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Assessment of the risk of unexplained recurrent spontaneous abortion based on the proportion and correlation of NK cells and T cells in peripheral blood

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Abstract.
BACKGROUND: Unexplained recurrent spontaneous abortion (URSA) is difficult to diagnose and treat clinically due to its unknown cause
OBJECTIVE: Changes in natural killer (NK) cells, T lymphocytes, and Th1(IFNγ)/Th2(IL-4) cytokines were investigated in the peripheral blood of patients with URSA to examine the pathogenesis, clinical diagnosis, and inform potential treatment strategies for this condition.
METHODS: For this study, we selected patients with URSA as the case group and included normal women in the control group. Flow cytometry was performed to detect lymphocytes and cytokines in the peripheral blood of all subjects.
RESULTS: The proportion of NK cells, Th1 cells, and the Th1/Th2 ratio were significantly higher in the URSA group compared to the control group; whereas the proportion of CD3+T cells was lower. Pairwise correlation analysis revealed a positive correlation between the percentage of NK cells and CD3+T cells, as well as CD3+CD4+T cells. Canonical correlation analysis indicated a significant correlation between NK cells and T cells, including their subgroups.
CONCLUSION: Patients with URSA have immune balance disorders, characterised by an increased proportion of peripheral blood NK cells, Th1, and Th1/Th2 ratio along with a decreased proportion of CD3+T cells. The proportion of NK cells and CD3+T may serve as predictive factors for URSA, while NK cells are closely related to the regulation of CD3+T cells and their subsets. By regulating the level of IFN-γ, NK cells can influence the proportion of CD3+T cells and induce a Th1 (IFNγ)/Th2 (IL-4) imbalance.

Keywords: Recurrent spontaneous abortion, immune balance, peripheral blood, leukocyte, natural killer cell, T cell

1. Introduction

Recurrent spontaneous abortion (RSA) refers to the occurrence of spontaneous abortion on two or more occasions with the same sexual partner before 20 weeks of pregnancy [1] and has an incidence as high as 5% in women of childbearing age [2]. The aetiology of RSA is complex and includes

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genetic factors, endocrine disorders, anatomical abnormalities, immune factors, and the prethrombotic state [3–6]. However, more than 50% of patients with RSA have an unknown aetiology, which is known as unexplained RSA (URSA). The pathogenesis of URSA has been suggested to be related to acquired immune disorders (including T/B lymphocytes, and cytokine abnormalities) and innate immune disorders (including increased numbers and activity of natural killer (NK) cells, abnormal function of macrophages and dendritic cells, and abnormal complement system) [7–9]. Studies have indicated that the regulation of peripheral blood NK (pNK) and various lymphocytes is associated with reproductive immunopathologies such as RSA, implantation failure, and preeclampsia, among other conditions [10]. Based on these hypotheses change that may be detectable in the peripheral blood of patients with URSA. This study aimed to analyze changes in T, B, and NK lymphocyte subsets and T-helper 1 (Th1)/T-helper 2 (Th2) cytokines in the peripheral blood of women with URSA and analysed the changes in lymphocytes and cytokines. Understanding the immunological contributors to the pathogenesis of URSA may provide a basis for clinical diagnosis and treatment.

2. Materials and methods

2.1. Subjects

2.1.1. URSA group

In total, 269 patients with URSA were admitted to the Family Planning Hospital of Guangdong Province from January 2014 to October 2021 and were enrolled in the study. Patients meeting the following criteria are considered URSA: (1) previously had two or more consecutive spontaneous abortions occurring at a gestational age of less than 12 weeks; (2) No abnormal chromosomal karyotype of parents; (3) No abnormality or infection of reproductive tract; (4) No abnormality in menstrual cycle, basic sex hormones, thyroid function, fasting blood sugar, etc.; (5) Autoantibodies (antinuclear antibodies, anti-Thyroid antibody, antiphospholipid antibody) were never positive; and (6) No abnormality was found in the father's semen.

2.1.2. Control group

Within the same period of time, 35 women at a similar gestational age who visited the hospital for routine check-up were enrolled as the control group. The individuals in the control group were selected based on the following criteria: (1) a history of $\geqslant 1$ normal birth; (2) No history of adverse pregnancy; (3) No complications during pregnancy; (4) No abnormal menstrual cycle; (5) No metabolic, immune-related diseases and family history.

