



Increased CD56⁺ NK cells and enhanced Th1 responses in human unexplained recurrent spontaneous abortion

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ABSTRACT. Recurrent spontaneous abortion (RSA) is reported to be associated with immune imbalance at the maternal-fetal interface. Immune cells in the decidual tissue are involved in maintaining immune tolerance during pregnancy; however, whether natural killer (NK) and T cells are altered in unexplained RSA (URSA) remains unknown. In this study, we compared the number and percentage of CD56⁺ NK cells, CD4⁺ T cells and CD8⁺ T cells by flow cytometry in 30 URSA patients and 30 normal pregnant controls. We found that there are a higher proportion of CD4⁺ T cells and CD16⁺CD56⁺ NK cells and a lower number of CD8⁺ T cells in the decidual tissue of URSA patients compared to normal controls. In addition, the number of T helper type 1 (Th1) cells and the Th1/Th2 ratio were higher in URSA patients compared to normal pregnant controls. In conclusion, our results indicate that the changes in the proportion of local T lymphocyte subsets, NK and Th1 cells, in the maternal-fetal interface may be related to occurrence of URSA.

Key words: Immune tolerance; Maternal-fetal interface; Natural killer cells; T helper cells; Unexplained recurrent spontaneous abortion

INTRODUCTION

Recurrent spontaneous abortion (RSA), defined as two or more spontaneous pregnancy losses 20 weeks before gestation, occurs in 1-5% of human pregnancies (Ford and Schust, 2009; Practice Committee of the American Society for Reproductive Medicine, 2012). Besides chromosome anomalies, anatomical malformations, infections and hormone-related diseases, unexplained reasons still account for over 70% of RSA cases. In recent years, unexplained RSA (URSA) was reported to be related to immunological imbalance at the maternal-fetal interface (Alijotas-Reig et al., 2015). From the perspective of immunology, the embryo expresses semi-allogeneic paternal antigens; therefore, establishing an immune-tolerant embryo with regard to the maternal immune system may be essential for a successful pregnancy.

Multiple types of immune cells are potentially involved in promoting immune tolerance during pregnancy and it is believed that inflammatory responses can result in embryo loss (Blois et al., 2005). While CD4⁺Foxp3⁺ regulatory T cells are abundant in decidual tissue during normal pregnancy to maintain the tolerance of the maternal-fetal interface, there are fewer T helper type 1 (Th1) cells secreting interferon- γ (IFN- γ) in decidual tissue during early pregnancy compared with levels in blood (Mjosberg et al., 2010). In addition, cytokine polarization in T helper type 2 (Th2) cells is crucial for ensuring tolerance at the maternal-fetal interface (Zhu et al., 2005). Interestingly, natural killer (NK) cells comprise the largest population of immune cells in human decidual tissue. Decidual NK cells differ from peripheral blood NK cells in phenotype, cytolytic activity and gene expression (Koopman et al., 2003), and they are essential for maintenance of pregnancy. Most NK cells in decidual tissue are CD56^{bright} while less than 10% of NK cells in the blood are CD56^{bright}. These NK cells promote immune tolerance by regulating inflammatory T helper type 17 (Th17) cells at the human maternal-fetal interface (Fu et al., 2013). However, whether the number of Th1, Th2 cells and NK cells change in URSA remains unknown.

In this study, we measured the frequency of T and NK cells in URSA patients and normal pregnant women by fluorescence-activated cell sorting (FACS) analysis. We found that CD16⁺CD56⁺ NK cells are preferentially accumulated in URSA patients. CD4⁺ T cells are increased but CD8⁺ T cells are decreased in decidual tissue in URSA patients compared to normal pregnant controls. Th1 cell responses are also enhanced in the deciduae of URSA patients. Our results indicate that increased number of Th1 and CD16⁺CD56⁺ NK cells may be related to URSA.

MATERIAL AND METHODS

Human samples

A total of 30 female patients with URSA from Guizhou Provincial People's Hospital were enrolled in this study from October 2009 to January 2014. Thirty women with normal pregnancies (no abortion or preterm birth anamnesis) were enrolled as control subjects in the same time period. URSA patients or healthy controls with endocrine diseases, immune diseases, or genital infections were excluded from this study. There were no significant differences in the age and weeks of gestation between the two groups (Table 1). Informed consent was obtained from all the subjects and the study was approved by the ethics committee from Guizhou Provincial People's Hospital.

Table 1. Comparison of clinical features between normal pregnancy and URSA groups.

