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Molecular detection of uterine innate lymphoid cells in the immunological mouse model of pregnancy loss

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ABSTRACT

Innate lymphoid cells (ILCs) are newly identified members of the innate lymphocyte family, which can function as adaptive T cells and act as critical modulators of inflammatory processes within different tissues and immune diseases. The role of uterine ILCs (uILCs) has recently been elucidated alongside changes associated with normal pregnancy. However, the proportions of uterine ILCs and their role in unsuccessful pregnancy remain unclear. We analyzed the characterization of uILC subsets and the expression of signature cytokines associated with ILCs in a mouse model of unsuccessful pregnancy induced by LPS, and we describe the dynamic changes they undergo during this process. We found that mice exposed to LPS display significantly higher levels of uNK cells, and uILC3s. However, a lower proportion of uILC2s and uILC1s were detected in abortion mice. In addition, we found that abortion mice display markedly higher expression of IFN-γ and IL-A17, and lower levels of IL-5. No significant differences in the expression of IL-13 and IL-22 were observed. The findings suggest that uILCs play distinct non-redundant roles during pregnancy, and uILCs may affect maternal-fetal tolerance via IL-17A, IL-5, and IFN-γ production.

1. Introduction

The harmonious regulation of the interplay between maternal immune microenvironment and semi-allograft fetus during the successful pregnancy is of significance. The disruption of the balance in maternal-fetal interface contributes to pregnancy loss has been confirmed [1–5]. The precise mechanisms of maternal immune, however, are poorly explained. So far as we know, various immunological factors are correlated with reproductive failures. Previous studies have shown the involvement of effector T helper cells (Th cells) Th1, Th2, Th17 and regulatory T (Treg) cells in maternal-fetal immune-regulation [6–8]. A transition from Th1- to Th2-type immune response in the maternal immune microenvironment during pregnancy is considered critical for the growth of the fetus [9]. Other immune cells, natural killer cells (NK cells), for instance, are involved in optimal pregnancy via creating the immune tolerance of the maternal-fetal interface [10]. In addition, recent reports have demonstrated the interaction between regulatory T cells and NK cells, which may play a vital immunosuppressive role in the uterine microenvironment [11].

Innate lymphoid cells (ILCs) are the most recently identified immune cell types, which attract high concern as the “mirror image” of T helper cell [12]. Mainly distributed at mucosal barriers, ILCs play vital roles in the defense against pathogens and in the maintenance of tissue or organ homeostasis [13]. They are divided into three subgroups based on the secretion of distinct cytokines and transcription factors: Group 1 ILCs include NK cells and ILC1 cells that produce IFN-γ and depend on T-bet for their differentiation; Group 2 ILCs produce type 2 cytokines (IL-4, IL-5, IL-9 and IL-13) and require GATA-binding protein 3 (GATA3) as well as retinoic acid receptor–related orphan receptor-α (RORα); Group 3 ILCs, as Th17 cells, produce IL-17 and/or IL-22 and express RORγt for their development [14–17].

In the uterus, roles of “helper” uterine ILCs (uILCs) are poorly understood. Different subgroups of ILCs were explored in both the human and mouse uterus during the early pregnancy [18,19]. In human decidua, ILC3s were detected as major ILCs subgroups. Interestingly, all the three subgroups were discovered in the uterus of virgin mice and

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the uterus at early-gestation. A new report confirmed the significance increase in murine uterine ILCs during pregnancy [20]. Recently, ILCs were detected at the human maternal-fetal interface, and their correlation with the pathological process of preterm labor was found [21]. However, their role in the pathological process of early pregnancy loss remains unclear.

This report aimed to further examine the existence of ILCs subgroups and their cytokines in the murine uterus during early pregnancy, as well as the causal connection between ILCs and abnormal inflammation relate to pregnancy loss. A well-established mice model of inflammation-mediated pregnancy loss was used which was induced by lipopolysaccharide (LPS) injection.