In the morning, 10 ml of peripheral blood (non-pregnancy, non-menstrual period) was collected without food and water, of which 5 ml was stored in an anticoagulation tube containing 2% EDTA, and aliquoted in EP tubes and stored at -80°C. The rest of the blood was left to stand for 1 hour, centrifuged at 1760 $\times$ g for 14 min in a low-speed centrifuge to separate the serum, and stored at -80°C.

2.2. Research methods

2.2.1. Instruments and reagents

Assays were performed using an ABD FACSCalibur Cell flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and its analysis software Cell Quest Pro. Detection reagents were obtained from BD Biosciences and included hemolysin, buffer (0.1% in phosphate buffered saline (PBS) sodium azide,

0. 1% sodium azide in PBS prepared with paraformaldehyde solution), T cells, B cells, NK cells and interferon (IFN)-γ/interleukin (IL)-4, 12-myristate 13-acetate, and isoniazid. Protein transport blocker (brefeldin A), lysate and membrane breaker, Multitest CD3FITC/CD8PE/CD45PerCP/CD4APC, Multitest CD3FITC/CD16+CD56PE/CD45PerCP/CD19APC, IFN-γ/IL-4 were obtained from BD, USA.

2.2.2. Detection of B cells, T cells, NK cells and related cell subtypes using flow cytometry

Blood samples were collected from all subjects during the non-pregnancy and non-menstrual periods, and 3–5 days after menstruation. Add 100 μll of whole blood into the test tube, add 10 μl of monoclonal antibody each, mix well, place at room temperature away from light for 15–30 min, add 2 ml of hemolysin to dissolve red blood cells, mix well, place at room temperature for 10 min, centrifuge at 1000 rpm for 10 min, discard supernatant. Add PBS buffer 1 ml to clean the cells, centrifuge 430 g for 10 min, dump the supernatant, add 300 μ L fixative to resuspend the cells, ABD FACSCalibur Cell Flow cytometer was detected. Cell Quest software was used to classify and calculate the percentages of T, B, and NK cells and lymphocyte populations.

2.2.3. Detection of Th1 (IFNγ)/Th2 (IL-4) using flow cytometry

To a 150-μL blood sample, 150 μL RPMI-1640 (without calf serum-foetal bovine serum), 10 μL 1 μg/mL phorbol 12-myristate 13-acetate, 10 μL 50 μg/mL ionomycin, and 10 μL 0.5 mg/mL brefeldin A working solution were added and mixed well. The samples were then incubated at 37°C in a 5% CO_2 atmosphere for 3–6 h. CD3PerCP (10 μL) and CD8APC (2 μL) were added for a further 15 min. After lysing the cells, the IFN-γ/IL-4 antibody was incubated with the samples at 24°C for 20 min, followed by centrifugation. Supernatant was taken and tested on ABD FACSCalibur Cell Flow cytometer

2.2.4. Statistical analysis

Data analysis was conducted using SPSS version 26 software (SPSS, Inc., Chicago, IL, USA). Normality of the data was assessed and data with normal distribution were tested for homogeneity of variance. Two-sample t-test was used for comparing the two datasets, while rank test was used for non-normally distributed data. Statistical significance was set at $P < 0.05$. Pearson correlation analysis was performed for evaluating the correlation between NK and T cells with normal distribution. Canonical correlation analysis was conducted for two sets of NK cells and T cells. Histograms and correlation diagrams were generated using GraphPad Prism6 software (GraphPad, Inc., La Jolla, CA, USA).

3. Results

3.1. General data comparison

As presented in Table 1, the age of URSA group patients ranged from 19 to 40 years, with a mean age of 28.59 $\pm$ 3.85 years. The control group patients were aged between 21 and 35 years, with a mean age of 28.29 $\pm$ 3.45 years. The difference in mean age between the two groups was not statistically significant. The body mass indices of the case and control groups were 18.7–23.9 (average 20.81 $\pm$ 1.46) and 19.1–23.7 (average 20.82 $\pm$ 1.39), respectively. The difference in the average body mass index between the two groups was not significant.

