



# The Ratio of Peripheral Blood Natural Killer Cells is not a Solid Surrogate Immune Index in Unexplained Recurrent Miscarriage

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## ABSTRACT

**Background:** Immunotherapies targeting peripheral natural killer (pbNK) cells in unexplained recurrent miscarriage (uRM) remain controversial. We hypothesized that the change in pbNK cell count might be a result of innate immune responses rather than a cause.

**Objective:** To explore whether the pbNK count is significantly different in women testing positive than those testing negative for commonly studied autoimmune markers.

**Methods:** Peripheral blood samples were collected from 302 eligible patients with uRM for the antinuclear antibody (ANA) testing determined by the enzyme-linked immunosorbent assay (ELISA), anti-thyroid peroxidase antibody (TPO-Ab) testing and anti-thyroglobulin antibody (Tg-Ab) testing determined by the chemiluminescent immunoassay, and pbNK cell testing determined by flow cytometry. The patients were divided into two groups according to the pbNK normal range, and the comparative analysis entailed an examination of the prevalence rates of autoantibodies within the high pbNK group and the normal pbNK group, followed by a comprehensive investigation into the potential correlations between autoantibodies and pbNK cells.

**Results:** There was a positive association between TPO-Ab positivity and high pbNK cells ( $p=0.016$ , OR=5.097, 95% CI 1.356–19.159), while there was a negative association between ANA positivity and high pbNK cells ( $p=0.013$ , OR=0.293, 95% CI 0.111-0.773). TPO-Ab-positive patients had a higher pbNK cell count compared with TPO-Ab-negative patients, while ANA-positive patients had a lower pbNK cell count compared with ANA-negative patients.

**Conclusion:** The change in pbNK cell count may be a consequence of immune responses, and there should be careful consideration in applying it as an immunotherapeutic index.

**Keywords:** Antinuclear Antibody, NK Cells, Recurrent Miscarriage, Thyroglobulin Antibodies, Thyroid Peroxidase Antibodies

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## INTRODUCTION

Recurrent miscarriage (RM) is defined as two or more miscarriages according to both the European Society of Human Reproduction and Embryology (ESHRE) and American Society for Reproductive Medicine (ASRM) guidelines (1, 2). The ASRM recommends that patients with RM receive an aetiological evaluation after two consecutive clinical miscarriages and that the epidemiological research of RM should begin at three consecutive miscarriages (2). There are various causes of RM: parental chromosome abnormalities, such as balanced translocation and Robertsonian translocation; immunological disorders, including autoimmune diseases such as antiphospholipid syndrome (APS), systemic lupus erythematosus (SLE) and connective tissue disorder (CTD); endocrine disorders, including polycystic ovary syndrome (PCOS) and hyperprolactinemia; metabolic disorders, including diabetes, hyperthyroidism and hypothyroidism; and genital disorders, including mediastinal uterus and endometrial polypus. However, in up to 69% of patients suffering from RM, the causes indicated above do not explain RM (3), indicating that there are unknown factors leading to RM, referred to as unexplained recurrent miscarriage (uRM).

The exploration of uRM is a challenge, with more than 30% of patients suffering one more miscarriage during the next pregnancy. A majority of scholars are supportive of an immunological basis for uRM, some of whom focused on the change in the number of pbNK cells, whereas others tended toward examining common autoantibodies including antinuclear antibody (ANA), anti-thyroid peroxidase antibody (TPO-Ab), and anti-thyroglobulin antibody (Tg-Ab). A gray market has flourished, aimed at normalizing the total pbNK cell count as a treatment for uRM, such as intravenous immunoglobulin (IVIG) and fat emulsion injection. However, the guidelines have not recommended any

therapeutic interventions to increase the live birth rate for couples with RM, with a potential immunological background. Thus, it is of great significance to evaluate the significance of the changing pbNK cell count in the uRM to provide a clinical therapeutic basis.

The pathogenesis of autoimmune diseases with dramatically increased antibody levels, such as SLE and autoimmune thyroiditis, has been investigated. Antiphospholipid syndrome secondary to SLE causes decreased placental function and miscarriage through placental vascular thrombosis (4, 5). Furthermore, permanent hypothyroidism that develops after autoimmune thyroiditis can lead to miscarriage (6, 7). However, the correlation between pbNK cells and autoantibodies isolated without confirmed autoimmune diseases with RM is still obscure. Several researchers have substantiated a robust correlation between the aforementioned immune markers and RM (8-13), whereas conflicting findings have been reported in other studies (14-18). Due to the differences in the obtained results, finding the best way to deliver intervention therapies for uRM is quite challenging.

