Thymic stromal lymphopoietin from trophoblasts induces dendritic cell-mediated regulatory T_{H2} bias in the decidua during early gestation in humans

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Running title: REGULATION OF TSLP ON T_{H2} BIAS IN HUMAN DECIDUA
Abstract

Thymic stromal lymphopoietins (TSLP) play critical roles in dendritic cell-mediated immune responses. In this study we found that human trophoblasts and decidual epithelial cells in maternal-fetal interface of early placentas express TSLP mRNA and protein, but only trophoblast cells secret soluble TSLP. Human decidual CD1c⁺ DCs (dDCs) highly express the functional TSLP receptor complex TSLPR and IL-7Rα. Recombinant human TSLP activates CD1C⁺ decidual DCs and peripheral Mo-DCs with increased co-stimulatory molecules, major histocompatibility complex (MHC) class II, and OX-40L. Human TSLP or supernatants from human trophoblasts specifically stimulate dDCs to highly produce IL-10 and T\(_h\)2-attracting chemokine CCL-17. The TSLP-activated dDCs prime decidual CD4⁺ T cells for T\(_h\)2 cell differentiation, involved in maternal-fetal immuno-tolerance. Interestingly, the protein expression of TSLP in normal pregnancy with significant T\(_h\)2 bias is much higher than that of miscarriage showing TH1 bias at the maternal-fetal interface. Therefore, human trophoblasts may contribute to maternal-fetal tolerance by instructing dDCs to induce regulatory T\(_h\)2 bias in human early pregnancy via TSLP.

Key words: TSLP; dDCs; Mo-DCs; T\(_h\)2 bias; Trophoblast.
Introduction

The maternal-fetal interface exhibits a Th2 bias characterized by IL-4, IL-5 and IL-10 secretion during pregnancy. Failure to establish such an immune milieu or exhibiting a Th1 bias, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α overexpression, is associated with miscarriage, and proposed to cause recurrent spontaneous abortion in humans1-3.

Thymic stromal lymphopoietin (TSLP) is an IL-7-like cytokine, originally cloned from the murine thymic stromal cell line Z210R.1, which supports B cell development4. The functional murine TSLP receptor (TSLPR) is a heterodimer consisting of the IL-7 receptor α (IL-7Rα) chain and a common γ receptor-like chain5. Both human TSLP and TSLPR were cloned in 2001 by computational analyses6. TSLP is produced by human epithelial cells such as skin keratinocytes, tonsil crypt epithelium, bronchial epithelial cells, and mediates such allergic inflammation as asthma and atopic dermatitis7-9. The TSLPR is mainly expressed on dendritic cells (DCs), and TSLP activation of DCs induces a Th2 type inflammatory response through OX40 ligand10.

TSLP treatment causes myeloid DCs to produce large amounts of CCL17 and CCL22, preferentially attracting Th2 cells. In allergic diseases, TSLP-DCs induces robust proliferation of naive allogeneic CD4+ T cells that subsequently differentiate into inflammatory Th2 cells producing high levels of IL-5 and IL-13, moderate IL-4 but little IL-10 and IFN-γ9. IL-25 enhances the expansion of chemoattractant receptor-homologous molecule (CRTH2) positive Th2 central memory cells induced
by TSLP-DCs, and further augments Th2 cytokine production. The enhanced IL-25-induced functions of Th2 memory cells are associated with sustained IL-4-independent expression of GATA-3. The peripheral blood monocytes-derived DCs (Mo-DCs) or gut DCs activated by mucosal epithelial cells normally produce a classical Th2 response with high IL-10 and IL-6 levels, and low IL-12 levels in human intestine. Whether a TSLP-TSLP receptors signaling pathway is associated with Th2 bias at the human maternal-fetal interface remains unknown.

Here, we showed TSLP protein expression in human trophoblasts and decidual epithelial cells at the maternal-fetal interface during early pregnancy, and found that decidual DCs (dDCs), expressing the highest level of TSLP receptors among all the decidual cells, are the main target cells of TSLP. Furthermore, we demonstrated that the TSLP-instructed dDCs strongly polarized decidual CD4+ T cells toward classical Th2 bias which is beneficial for pregnancy.
Material and Methods

Samples. The first-trimester human villous and decidual tissues were obtained from placentas of 15 clinically normal pregnancies [age: 27.30 ± 3.42 years, gestational age at sampling 8.18 ± 1.45 weeks (mean ± s.d.)], which were terminated for non-medical reasons, and of 15 miscarriages [age: 28.44 ± 3.45 years, gestational age at sampling 7.53 ± 1.78 weeks (mean ± s.d.)], which were classified as unexplained after the exclusion of maternal anatomic or hormonal abnormalities, or paternal and maternal chromosomal abnormalities. The endometrial samples were collected from the fertile women (5 cases) during the proliferative or secretory phase of normal menstrual cycle. Each subject completed a signed, written consent form in accordance with the Declaration of Helsinki approved by Human Investigation Committee in Hospital of Obstetrics & Gynecology, Fudan University.

Isolation and culture of human trophoblasts. The trophoblast cells were isolated by the trypsin-DNase I digestion and discontinuous Percoll gradient centrifugation, as described by our previous study, and cultured in Dulbecco modified Eagle medium (DMEM)-high glucose complete medium (2 mM glutamine, 25 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin) supplemented with 20% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) in 5% CO2 at 37 °C.

