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Human Trophoblasts Recruited T Lymphocytes and Monocytes into Decidua by Secretion of Chemokine CXCL16 and Interaction with CXCR6 in the First-Trimester Pregnancy

Yu Huang,*† Xiao-Yong Zhu,* Mei-Rong Du,* and Da-Jin Li2*‡

During human early pregnancy, fetus-derived trophoblasts come into direct contact with maternal immune cells at the maternofetal interface. At sites of placental attachment, invasive extravillous trophoblasts encounter decidual leukocytes (DLC) that accumulate within the decidua. Because we first found chemokine CXCL16 was highly expressed in and secreted by the first-trimester human trophoblasts previously, in this study we tested the hypothesis of whether the fetal trophoblasts can direct migration of maternal T lymphocyte and monocytes into decidua by secreting CXCL16. We analyzed the transcription and translation of CXCL16 in the isolated first-trimester human trophoblast, and examined the kinetic secretion of CXCL16 in the supernatant of the primary-cultured trophoblasts. We demonstrated that the sole receptor of CXCL16, CXCR6, is preferentially expressed in T lymphocytes, NKT cells, and monocytes, hardly expressed in two subsets of NK cells from either the peripheral blood or decidua. We further demonstrated the chemotactic activity of CXCL16 in the supernatant of the primary trophoblast on the peripheral mononuclear cells and DLC. Moreover, the CXCL16/CXCR6 interaction is involved in the migration of the peripheral T lymphocytes, γδ T cells, and monocytes, but not NKT cells. In addition, the trophoblast-conditioned medium could enrich PBMC subsets selectively to constitute a leukocyte population with similar composition to that of DLC, which suggests that the fetaldervived trophoblasts can attract T cells, γδ T cells, and monocytes by producing CXCL16 and interaction with CXCR6 on these cells, leading to forming a specialized immune milieu at the maternofetal interface. The Journal of Immunology, 2008, 180: 2367–2375.

A key cell of human placenta, the fetal cytotrophoblast plays an important role in successful pregnancy. These cytotrophoblast cells differentiate along either the villous or the extravillous trophoblast (EVCT) pathway (1). At the tip of the anchoring villi, they proliferate and differentiate into EVCT, which invades into decidua to form giant cells with two or three nuclei or replace the uterine spiral arterial endothelial cells; in contrast, the cytotrophoblasts on the border layer of the floating villi differentiate by cell-cell fusion into multinucleate syncytiotrophoblasts (ST) that cover floating villi, provide substance exchange between fetus and mother, and execute endocrine functions of placenta, such as the expression of hCG, leptin, hPL, and INSL4 (2–5). As a result, fetal cytotrophoblasts are not only in close proximity to, but are also in direct contact with, maternal decidual leukocytes (DLC) and peripheral immune cells in uterine spiral arteries.

The mechanisms by which the human allogeneic fetoplacental unit is not rejected by the maternal immune system have received intense attention, and it has now become clear that a large and specific population of immune cells, termed DLC, have special features in local cytokine production, down-regulatory cytotoxicity, endovascular formation, and placental development so as to keep pregnancy going smoothly (6–9). During decidualization, the uterine leukocytes appear to dramatically increase and account for at least 15% of all cells in the decidua from the early pregnancy through term. Moreover, they have an unusual composition: ~70% are CD56brightCD16− NK cells and the remainder include equal contributions of monocytes (~15%) and T cells (~15%) (10–11). Because chemokine and chemokine receptor interaction dominates the traffic of leukocytes, the mechanisms underlying the recruitment and maintenance of the DLC must involve the expression and secretion of chemokines at the maternofetal interface (12–15). To date, it has been found that several chemokines are expressed in, and are even secreted by, fetal cytotrophoblasts and decidual stroma, and the correspondent receptors are expressed in the DLC. The chemokines related to recruitment of decidual NK cells especially attract more attention: chemokines such as CCL3 (MIP-1α) and CXCL12 (stromal cell-derived factor-1α) expressed by cytotrophoblasts play a fundamental role in attracting decidual NK cells (16–19).
CXCL16, a ligand for CXCR6, is one of the only two known members of the plasma-membrane chemokines, consisting of a chemokine domain followed by a glycosylated mucin-like stalk and a single transmembrane helix followed by a short cytoplasmic tail (20). It is found as a membrane-bound and soluble form with completely different biological functions; the soluble CXCL16 (chemokine domain) induces homing of some leukocytes while the transmembrane molecule functions as a scavenger receptor for oxidized low-density lipoprotein and an adhesion molecule to CXCR6-expressing cells (21). Our previous research showed that first-trimester human cytotrophoblasts coexpressed CXCL16 and CXCR6 as well as secreted CXCL16, which induced their proliferation and invasion in an autocrine manner (22). In this study, we used an in vitro model to test whether CXCL16 derived from cytotrophoblasts also plays a role in maternal circular leukocytes trafficking to and residing in the first-trimester decidua. In this model, we first found that cytotrophoblast-conditioned medium (CM) attracted more PBMCs and DLC than control and then we used this model to show that CXCL16 from cytotrophoblasts functioned as one of the factors in the CM to recruit and maintain monocytes, T cells, and γδ T cells residing in human decidua.