In a previous study on autoimmune thyroiditis in anti-thyroid antibody-positive women with different kinds of reproductive failure, the authors confirmed an elevated NK cell ratio, suggesting an interplay between NK cells and autoimmune thyroid disorders (19-21). Anti-thyroid antibodies may be involved in thyroid cell necrosis through antibody-dependent cell-mediated cytotoxicity-natural killer cell mechanisms (22). Because NK cells are associated with the innate immune response and also involved in the prevention of autoimmune disease immunoregulators (23, 24), we suggest that it is likely that there are patients with autoimmunity among those with RM whose pbNK cell counts show a simultaneous variation. Furthermore, we raise the following question: is the increased number of pbNK cells itself in patients with RM worth being an immunotherapeutic index, or is this change in pbNK cell number merely

a response to the autoimmune antibodies derived from immune dysregulation that contribute to miscarriage. Given the absence of concurrent investigations encompassing both autoantibodies and pbNK cells in individuals experiencing uRM, this study specifically excluded patients previously diagnosed with autoimmune disorders. The primary objective was to explore the potential relationship between alterations in pbNK cell counts and the presence of autoimmune antibodies in patients diagnosed with uRM.

## MATERIALS AND METHODS

### *Subjects and Eligibility Criteria*

The records of 623 patients who visited the specialist clinic for RM of the First Affiliated Hospital of Sun Yat-Sen University from May 2014 to August 2021 were carefully reviewed (Fig. 1). Inclusion criteria were as follows: (1) 2 or more consecutive miscarriages; (2) gestational week <12 weeks; (3) missed abortion; (4) inevitable abortion; (5) incomplete abortion; (6) miscarriage confirmed by ultrasound or histopathological examination; (7) miscarriage without known aetiologic causes; (8) patients who were not pregnant when undergoing examinations in the first visit; (9) patients without a history of taking steroids within 3 months. Exclusion criteria were as follows: (1) 1 or inconsecutive miscarriages; (2) gestational week  $\geq$ 12 weeks; (3) biochemical pregnancy; (4) ectopic pregnancy; (5) placental trophoblastic diseases; (6) miscarriage with known

aetiologic causes; (7) patients pregnant while undergoing examinations in the first visit; (8) patients with a history of taking steroids within three months.

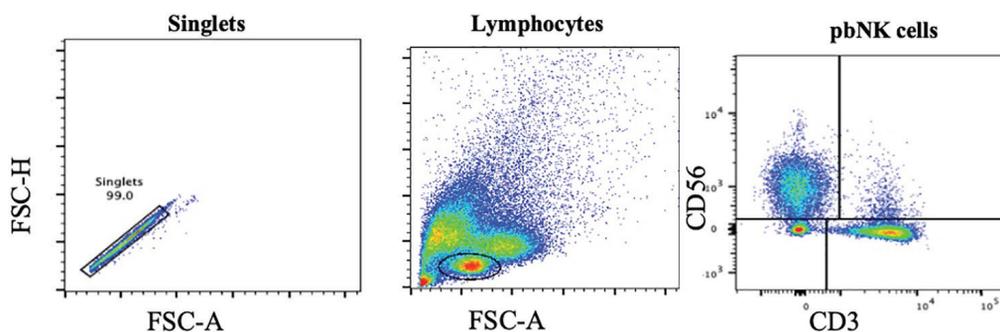
All women had a history of miscarriage(s) in the first trimester, 45 of them did not meet the diagnostic criteria for recurrent miscarriage and 11 patients did not finish the recommended etiologic examinations (1). A total of 593 women diagnosed with recurrent miscarriage had undergone parental karyotyping, screening for uterine abnormalities (3D US), hereditary thrombophilia, antiphospholipid antibodies (LA-ACA), thyroid function and antibodies, endocrine and metabolic disorders, and immunological tests. After finishing the above investigations, a total of 261 patients (45%) with recurrent miscarriage were excluded due to known miscarriage causes. Finally, 302 patients with uRM were recruited and their peripheral blood samples were collected within the early follicular phase of the menstrual cycle (days 2 to 5) for immune marker assays.

### *pbNK, TPO-Ab, Tg-Ab and ANA Assays*

Peripheral blood samples were collected in BD Vacutainer tubes, and all the samples were assayed using the same methods in a single laboratory. The titers and patterns were concordant in all cases. The readings were made independently by at least two experienced evaluators (blinded to the sample characteristics and time period).