2. Materials and methods

2.1. Animals

C57BL/6 and BALB/c mice aged 8–12 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China, and raised at 20–25 °C on a 12-h light and 12-h dark cycle. C57BL/6 female mice in natural cycling were mated with BALB/c males overnight at a ratio of 2:1, and the following morning of examining vaginal plugs was considered as gestational day (GD) 0.5. All animal protocols for experiments were conducted in accordance with the National Guidelines for Animal Use in Research (China).

2.2. Experimental protocol

Consequences of lipopolysaccharide (LPS; Sigma) on mouse pregnancy were conducted via intraperitoneal injection of LPS into pregnant C57BL/6 female mice mated with BALB/c males. The LPS-induced abortion was carried out as described previously [22]. Briefly, administration of LPS at a dose of 2.5 μg dissolved in 200 μl saline was injected intraperitoneally into the pregnant C57BL/6 female mice on GD8.5. The rates of abortion and fetal resorption were calculated 48 h after administration of LPS.

2.3. Tissue preparation

To detect serum cytokine production levels, blood was collected and stored at −80 °C for further analysis. Virgin uteri, pregnant uteri, fetus and placenta were entirely harvested from mated mice at designated gestation days for histology analysis.

2.4. Histology procedures

Placenta sites were harvested and fixed in 4% paraformaldehyde for histology analysis. After fixation, tissues were transferred to 70% ethanol at room temperature, dehydrated, and embedded in paraffin. Tissue sections on slides were stained with hematoxylin/eosin (H&E). The whole process was on basis of standard procedures.

2.5. Evaluation of embryo resorption

The numbers of fetuses and placentas per pregnant mouse, together with the fetal and placental weights were recorded for evaluation. The embryo abortion rate was designed as (resorbed embryos/sum of embryos) × 100%.

2.6. Cell isolation and flow cytometry

To obtain single-cell suspensions, the uteri were shredded and digested by RPMI 1640 containing 5 mg/ml collagenase IV (Sigma-Aldrich St. Louis, MO), 10% FCS, and 10 mM HEPES sodium salt. Then the cell suspension was filtrated through a 100-μm cell sterile nylon gauze strainer. Mononuclear cells from the uteri were purified by density gradient centrifugation over Ficoll-PaqueTM plus (GE Healthcare, Marlborough, MA, USA) and resuspended for flow cytometric analysis.

For the detection of ILCs, single-cell suspensions were stained by following Abs: lineage markers (CD3, CD14, CD19, CD11b, CD16/CD32, and B220) (BD Pharmingen), and anti-mouse CD45(BD Pharmingen), CD127(BD Pharmingen), RORγt (eBioscience), T-bet(BD Pharmingen), GATA3(BD Pharmingen), NK1.1(BD Pharmingen), IL33R (ST2) (BD Pharmingen), Eomes(eBioscience), CD49a(BD Pharmingen). The samples were acquired using BD FACSAria flow cytometer, and the data were analyzed by Flow Jo software (Tree Star, Ashland, OR, USA). For intranuclear staining for ILCs, Permeabilization procedure was carried out through a transcription factor staining Buffer Set (BD Pharmingen) as the manufacturer's instructions.

2.7. Analysis of Cytokine Production

Signature cytokines of ILCs in plasma were calculated using enzyme-linked immunosorbent assay kits (eBioscience). ELISA was performed as the manufacturer's instructions.

2.8. Statistical analysis

Data was presented as the mean ± SEM. Statistical analyses were made using Graph Pad Prism 6 software and IBM SPSS. Statistical significance was analyzed by *t*-test and one-way ANOVA. Post-test was performed by Tukey-test. Fetal and placental weights were analyzed using a mixed model access so that the impact of LPS on pregnancy could be tested. P value < 0.05 was considered significant.

