IL-25 promotes Th2 bias by upregulating IL-4 and IL-10 expression of decidual γδT cells in early pregnancy

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Abstract. Decidual immune cells (DICs), consisting of both innate and adaptive immune cells, have a pivotal role in maintaining immune tolerance for normal pregnancy. Our previous study demonstrated that interleukin (IL)-25 stimulates the proliferation of decidual stromal cells (DSCs) in an autocrine manner. However, the role of IL-25 in functional regulation of DICs is largely unknown. Flow cytometry was used to analyze the expression of IL-25 and its receptor (IL-17RB) in DICs, and the effect of IL-25 on the expression of Ki-67, IL-4, IL-10, interferon (IFN)-γ and transforming growth factor (TGF)-β in decidual γδT cells. In addition, ELISA assays were performed to detect the secretion of IL-10 and TGF-β in decidual γδT cells. The present findings indicated that decidual CD56 bright CD16-natural killer (NK) cells, natural killer T (NKT) cells, regulatory T (Treg) cells, $CD3^+$ T cells, macrophages and γδT cells co-expressed IL-25 and IL-17RB, particularly γδT cells. Recombinant human (rh) IL-25 protein upregulated the expression of Ki-67, IL-4, and IL-10, but downregulated the expression of IFN-γ in γδT cells; however, anti-human IL-25 or IL-17RB neutralizing antibody reversed these effects. These data suggest that IL-25 may promote IL-10 production by γδT cells as well as the proliferation of γδT cells, and possibly forms a positive feedback loop to maintain a T helper 2 cell bias at the maternal-fetal interface and further contributes to the maintenance of successful pregnancy.

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Introduction

Normal pregnancy is equivalent to allogeneic transplantation that requires maternal immune tolerance for the development of embryo (1). At the maternal-fetal interface, apart from over 70% $CD56^{bright}CD16^-$ natural killer (NK) cells, a few macrophages, $CD3^+$ T cells, dendritic cells but not B cells are also enriched to compose the decidual immune cells (DICs), thereby modulating immune response during pregnancy. As has been determined, T helper 2 (Th2) bias at the maternal-fetal interface, characterized by interleukin (IL)-4, IL-5, and IL-10 secretion, is pivotal for immune tolerance. In contrast, Th1/Th2 imbalance can lead to pathological pregnancy, such as miscarriage or preeclampsia (2-4).

γδT cells, composed of γ and δ chains, are abundant in epithelial tissues, including reproductive tract, skin, and intestine, but only make up a fraction of the peripheral and organic lymphocytes. As a type of innate-like lymphocytes, unlike αβ T cells γδT cells function without major histocompatibility complex restriction as well as CD4 or CD8 co-receptors, although a small proportion of human γδT cells express CD4 (5) or CD8 (6). Because of the widespread distribution of γδT cells, they are involved in a variety of disease conditions such as infection, autoimmunity, cancer, and miscarriage (7-9). Decidual γδT cells, expressing neither CD4 nor CD8 (double negative) markers, are increased significantly during pregnancy (10), and create a Th2 bias at the maternal-fetal interface by secreting IL-10 and transforming growth factor (TGF)-β (11-13). Moreover, a previous study has demonstrated that decidual γδT cells promote the proliferation and invasion of trophoblast cells as well as suppress the apoptosis via IL-10 (14).

As a member of the IL-17 family, IL-25, also known as IL-17E, seems to participate extensively in various immune-related diseases, such as allergy, asthma, and enteric nematode infection (15). On the one hand, IL-25 has a pathogenic effect on Th2-type diseases such as asthma; on the other hand, it plays a therapeutic role in Th1-type diseases such as

Crohn disease and Th17-type diseases such as autoimmune encephalomyelitis, thereby serving as a double-edged sword. For example, in asthma and airway high-reactivity disease, IL-25 secreted by airway epithelial cells or eosinophilic or basophilic granulocyte not only participates in the innate immune response to produce IL-4, IL-5, and IL-13, but also has a role in adaptive immune response to induce naïve T cells differentiation into Th2-type cells. In inflammatory bowel disease, antigen stimulates intestinal epithelial cells and macrophages to produce IL-25, which inhibits Th1 and Th17-type immune response by reducing the secretion of IL-12 and IL-23 by antigen-presenting cells, and protects the intestinal mucosal cells from damage. IL-17RB, a principal recognized IL-25 receptor, functions widely on diverse cells, including eosinophils, mast cells, monocytes, and T cells.