### *pbNK Assay*

For each patient, 2-3ml peripheral blood



**Fig. 1.** Gating method of pbNK cells. Singlets are removed by FSC-A and FSC-H; Lymphocytes are gated through FSC-A and SSC-A; NK cells are gated according to CD3 and CD56: CD3<sup>+</sup>CD56<sup>+</sup> lymphocytes.

was collected, and the samples were taken to the laboratory within 2 h. Peripheral blood was added on top of 3-4.5 ml lymphocyte separation solution (volume ratio of peripheral blood to Ficoll separation=1:1.5). After a 20-min centrifugation, the intermediate cloudy suspension WAS pipetted and put into a centrifuge tube for a 5-min centrifugation to obtain a single cell suspension of peripheral blood lymphocytes. Two milliliters of conditioned medium (RPMI1640+10% FCS+1% penicillin/streptomycin) were provided to resuspend and obtain peripheral blood lymphocyte single-cell suspensions. Markers were determined by flow cytometry, using CD3 and CD56 fluorochrome-conjugated monoclonal antibodies. Antibodies were purchased from BD Biosciences (San Jose, CA, USA) or eBioscience. Appropriate concentrations of antibodies were added to the cells ( $5 \times 10^5$  cells/tube) in 100  $\mu$ L flow cytometry staining buffer and incubated for 30 min at 4°C in the dark. Analysis was performed using a BD LSRFORTESSA 20x device. At least 50,000 lymphocyte-gated cells were obtained and analyzed for CD3<sup>-</sup>CD56<sup>+</sup> cells. The results and graphs were analyzed using Flowjo version 10A software (Flowjo, USA). NK cells were referred to as lymphocytes with the negative expression of CD3 and positive expression of CD56, i.e., CD3<sup>-</sup>CD56<sup>+</sup> lymphocytes, the total count of which represented the sum of all subtypes of pbNK cells. The normal range for a CD56<sup>+</sup> pbNK cell count was less than or equal to 18%, and >18% was considered a high CD56<sup>+</sup> pbNK number according to our laboratory standard.

#### *TPO-Ab and Tg-Ab Assays*

TPO-Ab is a protein that interferes with the action of enzymes crucial to producing thyroid hormones. Tg-Ab is an antibody directed against thyroglobulin, a protein present in the thyroid gland. TPO-Ab and Tg-Ab assays were performed by chemiluminescent immunoassay kits (Abbexa, UK). The sample was added to the

reaction vessel with paramagnetic particulates coated with thyroid antigens. After the first incubation, the materials binding to the solid phase were located in the magnetic field, while the unconjugated materials were washed off. Protein A-alkaline phosphatase conjugate was added and continuously bound to the thyroid antibodies. The reaction vessel was washed to remove unconjugated materials after a second incubation. Lumi-Phos530 was added to the reaction vessel to work as a chemiluminescent substrate, and the light generated by the reaction was measured by a luminometer. The light generation was proportionate to the levels of thyroid antibodies. A stored multipoint calibration curve was applied to determine the amount in the sample. The normal range of TPO-Ab was within 0-9.00 U/ml, and >9.00 U/ml was considered as TPO-Ab positivity. The normal range of Tg-Ab was within 0-3.99 U/ml, and >3.99 U/ml was considered as Tg-Ab positivity.

#### *ANA Assay*

ANA is a distinct category of autoantibodies that can bind to and demolish particular structures inside the cell nucleus, and ANA testing could be considered for explanatory purposes in managing RM according to ESHRE guidelines (1). ANA detection was performed by an enzyme-linked immunosorbent kit (FineTest, China). The microtiter well strips were marked and fixed for use on the plate. The samples (1:100) were diluted in 1x sample diluent. The controls supplied in the kit were put into use. One hundred  $\mu$ L of prediluted negative and positive controls were pipetted, and serum samples and sample diluent (blank controls) were diluted into proper well plates in duplicate. After gentle blending, the plate was covered and the mixture was incubated for 30 min at room temperature (24-28 °C). The wells were washed manually at least 3 times with a total of 300 $\mu$ L diluted wash buffer, and the plate was tapped over a paper towel between washings to ensure proper washing.

Then, 100 $\mu$ L of enzyme conjugate was added to each well. The plate was covered and incubated for 15 min at room temperature after gentle blending for 5-10 seconds. The wells were washed 4 times as above. Then, 100 $\mu$ L of TMB substrate was added to each well before gentle blending for 5 seconds. The plate was covered and incubated at room temperature for 15 min in the dark. The blue color developed in the positive wells. The reaction was terminated by adding 100 $\mu$ L of stop solution into all wells at the same time intervals. The blue turned into yellow after gentle blending for 5-10 seconds. The reaction was terminated by an ELISA reader. The upper limit value of ANA negativity was determined as 12 U/ml, >12 U/ml was considered ANA positivity.