Isolation and culture of decidual stromal cells and gland epithelial cells. The decidual tissue (4 to 6 g) was cut and digested in RPMI 1640 supplemented with collagenase type IV (1.0 mg/ml, CLS-1, Worthington Biomedical) and 1% FBS for 80
min at 37°C with gentle agitation. The total suspension was filtrated and enriched by discontinuous Percoll gradient centrifugation. The decidual stromal cells between densities of 1.042–1.062 g/ml were recovered, and cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The intact glandular organs retained on the 32-μm wire mesh were back-washed out, and cultured in DMEM/Ham’s F12 for 1-2 h in 5% CO₂ at 37°C. The floating glandular organs were transferred to a plate pre-coated with matrigel and cultured in complete medium of DMEM/Ham’s F12 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin.

Isolation and culture of CD4⁺ T cells and DCs. The decidual mononuclear cells between densities of 1.062–1.077 g/ml were collected, and the decidual CD4⁺ T cells were isolated by magnetic affinity cell sorting (MACS) using the CD4⁺ beads kit (Miltenyi Biotec).

The decidual cells between densities of 1.042–1.062 g/ml were collected, and decidual DCs were isolated by MACS with the BDCA-1 kit (Miltenyi Biotec). The purity of dDCs fraction was about 86.8% ± 7.8% as identified with PE-CD1c by FCM.

The CD14⁺ monocytes were isolated from the early pregnant PBMC by MACS. The cells passing through the LS columns were enriched for CD4⁺ T cells by MACS. Purified CD14⁺ monocytes were cultured with rhIL-4 (100U/ml, Peprotech) and rhGM-CSF (100U/ml, Peprotech) for 7 days. The floating cells with short dendrite were further isolated by using CD11c bead-based MACS, which were named
peripheral blood Mo-DCs with a purity of over 95%.

The DCs were cultured for 48 h with culture medium, or medium containing rhTSLP (15 ng/ml), rhIL-7 (50 ng/ml, Peprotech), LPS (10 μg/ml, Sigma-Aldrich). In other experiments, dDCs were co-cultured with trophoblasts, with or without neutralizing anti-TSLP antibody (50 μg/ml) in the co-culture.

**RT-PCR analysis.** The mRNA and cDNA templates for TSLP were prepared, and RT-PCR was performed as previously described\(^\text{13}\). The primer sequences were as follows: TSLP (131 bp), FW: 5'-GCT ATC TGG TGC CCA GGC TAT-3, RW: 5'-CGA CGC CAC AAT CCT TGT AAT-3; Housekeeping gene GAPDH (235 bp), FW: 5'-GGG GAG CCA AAA GGG TCA TCA TCT-3, RW: 5'-GAG GGG CCA TCC ACA GTC TTC T-3.

**qRT-PCR.** Real-time RT-PCR was performed as our previous methods\(^\text{14}\). The primers for human TSLP and GAPDH were the same as above. To compare the T-bet and GATA-3 mRNA in the primed dCD4\(^+\) T cells, the following primers were used: GATA-3 (79 bp), FW: 5'-GCG GGC TCT ATC ACA AAA TGA-3, RW: 5'-GCT CTC CTG GCT GCA GAC AGC-3; T-bet (75 bp), FW: 5'-GAT GTT TGT GGA CGT GGT CTT G-3, RW: 5'-CTT TCC ACA CTG CAC CCA CTT-3.

**Immunohistochemistry**

The villous and decidual tissue specimens were processed for immunohistochemical staining, and sheep anti-human TSLP, IFN-\(\gamma\), TNF-\(\alpha\), IL-4, or IL-10 primary antibody (R&D System) was used as primary antibodies, incubated overnight at 4°C, followed by incubation with a biotinylated rabbit anti-sheep (KPL) secondary antibody, then by the avidin-biotin-horseradish peroxidase (HRP) complex, and were stained with DAB.
and counterstained with haematoxylin. The immunocytochemistry experiments were performed with the same antibodies on the correspondent cells.

**Co-culture of DCs and CD4⁺ T cells.** The dDCs were cultured with culture medium, or medium containing rhTSLP (15 ng/ml), LPS (10 μg/ml, Sigma-Aldrich) or directly contacted with the primary trophoblast cells. After 24 h of culture, CD11c⁺ DCs were collected and washed three times with PBS to remove excess cytokines. The remaining cells were co-cultured with autologous or allogeneic decidual CD4⁺ T cells with different ratios of dendritic cell/T cell. The decidual CD4⁺ T cells were activated with 5 μg/ml anti-CD3, 1 μg/ml anti-CD28 and 20 U/ml rhIL-2 for 3 days, and collected, washed, then incubated with culture medium only, or medium containing various concentrations of rhTSLP (0.05, 0.5, 5.0 and 50.0 ng/ml). After 5 days of co-culture, the decidual CD4⁺ T cells were reactivated with anti-CD3 (OKT3) and anti-CD28 (28.2) (eBioscience) for 24 h before the supernatants were collected.