Moreover, our previous study has elucidated that human chorionic gonadotropin (hCG) derived from trophoblasts upregulates the expression of IL-25/IL-17RB in decidual stromal cells (DSCs), followed by further stimulating DSCs proliferation by activating c-Jun n-terminal kinase (JNK) and protein kinase B (AKT) signals, thus finally contributing to a successful pregnancy (16). However, whether DICs express IL-25/IL-17RB, and even whether IL-25 influences the local immune response at the maternal-fetal interface, are still unknown. Consequently, the present study was undertaken to investigate the expression of IL-25/IL-17RB in DICs and related functions in decidual γδT cells.

Materials and methods

Tissue collection. All tissue samples (n=42) were collected with informed consent according to the requirements of the Research Ethics Committee in the Obstetrics and Gynecology Institute, Fudan University Shanghai Medical College (Shanghai, China). All subjects completed informed consent forms for collection of tissue samples. In addition, this study was specifically approved by the Research Ethics Committee. Decidual samples were obtained from normal pregnant women (age 29.24±3.17 years; gestational age 8.11±1.37 weeks; mean ± standard deviation) whose pregnancies were terminated for non-medical reasons.

Cell isolation and culture. The tissues from the first-trimester pregnancy were placed immediately into cold Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA), transported to the laboratory within 1 h after surgery, and washed with Hank balanced salt solution for isolation of DICs. The DICs were isolated according to our previous procedures (17).

Isolation and culture of γδT cells. Decidual γδT cells were isolated using a magnetic activated cell-sorting human γδT cell positive selection isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) in accordance with the manufacturer's instruction. The purity of γδT cells was evaluated by flow cytometry with fluorescein isothiocyanate-γδTCR (eBioscience; Thermo Fisher Scientific, Inc.) antibody.

Monoclonal antibodies. Phycoerythrin (PE)-conjugated anti-IL-25 monoclonal antibody (mAb) and allophycocyanin (APC)-conjugated anti-IL-17RB mAb were purchased from R&D (R&D Systems, Inc., Minneapolis, MN, USA). Fluorescein-isothiocyanate-conjugated anti-CD56, CD14, CD25, and γδTCR mAbs, PE-conjugated anti-IL-4, TGF-β mAbs, PE-Cy5.5-conjugated anti-CD3, IL-10 mAbs, and APC-conjugated anti-interferon (IFN)-γ, Ki67 mAbs were purchased from eBioscience. APC-cy7-conjugated anti-CD45 and PE-cy7-conjugated anti-CD4 mAbs were purchased from BioLegend, Inc. (San Diego, CA, USA).

IL-25/IL-17RB expression in DICs. DICs were collected and washed with phosphate buffered saline. After blocking with 10% fetal bovine serum (Hyclone; GE healthcare, Logan, UT, USA)), DICs were divided into several parts for detecting the percentage of IL-25 or IL-17RB positive cells in different immune cells, including $CD45^+CD3^-CD56^+$ NK cells, $CD45^+CD3^+CD56^+$ natural killer T (NKT) cells, $CD45^+CD3^+$ T cells, $CD45^+CD3^+$γδT cells, $CD45^+CD14^+$ macrophages, and $CD45^+CD3^+CD4^+CD25^+$ regulatory T (Treg) cells. The recovered cells were immediately stained using a standard immunofluorescence assay with the different surface mAbs in darkness for 45 min at room temperature, followed by washing with phosphate buffered saline to remove unbound antibody, fixing, permeabilizing, and then incubated with anti-IL-25 antibody for another 1 h at room temperature. The isotype control was used as a negative control. After incubation, the cells were washed and analyzed immediately by flow cytometer (FACS Beckman; BD Biosciences, Franklin Lakes, NJ, USA).

Expression of cytokines in γδT cells. The isolated γδT cells were seeded in 24-well plates ($1x10^6$ cells per well) and treated with different concentrations of recombinant human (rh) IL-25 (R&D Systems, Inc.) (0, 0.1, 1, 10 ng/ml), 1 ng/ml rhIL-25 plus 10 μg/ml anti-IL-25 neutralizing antibody (anti-IL-25; R&D Systems, Inc.), or 1 ng/ml rhIL-25 plus 10 μg/ml anti-IL-17RB neutralizing antibody (anti-IL-17RB; R&D Systems, Inc.) for 24 h. Then, after treatment with PMA+BFA+ionomycin for 4 h, we detected the expression of IL-10, TGF-β, IL-4, and IFN-γ in γδT cells by flow cytometry following the aforementioned staining procedure.