3.2. Comparison of NK cells, B cells, and T cells and Th1 (IFNγ)/Th2 (IL-4) between control and URSA group

The percentage of NK cells was significantly higher in the URSA group compared to the control group (20.20 $\pm$ 5.34 vs 10.56 $\pm$ 2.71, $P = 0.000$). CD56dimCD16+ and CD56brightCD16-(21,22),

Table 1
Comparison of basic data between case group and control group

Group	Control ($n = 35$)	URSA ($n = 269$)	P
Age	28.29 ± 3.45	28.59 ± 3.85	0.661
BMI	20.82 ± 1.39	20.81 ± 1.46	0.958

Table 2
Comparison of NK cell, and B cell among control and URSA

Cohort	Control(35)				URSA(269)				
Variable	Mean	Median	Range	SD	Mean	Median	Range	SD	P
NK(%)*	10.56	10.24	6.10–18.64	2.71	20.20	19.91	3.34–40.50	5.34	0.000
CD3+CD56+(%)	5.7	4.9	1.02–23.17	4.48	5.33	4.68	0.38–27.64	3.37	0.971
CD56dimCD16+(%)*	92.71	95.12	74.75–99.17	6.91	95.86	97.27	70.87–99.72	4.08	0.006
CD56brightCD16-(%)	2.31	2.03	0.31–6.62	1.59	1.81	1.38	0.00–11.41	1.53	0.052
B-cell	13.05	12.21	6.06–24.58	4.32	12.17	11.86	1.78–41.99	4.57	0.207
CD19+CD5+B	18.68	11.66	2.80–59.49	15.55	14.16	12.23	0.52–59.62	9.86	0.409

Note. *: $P < 0.05$.

Table 3
Comparison of T cell, Th1(IFNγ), Th2(IL-4) and Th1(IFNγ)/Th2(IL-4) among control and URSA

Cohort	Control (35)				Mean URSA (269)				
Variable	Mean	Median	Range	SD	Mean	Median	Range	SD	P
CD3+T*	69.40	70.59	40.80–80.99	8.81	63.93	64.22	3.00–94.95	8.52	0.000
CD3+CD4+T	35.94	34.93	23.95–50.35	6.50	34.37	34.19	2.44–49.08	6.13	0.154
CD3+CD8+T	31.50	31.04	9.42–45.89	6.76	31.05	30.85	0.85–48.01	6.92	0.717
Th/Ts	1.33	1.2	0.52–3.10	0.51	1.43	1.37	0.58–3.46	0.50	0.273
Th1(IFNγ)*	16.56	14.71	7.60–34.39	6.44	22.62	21.86	0.16–61.42	10.60	0.000
Th2(IL-4)	2.17	1.5	0.39–13.40	2.25	2	1.39	0.03–10.45	1.77	0.379
Th1(IFNγ)/ Th2(IL-4)*	11.33	9.88	0.84–31.87	6.66	20.01	14.30	1.60–185	21.26	0.008

Note. *: $P < 0.05$.

two subgroups associated with cytotoxicity and cytokine production. Comparison of the NK cell subsets between the two groups (URSA vs control group) showed that CD3+CD56+(%) and CD56brightCD16-(%) did not significantly differ (5.33 ± 3.37 vs 5.70 ± 4.48, 1.81 ± 1.53 vs 2.31 ± 1.59, $P > 0.05$). The patient group exhibited a significantly lower ratio of CD3+T cells compared to the control group (63.93 ± 8.52 vs 69.40 ± 8.81, $P = 0.000$). However, there was no significant difference observed between the two groups in terms of CD3+CD4+T and CD3+CD8+T cells (34.37 ± 6.13 vs 35.94 ± 6.50, 31.05 ± 6.92 vs 31.50 ± 6.76, $P > 0.05$). The Th/Ts ratio was not significantly different between the two groups (1.43 ± 0.50 vs 1.33 ± 0.51, $P > 0.05$). The Th1 (IFNγ) ratio of the normal control group was significantly lower than that of the URSA group (2262 ± 10.60 vs 16.56 ± 6.44, $P = 0.031$). The Th1 (IFNγ)/Th2 (IL-4) ratio was higher than that of the normal control group (15.85 ± 11.01 vs 11.00 ± 8.51, $P = 0.000$), and there was no statistics for Th2 (IL-4) (2.00 ± 1.77 vs 2.17 ± 2.25, $P > 0.05$). There was no significant difference in B cells and CD19+CD5+B between the URSA group and the control group (Table 2, Table 3, Fig. 1).

3.3. Diagnostic value of the NK cell and CD3+T ratio for URSA

Comparison of the ratios of lymphocytes and their subgroups between the URSA group and normal control group showed that the ratios of NK cells and CD3+T were significantly different. To further

Table 4
The area under the curve (AUC) for NK cells and CD3+T cell

Group	AUC	Cut off	Sensitivity (%)	Specificity (%)	P
NK1	0.954	13.51	93.7	94.3	0.000
CD3+T2	0.717	69.86	79.9	62.9	0.000

Note. 1, The larger the value, the more likely it is URSA, 2, The smaller the value, the more likely it is to be URSA.