**Western blot for TSLP and its receptors.** Fifty micrograms of protein from the different cells were subjected to 10% or 15% SDS-polyacrylamide gel for TSLPR, IL-7Rα, TSLP and GAPDH, respectively. The proteins were transferred to PVDF membranes, which were blocked and immunoblotted with goat anti-TSLPR (1:1000, R&D System), mouse anti-IL-7Ra (1:1000, Abcam), sheep anti-TSLP (1:1000, R&D System) and rabbit anti-GAPDH (1:5000, KangChen, China) overnight at 4°C. The bounded antibodies were visualized using peroxidase-conjugated secondary antibodies (Santa Cruz) followed by detection using an ECL Kit (Pierce) on Las-300 (FujiFilm).
Enzyme-linked immunosorbent assay (ELISA). The supernatant from each indicated group was collected and centrifuged at 2000 $\times$ g and stored at -80°C. The amount of human cytokines including IL-4, IL-5, IL-10, TNF-$\alpha$ and IFN-$\gamma$ in the culture supernatants was quantified using cytokine-specific ELISA kit following the manufacturer’s instructions.

Flow cytometry. The freshly isolated decidual mononuclear cells were stained with FITC-conjugated mAb against human CD1c, CD3, CD14, CD19, CD56; PE-conjugated anti-human TSLPR, IL7R$\alpha$ or their corresponding isotype controls (eBioscience). The purified decidual and peripheral blood CD4$^+$ T cells were stained with FITC-conjugated mAb against human CD4 or CCR7, PE-conjugated anti-human CD45RA or CRTH2, PE/Cy5- conjugated anti-human CD45RO, and isotype control (eBioscience).

For intracellular cytokine production by quadruple labeling, CD4$^+$ CD45RO$^+$CCR7$^+$ T cells were gated and the expression of TNF$\alpha$ or IL-10 was analyzed by using FITC-conjugated anti-CD4 and PE/Cy5-conjugated anti-CD45RO, allophycocyanin-conjugated anti-CCR7, and PE-conjugated anti-TNF$\alpha$ or IL-10 mAbs. For triplicate labeling, CD4$^+$CRTH2$^+$ T cells were gated and analyzed for the expression of TNF$\alpha$ or IL10 with FITC-conjugated anti-CD4 and PE/Cy5-conjugated anti-CRTH2 and PE-conjugated anti-TNF$\alpha$ or IL-10 mAbs, and then analyzed in a FACS Calibur flow cytometer (BD Biosciences) using CellQuest software.

Statistical analysis. Statistical analysis was performed using the SPSS statistics software package (SPSS 11.5, Chicago, IL). Statistical comparisons for the TSLP
expression or cytokines in the supernatant used two-tailed Student \( t \) test or one-way ANOVA. The post-hoc Dunnett’s \( t \) test was used to compare the significance levels between control and various treatments. All error bars in the figures indicate standard deviations (s.d.). Statistical significance was accepted at \( P \) values < 0.05.

RESULTS

**TSLP is expressed at human maternal-fetal interface in early pregnancy**

We first screened samples by RT-PCR for TSLP mRNA expression in villi and decidua from women in early pregnancy. As shown in Fig 1a, TSLP mRNA is detected in all these tissues, as well as secretory-phase endometrium. The primary trophoblasts, decidual epithelial cells and stromal cells also express TSLP mRNA. TSLP mRNA levels in villi and decidua are significantly higher than that in secretory endometrium by semi-quantitative RNA analysis (\( P = 0.001 \) and \( P=0.041 \), respectively), and trophoblasts express significantly more TSLP mRNA than decidual stromal cells (\( P=0.0016 \)). The results are highly reproducible in four independent experiments.

The villous and decidual tissues from early pregnancy are positively stained for TSLP by immunohistochemistry, and syncytiotrophoblasts and cytrophoblasts in villi and invasive trophoblast cells in decidua expressed TSLP protein (Fig. 1b). However, the non-pregnant endometrium in either proliferative or secretory phase does not (Fig. 1b). Decidual epithelial cells are also positive for TSLP protein, while the large, pale stromal cells are TSLP-negative. These results were further confirmed
by immunocytochemistry that both primary trophoblasts and decidual epithelial cells express TSLP protein in cytoplasm, but decidual stromal cells do not (Fig. 1c).

The primary trophoblasts continually secret soluble TSLP into culture supernatant from 12–96 h, accumulating to 19.59 ± 1.12 pg/ml in 48 h of culture from cells at a density of 1×10^6/ml. However, TSLP is undetectable in supernatants of either the primary decidual epithelial or stromal cells (Data not shown).

**TSLP functional receptors, TSLPR and IL-7Rα, are expressed on decidual lymphocyte cells**

The cellular targets of TSLP have been identified to be lymphocytes and granulocytes. So we next detected the expression of TSLPR and IL-7Rα, the TSLP functional receptor on decidual mononuclear cells from early pregnancy by flow cytometry. As shown in Supplementary Fig. 1a, decidual CD14^+ monocyte/macrophage (Mo/Mph), an CD1c^+ DCs, are the main cells expressing surface TSLPR, while CD3^+ T cells and CD56^{bright} uterine NK cells express little TSLPR. The prevalence of TSLPR on the peripheral Mo/Mph and DCs in the freshly isolated decidual mononuclear cells is 25.34% ± 5.94% and 41.62% ± 6.84%, respectively (Fig 2a). IL7Rα is also observed to be expressed on peripheral CD14^+ Mo/Mph and CD1c^+ DCs, with 13.69±1.79% of peripheral CD14^+ Mo/Mph and 31.5±0.318% of decidual CD1c^+ DCs express IL7Rα, respectively (Fig 2a). In contrast to little TSLPR expression, 94.92±2.33% of CD4^+ T cells are IL7Rα-positive cells (Fig 2b). It is more surprising that activation with anti-CD3 and anti-CD28
antibodies significantly increases TSLPR expression level while remarkably decreases IL7Rα expression on CD4⁺ T cells (Fig 2b).