Ki-67 expression in γδT cells. γδT cells were seeded in 24-well plates ($1x10^6$ cells per well) and treated with different concentrations of rhIL-25 (0, 0.1, 1, 10 ng/ml), 10 ng/ml rhIL-25 plus 10 μg/ml anti-IL-25, or 10 ng/ml rhIL-25 plus 10 μg/ml anti-IL-17RB for 24 h. Subsequently, we used flow cytometry assay to detect the expression of Ki-67 in γδT cells following the aforementioned intracellular cytokine staining procedure.

Enzyme-linked immunosorbent assay (ELISA). γδT cells were seeded in 24-well plates ($1x10^6$ cells per well) and treated with 1 ng/ml rhIL-25, 1 ng/ml rhIL-25 plus 10 μg/ml anti-IL-25, 1 ng/ml rhIL-25 plus 10 μg/ml anti-IL-17RB for 48 h. The culture supernatants were then harvested, centrifuged to remove cellular debris, and stored at -80˚C. The concentrations of IL-10 and TGF-β were detected by ELISA according to the manufacturer's instructions (eBioscience; Thermo Fisher Scientific, Inc.).

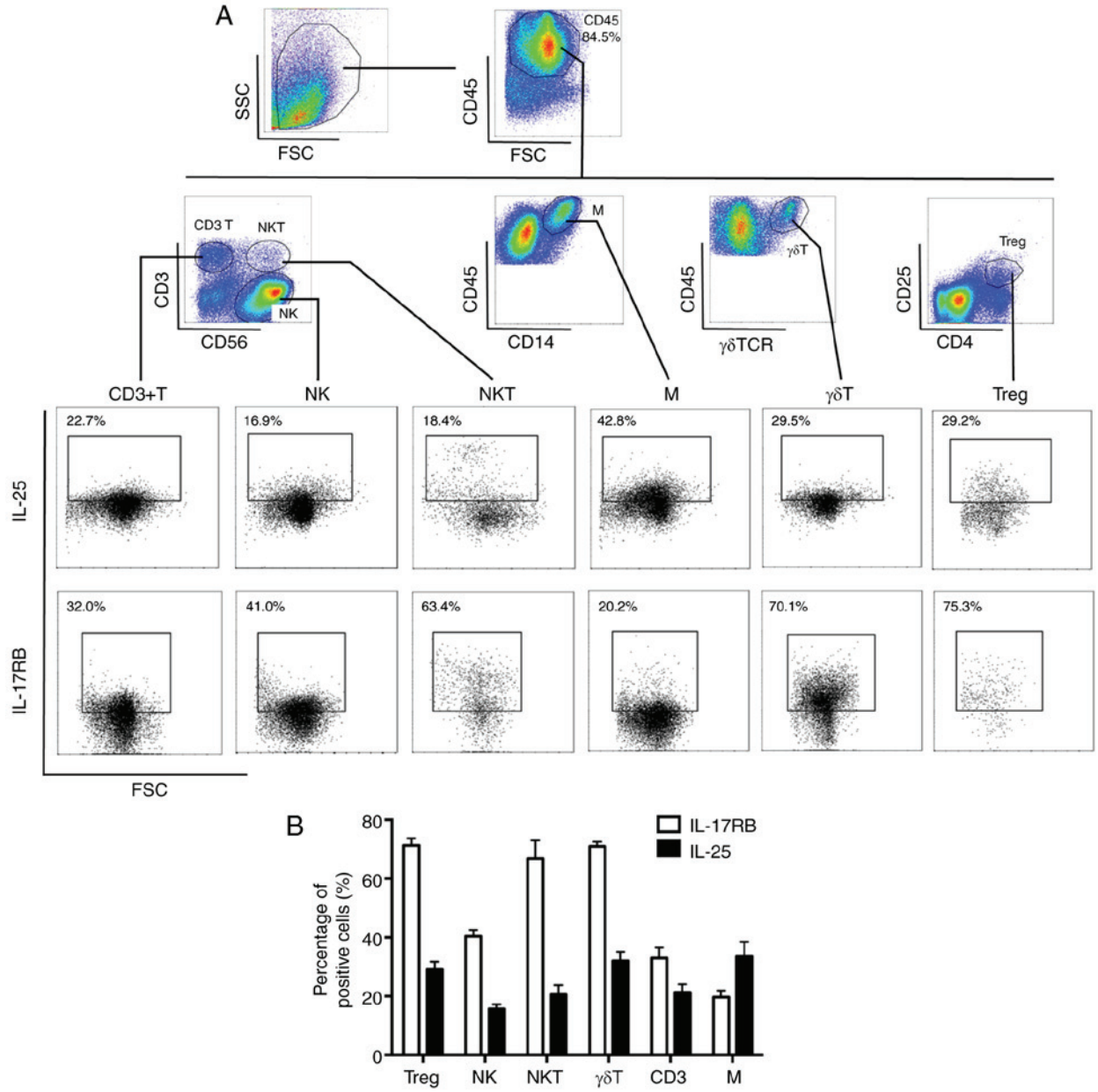

Figure 1. (A and B) Decidual γδT cells highly co-express IL-25 and IL-17RB. We used (A) flow cytometry to detect the percentage of (B) IL-25 or IL-17RB positive cells in different immune cells at the maternal-fetal interface (n=12). Data are presented as mean ± standard deviation. Experiments were repeated three times. IL, interleukin; Treg, decidual regulatory T cells; NK, decidual natural killer cells; NKT, decidual natural killer T cells; γδT, decidual γδTCR cells; CD3, decidual $CD3^+$ T cells; M, decidual macrophages.