Materials and Methods
Human placental tissue and blood collection in the first-trimester pregnancy

This study was approved by the ethical committee of Obstetrics and Gynecology Hospital (Fudan University, Shanghai, China), and informed consent was obtained from every woman participating in this study. The villi and decidua were obtained from elective termination of pregnancy (gestational age, 7–9 wk) for nonmedicinal cause at the Obstetrics and Gynecology Hospital (Fudan University). The tissues were immediately collected into DMEM and Ham’s F-12 medium (DMEM/F12; Invitrogen Life Technologies) with antibiotics (100 IU/ml penicillin, and 100 μg/ml streptomycin) and washed in HBSS for cytotrophoblast isolation and DLC isolation. The peripheral blood samples were obtained from healthy female control in the luteal phase.

Isolation and primary culture of human trophoblasts

The detailed procedure used to isolate cytotrophoblasts from the first-trimester placenta has been described previously (23). Briefly, the obtained placenta tissue was digested in four cycles of 10 min by 0.25% trypsin and 50 KU/ml DNase type I (Invitrogen Life Technologies) at 37°C with gentle agitation. The cell suspension was carefully layered over a discontinuous Percoll gradient (65–20%, in 5% step), and centrifuged at 2000 rpm for 20 min. The middle layer (density of 1.042–1.068 g/ml) was recovered and washed with DMEM/F12; the cells were diluted to 5 × 10⁶/ml and maintained in DMEM/F12 complete medium (2 mM glutamine, 25 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin), and supplemented with 10% FBS (Invitrogen Life Technologies), seeded on dishes precoated with matrigel, and incubated in 5% CO₂ at 37°C. After 24 h culture, the CM was recovered, passed through a 0.22-μm filter, and stored at −20°C before use.

The villous cytotrophoblast (VCT) and EVCT used in the immunohistochemical assay were isolated from the first-trimester placentas as previously described (24). After isolation, the EVCTs were seeded on dishes precoated with matrigel, VCTs were seeded on plastic dishes, and then incubated in 5% CO₂ at 37°C for 24 h before use. The human choriocarcinoma cell line JAR was cultured in DMEM/F12 complete medium supplemented with 10% FBS in 5% CO₂ at 37°C.

Isolation of PBMC

The PBMCs were isolated by Ficoll-Hypaque (Hua-jing Bio) density gradient centrifugation. PBMCs were washed in HBSS and resuspended at 10⁶ cells/ml in DMEM/F12 containing 1% FBS.

Isolation of DLC

The decidual tissue was digested with DNase type I (50 μg/ml; Invitrogen Life Technologies) and collagenase IV (300 U/ml; Worthington) in DMEM/F12 medium. Ten milliliters of this enzyme mixture was used per 1 g of wet weight of tissue, with digestion of 1 h at 37°C with agitation. The cell suspension was filtered through a 38-μm metal sieve and carefully layered on a discontinuous Percoll gradient consisting of 20, 40, 60% Percoll. After 20-min centrifugation at 2000 rpm, the cells from 40/60% interface were recovered and washed with HBSS. The DLC were resuspended at 10⁶ cells/ml in DMEM/F12 containing 1% FBS.

