



# NKG2D as a Cell Surface Marker on $\gamma\delta$ -T Cells for Predicting Pregnancy Outcomes in Patients With Unexplained Repeated Implantation Failure

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Maternal immune tolerance to semi-allogeneic fetus is essential for a successful implantation and pregnancy. Growing evidence indicated that low cytotoxic activity of  $\gamma\delta$ -T cells, which is mediated by activation and inhibitory receptors, is important for establishment of maternal immune tolerant microenvironment. However, the correlation between receptors on peripheral blood  $\gamma\delta$ -T cells, such as NKG2D, CD158a, and CD158b, and pregnancy outcome in patients with unexplained repeated implantation failure (uRIF) remains unclear. In this study, the association between the expression level of these receptors and pregnancy outcome in patients with uRIF was investigated. Thirty-eight women with uRIF were enrolled and divided into two groups: successful group and failed group, according to the pregnancy outcome on different gestational periods. The percentage of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in lymphocytes was significantly higher in uRIF patients who had failed clinical pregnancy in subsequent cycle, compared with those who had successful clinical pregnancy. However, there were no differences about the frequencies of CD158a<sup>+</sup> and CD158b<sup>+</sup>  $\gamma\delta$ -T cells between the successful and failed groups. The receiver operating characteristic curve exhibited that the optimal cut-off value of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells was 3.24%, with 92.3% sensitivity and 66.7% specificity in predicting clinical pregnancy failure in uRIF patients. The patients with uRIF were further divided into two groups, group 1 (NKG2D<sup>+</sup>  $\gamma\delta$ -T cells <3.24%) and group 2 (NKG2D<sup>+</sup>  $\gamma\delta$ -T cells  $\geq$ 3.24%), based on the cut-off value. The live birth rate of patients in the group 1 and group 2 were 61.5 and 28.0%, respectively. Kaplan-Meier survival curve further suggested that the frequency of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in lymphocytes negatively correlated with live birth rate in patients with uRIF. In conclusion, our study demonstrated that the frequency of peripheral blood NKG2D<sup>+</sup>  $\gamma\delta$ -T cells among lymphocytes is a potential predictor for pregnancy outcome in uRIF patients.

**Keywords:**  $\gamma\delta$ -T cells, NKG2D, unexplained repeated implantation failure, activation and inhibitory receptors, receiver operating characteristic curve

## INTRODUCTION

The current clinical definition of infertility is the state of a diminished capacity to conceive after 12 months of regular sexual intercourse. The prevalence of infertility is ~10% among men and 13% among women (1). Although assisted reproductive technologies helped numerous infertile couples in achieving a successful pregnancy, 10–15% of the infertile couples who were subjected to assisted reproductive technology treatment experienced failure to conceive after multiple *in vitro* fertilization-embryo transfer (IVF-ET) cycles (2). These patients are defined as repeated implantation failure (RIF) patients. The etiology of RIF is complex, mainly including genetic abnormalities, endocrine disorders, abnormal anatomy of the genital tract, presence of infection and/or some autoimmunity diseases, and thrombophilia conditions (3). After ruling out the RIF patients with above common etiology, there are still some patients with unknown etiology. These RIF patients are defined as unexplained RIF (uRIF) patients.

The maternal-fetal interface is an indispensable aspect to establish and maintain the successful pregnancy. On one hand the extra villous trophoblasts invade the decidua and directly interact with decidual immune cells, on the other hand the syncytiotrophoblast provides a connection with the uterus wall to allow the contact of the maternal circulation with the fetus for transport of nutrients (4). Therefore, an appropriate recognition of the semi-allogeneic fetus by the maternal immune system is required for establishing an immune tolerant microenvironment which allows a successful implantation and pregnancy (5). In this context,  $\gamma\delta$ -T cells, a group of unconventional T cells that express the T cell receptor containing a  $\gamma$  and  $\delta$  chain, display an important role due to their early and rapid response against cellular stress and infections in an major histocompatibility complex (MHC)-independent manner (6). Although  $\gamma\delta$ -T cells represent a minor population of T lymphocytes (1–10%) (7, 8), they exhibit multiple immunological functions, such as anti-viral infection, inflammation regulation, tissue homeostasis, and even in maintaining successful pregnancy (9–13). In the early 1990s, some studies suggested that peripheral  $\gamma\delta$ -T cells play an important role in decreasing natural killer activity through secreting anti-inflammatory cytokines during the whole pregnancy process (14, 15). In addition, the low cytotoxic activity of  $\gamma\delta$ -T cells is essential during a normal pregnancy (16). The  $\gamma\delta$ -T cell subsets with potent cytotoxic activity were associated with unexplained recurrent miscarriage (17). However, the correlation between  $\gamma\delta$ -T cells and uRIF still remains unknown.

Cytotoxic activity of  $\gamma\delta$ -T cells is mainly mediated by releasing soluble cytotoxic granules, such as perforin, granzyme and granulysin (18, 19). Most importantly, the degranulation process is regulated by the balance between activation and inhibitory receptors (20). For instance, NKG2D is an activation receptor expressed on most human NK cells and  $\gamma\delta$ -T cells. The interaction between NKG2D and its ligands can modulate the cytotoxic capacity of  $\gamma\delta$ -T cells (21–23). Indeed, the activation of the NKG2D pathway induces the secretion of cytotoxic granules, interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), finally promoting the cytotoxicity of  $\gamma\delta$ -T cells against

infected cells or tumor cells (24, 25). In contrast, regulatory receptors for self-MHC class I molecules, particularly killer cell immunoglobulin-like receptors (KIRs), negatively regulate  $\gamma\delta$ -T cells activation (26). KIR with two Ig domains (KIR2D), such as KIR2DL1 (CD158a) and KIR2DL2/L3 (CD158b), which can bind to the human leukocyte antigen (HLA)-C. This interaction activates the downstream immunoreceptor tyrosine-based inhibitory motifs to inhibit the signal activation (27). Previous studies suggested that CD158a<sup>+</sup>/CD158b<sup>+</sup>  $\gamma\delta$ -T cells were less cytotoxic than CD158a<sup>-</sup>/CD158b<sup>-</sup>  $\gamma\delta$ -T cells (28, 29). However, up to now, little is known regarding the relationship between expression pattern of NKG2D, CD158a and CD158b on  $\gamma\delta$ -T cells and pregnancy outcome in uRIF patients.