Statistical analysis. The data were analyzed using the least significant difference test after one-way analysis of variance (SPSS version 17.0; SPSS, Inc., Chicago, IL, USA). All values are shown as mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Decidual γδT cells highly co-express IL-25 and IL-17RB. Because of the complex components of DICs, we examined the expression of IL-25/IL-17RB in different types of cells by flow cytometry, including NK cells, NKT cells, $CD3^+$ T cells, γδT cells, macrophages, and Treg cells. The results of flow cytometer showed that 15.7±1.6% NK cells, 20.6±3.2% NKT cells, 29.1±2.6% Treg cells, 21.3±2.8% $CD3^+$ T cells, 32.9±3.4% macrophages, and 32.0±3.1% γδT cells expressed IL-25, whereas 40.4±2.0% NK cells, 66.8±6.2% NKT cells, 71.3±2.4% Treg cells, 33.0±3.6% $CD3^+$ T cells, 19.7±2.2% macrophages, and 70.9±1.6% γδT cells expressed IL-17RB (Fig. 1). Taken together, the expression levels of IL-25/IL-17RB in γδT cells were higher than other cells.

IL-25 promotes the proliferation of decidual γδT cells. Because of the high expression level of IL-25/IL-17RB in γδT cells, we intended to determine the effects of IL-25 on the functions of γδT cells. First, we isolated γδT cells from DICs by magnetic activated cell sorting, and the purity was examined by flow cytometry, which was approximately 90% (Fig. 2A). Next, we treated γδT cells with different concentrations of rhIL-25, rhIL-25 plus anti-IL-25, or rhIL-25 plus anti-IL-17RB for 24 h,