Group	N	Age (years)	Weeks of gestation
URSA	30	28.75 ± 4.58	8.19 ± 1.24
Normal pregnancy	30	27.12 ± 4.37	7.54 ± 1.66

Isolation of total lymphocytes from decidual tissue

Decidual sections (2 g) were obtained from each patient, cut into small pieces, and strained through a 50 µm gauge nylon mesh. The cell suspension was loaded on a Ficoll (Guizhou Hengyin Technology Co. Ltd., China) for lymphocyte purification and then washed with PBS, centrifuged and resuspended. The cell count was adjusted for no more than 1×10^6 /mL for flow cytometry analysis within eight hours.

Antibodies and FACS

The following antibodies were purchased from Becton-Dickinson (USA): FITC-conjugated anti-CD16, -CD56, -CD4; PE-conjugated IFN-γ; PerCP-conjugated anti-CD3; and APC-conjugated anti-interleukin-4 (IL-4). For T cell analysis, a combination of PerCP-CD3 and FITC-CD4 was used. For NK cell analysis, a combination of FITC-CD16 and -CD56 was used. Briefly, cells were stained with the cell surface antibody for 15 min. For intracellular staining, decidual cells were stimulated with phorbol myristate acetate (PMA) and ionomycin (Becton-Dickinson) for 4-6 hours, washed with PBS and stained with the cell surface marker as described above. Production of IFN-γ and IL-4 was evaluated using an intracellular staining assay (Becton-Dickinson), where cells were stained for 20 min. Data from the stained cells were collected using FACSCalibur (Becton-Dickinson) and analyzed with Flowjo software (Tree Star, OR, USA).

Statistical analysis

We used the SPSS 16.0 software for the unpaired two-tailed Student *t*-test to determine significant differences. Data are reported as means ± standard error of the mean (SE). $P < 0.05$ was considered to be statistically significant.

RESULTS

CD16⁺CD56⁺ NK cells preferentially accumulate in the deciduae of URSA patients

Previous studies have shown that although the percentage of human NK cells is less than 10% of total lymphocytes of peripheral blood (Beziat et al., 2011), they constitute a large proportion of lymphocytes in decidual tissue in pregnant women, where they play an immune-modulatory role in the early stages of pregnancy (Vacca et al., 2011). To investigate the possible role of NK cells in URSA, we compared the percentage of NK cells in decidual tissue from URSA patients and normal pregnant controls and found that CD16⁺CD56⁺ NK cells are accumulated in the deciduae of URSA patients compared to healthy controls (Table 2).

Table 2. Percentage of T cells and NK cells in decidual tissue between normal pregnant control and URSA patients.

Group	N	CD4 ⁺ (%)	CD8 ⁺ (%)	CD16 ⁺ CD56 ⁺ (%)
URSA	30	46.02 ± 8.45	34.96 ± 6.69	24.92 ± 8.63
Normal pregnancy	30	38.78 ± 7.84	44.02 ± 7.01	17.89 ± 6.24

CD4⁺ T cells are increased but CD8⁺ T cells are decreased in the deciduae of URSA patients

T cells have been reported to be present in the deciduae, where they take part in recognizing alloantigens at the maternal-fetal interface (Tilburgs et al., 2009). The total percentage of both CD4⁺ and CD8⁺ T cells by FACS analysis are summarized in Table 2. We found that the percentage of CD4⁺ T cells increased and CD8⁺ cells decreased in URSA patients compared to healthy controls.

Th1 cell responses are enhanced in URSA patients

Since we detected an increased population of CD4⁺ T cells in the deciduae of URSA patients, we sought to determine the specific subsets of increased CD4⁺ T cells. Thus, CD4⁺ T cells from decidual tissue in both groups were analyzed by intracellular staining of IFN- γ and IL-10. We observed that CD4⁺ T cells in URSA patients exhibited higher production of IFN- γ and lower expression of IL-10 compared to healthy controls. From the summarized data shown in Table 3, we concluded that Th1 cell responses are enhanced in the deciduae of URSA patients.

Table 3. Comparison of the percentage of Th1 and Th2 cells and their ratio (Th1/Th2) in decidual tissue between normal pregnant control and URSA patients.

Group	N	Th1 (%)	Th2 (%)	Th1/Th2
URSA	30	22.81 ± 4.07	2.17 ± 1.19	10.73 ± 4.66
Normal pregnancy	30	13.14 ± 2.40	3.56 ± 1.33	3.68 ± 1.97

DISCUSSION

The maternal-fetal interface consists of the placenta trophoblast and decidual tissue, and it is where maternal tissues come into direct contact with the embryo. Since the embryo carries the semi-allogeneic paternal antigen, it acts as an allograft. Therefore, establishing an immune-tolerant environment to protect the embryo from being rejected by decidual immune cells could be important to ensure a successful pregnancy. Although the mechanism of URSA is unknown, a number of studies have indicated that it may relate to the disruption of local immune cell function at the maternal-fetal interface. Loss of normal musculo-elastic structure converts tolerance towards the semi-allogeneic fetus and eventually leads to abortion.

