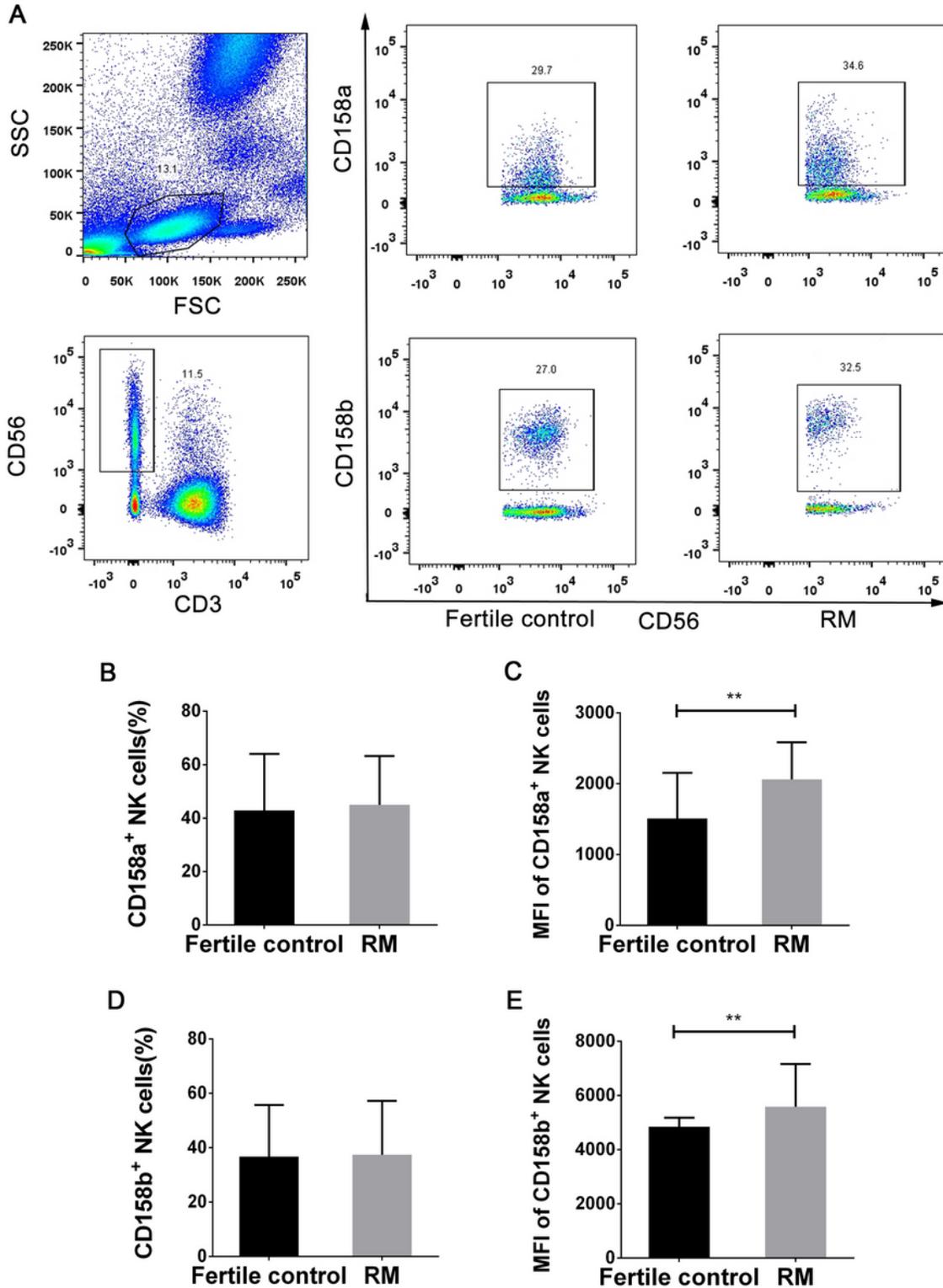
control and RM patients. Results are expressed as mean  $\pm$  SD. The independent t test was used for group comparison. \*P<0.05.



**Figure 3**

Expression of the activating receptors on the peripheral blood NK cells. (A) Flow cytometry images representing the expression of NKG2D, NKp30, and NKp46. The numbers indicate the percentage of the cells in the gate. The histograms shows the difference in the percentage and MFI of NKG2D+ (B, E),

NKp30+ (C, F), and NKp46+ (D, G) NK cells between the fertile control and RM patients. Results are expressed as mean  $\pm$  SD. The independent t-test was used to perform the comparison.



**Figure 4**

Expression of the inhibitory receptors of the peripheral blood NK cells. (A) Flow cytometry images representing the expression of CD158a and CD158b. The numbers indicate the percentage of the cells in the gate. The histograms show the difference in the percentage and MFI of CD158a+ (B, C) and CD158b+

(D, E) NK cells between the fertile control and RM patients. Results are expressed as mean  $\pm$  SD. The independent t-test was used to perform the comparison. \*\*P<0.005.