TSLP up-regulates the expression of surface molecules of human peripheral Mo-DCs and decidual DCs

As shown in Fig. 3, rhTSLP alone differently up-regulates expression of cell surface molecules, CD40, CD83, OX-40L, CD80, CD86 and HLA-DR on the decidual DCs compared to the control. There is no difference between TSLP treatment and LPS treatment, a potent DC activator. Similar results are obtained in the peripheral Mo-DCs stimulated by rhTSLP or LPS.

Trophoblast cells instruct decidual DCs to secrete high levels of Th2 cell-attractive CCL17 and IL-10 via TSLP secretion

We then investigated if TSLP could activate decidual DCs to produce high levels of Th2 cell-attractive CCL17 and IL-10 since it can increase the expression of co-stimulatory molecules, major histocompatibility complex (MHC) class II, and OX-40L on peripheral Mo-DCs and decidual DCs. The CD1c⁺ decidual DCs and peripheral blood Mo-DCs were treated with rhTSLP (15 ng/ml), or LPS (10 μg/ml) for 48 h, respectively. The results show that rhTSLP strongly stimulates decidual DCs to secrete CCL17 and IL-10 as measured by ELISA (Fig. 4a), but not TNF-α. IL-10 secreted by peripheral Mo-DCs from early pregnancy is significantly lower than that of decidual DCs, indicating the functional difference between these cells.
TSLPR is lowly expressed on the freshly isolated decidual CD4$^+$ T cells but significantly increases upon activation by anti-CD3 and anti-CD28. However, TSLP does not directly affect T$_H$ polarization, since the ratio of IL-4/IFN-$\gamma$ in decidual CD4$^+$ T cell supernatants does not alter significantly after rhTSLP treatment (data not shown). These results imply that TSLP may play a role in the decidual T$_H$2 cell differentiation by instructing decidual DCs.

To further determine if trophoblasts instruct decidual DCs to secrete high levels of T$_H$2 cell-attractive CCL17 and IL-10 via TSLP secretion, decidual DCs or peripheral Mo-DCs were co-cultured with human trophoblasts with or without neutralizing anti-hTSLP antibody (10 $\mu$g/ml) for 48 h. The trophoblasts strongly stimulate decidual DCs to secrete T$_H$2 cell-attractive chemokine CCL17 and IL-10 as detected by ELISA, but not TNF-$\alpha$ (Fig. 4b). The anti-TSLP neutralizing antibody significantly inhibits the trophoblast-induced secretion of IL-10 and CCL17, implying a TSLP-dependent process. Although trophoblasts increase IL-10 secretion by peripheral Mo-DCs in early pregnancy, the level is significantly lower than that of decidual DCs. The peripheral Mo-DCs also produce significant amounts of CCL17 in co-culture with trophoblasts without neutralizing TSLP antibody (Fig. 4b).

**TSLP-instructed decidual DCs polarize decidual CD4$^+$ T cells toward T$_H$2 bias**

We next investigated whether the TSLP-instructed dDCs (TSLP-dDCs) could polarize decidual CD4$^+$ T cells toward a T$_H$2 bias. The dDCs or Mo-DCs, pre-treated for 48 h with rhTSLP (15 ng/ml), rhIL-7 (50 ng/ml) or LPS (10 $\mu$g/ml) were
co-cultured with allogeneic decidual or peripheral CD4+ T cells for 5 days, respectively, and then IL-4, IL-5, IL-10, IFN-γ and TNF-α in the supernatant were determined by ELISA. The rhTSLP-dDCs markedly up-regulate IL-4 and IL-10, but only slightly up-regulate IFN-γ and TNF-α from decidual CD4+ T cells, compared with decidual CD4+ T cells co-cultured with dDCs but without TSLP (Fig. 5). However, no such trend is found for rhIL-7 or LPS-activated dDCs, suggesting that rhTSLP-dDCs can polarize decidual CD4+ T cells toward a TH2 biased profile.

The peripheral Mo-DCs from early pregnancy pre-treated by rhTSLP cause the peripheral CD4+ T cells to produce high levels of IL-4, IL-5 and TNF-α, but low levels of IL-10 and IFN-γ, consistent with other reports. These results indicate that decidual DCs and peripheral Mo-DCs play different roles in the TH1/TH2 balance at the maternal-fetal interface.