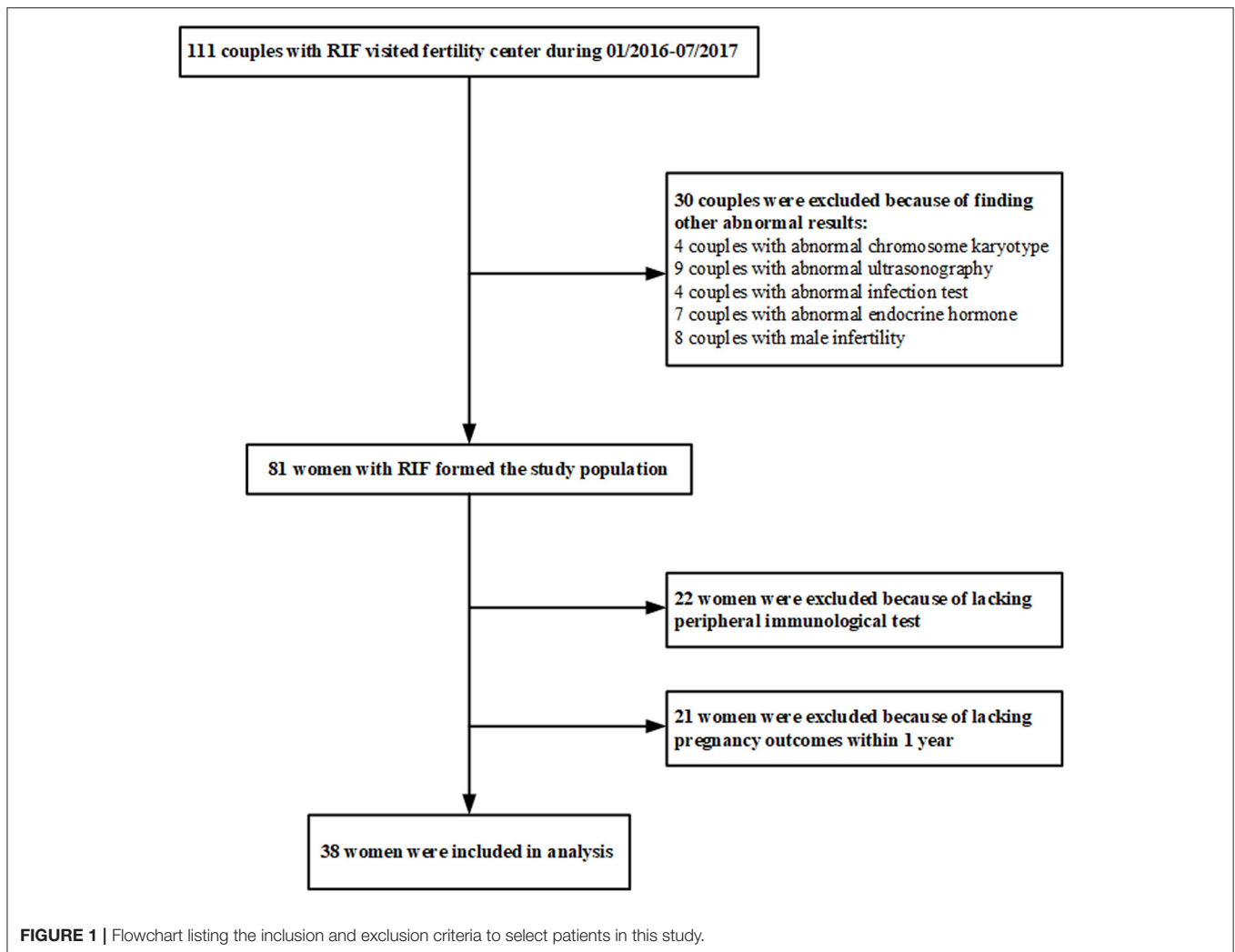
In this study, we demonstrated that high frequency of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in lymphocytes was inversely correlated with pregnancy outcomes in patients with uRIF. Our data suggest that the frequency of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in lymphocytes could be used to predict pregnancy outcome in uRIF patients.

## MATERIALS AND METHODS

### Study Population

Female infertile patients were selected from infertile couples who went to the Fertility Center of Shenzhen Zhongshan Urology Hospital, China, from January 2016 to July 2017. The inclusion criteria include: (1) age of 18–40 years; (2) met the diagnostic criteria of RIF: three or more IVF failures after the transfer of at least one high-quality embryo per cycle. The potential conventional factors causing RIF were not taken into consideration at the time of selection. The exclusion criteria include: (1) abnormal chromosome karyotype; (2) abnormal ultrasonography and hysterosalpingogram/hysteroscopy result; (3) infection of hepatitis B virus, hepatitis C virus, human immunodeficiency virus, toxoplasma, rubella virus, cytomegalovirus, or herpes virus; (4) abnormal basal endocrine hormone level, such as follicle-stimulating hormone, estradiol, luteinizing hormone, prolactin, or testosterone; (5) male infertility. The peripheral blood of the patients enrolled in this study was collected to perform immunological assays, and then these patients received an empirical therapy represented by the endometrium scratching operation. Women who did not provide the blood sample for immunological assays were excluded. To match the end-points of the study, women who were not subjected to embryo transfer operation within 1 year after the immunological tests were also excluded. A total of 111 couples met the diagnostic criteria of RIF and were enrolled in the study (Figure 1). After excluding 30 subjects with conventional factors, a total of 81 uRIF women comprised the study population. There were 22 subjects who failed immunologic testing and 21 women who did not undergo embryo transfer within 1 year after immunologic testing, resulting in a total of 38 uRIF women who participated in the statistical analysis.