#### *Sample Size Calculation and Statistical Methods*

Eligible patients were divided into two groups according to the normal pbNK range (less than 18%): the high pbNK group and the normal pbNK group. The positive rates of autoantibodies between the two groups were compared, and the associations between autoantibodies and pbNK cells were further explored. We calculated that a sample of 256.5 patients (161.5 in the normal pbNK group; and 95 in the high pbNK group) would provide the study with 80% power to detect a difference between the group proportions of -14% at a two-sided alpha of 0.05. The proportion in the normal pbNK group is assumed to be 10% and the proportion in the high pbNK group is 24%. Given an anticipated dropout rate of 5%, the total sample size required is 270 (170 in the normal pbNK group, and 100 in the high pbNK group) at least. Data were analyzed using the Statistical Package for Social Science (SPSS) version 23. The autoantibody examinations were obtained as both quantitative and qualitative results. The quantitative results are expressed as the median (25<sup>th</sup> to 75<sup>th</sup> percentile), and other measurement data are expressed as the mean $\pm$ SD. Independent sample t-test was

applied to compare the differences when normality and homogeneity of variance assumptions were satisfying; otherwise, the nonparametric Mann–Whitney U test was used to compare the numbers of CD56<sup>+</sup> NK cells between the groups. The relevant influencing factors of the pbNK cell count were determined via logistic analysis. The chi-square test was used to examine the relationship between the results of qualitative autoantibody testing and the pbNK cell count presented in a two-by-two contingency table. Differences were considered to be statistically significant when the *p*-value was <0.05.

## RESULTS

A retrospective analysis was conducted on the medical records of a cohort consisting of 623 patients. The patients' characteristics between the two groups are listed in Table 1. Age, BMI, basal levels of FSH, LH, and estradiol, and levels of TSH, fT3, fT4, tT3, and tT4 were compared between the two groups, and no significant differences were found.

Those individuals who did not undergo all aetiologic examinations (n=11), those with a single miscarriage (n=45), those with a prior history of successful live births (n=30), and those diagnosed with known aetiologic causes (n=563) were excluded from the study population. Finally, a total of 302 patients with uRM were included and further divided into a high-pbNK group (n=118) and a normal-pbNK group (n=184) according to the pbNK normal range for analysis (Fig. 2).

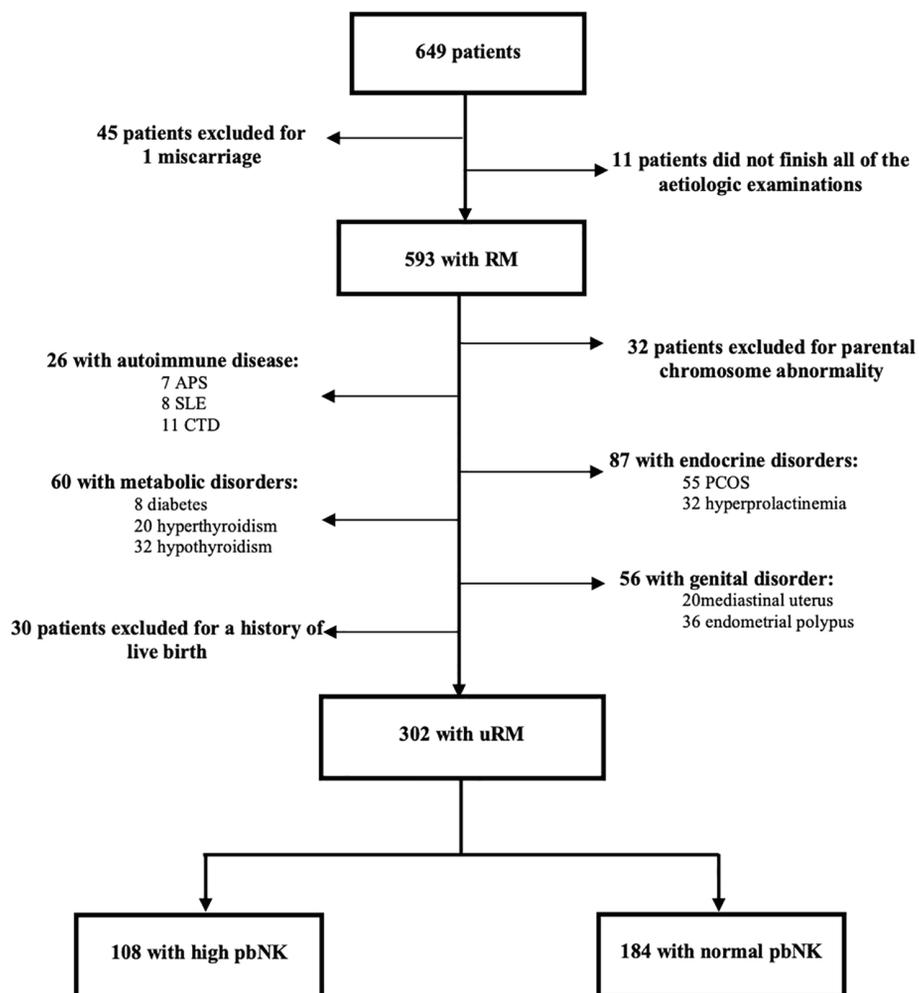
#### *Associations between Autoantibodies and pbNK Cells*

In the high-pbNK group, thirty-three subjects tested positive for autoantibodies, of which 8 were positive for ANA, 4 were positive for TPO-Ab, 2 were positive for Tg-Ab, 15 were positive for both TPO-Ab and Tg-Ab, 1 was positive for both TPO-Ab and ANA, 1 was positive for both Tg-Ab and ANA, and 2 tested positive for ANA, TPO-Ab, and Tg-Ab.