TH1 and TH2 cell differentiation is regulated by key transcriptional factors, such as T-bet for TH1 and GATA-3 for TH2 cells. TH1 cells express high levels of T-bet but low levels of GATA-3. We therefore examined the kinetics of GATA-3 and T-bet transcription by quantitative real-time PCR (qRT-PCR) in the decidual CD4+ T cells primed by the primary, TSLP-instructed or LPS-stimulated dDCs. Although decidual CD4+ T cells co-cultured with the primary dDCs express low levels of GATA-3 and T-bet at any time point, the allogeneic decidual CD4+ T cells primed by the TSLP-dDCs express high levels of GATA-3, but low T-bet (Supplementary Fig. 2). By contrast, the LPS-DCs induce decidual CD4+ T cells to express high levels of T-bet and low GATA-3.
Decidual and peripheral blood CD4+T cells have different surface phenotypes

Human CD4+ memory T cells are defined as CD45RO+CCR7+ central memory cells (Tcm) and CD45RO-CCR7- effector memory cells (Tem) by their distinctive surface phenotype, homing capacity, and effector functions\textsuperscript{16}. Tcm can undergo homeostatic proliferation and differentiate into effectors, and Tem with a reduced proliferative capacity can elicit immediate effector functions. Tcm and Tem can be further divided into subsets producing Th1 or Th2 cytokines with differential chemokine and tissue-homing receptor expressions, linking memory T cell subsets with polarized Th1 or Th2 function\textsuperscript{17}. CD4+CRTH2+ T cells produce IL-4, IL-5, and IL-13 but not IFN-\(\gamma\) upon T cell receptor (TCR) triggering, and represent Th2 central memory cells. Because the positively isolated total CD4+ T cells in this study produce different Th cytokines, especially IL-10 and TNF-\(\alpha\), we thus examined the CD4+ T cell phenotypes to understand this difference. The decidual CD4+ T cells are consisted of 77.57% ± 11.92% of CD45RO+ memory T cells, Tcm constitute 3.64% ± 0.62% of total decidual CD4+ T cells, while the decidual CD4+CRTH2+ T cells are only 1.24% ± 0.13% (Fig 6a, c). However, the minority of peripheral blood CD4+ T cells is CD45RO+ memory T cells which occupy 25.14% ± 4.22%; the Tcm are 10.75% ± 4.35%, and the peripheral CD4+ CRTH2+ T cells are 3.18% ± 2.42% (Fig. 6b, c). These results suggest that two groups of CD4+ T cells exist in human decidua. Subsequently, we investigate which population produces IL-10 and TNF-\(\alpha\) upon TSLP stimulation. The results in Fig. 6d show that 60.03±1.21% of TSLP-treated CD4+CD45RO+CCR7+ T cells express TNF-\(\alpha\) and 3.24±1.31% of these cells express...
IL-10. In contrast, 20.24±4.20% of CD4⁺CRTH2⁺ T cells produce TNFα and 3.8±0.93% of these cells express IL-10. These data have demonstrated TSLP treatment induces IL-10 production in both groups of CD4⁺T cells, while induces TNFα production mainly by Tcm. These results indicate that alteration of IL-10 and TNF-α production may be due to functional differences between dDCs and peripheral Mo-DCs, and relatively less Tcm and more CD4⁺CRTH2⁺ T cells at the maternal-fetal interface contribute to the generation of Th2 bias.

Lower TSLP and its receptor expression and Th2 bias at the maternal-fetal interface from miscarriage

Since successful pregnancy is characterized by Th2 bias at maternal-fetal interface, and failure to establish such an immune milieu or exhibiting a Th1 bias is associated with miscarriage, we then investigate if there is some difference on the expression of TSLP and its receptors at the maternal-fetal interface between the normal pregnancy and miscarriage.

We first analyzed the TSLP mRNA level in the placenta by qRT-PCR. The villous tissues from early normal pregnancy present significantly higher TSLP mRNA level than that of the unexplained miscarriage (P < 0.01). The same result is obtained in the decidua (Fig. 7a).

TSLP protein expression in trophoblasts, decidual stromal cells and epithelial cells from the normal early pregnancy or unexplained miscarriage was also evaluated by Western blot (Fig. 7b). The ratio of hTSLP/GAPDH in trophoblasts from the normal
early pregnancy is 0.58 ± 0.02, significantly higher than that of the miscarriage, 0.32 ± 0.02. The ratio of decidual epithelial cells from normal pregnancy was 0.29 ± 0.01, also significantly higher than the 0.14 ± 0.01 of miscarriage.

We also examined the expression of TSLP receptors, TSLPR and IL-7Rα, in these cells from normal pregnancy and miscarriage. As shown in Figure 7c, the ratio of TSLPR/GAPDH in trophoblasts and DECs from normal pregnancy is significantly higher than that of the miscarriage. The similar change is observed on IL-7Rα expression. There is no expression of TSLP and its receptors TSLPR/IL-7Rα protein in DSCs either from normal pregnancy or miscarriage.