To assess the power value of the receptors on the  $\gamma\delta$ -T cell in predicting the outcome of pregnancy failure in patients with uRIF on different gestational periods, implantation, clinical pregnancy, ongoing pregnancy, and live birth were followed up. Potential successful implantation was monitored at ~14 days after embryo



transfer and was defined as the presence of seropositive-human chorionic gonadotropin. Otherwise, implantation failure was defined. A successful clinical pregnancy was defined as the presence of a gestational sac as shown by ultrasound 4–5 weeks after embryo transfer. If absent, a failed clinical pregnancy was defined. Successful sustained pregnancy was defined as the presence of a live fetus of ~12 weeks gestational age. Otherwise, it was defined as a failure of the ongoing pregnancy. Successful live birth was defined as the delivery of a live fetus by the mother at full term gestation, otherwise, it was defined as failure of live birth.

This study was performed in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Shenzhen Zhongshan Urology Hospital. All participants gave their informed consent for the inclusion in this study before their participation.

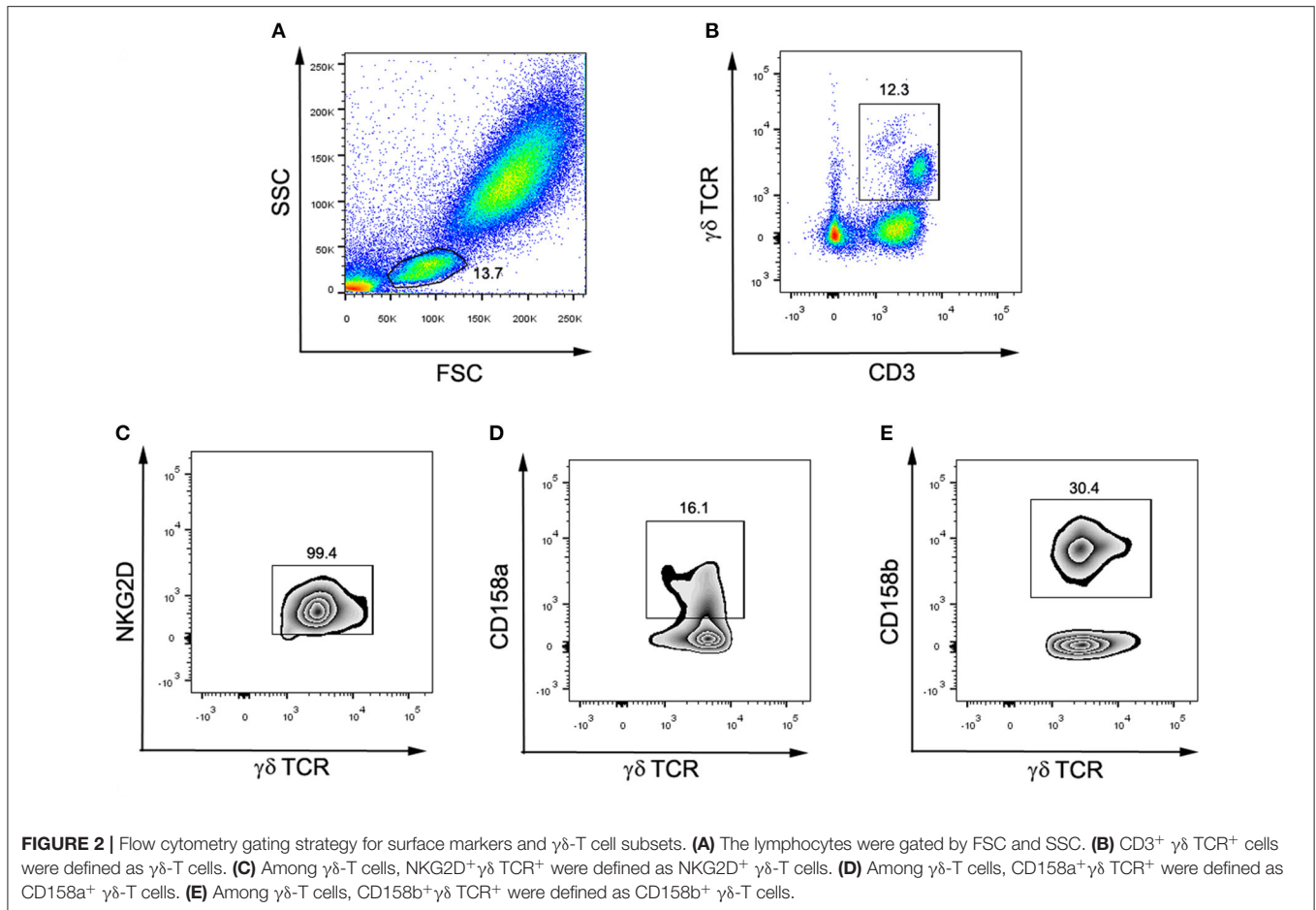
## Flow Cytometry

Immunological phenotypes of  $\gamma\delta$ -T cells and their subpopulations were determined by flow cytometry. The following fluorophore-conjugated mouse monoclonal antibodies

anti-human antibodies were used: PerCP-conjugated anti-CD3 (347344; BD Pharmingen, USA), PE-cy7-conjugated anti-TCR $\gamma\delta$  (331222; Biolegend, USA), FITC-conjugated anti-NKG2D (11-5878; eBioscience, USA), FITC-conjugated anti-CD158a (556062; BD Pharmingen, USA), PE-conjugated anti-CD158b (559785; BD Pharmingen, USA).