3. Results

3.1. Maternal LPS administration results in fetal and placental resorption

To confirm the impacts of LPS on reproductive outcomes, pregnant mice were intraperitoneally injected by LPS (2.5 μg/20 g) or saline on GD 8.5, and sacrificed 48 h later. And then the percentage of fetal resorption per mouse, viable implantation sites, and the number of total implantation sites were measured. Compared with the normal pregnancy group and saline group, LPS-induced abortion group experienced a significant decrease in viable implantation sites and a significantly high rate of embryo resorption. However, there was no significant difference in total implantation sites of normal pregnancy group, saline group and abortion group (Fig. 1). In addition, histopathology of the placenta between pregnant and abortion mice was analyzed as well. Compared to the pregnant mice, abortion mice exhibited the apparent hemorrhages and blurry tissue structures as shown in Fig. 2.

3.2. The differential proportions of uILCs in virgin, pregnant and abortion mice

Here, the proportions of CD45 + Lin − ILCs in pregnant and abortion mice were examined respectively. As reported before, no significant different resorption rates were detected between pregnant and saline-treated mice. Thus the percentage of uILCs in normal pregnant mice and LPS-treated female mice was determined. Afterwards, the proportion changes of uILCs subgroups in pregnant and abortion mice were explored.

The gating strategy of CD45 + Lin − T-bet + Eomes− was used to describe uILC1s. As shown in Fig. 3, compared with pregnant mice, abortion mice displayed a significant decrease in the proportion of uILC1s (CD45 + Lin − T-bet + Eomes−), but a much higher expression of uNK (CD45 + Lin − T-bet + Eomes+) cells (Fig. 3B).

The previous study reported that uILC2s increased most during the pregnancy [20]. Here the expression of uILC2s (CD45 + Lin − ST2 + GATA-3+) in pregnant and abortion mice was