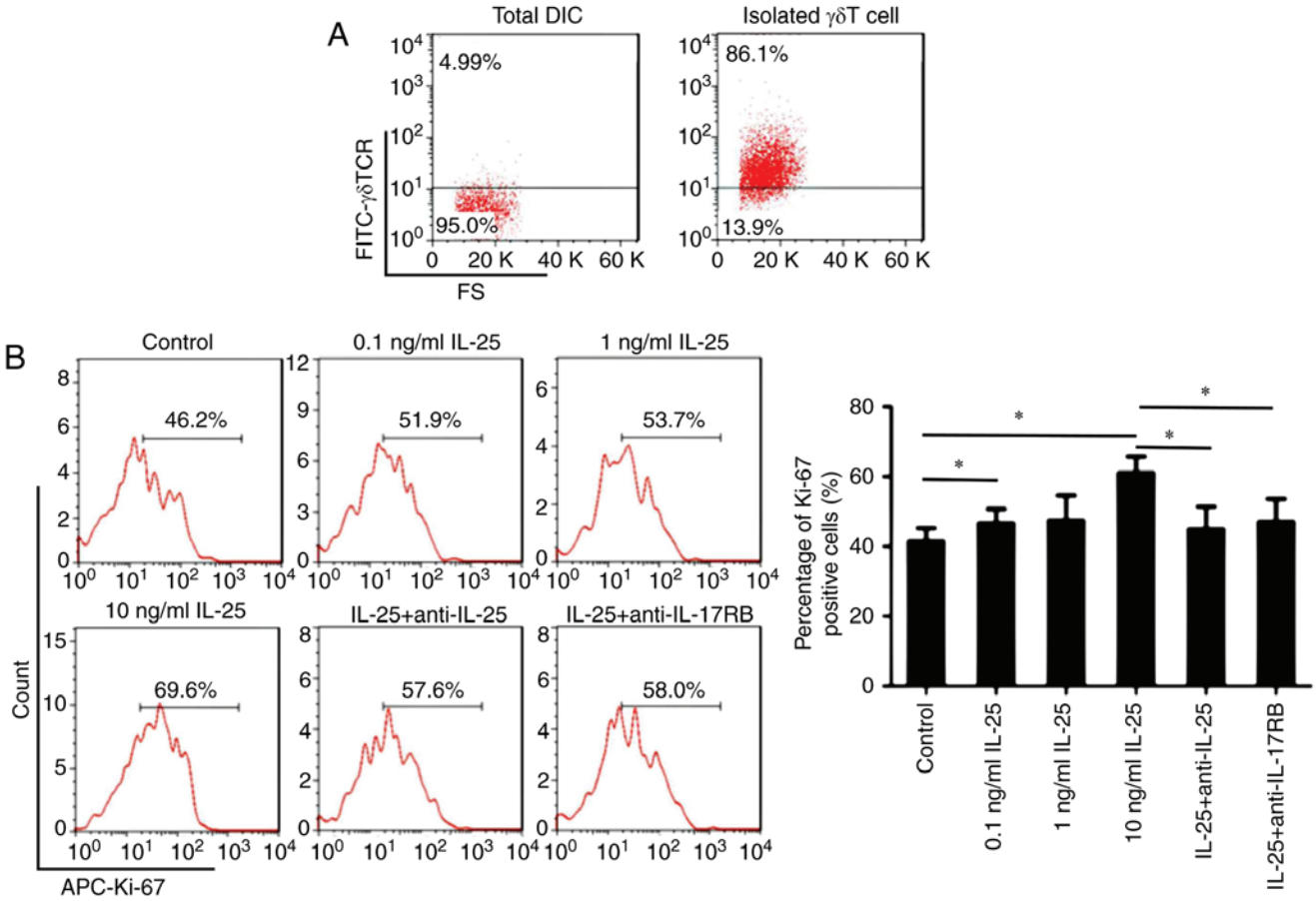

Figure 2. IL-25 promotes the proliferation of decidual γδT cells. (A) Isolation and purity identification of γδT cells. DICs were collected and isolated for γδT cells by magnetic activated cell sorting, the purity of which was detected by flow cytometry. (B) After treatment with different concentrations of rhIL-25 (0, 0.1, 1, 10 ng/ml), 10 ng/ml rhIL-25 plus 10 μg/ml anti-IL-25 or 10 ng/ml rhIL-25 plus 10 μg/ml anti-IL-17RB for 24 h, we used flow cytometry assay to examine the expression of proliferation-related molecule Ki-67 in γδT cells (n=9). IL, interleukin; IL-25, recombinant human (rh) IL-25; anti-IL-25, anti-IL-25 neutralizing antibody; anti-IL-17RB, anti-IL-17RB neutralizing antibody. *P<0.05 compared with control. Data are presented as mean ± standard deviation.

after which we detected the expression of proliferation-related molecule Ki-67 in γδT cells. As shown, IL-25 upregulated the percentage of Ki-67 positive γδT cells, whereas anti-IL-25 or anti-IL-17RB abrogated this effect (Fig. 2B). These results indicated that IL-25 stimulates the proliferation of γδT cells remarkably, and may lead to the accumulation of γδT cells in decidua.

IL-25 stimulates the production of IL-4 and IL-10, and suppresses the production of IFN-γ in γδT cells. To explore the effect of IL-25 on the expression of cytokine in decidual γδT cells, we treated these cells with rhIL-25, rhIL-25 plus anti-IL-25, and rhIL-25 plus anti-17RB for 24 h, and then detected the expression of IL-10, TGF-β, IL-4, and IFN-γ in γδT cells by flow cytometry (Fig. 3). As shown, IL-25 could upregulate the percentage of IL-10 positive γδT cells in a concentration-dependent manner, whereas anti-IL-25 or anti-IL-17RB could abrogate this effect (Fig. 3A and D). Meanwhile, IL-25 had no influence on the percentage of TGF-β positive γδT cells (Fig. 3A and E). Additionally, IL-25 also promoted the expression of IL-4 in γδT cells (Fig. 3B and F), while inhibiting the expression of IFN-γ (Fig. 3C and G). IL-25 obviously increased the ratio of IL-4 to IFN-γ (Fig. 3H). In addition, the ELISA results showed that IL-25 could facilitate the secretion of IL-10 by γδT cells, which was abrogated by anti-IL-25 or anti-IL-17RB, but had no influence on that of TGF-β (Fig. 3I and J). Therefore, these results suggest that IL-25 significantly increases the expression of IL-10 and IL-4, decreases the level of IFN-γ in γδT cells, and thus is beneficial to form a Th2 immune bias at the maternal-fetal interface.