## Phenotypic Characteristics of $\gamma\delta$ -T Cells

To determine the surface expression of activation markers on the surface of  $\gamma\delta$ -T cells, the heparinized whole blood (100  $\mu$ L) was collected during the mid-luteal phase of the menstrual cycle and was stained with anti-CD3 (5  $\mu$ L), anti-TCR $\gamma\delta$  (5  $\mu$ L), and anti-NKG2D (10  $\mu$ L) antibodies. To determine the surface expression of inhibitory markers on  $\gamma\delta$ -T cells, the heparinized whole blood (100  $\mu$ L) was incubated for 15 min with antibodies against CD3 (5  $\mu$ L),  $\gamma\delta$  TCR (5  $\mu$ L), CD158a (10  $\mu$ L), and CD158b (10  $\mu$ L). After 15 min of incubation in the dark, the whole blood was lysed using 1 $\times$ FACS lysis solution, and the cells were subsequently washed three times with PBS. Finally, cells were analyzed with a BD FACSCanto II flow cytometer (BD, USA). According to the gating strategy (Figure 2), the lymphocytes were gated by



FSC and SSC first. Then  $CD3^+ TCR\gamma\delta^+$  cells were defined as  $\gamma\delta$ -T cells. In the population of  $\gamma\delta$ -T cells,  $TCR\gamma\delta^+NKG2D^+$  cells,  $TCR\gamma\delta^+CD158a^+$  cells, and  $TCR\gamma\delta^+CD158b^+$  cells were defined as  $NKG2D^+ \gamma\delta$ -T cells,  $CD158a^+ \gamma\delta$ -T cells and  $CD158b^+ \gamma\delta$ -T cells, respectively. The percentage of receptor-positive  $\gamma\delta$ -T cells in total  $\gamma\delta$ -T cells,  $CD3^+$  T cells, and overall lymphocytes was respectively calculated.

## Statistical Analysis

Statistical analysis was performed using SPSS 20.0 (IBM, USA). Kolmogorov-Smirnov test was used to determine the normal distribution of the data. Data were presented as mean  $\pm$  SD when normally distributed, or as median with quartiles when not normally distributed. Inter-group differences in immune markers were examined using the independent *t*-test when the data were normally distributed, or using the Mann-Whitney *U*-test when they were not normally distributed. The categorical variables were analyzed by chi-square test or Fisher's exact test. Receiver operating characteristic (ROC) curve and the area under the curve (AUC) were used to establish the power value of the immune markers in predicting the pregnancy outcome in patients with uRIF. The overall live birth rate and gestational age of the patients with uRIF were estimated by the Kaplan-Meier analysis. The time to event was referred to the length in weeks from the date of the embryo transfer to the date of the patient

undergoing pregnancy failure, or to the date of the end point of the pregnancy in patients who gave live birth, or to the date of loss to follow-up (censored). A two-tailed *P*-value of  $< 0.05$  was considered statistically significant.

## RESULTS

### Clinical Characteristics for uRIF Patients Categorized by Pregnancy Outcomes

To investigate the relationship between basic clinical characteristics and pregnancy outcomes, basic clinical characteristics were compared between uRIF patients with successful pregnancy outcome and patients with failed pregnancy outcome on different gestational periods. As shown in **Table 1**, regardless of the gestational period, there was no evident difference in the clinical characteristics, such as age, body mass index, number of previous failed pregnancy, content of estradiol, progesterone, follicle-stimulating hormone, luteinizing hormone, prolactin, and testosterone (all  $P > 0.05$ ), between the successful group and failed group. Other factors which potentially affect the pregnancy outcome during IVF-ET treatment process, such as percentage of blastocyst in the transferred embryos, number of transferred embryos, number of

**TABLE 1** | Clinical characteristics of uRIF patients categorized according to the pregnancy outcome.

Factors	Implantation		P	Clinical pregnancy		P	Ongoing pregnancy		P	Live birth		P
	Successful (n = 23)	Failed (n = 15)		Successful (n = 20)	Failed (n = 18)		Successful (n = 16)	Failed (n = 22)		Successful (n = 15)	Failed (n = 23)	
Age (years)	34.7 ± 4.1	36.3 ± 3.5	0.221	34.8 ± 4.4	35.9 ± 3.4	0.380	34.6 ± 4.6	35.8 ± 3.3	0.338	34.5 ± 4.8	35.8 ± 3.3	0.346
BMI (kg/m <sup>2</sup> )	21.3 ± 2.3	21.7 ± 2.0	0.502	21.1 ± 2.4	21.7 ± 1.9	0.435	20.9 ± 2.3	21.8 ± 2.0	0.206	20.9 ± 2.4	21.8 ± 1.9	0.233
No. of previous pregnancy failure	4.9 ± 2.0	4.1 ± 2.4	0.244	4.9 ± 2.0	4.2 ± 2.4	0.342	5.3 ± 2.0	4.1 ± 2.2	0.104	5.2 ± 2.0	4.2 ± 2.2	0.156
E2 (pg/mL)	40.5 ± 11.9	40.3 ± 20.4	0.963	42.3 ± 11.4	38.3 ± 19.3	0.434	42.7 ± 12.8	38.8 ± 17.4	0.449	42.9 ± 13.2	38.9 ± 17.0	0.445
P (ng/mL)	0.3 ± 0.1	0.3 ± 0.2	0.616	0.3 ± 0.1	0.3 ± 0.2	0.513	0.3 ± 0.1	0.3 ± 0.2	0.491	0.3 ± 0.1	0.3 ± 0.2	0.487
FSH (mIU/mL)	6.8 ± 1.7	6.8 ± 2.0	0.953	6.5 ± 1.2	7.0 ± 2.3	0.403	6.4 ± 1.3	7.0 ± 2.1	0.360	6.4 ± 1.4	7.0 ± 2.0	0.355
LH (mIU/mL)	5.1 ± 1.8	4.0 ± 1.5	0.050	4.9 ± 1.6	4.4 ± 1.9	0.373	4.7 ± 1.5	4.6 ± 1.9	0.912	4.7 ± 1.5	4.6 ± 1.9	0.911
PRL (ng/mL)	19.5 ± 5.2	18.1 ± 5.5	0.408	19.8 ± 5.2	18.1 ± 5.4	0.331	19.7 ± 5.8	18.4 ± 5.0	0.470	19.7 ± 6.0	18.4 ± 4.9	0.465
T (ng/mL)	0.6 ± 0.3	1.1 ± 2.1	0.380	0.6 ± 0.3	1.0 ± 2.0	0.430	0.6 ± 0.3	0.9 ± 1.8	0.513	0.6 ± 0.3	0.9 ± 1.7	0.509
Blastocyst/transferred embryos	52.2%	33.3%	0.326	50.0%	38.9%	0.532	56.2%	36.4%	0.324	60.0%	34.8%	0.185
No. of transferred embryos	1.9 ± 0.5	2.2 ± 0.7	0.106	1.9 ± 0.6	2.2 ± 0.6	0.114	1.8 ± 0.7	2.1 ± 0.6	0.110	1.9 ± 0.6	2.1 ± 0.6	0.287
No. of high-quality embryos	1.4 ± 0.8	1.6 ± 0.9	0.445	1.4 ± 0.8	1.6 ± 0.9	0.575	1.3 ± 0.9	1.6 ± 0.8	0.343	1.4 ± 0.9	1.6 ± 0.8	0.445