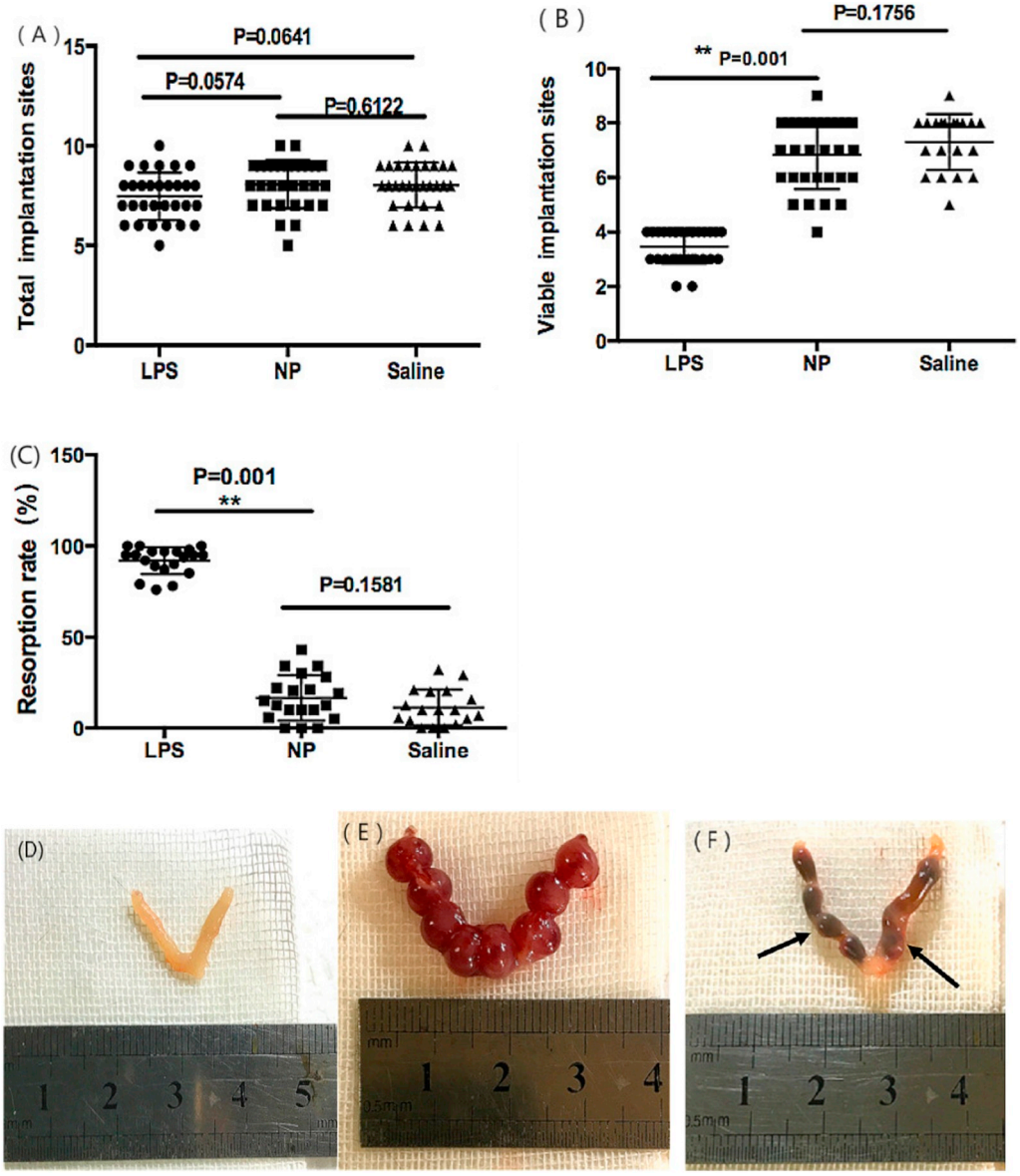

Fig. 1. Maternal LPS administration results in fetal and placental resorption. The number of total implantation sites (A), viable implantation sites (B) and the embryo resorption rates (C) in normal pregnancy group, saline group and abortion group. Photographs of uterine represent pregnancy status: (D) the uterine of virgin mice; (E) the uterine of normal pregnancy mice;(F) the uterine of abortion mice. Arrows indicate the absorbed embryos. n = 15–20 per group.

studied. Compared with pregnant mice, abortion mice showed significantly lower proportions of uILC2s (Fig. 3C).

Moreover, the strategy of CD45 + Lin − CD127 + RORγt+ was employed to define uILC3s and to explore the expression of uILC3s. The proportion of uILC3s was found significantly increased in abortion mice (Fig. 3D). Furthermore, we also examined the percentages of uNK cells, uILC1s, uILC2s, and uILC3s among CD45+ lymphocytes in two groups. Compared with normal pregnant mice, abortion mice displayed much higher percentages of uNK cells and uILC3s, but significantly lower percentages of uILC1s and uILC2s (Fig. 3E).

These findings probably highlighted the non-negligible roles of uILC1s, uILC2s, and uILC3s during pregnancy.

3.3. Signature cytokines produced by uILCs in abortion mice

Then the expression of serum signature cytokines associated with ILCs in pregnant and abortion mice was determined. Compared with pregnant mice, abortion mice exhibited a significant increase in the expression of IFN-γ, IL-17A, IL-22, in particular, IFN-γ and IL-17A, but a significant decrease in the expression of IL-5 and IL-13 (Fig. 4).

4. Discussion

The current study focused on the differential expression of uILCs subgroups in abortion mouse model induced by LPS for the first time. In abortion mice, a significant increase in Group 1ILCs, uNK cells, and uILC3s, and a much lower proportion of uILC2s were found; in addition, a markedly higher expression of IFN-γ and IL-A17, and a lower expression of IL-5 were showed. However, no significant alterations in expression of IL-13 and IL-22 were identified in pregnant and abortion mice. The findings showed that uILCs played a non-redundant role in pregnancy.