Quantification of CXCL16 and CXCR6 mRNA by TaqMan RT-PCR

Real-time RT-PCR was performed according to the published methods (22). Total RNA from trophoblasts or JAR was extracted and reverse transcribed, then cDNA was amplified by real-time PCR in a final volume of 50 μl containing 25 μl of Hot-start PCR Master Mix (RuiCheng Bio) and 200 nM of each primer probe. The primers and probes in the study were as follows: human CXCL16, forward primer: 5’-GGC CCA CCA GAA GCA TTG AC-3’, reverse primer: 5’-CTG AAG ATG CCC CCT CTG AG-3’, and TM: 5’-CCT ACC AGC CCC CCA ATT TCT CAG G-3’; human cyclophilin A, forward primer: 5’-GTC ACC CCC ACC GTG TCC TT-3’, reverse primer: 5’-CTG CTG TCT TTG GGA CCT TGT-3’, and TM: 5’-AGC TCA AAG GAC GAC GGC GCC A-3’ (DaAn Gene). Each sample was analyzed in duplicates using ABI Prism 7000 Sequence Detector (Applied Biosystems). The PCR amplification was correlated against a standard curve. Three independent experiments were done (including 15 placental samples), and the results were reproducible.

Immunohistochemical staining

Immunocytochemical and immunohistochemical assay were performed as previously published (22). Briefly, the isolated trophoblasts were cultured for 24 h, and then they were fixed in 4% PFA and washed in PBS. The cells were blocked with 7% horse serum in PBS and incubated with primary Abs (or matched control Ig) overnight at 4°C. Anti-human cytokerin-7 mAb (Zymed Laboratories) and anti-human vimentin mAb (Sino-America) were used as identification markers for cells of trophoblast lineage. Anti-human CXCL16 Ab (R&D Systems) were administrated to detect whether cytotrophoblasts express CXCL16. The cells were incubated with a biotinylated secondary Ab for 30 min followed by streptavidin-HRP incubation for another 30 min (Avidin-Biotin Histostain kit; Sino-America).

Cryosections of villous tissues were fixed with cold acetone for 5 min and then blocked with methanol containing 3% H₂O₂, sequential 7% horse serum, and incubated with anti-CXCL16 Ab and goat IgG, respectively, overnight at 4°C. The sections were then improved with the Avidin-Biotin Histostain kit.

Cell and tissue slides were stained with 3, 3-diaminobenzidine and counterstained with hematoxylin. Immunohistochemical results were evaluated by a pathologist. The experiments were repeated five times with five placenta samples, respectively.

An ELISA

The purified trophoblasts were seeded in a 24-well plate (600-μl cell suspension/well) at various densities of 1 × 10⁴, 5 × 10⁴, 1 × 10⁵, 5 × 10⁵, 1 × 10⁶, 5 × 10⁶ cells/ml. The culture plates were precoated with matrigel. The trophoblast supernatants were collected in 12, 24, 36, 48, 60, 72, 100 h of culture. Each supernatant was centrifuged at 2000 × g and stored at −20°C. The human CXCL16 ELISA kit (R&D Systems) was used to measure chemokine production in each supernatant and CM according to the manufacturer’s instructions. The CXCL16 assay demonstrated a sensitivity of 0.007 ng/ml and an intra-assay coefficient of variation of 3.5–4.9%. ELISA was done in duplicates of two separate experiments.

Ab labeling and flow cytometry

To characterize the cell populations, we analyzed the PBMCs and DLC using multicolor flow cytometry to directly test the composition and CXCR6 expression with 10 blood and decidual samples, respectively. The fluorescence-conjugated Abs and their controls used in this study were summarized in Table I. The PBMCs or DLC were blocked by treatment with normal horse serum, 10% in PBS, for 15 min at room temperature before staining. All the fluorescence-conjugated Abs and isotype-matched controls were incubated with the cells in recommended usage for 30 min at room temperature in the dark. The cells were then washed twice with 1 ml of PBS by centrifugation at 1000 × g for 5 min and analyzed by FACScalibur flow cytometry and CellQuest software (BD Biosciences).

For double labeling, monocytes (CD14⁺) were gated and analyzed for the expression of CXCR6 with FITC-conjugated anti-CD14 and PE-conjugated anti-CXCR6 mAbs. For triplcate labeling, T cells (CD3⁺) and γδ T cells (CD3⁺ γδTCR⁺) were gated and analyzed for the expression of CXCR6 with allophycocyanin-conjugated anti-CD3, FITC-conjugated anti-CD8, and PE-conjugated anti-CXCR6 mAbs.
anti-γδ TCR, and PE-conjugated anti-CXCR6 mAbs. For quadruple labeling, NKT cells, CD3−CD56+, and NK cells, CD3−CD56−CD16+ and CD3−CD56−CD16−, were gated and analyzed for the expression of CXCR6 with allophycocyanin-conjugated anti-CD3, FITC-conjugated anti-CD56, PE/Cy5-conjugated anti-CD16, and PE-conjugated anti-CXCR6 mAbs (25). In chemotaxis assay, the migrated cells were labeled with similar phenotypic Abs except PE-conjugated anti-CXCR6 Ab and followed by flow cytometry as mentioned above to characterize leukocyte subsets.