BMI, body mass index; E2, estradiol; P, progesterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PRL, prolactin; T, testosterone.

**TABLE 2** | Comparison of frequency of  $\gamma\delta$ -T subsets in uRIF patients with successful pregnancy and those patients with pregnancy failure.

Cell populations	Implantation		P	Clinical pregnancy		P	Ongoing pregnancy		P	Live birth		P
	Successful (n = 23)	Failed (n = 15)		Successful (n = 20)	Failed (n = 18)		Successful (n = 16)	Failed (n = 22)		Successful (n = 15)	Failed (n = 23)	
<b>In <math>\gamma\delta</math>-T cells (%)</b>												
NKG2D <sup>+</sup> $\gamma\delta$ -T cells	79.2 ± 25.0	91.1 ± 5.2	0.078	81.3 ± 21.0	86.8 ± 20.0	0.077	82.9 ± 21.0	84.6 ± 20.4	0.438	82.7 ± 21.7	84.7 ± 20.0	0.595
CD158a <sup>+</sup> $\gamma\delta$ -T cells	13.1 ± 10.6	11.9 ± 11.2	0.734	13.4 ± 11.2	11.8 ± 10.5	0.660	11.7 ± 9.2	13.3 ± 11.9	0.660	12.2 ± 9.3	12.9 ± 11.7	0.836
CD158b <sup>+</sup> $\gamma\delta$ -T cells	18.5 ± 16.4	17.0 ± 13.6	0.778	18.5 ± 17.3	17.2 ± 13.0	0.789	22.1 ± 17.6	14.8 ± 12.7	0.149	23.3 ± 17.5	14.3 ± 12.6	0.075
<b>In CD3<sup>+</sup> T cells (%)</b>												
$\gamma\delta$ -T cells	8.4 ± 3.7	13.0 ± 10.5	0.120	8.2 ± 3.8	12.5 ± 9.6	0.090	8.4 ± 4.2	11.5 ± 8.9	0.216	8.8 ± 4.1	11.1 ± 8.9	0.352
NKG2D <sup>+</sup> $\gamma\delta$ -T cells	8.0 ± 3.8	12.4 ± 10.4	0.140	7.7 ± 4.0	12.0 ± 9.5	0.093	8.1 ± 4.3	10.9 ± 8.9	0.254	8.5 ± 4.2	10.5 ± 8.9	0.405
CD158a <sup>+</sup> $\gamma\delta$ -T cells	1.3 ± 1.1	2.1 ± 2.9	0.378	1.4 ± 1.1	1.9 ± 2.7	0.418	1.3 ± 1.1	1.8 ± 2.5	0.432	1.4 ± 1.1	1.8 ± 2.4	0.584
CD158b <sup>+</sup> $\gamma\delta$ -T cells	1.6 ± 1.8	2.5 ± 3.2	0.298	1.6 ± 1.9	2.4 ± 2.9	0.357	1.9 ± 2.0	2.0 ± 2.8	0.972	2.1 ± 2.0	1.9 ± 2.7	0.359
<b>In lymphocytes (%)</b>												
CD3 <sup>+</sup> T cells	60.1 ± 22.4	62.2 ± 7.3	0.064	59.5 ± 24.0	62.5 ± 6.9	0.016*	61.3 ± 26.5	60.6 ± 7.7	0.258	61.4 ± 27.5	60.6 ± 7.5	0.887
$\gamma\delta$ -T cells	4.8 ± 2.0	8.4 ± 7.3	0.079	4.5 ± 2.1	8.0 ± 6.7	0.047*	4.7 ± 2.2	7.3 ± 6.3	0.095	4.9 ± 2.1	7.0 ± 6.2	0.224
NKG2D <sup>+</sup> $\gamma\delta$ -T cells	4.0 ± 2.1	8.0 ± 7.1	0.050	3.8 ± 2.1	7.6 ± 6.5	0.025*	4.0 ± 2.1	6.7 ± 6.2	0.067	4.2 ± 2.1	6.5 ± 6.1	0.166
CD158a <sup>+</sup> $\gamma\delta$ -T cells	0.8 ± 0.5	1.4 ± 1.6	0.164	0.8 ± 0.5	1.3 ± 1.5	0.197	0.8 ± 0.5	1.2 ± 1.3	0.199	0.8 ± 0.5	1.2 ± 1.3	0.211
CD158b <sup>+</sup> $\gamma\delta$ -T cells	1.1 ± 1.2	1.7 ± 2.1	0.240	1.0 ± 1.2	1.7 ± 1.9	0.190	1.2 ± 1.2	1.4 ± 1.8	0.641	1.2 ± 1.3	1.4 ± 1.8	0.739

\*P < 0.05.