Notably, recent studies confirmed that different ILCs subgroups could be detected in murine and human uterine [22–25]. The alterations of the proportion of uILCs subgroups in early pregnancy in mice and human were also reported [20,23]. However, there was no research

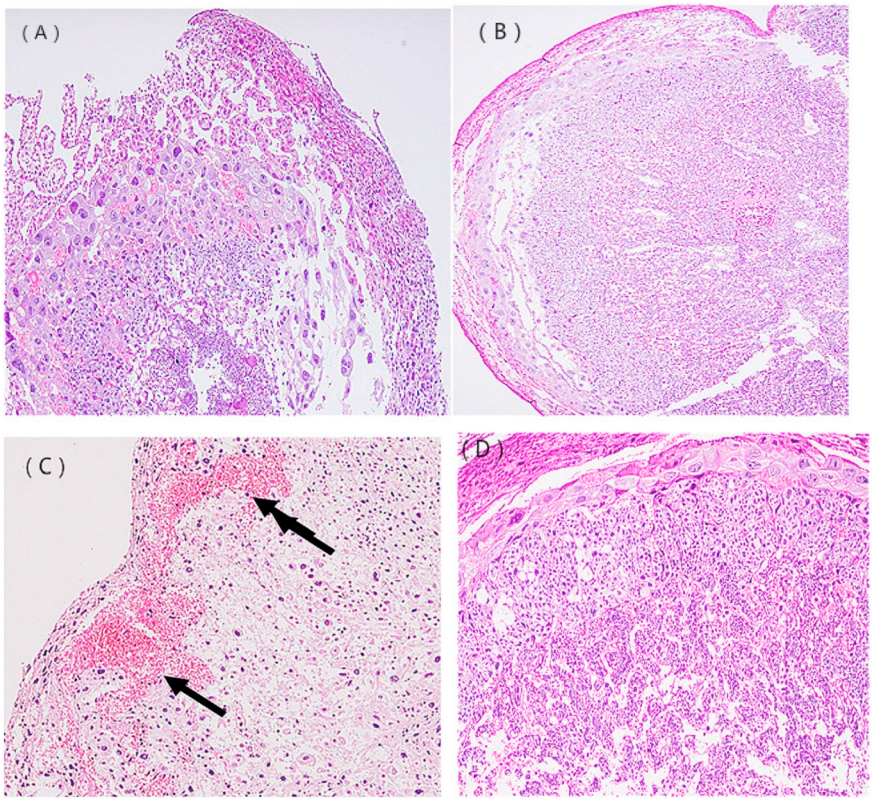

Fig. 2. Apparent hemorrhages and blurry tissue structures were detected in abortion mice. Representative HE stained sections of the placenta in normal pregnancy group and abortion group. (A) the placenta of abortion (40×); (B) the placenta of normal pregnancy (40×); (C) the placenta of abortion (100×); (D) the placenta of normal pregnancy (100×). Arrows indicate the apparent hemorrhages.

on uILCs subgroups in early pregnancy loss. In this study, the alterations in the expression of uILCs subgroups in abortion mice model were reported for the first time. Group 1 ILCs, uNK cells and uILC3s showed markedly increased alterations in abortion mice but decreased in uILC1 and uILC2. It has been widely accepted that pregnancy should be a peculiar situation in which the semi-allogeneic fetus must be tolerated by the maternal immune system [26,27]. A transition from Th1- to Th2-type immune response and Th17/Treg balance in the maternal immune microenvironment during pregnancy is thought critical for the growth of the fetus. Group 1ILCs and Group 2 ILCs respond to type1 and type 2 inflammation respectively. The balance of Th1/Th2 immune response could be altered toward Th1-type immune response by LPS-mediated inflammation in abortion mice. In addition, as the IFN-γ – producing cells, Group1 ILCs are composed of two major cells, ILC1s and NK cells, which regulate the immune tolerance in the maternal-fetal interface. Furthermore, during the early pregnancy, uILC3s may promote proliferation and invasion of trophoblast. When LPS-mediated abortion occurs, the alterations in uILC3 will be inevitable.