**Table 1. Comparison of baseline characteristics and autoantibody-positive rates between high-pbNK and normal-pbNK groups in patients with unexplained recurrent miscarriage**

| Characteristics          | High-pbNK Group | Normal-pbNK Group | p-value |
|--------------------------|-----------------|-------------------|---------|
| Age (years)              | 31.1±4.3        | 31.5±4.9          | 0.476   |
| BMI (kg/m <sup>2</sup> ) | 21.3±2.5        | 21.6±2.8          | 0.317   |
| Basal FSH (IU/L)         | 5.6±2.3         | 6.2±3.7           | 0.148   |
| Basal LH (IU/L)          | 3.5±1.8         | 3.5±1.7           | 0.865   |
| Basal estradiol (pg/mL)  | 33.0±14.2       | 36.5±18.3         | 0.112   |
| TSH (uIU/mL)             | 2.0±1.2         | 1.9±0.9           | 0.567   |
| FT3 (pmol/L)             | 4.6±0.6         | 4.5±0.7           | 0.627   |
| FT4 (pmol/L)             | 12.4±1.9        | 12.6±2.2          | 0.467   |
| Tg-Ab positive rate      | 17.8%           | 15.8%             | 0.87    |
| TPO-Ab positive rate     | 20.3%           | 10.9%             | 0.04    |
| ANA positive rate        | 11.9%           | 22.3%             | 0.02    |

BMI: body mass index; FSH: follicle stimulating hormone; FT3: free triiodothyronine; FT4: free thyroxine; LH: luteinizing hormone; TSH: thyroid-stimulating hormone; TPO-Ab: anti-thyroid peroxidase antibody, ANA: antinuclear antibody, Tg-Ab: anti-thyroglobulin antibody The data of age, BMI, basal sex hormone, and thyroid function are expressed as the mean±SD, and the data of the two groups were analyzed by independent sample t-test. The data of autoantibody rates are expressed as percentages and the data of the two groups were analyzed by chi-square test. *P*<0.05 is considered to indicate a significant difference.



**Fig. 2.** Flow chart of the study participants. APS: antiphospholipid syndrome; CTD: connective tissue disorders; PCOS: polycystic ovary syndrome; pbNK: peripheral blood Natural Killer cell; RM: recurrent miscarriage; SLE: systemic lupus erythematosus; uRM: unexplained recurrent miscarriage

In the normal-pbNK group, fifty-nine subjects tested positive for autoantibodies, of which 26 were positive for ANA, 1 was positive for TPO-Ab, 10 were positive for Tg-Ab, 6 were positive for both TPO-Ab and Tg-Ab, 2 were positive for both TPO-Ab and ANA, 2 were positive for both Tg-Ab and ANA, and 10 were positive for ANA, TPO-Ab, and Tg-Ab.

The positive rates of autoimmune antibodies between the two groups are compared in Table 1. The positive rates of TPO-Ab in the high-pbNK group were significantly higher than in those in the normal-pbNK group (20.3% vs. 10.9%,  $p=0.04$ ), and the positive rates of ANA in the normal-pbNK group were significantly higher than in those in the high-pbNK group (22.3% vs. 11.9%,  $p=0.02$ ). However, the positive rates of Tg-Ab were not significantly different between the two groups ( $p>0.05$ ).

#### *Risk Factor Analysis for High-NK Cells in the uRM*

Factors associated with high-pbNK count were examined using multivariable logistic regression. Candidate variables for the logistic regression included age, BMI, basal levels of FSH, basal levels of LH and estradiol, TSH, fT3, fT4, tT3, tT4, and qualitative ANA, TPO-Ab, and Tg-Ab (positive or negative).