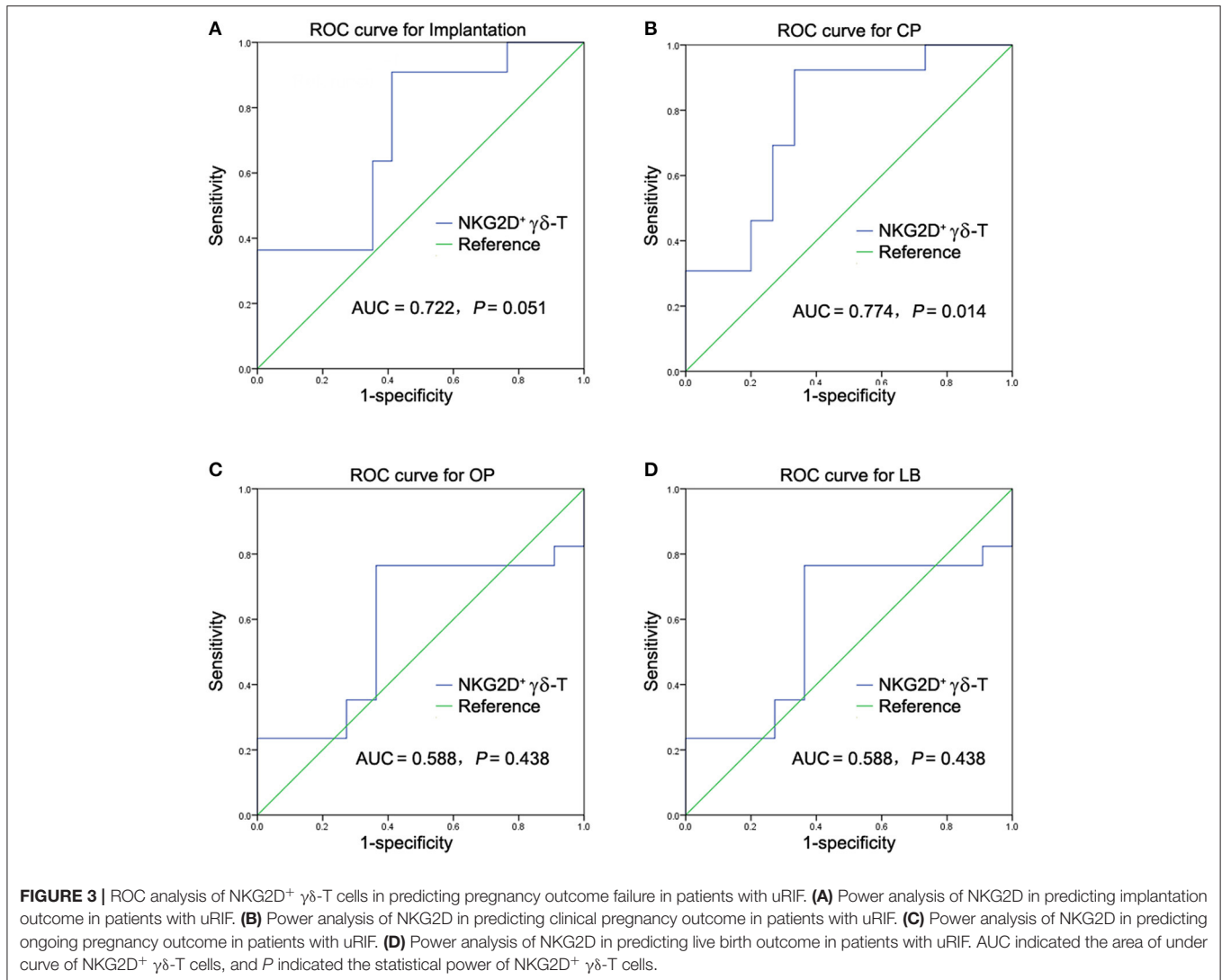
transferred high-quality embryos were also comparable between the successful group and failed group.

## Expression of NKG2D on $\gamma\delta$ -T cells Were Increased in uRIF Patients With Clinical Pregnancy Failure

To investigate the relationship between surface receptors on  $\gamma\delta$ -T cells and pregnancy outcome in patients with uRIF, the percentage of NKG2D<sup>+</sup>, CD158a<sup>+</sup> or CD158b<sup>+</sup>  $\gamma\delta$ -T cells in  $\gamma\delta$ -T cells, CD3<sup>+</sup> T cells or overall lymphocytes were compared between the successful group and failed group, respectively. As

shown in **Table 2**, there were no significant differences in the percentages of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells, CD158a<sup>+</sup>  $\gamma\delta$ -T cells and CD158b<sup>+</sup>  $\gamma\delta$ -T cells in total  $\gamma\delta$ -T cells between the successful group and failed group. The percentages of total  $\gamma\delta$ -T cells, NKG2D<sup>+</sup>  $\gamma\delta$ -T cells, CD158a<sup>+</sup>  $\gamma\delta$ -T cells and CD158b<sup>+</sup>  $\gamma\delta$ -T cells in CD3<sup>+</sup> T cells were similar between the successful group and failed group. In addition, no significant differences were found on the percentages of CD158a<sup>+</sup>  $\gamma\delta$ -T cells and CD158b<sup>+</sup>  $\gamma\delta$ -T cells in the overall lymphocytes between the successful group and failed group. However, the percentages of CD3<sup>+</sup> T cells [(62.5 ± 6.9)% vs. (59.5 ± 24.0)%, P = 0.016],  $\gamma\delta$ -T cells [(8.0 ± 6.7)% vs. (4.5 ± 2.1)%, P = 0.047], and NKG2D<sup>+</sup>  $\gamma\delta$ -T cells





[(7.6 ± 6.5)% vs. (3.8 ± 2.1)%, *P* = 0.025] in lymphocytes were significantly increased in uRIF patients with clinical pregnancy failure, when compared to that in uRIF patients with successful clinical pregnancy. Taken together, our results suggested that the increase of NKG2D<sup>+</sup> γδ-T cell frequency in lymphocytes might be associated with clinical pregnancy failure in uRIF patients.