Discussion

Accounting for the majority of decidual $CD3^+$ T cells, γδT cells, composed of Vγ9/Vδ1 chains, are functionally polarized to be Th2 cells, whereas human peripheral lymphocytes preferentially expressing Vγ9/Vδ2 chains function as Th1 cells (18-21). Based on previous research, chemokine CXCL16/CXCR6 at the maternal-fetal interface recruits γδT cells to the local decidua, the proportion of which increases significantly higher than that of the peripheral blood. In addition, the number of γδT cells in the peripheral blood and decidua of normal early pregnancy is remarkably more than that during nonpregnancy (22). Decidual γδT cells express high levels of IL-10 and TGF-β, low levels of TNF-α and IFN-γ, but almost no IL-2 and IL-4, contributing to normal pregnancy. Interestingly, we have previously discovered that decidual γδT cells promote the proliferation and invasion of trophoblast cells as well as inhibit apoptosis via IL-10, thus facilitating the formation of placenta (14). Currently, two classical mechanisms are

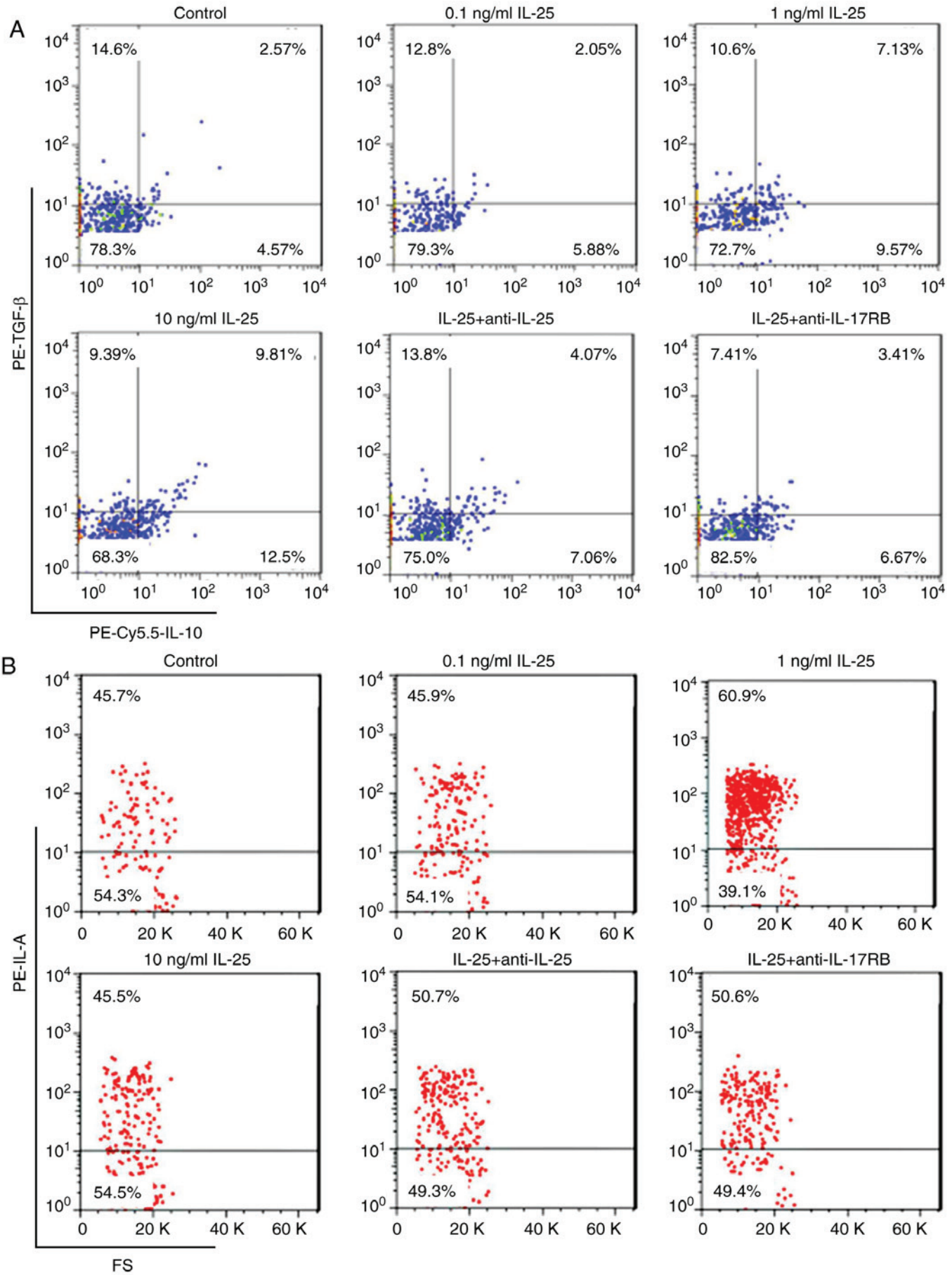

Figure 3. IL-25 stimulates the production of IL-4 and IL-10, and suppresses the production of IFN-γ in γδT cells. (A and B) Detection of the expression of several cytokines in γδT cells.Cells received treatment with different concentrations of rhIL-25 (0, 0.1, 1, 10 ng/ml), 1 ng/ml rhIL-25 plus 10 μg/ml anti-IL-25, 1 ng/ml rhIL-25 plus 10 μg/ml anti-IL-17RB for 24 h. IL, interleukin; IFN, interferon.