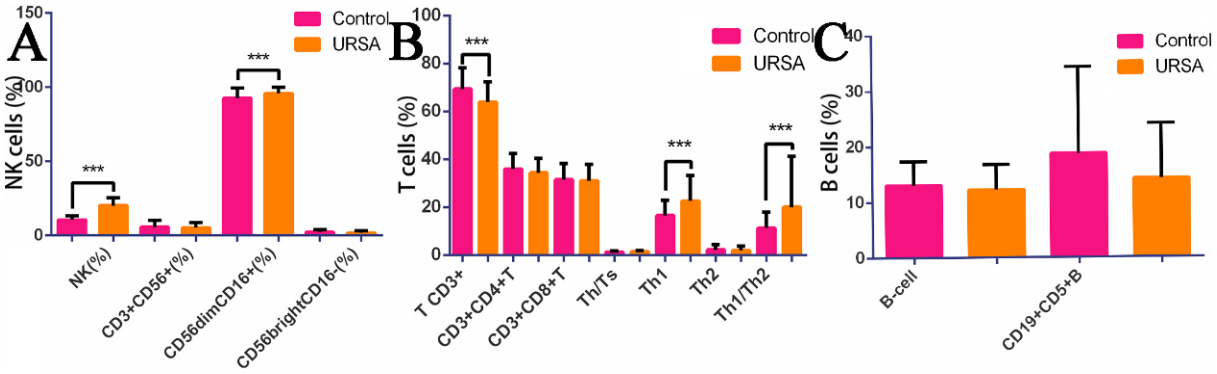

Fig. 1. Histogram of NK cell, T cell, B cell, Th1(IFNγ) and Th2(IL-4).

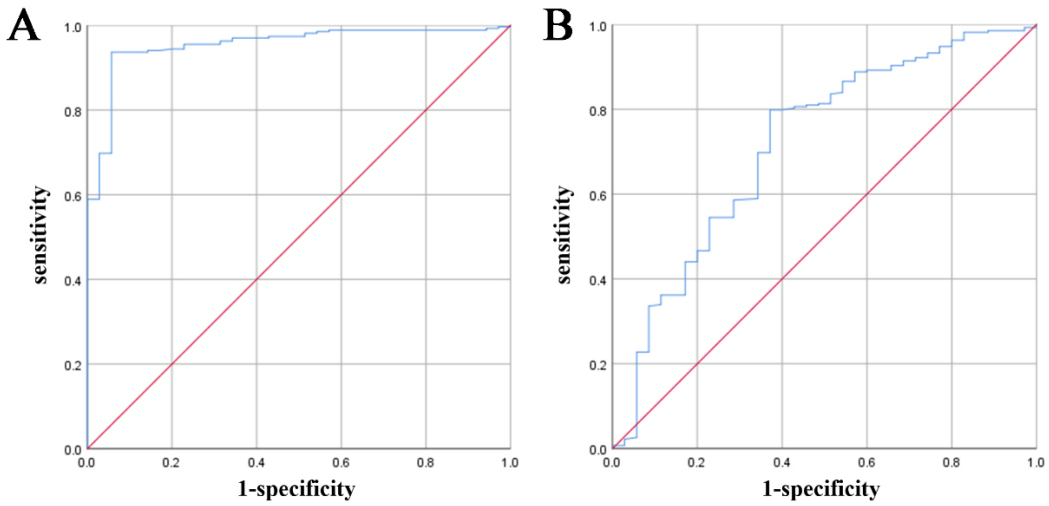

Fig. 2. ROC map of NK cells and CD3+T.

analyse the diagnostic value of NK cells and CD3+T lymphocytes in recurrent miscarriage, we draw a receiver operating characteristic (ROC) curve, as shown in Table 4 and Fig. 2. The area under the ROC curve of NK cells was 0.954 ($P = 0.000$), which is greater than the area under the chance reference line 0.5, and thereby has diagnostic value. Analysis of the ROC curve showed that the cut-off value of URSA predicted by NK cells was 13.51%, sensitivity was 93.7%, and specificity was 94.3%, In contrast, the area under the ROC of CD3+T was 0.717 ($P = 0.000$), which is greater than the chance reference line and area of 0.5, indicating diagnostic value. The predicted cut-off value of URSA was 69.86%, sensitivity was 79.9%, and specificity was 62.9%.