Meanwhile, the T\textsubscript{H}1 cytokines, IFN-γ and TNF-α, or T\textsubscript{H}2 cytokines, IL-4 and IL-10, at the maternal-fetal interface were also evaluated by immunohistochemistry, and show a significant T\textsubscript{H}2 bias in the early pregnancy compared with the T\textsubscript{H}1 bias in the miscarriage (Fig. 7d, 7e).
DISCUSSION

The human maternal-fetal interface is characterized by intimate contact between the maternal tissue and the extravillous trophoblast cells that invade the decidua. High amounts of different leukocytes are present within the stromal compartment of the luteal phase endometrium which increase in the first trimester decidua\textsuperscript{18}. The most abundant leukocytes are CD56\textsuperscript{bright}CD16\textsuperscript{-} NK cells, CD38\textsuperscript{+}CD2\textsuperscript{+/-}CD3\textsuperscript{+}CD16\textsuperscript{-}CD68\textsuperscript{+} macrophages and CD3\textsuperscript{+} T cells (including both CD4\textsuperscript{+} and CD8\textsuperscript{+}); whereas B cells are virtually absent\textsuperscript{19}, dendritic cells are also present within the decidua\textsuperscript{20}. These various cells interact and play critical roles in modulation of placentation and fetal development\textsuperscript{21}.

We have found that only trophoblasts secret soluble TSLP at the maternal-fetal interface. Both TSLP and its functional receptors TSLPR and IL-7R\textsubscript{α} are expressed on human trophoblasts and DECs, but not in DSCs, and the TSLP and its receptors expression in the unexplained miscarriage are significantly decreased, suggesting that TSLP plays a role in human normal early pregnancy.

The resident DCs in the decidua are in close contact with extravillous trophoblasts usually at the decidua basalis\textsuperscript{22}. Moreover, the dDCs are the main cells expressing both TSLPR and IL-7R\textsubscript{α}, forming functional TSLP receptors. TSLP secreted by trophoblasts is a DC instructor different from other DC activation factors, such as LPS. TSLP induces decidual DCs to secrete high levels of IL-10 and T\textsubscript{H}2 cell-attractive chemokine CCL17, but not the proinflammatory cytokine TNF-\textsubscript{α}. By secreting TSLP, the trophoblasts endow decidual DCs with the ability to prime total decidual CD4\textsuperscript{+} T
cells to produce higher IL-5, IL-4 and IL-10 levels while minimal inflammatory cytokines such as TNF-α and IFN-γ. However, rhTSLP does not induce peripheral Mo-DCs from early pregnancy to produce IL-10 and TNF-α. The rhTSLP-DCs also prime peripheral naive CD4+ T cells to produce high levels of TNF-α, IL-5 and moderate IL-4, but not IL-10 or IFN-γ. These findings suggest that TSLP at the maternal-fetal interface may play a critical role in maternal-fetal immuno-tolerance that is different from allergic inflammation.

TSLP strongly activates CD11c+ peripheral DCs to produce Th2-attracting chemokines CCL17 and CCL22. Our results also show that the TSLP-primed CD1c+ decidual DCs and CD11c+ Mo-DCs to produce high levels of CCL17, which may attract blood CCR4+CD4+ T cells into the maternal-fetal interface. Human trophoblasts in early pregnancy also secrete CCL17 to regulate the infiltration of CD4+ T cells into the decidua, but at significantly lower levels than that of trophoblasts co-cultured with decidual DCs. These two sources of CCL17 at the maternal-fetal interface may play a similar role in attracting CD4+ T cells into the decidua.

Human trophoblast cells in first trimester pregnancy induce dDCs to release high IL-10 but low TNF-α level, a status protective for pregnancy. IL-10 directly suppresses T cells or indirectly induces tolerogenic DCs and regulatory T cells. The dDCs in human uterine decidua likely regulate immune responses to both uterine infections and trophoblast cells, through engagement of human inhibitory receptor ILT4 by its natural ligand, HLA-G. Nevertheless, how decidual DCs control Th1 or
TH2 differentiation is not well-understood.

Activated CD4+ cells can differentiate into at least two distinct TH phenotypes: TH1 cells producing IFN-γ that orchestrate the T cell-dependent cytotoxicity in certain autoimmune diseases, allograft rejection, and delayed-type hypersensitivity; and TH2 cells secreting IL-4 to facilitate antibody-producing B cells. Certain cross-regulating cytokines are also key in the TH1 and TH2 differentiation from CD4+ T cells. Pregnancy is known to produce a TH2 bias1, 2, 29. In mice, TH2 cytokines such as IL-10 and IL-4 are beneficial, while TH1 cytokines such as IFN-γ and TNF-α are harmful in feto-placental growth.

Although activated decidual CD4+ T cells express functional TSLP-receptors, TSLP does not directly induce TH1 or TH2 differentiation. Rochman has also reported that human peripheral CD4+ T cells proliferate significantly in response to rhTSLP30. However, Zhou et al. demonstrated that TSLP directly promoted murine CD4+ T cell to differentiate into TH2 cells31. The TSLPR expression on activated decidual CD4+ T cells is very low; thus, TSLP-TSLPR signaling pathway may be involved in the crosstalk between the decidual DCs and CD4+ T cells, and TH2 cell expansion may contribute to successful pregnancy.

Compared with normal early pregnancy, trophoblast cells from miscarriage express much lower TSLP. In the intestine low TSLP expression is associated with Crohn’s disease. However, TSLP over-expression in the skin or bronchial tube leads to atopic dermatitis or asthma3, 31. The local homeostasis of TH1/TH2 is broken when the TSLP expression level is changed, resulting in such TH1-mediated intestinal
pathologies or Th2-mediated allergic disease\textsuperscript{12,9}. We propose that the Th2 bias at the maternal-fetal interface maintained by the TSLP-dDCs is important for maternal tolerance to the fetus during early pregnancy.