**Chemotaxis**

Chemotaxis was performed according to the published methods (26). The CXCL16 concentration of CM was adjusted to 1.6–1.8 ng/ml by ELISA before use. We used transwell plates (24-well, 6.5-mm diameter; Corning) containing polycarbonate filters of 5.0-μm pore size. The trophoblast CM of 600 μl, control medium, or control medium supplemented with recombinant human (rh) CXCL16 (catalog number: 976-CX; R&D Systems), at concentrations of 10 and 100 ng/ml, were added to the bottom chamber of each well, and 100 μl of PBMC or DLC suspensions containing 10⁶ cells was added to the upper chamber. Where indicated, 30 μg/ml control goat IgG (Hua-Mei Bio) or CXCL16-neutralizing Ab (R&D Systems) was added to the cell suspension. After incubation for 3 h at 37°C in a standard tissue-culture incubator, all the cells migrated into each lower chamber were collected and labeled with fluorescence-conjugated Abs. The total number of migrated cells was counted manually by using a hemacytometer three times. The absolute number and composing percentage of migrated peripheral or decidual NK subsets, T cells, γδ T cells, NKT cells, and monocytes were analyzed by flow cytometry as mentioned previously. All the assays were done in triplicates of four separate experiments.

**Statistical analysis**

The post-hoc Dunnett’s t test was used to compare the significance levels between control and various treatments in chemotaxis. All the error bars in the figures indicated SE. The differences were accepted as significant at $p < 0.05$. Linear regression analysis and $R^2$ values were applied to evaluate the correlation between surface expression of CXCR6 and chemotaxis by CXCL16.

**Results**

**Immunocytochemical characterization for the purity of trophoblasts**

At 24 h of culture, we characterized the expression of cytokeratin-7 and vimentin in these cells. The isolated cells were almost all stained for cytokeratin-7, whereas few cells were found stained with anti-vimentin Ab. We observed that the purity of isolated trophoblasts exceeded 95%.

![FIGURE 1.](image_url)

**FIGURE 1.** The transcription and translation of chemokine CXCL16 in human first-trimester trophoblasts. The real-time quantitative PCR was used to analyze transcription of CXCL16 in trophoblasts and JAR cells with cyclophilin A (CyP) as positive control. Three independent experiments were done (including 15 placental samples) and the results were reproducible (A). The specific brown-colored stainings for CXCL16 were recognized in the cytoplasm and cytomembrane of the primary-cultured villous cytotrophoblasts (B), and extravillous cytotrophoblasts (C) by immunocytochemistry, and the positive stainings were recognized by villous cytotrophoblasts and ST (E) and by extravillous cytotrophoblasts (F) by immunohistochemistry. No background staining was observed in goat isotype controls (D and G). The experiments were repeated five times with five placenta samples, respectively. The picture is a representative one. Magnification: B, D, E, and G, ×200; C and F, ×400.
trophoblasts was above 95% (22). In the experiments, mononuclear cytotrophoblasts aggregated and fused to ST if seeded on plastic culture dishes within 24–48 h, but they migrated and invaded the matrigel without fusion if seeded on matrigel-coated culture dishes. Because the CM supernatants were harvested after 72 h of culture on matrigel, chemokines in the CM were derived mainly from extravillous cytotrophoblasts.

Expression of chemokine CXCL16 in the first-trimester human cytotrophoblasts

To validate the transcription of CXCL16 in trophoblasts, we analyzed the transcriptional levels for both the isolated trophoblast and the JAR line by quantitative real-time RT-PCR. Fig. 1A showed mRNA copies for $3.45 \times 10^5$ CXCL16 and $3.78 \times 10^6$ cyclophilin A in the isolated trophoblasts, and human trophoblasts expressed an increase that was 102-fold more in CXCL16 mRNA than in the JAR cell.