Table 5
Relationship between NK cell and T cell in Ursa group

Group		NK(%)	CD3+T	CD3+CD4+T	CD3+CD8+T	Th/Ts	Th1(IFγ)
NK(%)	r	1	−0.500	−0.396	−0.32	0.080	−0.012
	P	–	0.000	0.000	0.597	0.192	0.845
CD3+T	r	−0.500	1	0.471	0.438	−0.184	−0.024
	P	0.000	–	0.000	0.000	0.002	0.702
CD3+CD4+T	r	−0.396	0.471	1	−0.304	0.571	−0.144
	P	0.000	0.000	–	0.000	0.000	0.023
CD3+CD8+T	r	−0.032	0.438	−0.304	1	−0.680	0.048
	P	0.597	0.000	0.000	–	0.000	0.449
Th/Ts	r	0.080	−0.184	0.571	−0.680	1	−0.166
	P	0.192	0.002	0.000	0.000	–	0.009
Th1(IFγ)	r	−0.012	−0.024	−0.144	0.048	−0.166	1
	P	0.845	0.702	0.023	0.449	0.009	–

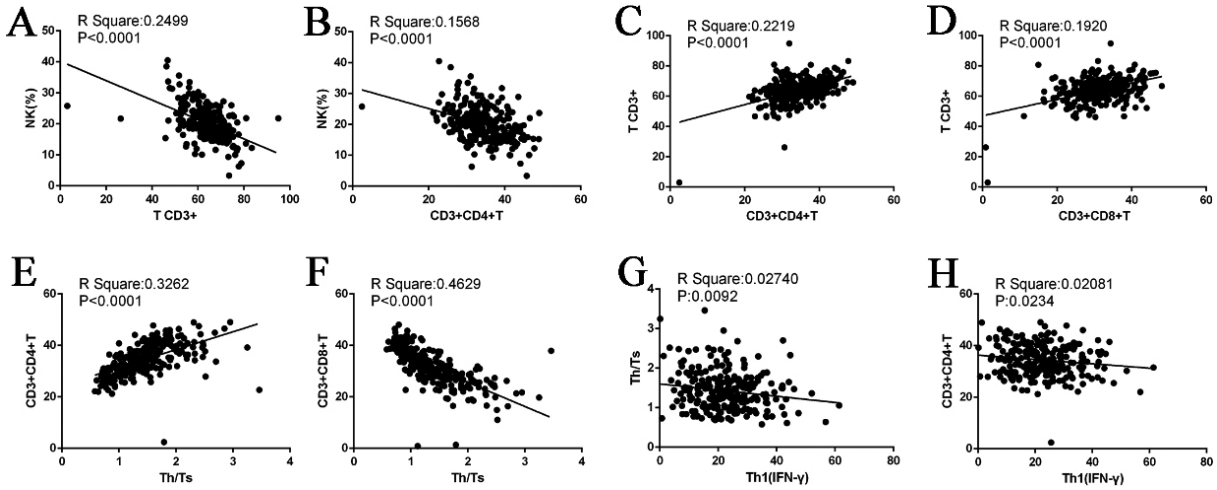

Fig. 3. Correlation diagram.

3.4. Correlation analysis of NK cells, T cell subsets, and cytokines

3.4.1. Pairwise comparison between NK cell and T cell subsets

As described in Section 3.1, we compared the ratio of lymphocytes and subgroups between the URSA and control groups. The results revealed significant differences in NK cells, CD3+T, and Th1(IFNγ). Of these cell types, NK cells, CD3+T, and their subgroups were normally distributed. Pairwise correlation analysis of indicators conforming to the normal distribution was performed, and the results are shown in Table 5 and Fig. 3.

3.4.2. Correlation between NK and T cell groups

Correlation analysis showed that NK cells are significantly related to T cells. Therefore, NK cells and their three subgroups U1 (including NK, CD3+ CD56+, CD56dimCD16+, CD56brightCD16-) and T cells and their two subgroups V1 (including CD3+T, CD3+CD4+T, CD3+CD8+T) were analysed to determine their canonical correlation. Through canonical correlation analysis, two pairs of variables were generated. The two pair coefficients were 0.553 and 0.349, respectively, which were significant ($P < 0.001$) (Table 6), indicating a correlation between NK cells, subgroups, T cells, and subgroups. As

Table 6
Canonical correlation variables and coefficients. (NK cell and T cell)

Typical variable	Canonical correlation coefficient	Wilk's	F	P
1	0.553	0.568	13.767	0
2	0.349	0.818	9.241	0
3	0.261	0.932	–	–