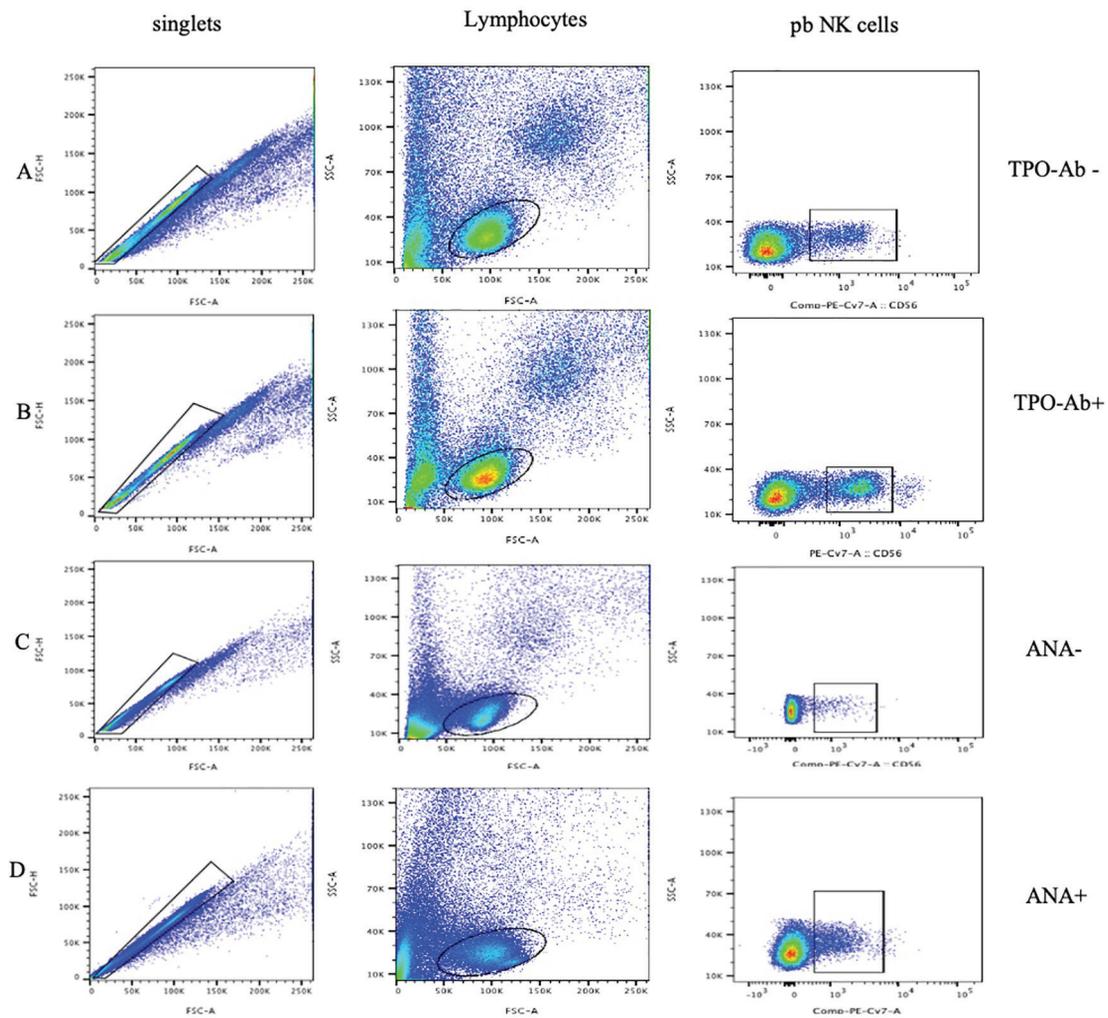
No significant association between age, BMI, basal levels of FSH, LH, estradiol, or average levels of TSH, fT3, fT4, tT3, or tT4 and high pbNK cells was found. There was a statistically significant positive association between TPO-Ab positivity and high pbNK cells ( $p=0.016$ , OR=5.097, 95% CI 1.356–19.159), while there was a statistically significant negative association between ANA positivity and high pbNK cells ( $p=0.013$ , OR=0.293, 95% CI 0.111–0.773) (Table 2).

TPO-Ab-positive patients had a higher pbNK cell count compared with TPO-Ab-negative patients, and ANA-positive patients had a lower pbNK cell count compared with ANA-negative patients (Fig. 3).

**Table 2. Risk factor analysis for high-NK cell count in unexplained recurrent miscarriage**

| Characteristic                        | Increased pbNK cell count |                     |                              |
|---------------------------------------|---------------------------|---------------------|------------------------------|
|                                       | OR <sup>a</sup>           | 95% CI <sup>a</sup> | <i>p</i> -value <sup>a</sup> |
| Age of female patients (years)        | 0.990                     | 0.920-1.065         | 0.782                        |
| BMI (kg/m <sup>2</sup> )              | 0.924                     | 0.815-1.049         | 0.222                        |
| Basal FSH concentration (IU/L)        | 0.853                     | 0.728-0.999         | 0.059                        |
| Basal LH concentration (IU/L)         | 1.076                     | 0.870-1.330         | 0.500                        |
| Basal estradiol concentration (pg/mL) | 0.978                     | 0.957-0.999         | 0.052                        |
| TSH concentration (uIU/mL)            | 1.086                     | 0.740-1.593         | 0.673                        |
| fT3 concentration (pmol/L)            | 0.536                     | 0.281-1.023         | 0.058                        |
| fT4 concentration (pmol/L)            | 1.037                     | 0.873-1.232         | 0.676                        |
| Qualitative TPO-Ab                    |                           |                     |                              |
| Negative                              | Reference                 |                     |                              |
| Positive                              | 5.097                     | 1.356-19.159        | 0.016                        |
| Qualitative Tg-Ab                     |                           |                     |                              |
| Negative                              | Reference                 |                     |                              |
| Positive                              | 0.437                     | 0.119-1.604         | 0.212                        |
| Qualitative ANA                       |                           |                     |                              |
| Negative                              | Reference                 |                     |                              |
| Positive                              | 0.293                     | 0.111-0.773         | 0.013                        |