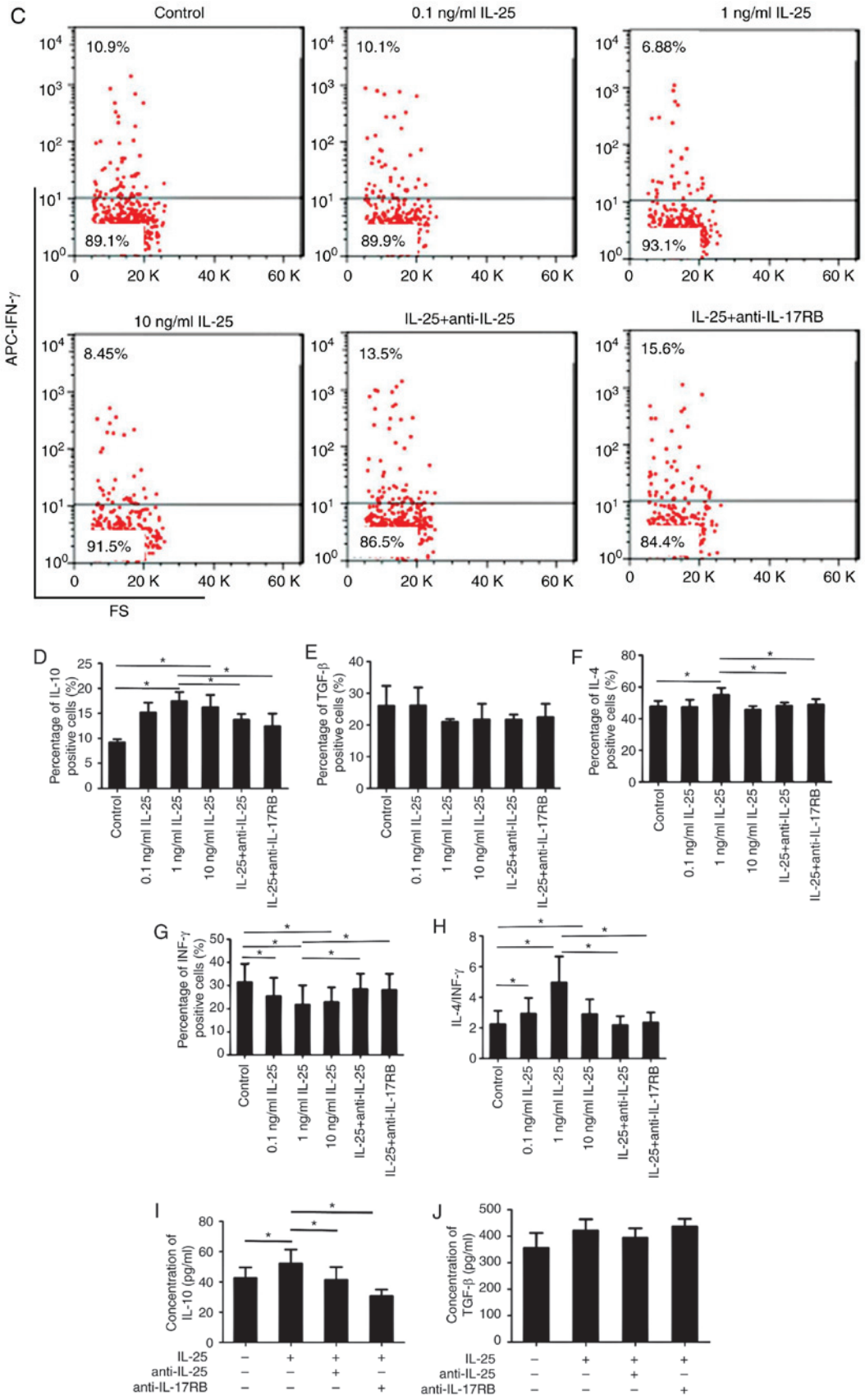

Figure 3. Continued. IL-25 stimulates the production of IL-4 and IL-10, and suppresses the production of IFN-γ in γδT cells. (C-H) Detection of the expression of several cytokines in γδT cells. After treatment with different concentrations of rhIL-25 (0, 0.1, 1, 10 ng/ml), 1 ng/ml rhIL-25 plus 10 μg/ml anti-IL-25, 1 ng/ml rhIL-25 plus 10 μg/ml anti-IL-17RB for 24 h, the expression of (D-G) IL-10, TGF-β, IL-4, and IFN-γ in γδT cells was detected. In addition, the ratio of (H) IL-4 to IFN-γ in γδT cells was also elevated obviously after treatment with rhIL-25, n=12; *P<0.05 compared with control. (I and J) The effect of IL-25 on the expression of IL-10 in γδT cells was confirmed. We used 1 ng/ml rhIL-25, 1 ng/ml rhIL-25 plus 10 μg/ml anti-IL-25, 1 ng/ml rhIL-25 plus 10 μg/ml anti-IL-17RB to treat γδT cells for 48 h, after which the secretion levels of IL-10 and TGF-β by γδT cells were detected by enzyme-linked immunosorbent assay. n=9; *P<0.05 compared with rhIL-25 treatment group. Data are presented as mean ± standard deviation. IL, interleukin; IFN, interferon; TGF, transforming growth factor.

thought to induce immune tolerance by decidual γδT cells, either exerting immunosuppression directly through cytokine production or inducing the differentiation of naïve $CD4^+$ T cells into inhibitory/regulatory T cells via IL-10 and TGF-β indirectly (18).

Growing evidence has shown that IL-25 not only stimulates human umbilical vein endothelial cell proliferation and the length, number, and area of microvessel structures in a concentration-dependent manner, but also promotes angiogenesis (23,24). However, downregulation of IL-25 in villus from patients with recurrent miscarriages may alter the local Th1/Th2 ratio (25), giving IL-25 an important role in normal pregnancy. In this study, we found that IL-25/IL-17RB were widely expressed in both the innate immune cells and adaptive immune cells of DICs, such as NK cells, NKT cells, $CD3^+$ T cells, macrophages, and Treg cells, especially γδT cells, indicating that decidual γδT cells are not only a source of IL-25 at the