In normal pregnancy, adaptive immune responses are suppressed and CD4⁺Foxp3⁺ regulatory T cells are increased to prevent the rejection of the semi-allogeneic fetus (Leber et al., 2010). T cells, classified as CD4⁺ and CD8⁺ cells, are a key component of the adaptive immune system. In early pregnancy, T cells constitute 5-20% of total lymphocytes in decidual tissue and their numbers increase to 40%-80% at full-term pregnancy (Tilburgs et al., 2010). In contrast to peripheral blood, where CD4⁺ T cells are the most abundant T cell subset, CD8⁺ T cells form the

predominant T cell subset in decidual tissue at full-term pregnancy, suggesting a positive role in normal pregnancy. Unlike conventional cytotoxic CD8⁺ T cells in peripheral tissues, CD8⁺ T cells in decidual tissue are important for providing protective immunity and secreted cytokines in early pregnancy to promote the development of the fetus. These CD8⁺ T cells are considered to be regulatory or suppressor CD8⁺ T cells, co-expressing PD-1 and Tim-3 (Wang et al., 2015). CD4⁺ effector T cells, particularly the recently described Th17 cells, may play an important role in the pathogenesis of URSA (Wang et al., 2015). It has been reported that the proportion of Th17 cells, the Th17-inducing cytokine IL-23, and RAR-related orphan receptor C (RORC), an essential transcription factor in Th17 cells, are higher in decidual tissue from URSA patients compared to normal pregnant controls (Wang et al., 2010). In our study, we show that the percentage of CD4⁺ T cells is increased while CD8⁺ T cells are decreased in URSA patients compared to normal controls. An elevated CD4⁺ to CD8⁺ ratio indicates that the activity of effector T cells may be upregulated and immune tolerance at the maternal-fetal surface may be disrupted, resulting in rejection of the fetus.

In addition to T cells, NK cells constitute a large proportion of total lymphocytes in decidual tissue. NK cells are programmed to kill syngeneic and allogeneic tumor cells without prior sensitization (Arjona and Sarkar, 2008). However, during normal pregnancy, the cytolytic function of decidual NK cells is substantially reduced. In mouse models, it has been shown that NK cells display a regulatory function during reproduction (Koopman et al., 2003). Moreover, several studies have revealed that human decidual NK cells could inhibit trophoblast invasion and vascular remodeling through secretion of a variety of angiogenic factors, cytokines, and chemokines (Bilinski et al., 2008; Hanna et al., 2009; Kalkunte et al., 2009). Human NK cells can be divided into CD56^{bright} and CD56^{dim} subsets based on the expression of CD56 and CD16. CD56^{dim} NK cells display high cytotoxicity and poor cytokine secretion. CD56^{bright} NK cells have the capacity to produce cytokines abundantly following activation of monocytes but have low natural cytotoxicity; in addition, they are CD16^{dim} or CD16⁽⁻⁾ (Cooper et al., 2001). Several studies have shown that increased CD56^{bright} NK cells may lead to fetal loss. Ghafourian et al. (2015) found that CD16⁺CD56⁺ NK cells were significantly increased in women with RSA compared to normal pregnant controls. Andalib et al. (2005) also found a higher percentage of CD16⁺CD56⁺ NK cells in the peripheral blood leukocytes of women with RSA. In accordance with these results, we observed an increased number of CD16⁺CD56⁺ NK cells in the decidual tissue of URSA patients.

Besides immune cells, cytokines could also be involved in interactions between the maternal immune and reproductive systems during pregnancy. During normal pregnancy, maternal immunological tolerance of the allogeneic fetus is associated with a shift in maternal T cell subsets from a Th1 to Th2 phenotype. CD4⁺ T cells can be grouped by cytokine secretion into IFN- γ -producing Th1 cells and IL-4-producing Th2 cells. Marzi et al. (1996) demonstrated lower IFN- γ and higher IL-4 production in phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) from pregnant women compared to non-pregnant controls. Studies by Reinhard et al. (1998) and Saito et al. (1999) reiterated these results. In all, these studies provide strong support for the idea that pregnancy is a Th2 cytokine-based condition. In our study, IFN- γ secretion was enhanced and IL-4 secretion was impaired in URSA patients compared to normal controls. The ratio of Th1/Th2 was significantly higher in URSA decidual tissue, illustrating a shift from Th2 to Th1 in URSA.

In conclusion, the immune tolerance at the maternal-fetal interface could be an essential factor for normal pregnancy. Our results highlight the importance of maintaining an immune-suppressive environment in decidual tissue during pregnancy, potentially providing new insight for developing treatments for URSA.

Conflicts of interest

The authors declare no conflict of interest.

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