BMI: body mass index; CI: confidence interval; FSH: follicle stimulating hormone; fT3: free triiodothyronine; fT4: free thyroxine; LH: luteinizing hormone; TSH: thyroid-stimulating hormone; TPO-Ab: anti-thyroid peroxidase antibody, ANA: antinuclear antibody, Tg-Ab: anti-thyroglobulin antibody; OR: odds ratio. Adjusted for the age of female patients, BMI, basal FSH concentration, basal LH concentration, basal Estradiol concentration, TSH concentration, fT3 concentration, fT4 concentration, qualitative TPO-Ab (Negative, Positive), qualitative Tg-Ab (Negative, Positive), qualitative ANA (Negative, Positive).  $P<0.05$  is considered to indicate a significant difference.



**Fig. 3.** Gating method for NK cells in the autoantibody-positive and autoantibody-negative cohort. A. gating NK cells in TPO-Ab negative patients; B. gating NK cells in the TPO-Ab positive cohort; C. gating NK cells in ANA negative cohort; D. gating NK cells in ANA positive cohort. TPO-Ab<sup>-</sup>: anti-thyroid peroxidase antibody-negative, TPO-Ab<sup>+</sup>: anti-thyroid peroxidase antibody-positive, ANA<sup>-</sup>: antinuclear antibody-negative, ANA<sup>+</sup>: antinuclear antibody-positive. The pbNK cell count in the TPO-Ab positive cohort is higher than that in the TPO-Ab negative cohort, while the pbNK cell count in the ANA positive cohort is lower than that in the ANA negative cohort. ANA: antinuclear antibody; TPO: anti-thyroid peroxidase antibody

## DISCUSSION

To the best of our knowledge, this is the first study on the correlation between pbNK and autoimmune antibodies in unexplained recurrent miscarriage. This finding suggests an underlying cause of the change in pbNK cell number and an indirect association of pbNK cell count with uRM, and enables clinicians to look at the role of pbNK cells from a new perspective that the change in pbNK cell number may be considered as a result rather than a causal factor in RM,

with a main focus on exploring potential autoimmune disorders. Therefore, there should be careful consideration on applying the change in the pbNK cell count as an immunotherapeutic index.

Thyroid-stimulating hormone (TSH) acts in an endocrine and paracrine manner on the number of NK cells by augmenting NK cells' response to IL-2, increasing their proliferative response (25, 26). This study was based on an RM population and excluded patients with autoimmune diseases and mild thyroid abnormalities but included patients who

simply tested positive for antibodies, which makes it produce more reliable results. These patients did not meet the diagnostic criteria for miscarriage-associated autoimmune diseases consisting of autoimmune thyroiditis, APS, SLE, immune vasculitis, Sjogren's syndrome, or CTD after a medical consultation with the Department of Rheumatology but had only one or more test-positive antibodies.

The baseline characteristics suggested similar endocrinological status between high-pbNK and normal-pbNK patients which reduced the bias of the effect of hormone fluctuations on immune markers. The positive rates of TPO-Ab in patients with high-pbNK count were significantly higher than those in patients with normal-pbNK count, while the positive rates of ANA were the opposite. Our results were consistent with the findings that show evidence of the relationship of elevated pbNK cells with thyroid autoimmunity (19, 20) and reduced pbNK cells in patients with autoimmune arthritis or juvenile rheumatoid arthritis (27, 28). The strengths of our studies are that the sample size was relatively large among the published studies. In addition, we excluded all known effects on pbNK cells, including subclinical hypothyroidism, because the association between subclinical hypothyroidism and the risk of RM is limited (29) and may not be regarded as the cause of RM. However, the level of pbNK cells could be influenced by TSH in the presence of SCH (30, 31). Patients with SCH were excluded from our study to make the results more convincing. Additionally, this study reduced the interference of factors that may lead to changes in the number of pbNK cells. The level of pbNK cells has been confirmed to be affected by factors such as the menstrual cycle or steroid use (32). Therefore, patients taking steroids within three months were excluded from this study. Our study has some limitations; patients who underwent pbNK cell examination on the same day of the menstrual cycle were not guaranteed, which was a possible defect of this study. However, we set a time limit for the peripheral blood

collection within the early follicular phase of the menstrual cycle (days 2 to 5), when the levels and fluctuations of reproduction-related hormones exert little effect on the NK cells. Furthermore, the positive results obtained from ANA testing cannot be used to accurately reflect the entire population of antibody-positive women with uRM since ANA testing demonstrates both sensitivity and specificity. As a result, we can only use this indicator to define a portion of the population in the management of RM, as recommended by ESHRE guidelines (1).