The resident dDCs in close contact with trophoblasts are accurately regulated. Trophoblasts expressing paternal antigens are not simply tolerated by the maternal immune system, but instead may actively express TSLP and other bioactive molecules to instruct dDCs to induce a favorable Th2-biased immune milieu\textsuperscript{9,12,32,33}. The embryo-derived trophoblasts may modulate the function of the decidual immunocompetent cells in a DC-dependent manner via TSLP-TSLPR signaling.

Here, we compared the isolated dDCs and \textit{in vitro} generated monocyte-derived DCs in a series of experiments. Due to the low frequency, direct isolation of sufficient amounts of peripheral CD11c\textsuperscript{+} DCs from one donor was not possible. Although we have demonstrated TSLP expression in decidual epithelial cells by immunohistochemistry, the primary decidual epithelial cells fail to secrete soluble TSLP. Previous reports suggest that DCs and NK cells may also mediate TSLP-mediated cell signaling, and generate a similar Th2-bias status, characterized by expanded IL-10\textsuperscript{+} NK cells\textsuperscript{34,35}. This may partially occur because some T cells and certain NK cells share the same progenitors\textsuperscript{36,37}.

Indeed, the Th1/Th2 paradigm in pregnancy is too simplistic, as implantation and early pregnancy are clearly accompanied by inflammatory (i.e. Th1) processes that may actually benefit pregnancy\textsuperscript{38,39}. However, maintaining at least a Th1/Th2 balance is critical to a successful pregnancy. Additionally, Th1 bias is commonly recognized at
the peri-implantation stage of embryos, and thereafter, the Th2 bias is apparent in the early gestation, which is necessary for fetal and placental development\textsuperscript{40}.

In conclusion, our study shows that trophoblast-derived TSLP induces a classic $T_H2$ bias at human maternal-fetal interface through instructing decidual DCs, which may play an important role in establishing and maintaining maternal-fetal immunotolerance.

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**AUTHOR CONTRIBUTIONS**

P.-F. G. and H.-X. W. performed the major part of the experiments, and wrote the manuscript; M.-R.D. replenished experiment and revised the manuscript; Y. L. contributed to immunohistochemistry of $T_H1$ and $T_H2$ cytokines, and reviewed the manuscript; L.-P. J. contributed to RT-PCR, and FCM; and D.-J. L. designed and supervised the study, and edited and revised the manuscript. The authors have no conflicts of interest to disclose.
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Figure legends

Figure 1. TSLP is expressed at human maternal-fetal interface in the early pregnancy. TSLP expression was evaluated by RT-PCR (a), immunohistochemistry (b), and immunocytochemistry (c). Both villi and decidua from the early pregnancy and endometrial tissues from non-pregnant women expressed TSLP mRNA. The primary human trophoblasts (Tros), decidual epithelial cells (DECs) and decidual stromal cells (DSCs) also expressed TSLP mRNA (a). TSLP protein was localized in the cytoplasm of Tros (single arrow, cytotrophoblast cells; double arrow, syncytiotrophoblast cells) in villi and the cytoplasm of DECs (↑) in decidua (b), but absent in DSCs (▲). TSLP protein couldn’t be detected in endometrium of the non-pregnancy, including proliferative and secretory phase (b). These results were further confirmed in the primary trophoblasts, DECs and DSCs (c). Data are shown as mean ± s.d. The experiments were repeated four times with four placenta, decidua and endometrial tissue samples, respectively. The picture is a representative one.

Figure 2. TSLP receptor is expressed on decidual CD14⁺ Mo/Mph, CD1c⁺ DCs and activated dCD4⁺ T cells. FCM was used to analyze the expression of TSLP receptor (TSLPR and IL-7Rα) on decidual CD14⁺ Mo/Mph, CD1c⁺ DCs. Both TSLPR and IL-7Rα are expressed on decidual CD14⁺ Mo/Mph, CD1c⁺ DCs (a). Stimulation with anti-CD3 and anti-CD28 antibodies significantly increased TSLPR expression level while remarked decreased IL7Rα expression on CD4⁺ T cells (b). Each bar shows the mean ± s.d. of four repeated experiments from four cases.

Figure 3. The rhTSLP promotes DC maturation.
Decidual DCs and peripheral blood Mo-DCs were stimulated with TSLP or LPS, or cultured in medium only. CD40, CD83, OX-40L, CD80, CD86 and HLA-DR expression was determined by flow cytometry. Filled histograms represent staining of the cells with the markers on the top of histograms; Open histograms represent the isotype control (a). The numbers in the histograms indicate the percentage of positive-staining cells in DCs (b). TSLP up-regulates CD40, CD83, OX-40L, CD80, CD86 and HLA-DR expression on DCs compared to the control. The phenotypic changes on Mo-DCs were similar to dDCs. Numbers indicate the percent of positive-staining cells on DCs (b). The results shown are from four independent experiments with four cases. Data are shown as mean ± s.d. The picture is a representative one.