LPS is the most potent antigenic component of the Gram-negative bacteria cell wall and is known to modulate the expression of various proinflammatory cytokines. In mice model, embryos are very sensitive to inflammation. The dose of LPS in the study was cited from previous studies which confirmed that the right dose of LPS do not endanger the survival of pregnant mice but produce pregnancy loss [5,28,29]. In the study, LPS-induced abortion mice model was selected by our group to determine the characterization and expression of uILC subsets in unsuccessful pregnancy. Another well-established abortion-prone mice model by mating CBA/J females with DBA/2J males has been reported in previous studies to analyze the characteristics of idiopathic abortion [28–30]. However, the expression of uILC subsets in the abortion-prone mice model has not been studied yet. Therefore, the different abortion mice models will be utilized in our further research to investigate the proportions of uILC subsets and potential mechanisms of these innate lymphoid cells.

In addition, the present research didn't investigate the differential expression of uILCs subgroups in women with recurrent miscarriage. Successful pregnancy is dependent on very specific and dynamic immune adaptations, with different local and systemic immune responses taking place during human pregnancy. At the maternal-fetal interface, fetal cells come into close contact with maternal immune cells. Fetal antigens can be recognized by the maternal immune system but do not trigger rejection of the semi-allogeneic fetus. Various immunological factors are correlated with human inflammation-mediated pregnancy loss [31,32]. Previous studies have reported that well-established LPS-induced abortion mice model could be applied to explore potential mechanisms of inflammation-mediated pregnancy loss [5,28,29]. In future study, more human decidual tissues would be obtained for further research.

Inflammatory cytokines, such as IFN-γ, favor successful embryo implantation by modulating angiogenesis and tissue remodeling [33]. Furthermore, IL-17 may induce trophoblast invasion via increasing progesterone secretion [26], IL-13, IL-5 and IL22 contribute to implantation and successful pregnancy via artery and tissue remodeling [28]. Hence, the alterations in these cytokines secreted were analyzed by ILCs in pregnancy loss mice model. Unsurprisingly, the expression of IFN-γ, IL-5 and IL-17A altered with the change of ILCs in abortion mice. It implied that uILCs could boost maternal-fetal tolerance via IL-17A, IL-5 and IFN-γ production. However, inflammatory cytokines detected in this article could also be produced by other immune cells, such as Th2 cells, Th17 cells, NK cells, et al. In addition, IFN-γ can be produced by not only ILC1s but also ILC3s [12]. Therefore, we should have a thorough detection of cytokines produced by uILCs in future study.

In conclusion, this study explored the changes in proportions of uILCs subgroups through pregnancy loss mice model for the first time. The marked alterations in the proportion of uNK, uILC1, uILC2, and uILC3 in abortion mice confirmed the significance of uterine ILCs in pregnancy. Also it was inferred that uILCs posed an impact on maternal-fetal tolerance via IL-17A, IL-5, and IFN-γ production. These findings