Table 7
Normalized canonical correlation coefficient of set 1

Group	1	2	3
NK	0.962	−0.160	0.014
CD3+CD56+	−0.194	−0.855	0.466
CD56dimCD16+	−0.059	0.455	1.007
CD56brightCD16-	−0.049	0.397	0.167
CD3+T	−1.052	−1.057	−0.489
CD3+CD4+T	−0.065	1.413	0.440
CD3+CD8+T	0.304	0.592	1.292

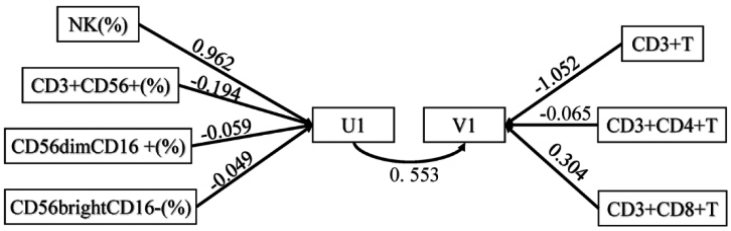

Fig. 4. Path map of canonical correlation analysis.

the correlation coefficient of the second pair of typical variables was relatively small, indicating a low correlation, it was not further analysed. According to Table 7, the composition of the first pair of typical variables is as follows:

$$\text{U1} = 0.962 \times \text{NK} - 0.194 \times (\text{CD3+CD56+}) - 0.059 \times (\text{CD56dimCD16+}) - 0.049 \times (\text{CD56brightCD16-})$$

$$\text{V1} = -1.052 \times (\text{CD3+T}) - 0.065 \times (\text{CD3+CD4+T}) + 0.304 \times (\text{CD3+CD8+T})$$

For the first pair of typical variables, U1 was positively correlated with the original NK data with a correlation coefficient r of 0.962. Thus, U1 mainly describes the total number of NK cells. V1 was negatively correlated with CD3+T with a correlation coefficient r of −1.052, and thus V1 mainly describes CD3+T. The total number of NK cells was closely related to CD3+T and, within a certain range, CD3+T decreases with an associated increase in the total number of NK cells. A typical correlation analysis path diagram is shown in Fig. 4.

4. Discussion

Pregnancy is a process of immune tolerance during allogeneic transplantation. The process from embryo implantation to successful delivery largely depends on the formation of immune tolerance at the maternal – foetal interface [11,12]. The establishment and maintenance of maternal-foetal immune balance are thought to be closely related to Th1 (IFNγ)/Th2 (IL-4) cell differentiation and dysfunction,

and the imbalance of maternal-fetal immune tolerance is the main cause of URSA [13–15]. In this study, peripheral blood T cells, B cells, NK cells, and Th1 (IFNγ)/Th2 (IL-4) cytokines were detected and compared with a control group to observe changes in the immune status of patients with URSA. B cells and their subsets represent humoral immunity; there was no difference in the proportion of B cells between the control and URSA groups. This may be because we excluded autoimmune factors in this study, and instead focused on the influence of T cells, NK cells, and Th1 (IFNγ)/Th2 (IL-4) cytokines in URSA and their interactions.

4.1. Role and predictive value of T cells in URSA

T cells play a key role in immune responses. According to their phenotypes, they can be divided into CD3+CD4+T cells and CD3+CD8+T cells, which are responsible for different immune effects. CD3+CD4+T-mediated cellular immunity accounts for approximately 65% of total T lymphocytes in the normal peripheral blood and increased or hyperfunction leads to enhanced maternal cellular immunity [16, 17]. Pregnancy is considered a type of allotransplantation; when cellular immunity is enhanced, the embryo is strongly rejected, resulting in pregnancy failure. CD3+CD8+T cells can inhibit B cell-mediated humoral immunity, inhibit the delayed anti-allergy response mediated by CD4+T cells, and interact with CD3+CD4+T cells to reach a relative equilibrium state [18]. Hence, the ratio of CD4+T/CD8+T cells can serve as an indicator of the body's immune function status. During the initial phase of normal pregnancy, the number of CD8+T cells increases and the ratio of CD4+T/CD8+T decreases, which helps in preventing the maternal immune system from rejecting the embryo. Zhao et al. reported that the percentage of CD3+CD8+T cells in the peripheral blood of patients decreased significantly and the ratio of CD4+T/CD8+T increased significantly, which was reversed by effective treatment. This indicates that the ratio of CD4+T/CD8+T in the normal range is very important for maintaining pregnancy. Previous studies demonstrated no difference in the percentage levels of peripheral blood CD3+T lymphocytes and CD3+CD4+T cells in non-pregnant patients with RSA and non-pregnant women with normal fertility, whereas the increase in the percentage of CD3+CD8+T cells was significant [19]. In this study, a decrease in the ratio of CD3+T lymphocytes was observed in the URSA group compared to the control group, while no significant differences were found in the CD3+CD4+T cell subset, CD3+CD8+T cell subset, and CD4+T/CD8+T ratio between the two groups. To evaluate the predictive value of CD3+T in URSA, a ROC curve was drawn. The results showed that the area under the ROC curve of CD3+T was 0.717, the critical value for predicting URSA was 69.86%, sensitivity was 79.9%, and specificity was 62.9%, suggesting that the percentage of CD3+T can be used to predict URSA.