Figure 4. rhTSLP and trophoblast cells induces decidual DCs to produce high levels of IL-10 and CCL17. The peripheral monocytes from normal early pregnant women were cultured with rhIL-4 (100 U/ml) and GM-CSF (100 U/ml) for 5 to 9 days. At day 7, the cells became star-like morphologically. The CD1c⁺ dDCs or peripheral Mo-DCs from the early pregnancy were treated with rhTSLP (15 ng/ml), or LPS (10 μg/ml), respectively, for 48 h. The rhTSLP significantly stimulates dDCs to release higher level of T helper 2 cell-attractive CCL17 and IL-10. TNF-α production by dDCs remains at a lower level except for LPS-dDCs, whereas the rhTSLP-treated Mo-DCs secret lower levels of TNF-α and IL-10 (a). The dCD1c⁺ DCs or Mo-DCs from the early pregnancy were co-cultured with trophoblast cells, with or without 10 μg/ml anti-human TSLP neutralizing antibody (trophoblasts + αTSLP) for 48 h.
Human trophoblast cells stimulate the decidual DCs to secrete high levels of IL-10 and CCL-17, which can be effectively inhibited by neutralizing anti-TSLP antibody. The amount of TNF-α secreted by the trophoblast-regulated Mo-DCs is not significantly different from that of the trophoblast-regulated dDCs, and αTSLP has no significant effect on them. Data are from four independently conducted experiments with four cases. Data are shown as mean ± s.d.

Figure 5. TSLP-treated DCs induce CD4⁺ T cells to polarize toward a T_{H2} bias. The two kinds of DCs were exposed respectively to 15 ng/ml rhTSLP (TSLP-DCs), 50 ng/ml rhIL-7 (IL-7-DCs), or 10 μg/ml LPS (LPS-DCs) for 24 h, washed three times, and then co-cultured respectively with decidual CD4⁺ T (dCD4⁺ T) or peripheral CD4⁺ T (pCD4⁺ T) cells for 5 d. Thereafter, the T cells were transferred to a new 96-well round-bottom plate pre-coated with anti-CD3 and anti-CD28, and cultured for 24 h. The cytokines secretion of the CD4⁺ T cells was determined by ELISA. IL-4, IL-5 and IL-10 secretions of the decidual CD4⁺ T cells co-cultured with the TSLP-treated decidual DCs are highly up-regulated, but no such trend is observed for IFN-γ and TNF-α production, as compared with the primary DCs. The Mo-DCs pre-treated by rhTSLP induce peripheral CD4⁺ T cells (pCD4⁺ T) to produce higher levels of IL-4, IL-5 and TNF-α, but lower levels of IL-10 and IFN-γ. Data are the means ± s.d. from four independently conducted experiments with four samples.

Figure 6. Decidual and peripheral CD4⁺ T cells show different surface phenotypes and function. Flow cytometry was used to investigate the surface phenotype and cytokine production of the decidual and peripheral CD4⁺ T cells (a, b, c, d). The
decidual CD4+ T cells constitute 77.57% ± 11.92% of CD45RO+ memory T cells, 3.64% ± 0.62% of CD45RO+CCR7+CD4+ T cells, and 1.24% ± 0.13% of dCD4+CRTH2+ T cells (a, c). The CD45RO+ memory T cells include about 25.14% ± 4.22% of the pCD4+ T cells, the CCR7+CD45RO+ Tcm are 10.75% ± 4.35%, and pCD4+CRTH2+T cells are about 3.18% ±2.42% (b, c). Cytokine production by decidual CD45RO+CCR7+CD4+ T cells and CD4+CRTH2+ T cells was determined by FCM (d). Data are the means ± s.d. from four independently conducted experiments with four samples, and compared in the histogram (c, d).

**Figure 7. Higher TSLP and its receptor expression and Th2 bias at maternal-fetal interface in the early pregnancy than miscarriage.** qPCR shows that TSLP expression in villi and decidua from placenta of normal early pregnancy was significantly higher than that of unexplained miscarriage (a). Western blot analysis of TSLP, TSLPR and IL-7Ra in freshly isolated trophoblast cells, decidual stromal cells (DSCs), decidual epithelial cells (DECs) and DSCs from normal early pregnancy and unexplained miscarriage were performed (b, c ). The bands were scanned and analyzed on Las-300 (FujiFilm), and the ratio of TSLP, TSLPR or IL7Ra to GAPDH indicates the relative protein levels in each group, respectively. The Th1 cytokines, IFN-γ and TNF-α, or Th2 cytokines, IL-4 and IL-10, at the maternal-fetal interface were evaluated by immunohistochemistry (d). Histogram shows the relative expression level of the indicated cytokines as determined by densitometric analysis (e). Data are mean ± s.d from four repeated experiments with four placenta samples, respectively. Original magnification in the representative results is 200-fold (e).
Figure 1
Figure 2

(a) 

(b) 

Fresh decidual CD4+ T cells

Activated decidual CD4+ T cells

TSLPR-PE

IL7Rα-PE

CD4-FITC

CD14-FITC

% of indicated cells

CD14+ dMo/Mph

CD1c+ dDC

TSLPR

IL7Rα

% of indicated cells

Fresh decidual CD4+ T cells

Activated decidual CD4+ T cells
Figure 4
Figure 5
Figure 6
Figure 7
Thymic stromal lymphopoietin from trophoblasts induces dendritic cell-mediated regulatory T H2 bias in the decidua during early gestation in humans

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