After identifying CXCL16 transcription in the first-trimester trophoblasts, we analyzed CXCL16 expression in the cultured first-trimester VCT, EVCT, and placental cryosections by immunocytochemistry and immunohistochemistry, respectively. The results showed that the cytoplasm and cytomembrane of trophoblasts were strongly stained for CXCL16; moreover, both multinucleated ST and mononucleated trophoblasts expressed CXCL16 positively (Fig. 1B). In addition, immunohistochemical staining for CXCL16 in the first-trimester placental tissues showed specific brown-colored staining in the cytoplasm and cytomembrane of villous cytotrophoblasts, ST, and extravillous cytotrophoblasts (Fig. 1, D and E).

FIGURE 2. The accumulated concentration of CXCL16 in culture medium of first-trimester cytotrophoblasts was examined by ELISA. Purified cytotrophoblasts were seeded at $1 \times 10^6$, $5 \times 10^5$, $1 \times 10^5$, $5 \times 10^4$, $1 \times 10^4$, and $5 \times 10^3$ cells/ml, and then the supernatants were collected and measured after 12, 24, 36, 48, 60, 72, and 100 h of culture. The results showed CXCL16 accumulation in the medium during the course of culture. Each point represents the mean ± SD obtained from duplicate dishes, and the data are representative of two independent experiments.

FIGURE 3. Flow cytometry analysis for expression of chemokine receptor CXCR6 on PBMC and DLC populations. A, Fresh PBMC (left) and DLC (right) were stained with fluorescent-conjugated Abs to distinguish individual leukocyte populations and detect CXCR6 expression. NKT cells were gated on the basis of the expression of CD56 and CD3; NK cells were gated on the expression of CD56 and the lack of CD3 expression, and those positive or negative for CD16 expression were used to indicate two subsets of NK cells (B). Monocytes were gated on the expression of CD14 (C). T lymphocytes were gated on expression of CD3, and γδ T lymphocytes were gated on expressions of CD3 and γδ TCR (D). B–D, A typical analysis of CXCR6 expression on the surface of DLC populations. E, CXCR6 distributions on the surface of some DLC populations were significantly different from that of pregnant periphery leukocytes. Bars represent the percent of CXCR6-positive cells with fluorescence exceeding the isotype control. Each bar shows the mean ± SE of 10 cases. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, as compared between PBMC and DLC. Error bars depict the SEM.
Secretion in vitro of CXCL16 by the first-trimester trophoblasts

Human trophoblasts had a weak proliferative ability and a short survival period in vitro. When seeded on the matrigel-coated plate, they can survive for \( \frac{1}{1022} \) wk. We cultured the isolated trophoblasts in a matrigel-coated 24-well plate for 12–100 h in different densities, and examined the release of soluble CXCL16 in culture medium of trophoblasts by ELISA. The primary-cultured trophoblasts secreted CXCL16 continuously at nearly a constant rate, and the production of CXCL16 correlated positively to the cell density. When the trophoblasts were seeded in \( \frac{1}{1003} \) \( 10^6 \) cells/ml, the accumulated concentration of CXCL16 was \( 2.690 \pm 0.180 \) ng/ml after culture for 100 h. The 100-h accumulated levels of CXCL16 were \( 0.190 \pm 0.014 \) and \( 0.170 \pm 0.04 \) ng/ml, respectively, when seeded in \( 5 \times 10^4 \) and \( 1 \times 10^6 \) cells/ml. When seeded in \( 5 \times 10^5 \) cells/ml, the level of CXCL16 was undetectable because of little cell survival (Fig. 2).

The expression of CXCR6 on decidual and peripheral immunocyte

Following isolation of PBMC and DIC, we used multiple-color flow cytometry to analyze CXCR6 expression on peripheral and decidual CD56\(^+\)CD16\(^-\)NK cells, CD56\(^-\)CD16\(^+\)NK cells, T cells, γδ T cells, NKT cells, and monocytes (Fig. 3). On average, peripheral monocytes and γδ T cells expressed high levels of CXCR6 (87.92 ± 6.61%, 58.22 ± 7.84%), and T cells and NKT cells expressed CXCR6 in 15.28 ± 3.43% and 15.12 ± 4.41%, while both CD56\(^+\)CD16\(^-\)NK and CD56\(^-\)CD16\(^+\)NK cells expressed CXCR6 at very low levels of 5.93 ± 1.31% and 5.80 ± 2.27%, respectively.