### Power Analysis of NKG2D<sup>+</sup> γδ-T cells in Predicting Pregnancy Failure in uRIF Patients

To further evaluate the predictive power and cut-off value of NKG2D<sup>+</sup> γδ-T cells in predicting pregnancy failure in uRIF patients, 28 patients were randomly selected from the study population and analyzed by the ROC curve, while the remaining 10 patients were used to validate the power. The ROC curve showed that the accuracies of NKG2D<sup>+</sup> γδ-T cells in predicting implantation, clinical pregnancy, ongoing pregnancy and live birth, measured by AUC, were 0.722 (95% CI: 0.526-0.917, *P* = 0.051, **Figure 3A**), 0.774 (95% CI: 0.597-0.951, *P* = 0.014, **Figure 3B**), 0.588 (95% CI: 0.366-0.811, *P*

= 0.438, **Figure 3C**), and 0.588 (95% CI: 0.366-0.811, *P* = 0.438, **Figure 3D**), respectively. These results indicated that the frequency of NKG2D<sup>+</sup> γδ-T cells in lymphocytes could be used as a potential indicator to predict clinical pregnancy failure in patients with uRIF. At a cut-off value of 3.24% referred to the percentage of NKG2D<sup>+</sup> γδ-T cells in lymphocytes, the sensitivity and specificity were 92.3 and 66.7%, respectively. In the validation set, the sensitivity, specificity, positive predictive value, and negative predictive value of NKG2D<sup>+</sup> γδ-T cells for clinical pregnancy failure were 80.0, 20.0, 50.0, and 50.0%, respectively. The high accuracy and sensitivity in predicting clinical pregnancy failure further indicated that the percentage of NKG2D<sup>+</sup> γδ-T cells in lymphocytes was a potential prognostic indicator for clinical pregnancy failure in uRIF patients.

### Kaplan-Meier Survival Analysis Based on NKG2D<sup>+</sup> γδ-T cells in Patients With uRIF

The frequency of NKG2D<sup>+</sup> γδ-T cells in lymphocytes allowed us to classify the uRIF patients into subgroups (group 1: NKG2D<sup>+</sup>

**TABLE 3** | Clinical characteristics of uRIF patients categorized based on the cut-off value of percentage of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in lymphocytes.

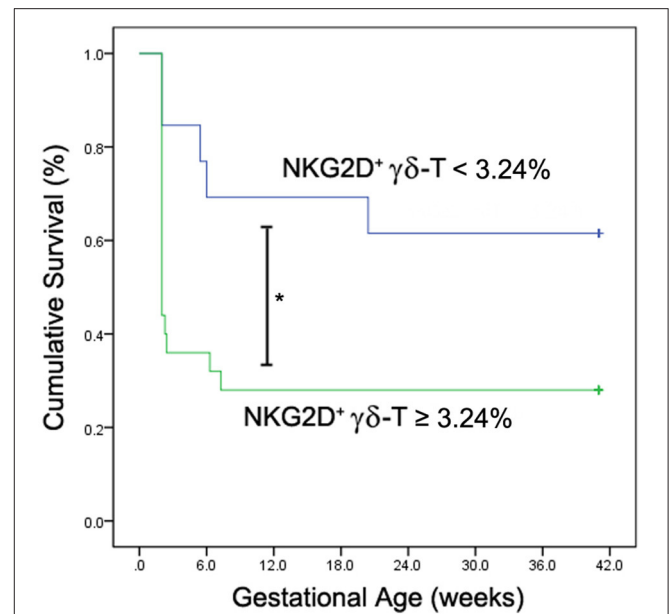
Factors	NKG2D <sup>+</sup> $\gamma\delta$ -T cells		P
	Low group (n = 13)	High group (n = 25)	
Age (years)	35.6 ± 3.9	35.1 ± 4.0	0.718
BMI (kg/m <sup>2</sup> )	22.3 ± 1.6	21.0 ± 2.3	0.083
No. of previous pregnancy failure	4.8 ± 2.6	4.5 ± 2.0	0.702
E2 (pg/mL)	43.3 ± 14.3	38.9 ± 16.2	0.414
P (ng/mL)	0.3 ± 0.1	0.3 ± 0.2	0.767
FSH (mIU/mL)	6.5 ± 1.8	6.9 ± 1.8	0.541
LH (mIU/mL)	4.9 ± 2.1	4.5 ± 1.6	0.546
PRL (ng/mL)	20.4 ± 7.0	18.2 ± 4.1	0.225
T (ng/mL)	0.5 ± 0.3	1.0 ± 1.6	0.286
Blastocyst/transferred embryos	61.5%	36.0%	0.178
No. of transferred embryos	1.9 ± 0.6	2.0 ± 0.6	0.585
No. of high-quality embryos	1.4 ± 0.8	1.6 ± 0.9	0.555

BMI, body mass index; E2, estradiol; P, progesterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PRL, prolactin; T, testosterone.

$\gamma\delta$ -T cells  $\leq 3.24\%$ ; group 2: NKG2D<sup>+</sup>  $\gamma\delta$ -T cells  $> 3.24\%$ ; cut-off value was determined with the ROC curve). The clinical characteristics of these two groups are listed in **Table 3**, and the results showed no evident difference among the basic parameters in these two groups. The live birth rate of patients in the group 1 (NKG2D<sup>+</sup>  $\gamma\delta$ -T cells  $< 3.24\%$ ) and group 2 (NKG2D<sup>+</sup>  $\gamma\delta$ -T cells  $\geq 3.24\%$ ) were 61.5% and 28.0%, respectively. Kaplan-Meier survival curve (**Figure 4**) and the test of the equality of survival distribution (**Supplementary Table 1**) further demonstrated that the frequency of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in lymphocytes negatively correlated with live birth rate in patients with uRIF. In addition, the estimated mean gestational age was 28 weeks in the group 1, while it was only 13 weeks in the group 2, suggesting that the presence of a low frequency of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in lymphocytes corresponded to a significantly increased chance of successful live birth (**Supplementary Table 2**). Taken together, these data suggested that the frequency of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in lymphocytes was negatively associated with the live birth rate and gestational age in uRIF patients.