4.2. Role and predictive value of NK cells in URSA

Peripheral NK (pNK) cells account for 10–15% of lymphocytes and are important intrinsic immune cells in the body [20]. According to the surface expression of CD56 and CD16 markers, mature human NK cells can be divided into two subsets, CD56dimCD16+ and CD56brightCD16- [21,22], which are related to cytotoxicity and cytokine production [23]. CD56dimCD16+NK cells dominate in the peripheral blood and are rich in granulysin and perforin, which can effectively lyse cells and are highly cytotoxic [24]. NK cell parameters, including absolute numbers or proportions (%), subsets, and functional activities such as cytotoxicity or secretion of cytokines, and other factors, have been extensively investigated in patients with URSA. The increase in the proportion and killing activity of NK cells in peripheral blood are among the parameters that may predict the risk of miscarriage, and are considered causal and prognostic factors for pregnancy loss [25].

Therefore, high levels of NK cells may play an important role in RSA. Current research has demonstrated that the use of immunoglobulin as a treatment for URSA patients has been effective. Studies have compared the percentage of peripheral blood NK cells before and after treatment and found that the number and toxicity of NK cells were reduced, leading to an improved success rate of pregnancy [26]. Ebina et al. conducted a cohort study that found an association between increased activity of peripheral blood NK cells before pregnancy and RSA, with a relative risk of 3.4 and a 95% confidence interval of 1.3–8.7 [27]. The levels of NK cells in the peripheral blood of normal pregnant women were similar to those of non-pregnant women, suggesting that NK cell levels in the peripheral blood are lower during pregnancy (similar to those before pregnancy). In addition, a meta-analysis evaluating the number and proportion of NK cells in peripheral blood of women with RSA showed that the proportion of NK cells in patients with RSA was significantly higher than the control group [25].

King et al. also reported that the total number of PNK in patients who had an abortion was higher than that in normal pregnant women [7,28]. The findings from these studies indicate a potential link between NK cell changes and pregnancy loss. It is suggested that the alteration in the number of circulating NK cells could be a primary event that plays a crucial role in the development of RSA In this study, the proportion of NK cells was significantly increased in the URSA group compared to in the normal control group, which is consistent with the results of Ly Zhu et al. [29]. To further evaluate the predictive value of NK cell ratio on URSA, we performed ROC analysis, and the results showed that the area under the ROC curve was 0.954, indicating an excellent screening effect. The Youden index was determined to be 13.51. The sensitivity and specificity at this time were 0.937 and 0.943, respectively, indicating their usefulness as predictors of URSA. By comparing the ROC curve (cut-off value, 18%) of the percentage of NK cells in the RSA group and control group, King et al. [28] suggested that the proportion of NK cells had a predictive value in evaluating patients with RSA, which is different from URSA, and thus the results of this study differ from those of the previous study.

4.3. Th1 (IFNγ)/Th2 (IL-4) balance and URSA

Immune cells at the maternal-foetal interface mainly exert immune effects by secreting cytokines. In recent years, studies of Th1 type cytokines have increased, with the results showing that IFN-γ, tumour necrosis factor-β and IL-2 have immuno-inflammatory effects and that the main Th2 type cytokines, IL-4, IL-5, and IL-10, have anti-inflammatory roles [30]. Animal experiments showed that normal pregnancy depends on Th2 type cytokines, with *in vitro* induction of Th2 type cytokines to Th1 type cytokines adversely affecting pregnancy. Majid et al. conducted a study where they observed a significant reduction in the count of Th1 lymphocytes, transcription factor expression, and cytokine levels in patients with miscarriage after immunoglobulin therapy, and significantly reduced Th1/Th2 ratio at the end of treatment, improving pregnancy success by reducing Th1/Th2 ratio [26,31]. In this study, the Th1 type cytokine detected was IFN-γ and the Th2 type cytokine was IL-4. Compared with the control group, the Th1 (IFNγ) levels and Th1 (IFNγ)/Th2 (IL-4) ratio of the URSA group were significantly higher than that of the normal control group ($P < 0.05$), whereas Th2 (IL-4) type cytokines showed no significant difference. It has been suggested that an imbalance in Th1 (IFNγ)/Th2 (IL-4) in patients with URSA is related to an increase in IFNγ, which leads to miscarriage.