maternal-fetal interface, but also one of the target cells of IL-25. Then, we further investigated the effect of IL-25 on the function of decidual γδT cells, finding that IL-25 could promote the proliferation of decidual γδT cells as well as the secretion of Th2 cytokines, especially IL-10. However, the mechanism by which IL-25 promotes decidual γδT cells proliferation is currently unknown, which needs to be further explored in the future.

IL-10, an anti-inflammatory cytokine, is critical for preventing inflammatory and autoimmune pathologies. Because of the deficient IL-10 expression in CBA x DBA/2 abortion mice, supplementation of exogenous IL-10 could reduce the embryo absorption rate (26). Likewise, the expression of IL-4 and IL-10 in decidual T cells of patients who have had an abortion is lower than that during normal pregnancy, whereas the expression of IFN-γ and IL-2 is in contrast (27), indicating that Th2 bias at the maternal-fetal interface is critical for a successful pregnancy. The reduction of IL-4 and IL-10 during pregnancy also leads to preeclampsia, fetal growth restriction, premature labor, and other conditions (28). In addition, it has been discovered that trophoblast cells promote the proliferation of γδT cells to secrete IL-10 and TGF-β through CXCL16, thus in turn increasing the proliferation and invasion of trophoblasts and eventually contributing to the development of placenta and the maintenance of normal pregnancy.

In summary, based on the aforementioned and our previous study results, some molecules at the maternal-fetal interface, such as CXCL16, may recruit peripheral γδT cells to local decidua, which not only produce Th2 cytokines (e.g., IL-10, IL-4) but also reduce the production of Th1 cytokines (e.g., IFN-γ). In addition, IL-25 secreted by various cells (e.g., DSCs and DICs) accelerates the proliferation of γδT cells, whereas the apoptosis of which could be inhibited by thymic stromal lymphopoietin (TSLP) (29), resulting in a positive feedback loop and regulatory Th2 bias at the maternal-fetal interface.

Acknowledgements

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