## DISCUSSION

Our study demonstrated that the frequency of peripheral NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in lymphocytes was significantly increased in the uRIF patients with failure of implantation or clinical pregnancy compared to that in the uRIF patients with corresponding successful pregnancy. The ROC analysis for the diagnostic power of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells resulted in an AUC of 0.774, with 92.3% sensitivity and 66.7% specificity in differentiating uRIF patients with successful clinical pregnancy from those with clinical pregnancy failure. Moreover, K-M survival curve analysis showed that the frequency of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells was negatively associated with live birth rate and



**FIGURE 4** | Kaplan-Meier survival analysis of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells for live birth rate in uRIF patients. The cumulative survival rate (live birth rate) was compared between uRIF patients with low frequency of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells ( $< 3.24\%$ , blue line) and those with high frequency of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells ( $\geq 3.24\%$ , green line). \* $P < 0.05$ .

gestational age. These results suggest that the NKG2D on  $\gamma\delta$ -T cell is a potential biochemical predictor for pregnancy outcome in uRIF patients.

Previous studies showed that peripheral  $\gamma\delta$ -T cells may be involved in recurrent miscarriage through the increased pro-inflammatory factors such as interleukin (IL)-12, IL-17, and IFN- $\gamma$ , and the decreased anti-inflammatory factors such as IL-10 (17, 30). The increased NKG2C and decreased NKG2A on peripheral  $\gamma\delta$ -T cells were also reported to be associated with premature birth (31) and pre-eclampsia (32). In current study, we showed that the percentage of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in lymphocytes was significantly increased in the uRIF patients with a failure in implantation or clinical pregnancy, when compared to that in the uRIF patients with successful implantation or successful clinical pregnancy. Taken together, these data indicated that abnormal expression of molecules regulating cytotoxicity of  $\gamma\delta$ -T cells was associated with adverse pregnancy outcomes. However, these studies were mainly based on the comparisons of the  $\gamma\delta$ -T cell status between women with adverse pregnancy and normal pregnancy, the prognosis value of these markers in reproductive failure was not investigated. Therefore, the clinical significance of these conclusions is greatly reduced. In this study, we demonstrated that the AUC of the frequency of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in lymphocytes to predict the clinical pregnancy failure was 0.774, and the optimal cut-off value was 3.24% with 92.3% sensitivity and 66.7% specificity. Moreover, further K-M survival analysis showed that the lower live birth rate and shorter gestational age were observed in uRIF patients with a high frequency of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells. Our results

indicate that NKG2D on  $\gamma\delta$ -T cell is a potential predictor for pregnancy outcome in uRIF patients. It is known that the cytotoxic activity of  $\gamma\delta$ -T cells against virus or cancer cells was determined by the expression of NKG2D and granzyme B (GrB) (22, 33–35). Recently we also found that the percentage of GrB<sup>+</sup>  $\gamma\delta$ -T cells in lymphocytes was negatively associated with successful clinical pregnancy (36). These data suggest that the increase of  $\gamma\delta$ -T cell cytotoxicity is deleterious to the pregnancy.

The limitations of this study are reflected in the fact that, although the sensitivity of NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in predicting clinical pregnancy failure was as high as 80.0% during the validation phase, the specific, positive, and negative predictive values were as low as 20.0, 50.0, and 50.0%, respectively. The following three factors may contribute to them. First, the relatively small sample size in this study. The larger sample size, the greater statistical power and accuracy of the prediction. Therefore, a study with a larger sample size should be further performed to confirm this conclusion. Second, other receptors related to the cytotoxicity of  $\gamma\delta$ -T cells, such as NKG2C (31) and NKG2A (32), should be investigated in further study. Third, the appropriate interaction between receptors on immune cells and corresponding ligands on trophoblasts should be also considered because they are also associated with a successful pregnancy (37). The role of NKG2D at the maternal-fetal interface might be modified by the corresponding ligands expressed on trophoblasts. Previous study reported that the KIR genotype and haplotype is associated with pregnancy loss during IVF cycles. KIR B haplotype carriers experienced more pregnancy failure than KIR A haplotype carriers after embryo transfer. However, this risk was significantly influenced by their corresponding ligand, such as HLA-C alleles, present in the embryo. The high-risk combinations (KIR B/HLA-C1) resulted in a 51% increased risk of pregnancy failure (38). Therefore, the overall prognostic value of combined indicator with NKG2D and potential ligands on trophoblasts in predicting pregnancy outcomes in uRIF patients should be also further investigated.

Taken together, our study suggests that the increased frequency of peripheral NKG2D<sup>+</sup>  $\gamma\delta$ -T cells in lymphocyte is significantly associated with pregnancy failure in uRIF patients. The ROC and K-M survival analysis indicate that NKG2D on  $\gamma\delta$ -T cell is a potential biomarker in predicting pregnancy outcome in uRIF patients. It is worth noting that finding effective treatment and evaluating their efficacy against NKG2D on  $\gamma\delta$ -T cells is a necessary step before this predictor can be applied in clinical translation.

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## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Shenzhen Zhongshan Urology Hospital, Shenzhen, Guangdong Province, PR China. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

WT and CH designed the study, performed the data analysis, and wrote the manuscript. ZX performed the data analysis and critically reviewed the manuscript. YZh and YL performed the experiments and assisted in the preparation of the figures and tables. JX and HZ enrolled the subjects and collected the peripheral blood. YZe contributed to the study design, and critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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