Furthermore, the activation or inhibition of NK cells can be influenced by imbalances in the Th1/Th2 response, as an imbalance in proinflammatory and anti-inflammatory cytokines may occur. This creates an environment in which Th1 cytokines are prevalent in the periphery, which may lead to NK cell activation and proliferation. Thus, NK cells and Th1/Th2 interact and participate in the pathogenesis of abortion.

4.4. Correlation between NK cells and T cells in patients with URSA

Traditionally, NK cells were thought to constitute an important part of the innate immune system. However, as studies have advanced, new roles for NK cells in adaptive immunity have been continuously revealed. pNK cells act not only as killers but also as regulators of adaptive immunity. Immune disorders in patients with URSA may be related to the regulation of multiple lymphocytes. In this study, a two-by-two analysis of the ratios of NK cells, CD3+T, and Th1 (IFNγ) showed that the percentage of NK cells was positively correlated with CD3+T; CD3+CD4+T showed a negative correlation ($P = 0.000$). NK cells are innate immune cells that can affect the body's adaptive immune response in various manners, including by causing cytolysis, secretion of cytokines, and changes in antigen presentation, which affect CD3+CD4+T and CD3+CD8+T cells to different antigens. NK cells involved in the immune response, particularly those expressing CD56+ and low CD16+, can secrete a variety of cytokines that influence the immune response of T cells [32–40]. In this study, the percentage of NK cells was positively correlated with its subset, suggesting that when the total number of NK cells increases, the proportion of CD56dimCD16+ (%) cells also increased; CD56dimCD16+ (%) had a high degree of cytotoxicity and can thus negatively affect pregnancy. IFN-γ secreted by NK cells can limit the proliferation of T cells and inhibit the differentiation of CD4+T cells [40–42], in turn leading to the exhaustion of T cells and peripheral immune tolerance [43,44]. The perforin/granzyme pathway is another important pathway for NK cells to kill target cells [45]. NK cells can directly eliminate CD3+CD4+T cells through perforin, causing CD3+CD8+T cells to lack CD3+CD4+ cells. T cells cannot exert antiviral effects, making them incompetent [46]. In animal models with perforin deficiency, NK cells have not been found to directly kill or eliminate CD3+CD4+T or CD3+CD8+T cells, and virus-specific T cells can secrete more IFN-γ [46,47]. Animal studies have shown that in a virus-infected mouse model with NK cells removed, the proliferation ability of T cells was enhanced, and the number of T cells expressing IFN-γ increased. Thus, NK cells may affect T cell regulation by secreting IFN-γ. In this study, compared with the normal control group, the proportion of peripheral blood NK cells and IFN-γ in the URSA group was increased, but the proportion of CD3+T cells decreased significantly.

5. Conclusion

Our results indicate that the secretion of IFN-γ by NK cells plays a limiting role in the proliferation of T cells. Additionally, excessive IFN-γ shifts the balance between Th1 (IFNγ)/Th2 (IL-4) and causes miscarriage. At present, the regulation of T cells by NK cells involves different mechanisms of action. However, whether NK cells use multiple mechanisms to regulate T cells simultaneously or whether different NK cell subgroups are regulated by different strategies requires further analysis.

We found that patients with URSA have immune dysfunction reflected as changes in peripheral blood lymphocytes. The main manifestations are increases in the proportion of NK cells and Th1 and Th1/Th2 ratio and a decrease in the proportion of CD3+T cells. The proportion of NK cells and CD3+T cells appear to have a predictive value for URSA. In addition, different types of immune cells are correlated, making it difficult to accurately evaluate the immune disorder using a single index. Further studies are needed to confirm the mechanism underlying the mutual regulation of various immune cells.

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Ethics statement

The experimental protocol was approved by the Research Ethics Committee of the Family Planning Research Institute of Guangdong Province.

Conflict of interest

The authors declare that no conflict of interest could be perceived as prejudicing the impartiality of the research reported.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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