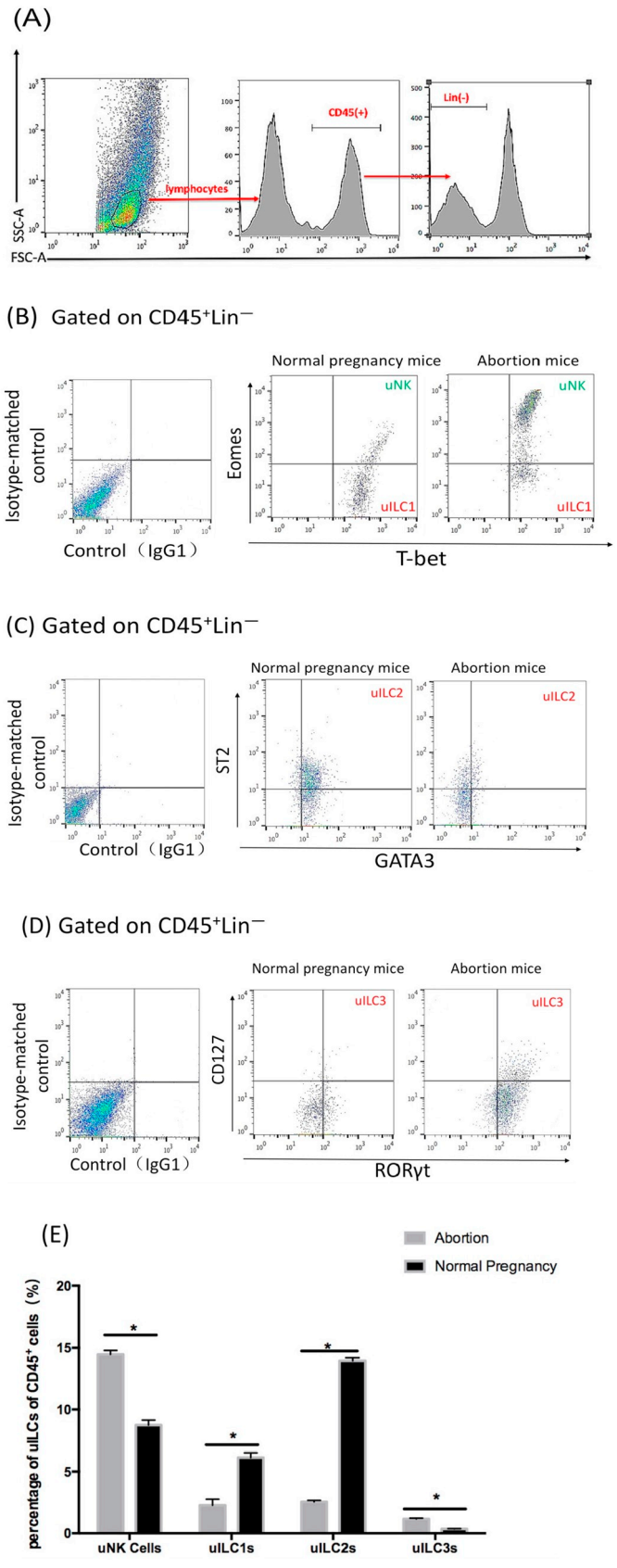

Fig. 3. Different proportions of uILCs subsets in pregnant and abortion mice. The gating strategy of CD45 + Lin − T-bet + Eomes − was used to describe uILC1s; uNK cells were described as CD45 + Lin − T-bet + Eomes +; uILC2s were defined as CD45 + Lin − ST2 + GATA-3 +; uILC3s were identified by the gating strategy of CD45 + Lin − CD127 + RORγt +. (A) The gating strategy used for identifying of uILCs; (B) The flow cytometry blot shows the expression of uILC1s and uNK cells in two groups. (C), (D) indicate the proportions of uILC2s and uILC3s in normal pregnancy and abortion mice respectively. (E) The percentages of uNK cells, uILC1s, uILC2s, and uILC3s among CD45 + cells in two groups. $*P < 0.05$. $n = 10–15$ per group.(Lin −;CD15 − CD14 − CD3 − CD19 − CD56 − CD11b −).

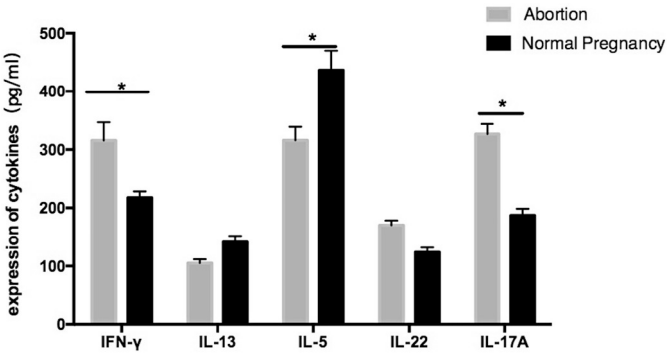

Fig. 4. Serum Cytokines produced by ILCs in normal pregnancy and abortion mice. IFN-γ, IL-5, IL13 and IL17A, IL22 are the signature cytokines expressed by ILC1s, ILC2s and ILC3s respectively. $*P < 0.05$. $n = 24$.

provide a new perspective for maternal-fetal immune response and pregnancy failure research. The vital roles of ILCs in pregnancy need to be determined in further studies.

Conflict of interests

All authors declare no competing interests.

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