In the first-trimester decidua, CXCR6 was expressed 87.29 ± 6.95% on decidual γδ T cells, 47.74 ± 6.64% on monocytes, 44.14 ± 4.40% on NKT cells, and 30.39 ± 5.23% on T cells. However, very low percentages of CD56\(^-\)CD16\(^+\)NK cells (4.84 ± 0.77%) and CD56\(^+\)CD16\(^+\)NK cells (4.79 ± 1.30%) expressed CXCR6. It was found that monocytes in decidua expressed lower levels of CXCR6 than that in peripheral blood (\( p < 0.001 \)), but NKT cells, γδ T cells, and T cells in decidua expressed higher levels of CXCR6 than that of the peripheral blood, respectively (\( p < 0.001, p < 0.01, p < 0.05 \)). No significant difference was found in CXCR6 expression between decidual and peripheral NK subsets (Fig. 3E).

The experimental system also allowed us to get the composing percentage of lymphocyte subsets in PBMC and DLC. The results showed that CD56\(^+\)CD16\(^-\)NK cells constituted the major subset of the DLC population by 67.02 ± 18.33%, T lymphocytes made up 11.05 ± 7.22%, and monocytes made up 5.28 ± 0.29% of DLC (data not shown).
CXCL16 in the trophoblast CM induced migration of the peripheral immunocyte

We assayed the chemotactic activity of CM from trophoblasts and CXCL16 on PBMCs. In this experiment, CXCL16 in the trophoblast CM was quantified and adjusted to 1.6–1.8 ng/ml by ELISA. First, we counted the total number of PBMCs migrated to the lower chambers containing CM or rhCXCL16. The results showed that the trophoblast CM or CXCL16 at 100 ng/ml induced 73.1 and 45.3% increases, respectively, in the migration of PBMCs compared with the control medium, while treatment with neutralizing Ab of CXCL16 could inhibit the chemotactic activity of CXCL16 completely, and of CM partly, as compared with the isotypic IgG control (Fig. 4A, the chemotaxis data of the isotype control not shown here).

As reported by other investigators, the trophoblast CM is capable of attracting larger numbers of NK cells, monocytes, and T lymphocytes (16). Our results showed that CM induced a 4.60-fold increase in the migration of CD56<sup>+</sup>CD16<sup>+</sup>NK cells and a 5.15-fold increase in the migration of CD56<sup>+</sup>CD16<sup>+</sup>NK cells. The cytotrophoblast CM increased monocyte migration by 5.18-fold over basal levels, and increased T cell and γδ T cell migration by 1.47- and 1.81-fold, respectively, exhibiting a relatively modest response of T lymphocytes compared with that of monocytes and NK cells. But we did not find the migration of NKT cells in our experimental system (Fig. 4B–D).

In the above experiments, we also assessed the contribution of chemokine CXCL16 to induction of the peripheral immunocyte chemotaxis. We found that both monocytes and γδ T cells could be attracted significantly by CXCL16 at a concentration of 10 ng/ml, and their responses increased to 5- and 1.92-fold of control, respectively, when treated with rCXCL16 at 100 ng/ml (Fig. 4C and D). CXCL16 at 100 ng/ml could also be enriched by a 92% increase of peripheral T lymphocytes (Fig. 4C). Moreover, CD56<sup>+</sup>CD16<sup>+</sup>NK, but not CD56<sup>+</sup>CD16<sup>+</sup>, cells also responded moderately to a high dose of CXCL16 (100 ng/ml) in an ~2-fold increase; thus, the two peripheral blood NK subsets appeared different in chemotaxis to CXCL16 although they had similarly lower CXCR6 expression (Fig. 4B). However, despite the fact that NKT cells expressed moderate levels of CXCR6, CXCL16 was found to hardly attract NKT cells in our experimental system (Fig. 4D). Furthermore, the results showed that CXCL16 Ab (30 μg/ml) completely inhibited monocytes, T cells, γδ T cells, and CD56<sup>+</sup>CD16<sup>+</sup>NK cell migration induced by CXCL16 at 100 ng/ml to baseline, and completely inhibited the CM-induced migration of T and γδ T cells to baseline, but slightly reduced the CM-induced migration of NK cell and monocyte (Fig. 5A–D).