Multivariable logistic regression suggested that positive TPO-Ab was a promoting factor for the high pbNK cell count, while ANA was an inhibiting factor. Logistic regression using candidate variables including age, BMI, basal levels of FSH, basal levels of LH and estradiol, TSH, fT3, fT4, tT3, and tT4, and quantitative results of ANA, TPO-Ab, and Tg-Ab, was also performed, and no significant difference was found (Supplementary Table 1). The results suggested that the production of autoantibodies rather than their titers influence the fluctuation of pbNK cells. Changes in the pbNK cell count are a result of the miscarriage-associated autoimmune response rather than a causal factor for RM. One of the underlying mechanisms is that because NK cells are the effectors of innate immune responses, the presence of autoantibodies is indicative of a widespread activation in the immune system, with potential dysregulation of the pregnancy environment impacting the clinical outcome (33). Our results also suggest that the association between pbNK cell count and RM can be indirect, and the changing pbNK cell count reflects immune dysfunction, which is the cause of the relationship between RM and autoimmune antibodies. TPO Ab has been proven to lead to thyrocyte death through antibody-dependent cytotoxicity involving NK cells (34). This may result in damage to reproductive organs expressing thyroid peroxidase, and the presence of autoantibodies indicates preexisting immune abnormalities that may contribute to RM. In the initial

stages of autoimmunity, when autoantibody levels are low and physiological function is normal, the primary effect of the antibodies is the creation of a hostile immune environment at the level of the organs; as the autoimmune process advances, there is also a noticeable impairment in the response to human chorionic gonadotropin (35), which is largely responsible for miscarriage. We presume that patients with positive anti-thyroid antibodies are potential patients with autoimmune diseases, and clinical manifestations often proceed by the presence of characteristic organ-specific antibodies that might occur in serum even a few years before symptom onset and diagnosis (36). The changes in pbNK cell counts are likely to be the responses to autoimmune abnormalities, and the production of TPO-Ab and ANA indicates the onset of symptoms before clinically diagnosed thyroid or immune diseases responsible for RM emerge over time, when the normal pbNK cell count of the patients with RM may be a compensatory response rather than a real pathological change.

Hence, the increased number of pbNK cells is not a factor of uRM and cannot be applied as a therapeutic index in patients with uRM. In the process of diagnosis and treatment for RM, when there is a change in NK cell count, it should be considered whether it is the consequence of certain immune disease factors, either the progression or the complication of which can lead to miscarriage. The focus of treatment is on the immune disease and its complications rather than on NK cells. Endocrine therapy targeted at thyroid function under the condition of dynamic monitoring during pregnancy in uRM patients with both simple positive TPO-Ab and an increased number of pbNK cells may be of more clinical value. Further full-scale follow-up of pregnancy outcomes is needed to reach clearer conclusions to guide our daily clinical work. We hoped to know the pregnancy outcomes of uRM patients with simple positive TPO-Ab and elevated pbNK cell counts after treatment compared with the untreated patients; however, we found that

driven by fear of recurrence of miscarriage and excessive anxiety, most of the patients who have been followed up have undergone diverse and inconsistent therapies such as immunoglobulin, fat emulsion, hormones or immunosuppressants in different medical institutions, with few patients remaining untreated, which increases the complexity and bias of the results and makes it difficult to conduct further analysis as planned. In subsequent studies, dynamic monitoring of thyroid function before and during pregnancy requires more attention than a decline in pbNK cell count or autoantibody titers, to determine the diversion from immunotherapy to endocrine therapy directed at thyroid function in uRM patients with both simple positive TPO-Ab and an increased number of pbNK cells, as this would be of more clinical value.

## CONCLUSION

In conclusion, there is no parallel changing trend of pbNK cell count and positive autoantibody in uRM patients. The change in the pbNK cell number may be a consequence of immune responses in autoantibody-positive recurrent miscarriage patients, and there should be careful consideration in applying it as an immunotherapeutic index.

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## AUTHORS' CONTRIBUTION

NY, PL, and YY conceptualized framework and designed the study; CZ and YY investigated and supervised the research findings; NY and YY managed the data; NY and PL conducted data analysis; CZ facilitated funding acquisition; CZ, YY, HJ, PL, and NY conducted the research; NY, PL, and HJ planned the methodology of the work; YY managed the project and provided the resources; PL and HJ performed statistical analyses; NY, PL, and YY visualized and validated data; NY, PL, HJ, and YY wrote the original draft; CZ and YY edited and revised the manuscript. All authors reviewed the final version and approved the paper to be published.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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