The process allowed us to investigate a given PBMC subtype chemotaxis by the CM or rhCXCL16 (Fig. 4E). We found a dramatic difference among PBMC subtypes with respect to their responses to the CM and CXCL16. Although the CM could attract most PBMC, the NK cells and monocytes were dramatically enriched in the CM-induced population, comprising 53.65 and 22.58%, respectively, in contrast to 27.44 and 13.30% of the spontaneous population. On the contrary, T lymphocytes were decreased in the CM-induced population, comprising only 14.92% in contrast to 34.20% of the control. Monocytes were also somewhat enriched in the populations attracted to CXCL16 at a concentration of 10 and 100 ng/ml, comprising 24.89 and 28.18% in contrast to...
13.3%, of control. The T lymphocyte population was decreased slightly in CXCL16 treatment, comprising 22.77% at the CXCL16 concentration of 10 ng/ml and 25.29% at 100 ng/ml, in contrast to 34.20% of control. NK cell and γδ T cell population percentages in the CXCL16-induced group were similar to that of control.

Finally, we analyzed the correlation between CXCR6 expression and CXCL16-mediated chemotactic effects on PBMC populations (Fig. 6A). CXCL16 strongly attracted the peripheral monocyte, T cell, and γδ T cell, but had a little effect on the NK cell, which correlated well with the expression level of CXCR6 on those cells (p < 0.05). The only exception was the NK cell that expressed CXCR6 moderately but was not recruited by CXCL16.

**CXCL16 in the cytotrophoblast CM induced migration of DLC**

Having shown that CXCL16 in cytotrophoblast CM induces the migration of PBMC, we then assayed the chemotactic activity of CM or CXCL16 to the DLC. Both the CM from cytotrophoblasts and rhCXCL16 at 100 ng/ml recruited higher total numbers of DLC, 136.2 and 70.5% increases, respectively, than the medium control; DLC appeared to have stronger responses to the CM and CXCL16 than PBMC. Also, we observed that addition of anti-CXCL16 could reverse CXCL16 chemotaxis and slightly reduce CM chemotaxis (Fig. 5A).

Similarly, we assayed the chemotactic activity of the CM and CXCL16 to the decidual CD56⁺CD16⁻ NK, CD56⁺CD16⁺ NK, T, γδ T, and NK T cells, and monocytes. Our results showed that the CM induced a 2.64-fold increase in the migration of CD56⁺CD16⁻ NK cells and a 3.67-fold increase in the migration of CD56⁺CD16⁺ NK cells. The CM induced monocyte migration by a dramatic increase of 8.93-fold, and induced T cell and γδ T cell migration by 2.33- and 2.50-fold increases, respectively. As to the chemotactic activity of CXCL16, we found that the migration of the decidual γδ T cells increased to 2.04- and 3.96-fold of control, respectively, when treated by the rCXCL16 at 10 and 100 ng/ml, and the responses of the decidual T cells and monocytes to CXCL16 at 100 ng/ml increased significantly to 2.09- and 3.19-fold of the baseline. CD56⁺CD16⁻ and CD56⁺CD16⁺ NK cells were not enriched by an experimental dose of CXCL16 (10 and 100 ng/ml), nor were NK T cells. Addition of anti-CXCL16-neutralizing Ab (30 μg/ml) could completely inhibit the decidual monocyte, T cell, and γδ T cell migration induced by CXCL16 at 100 ng/ml to baseline, as well as the CM-induced migration of T cells to baseline, but slightly inhibit the CM-induced migration of NK cells, γδ T cells, and monocytes (Fig. 5, B–D).

Being different from PBMC, the DLC subtypes were maintained after migration upon the CM or CXCL16. CD56⁺CD16⁻ NK cells contributed the largest population of DLC or migrated DLC by 70–80%, with T lymphocytes and monocytes comprising equal percentages of 10% (Fig. 5E). Chemotactic responses of DLC subtypes to CXCL16 were largely parallel to their expression level of receptor CXCR6, with the only exception being the decidual NKT cell.

**Discussion**

The presence of a semiallogeneic fetus in the maternal uterus presents a major challenge to the immune system. One of the ways in which the immune system deals with the challenge is the unique distribution of DLC. It has been reported that >70% of decidual lymphocytes are CD56⁻CD16⁻ NK cells in contrast to only ~1% CD56⁺CD16⁻ NK cells in peripheral blood lymphocytes, whereas T cells constitute only 10% of decidual cells. We confirmed the characteristic composition of the leukocytes by multiple-color labeling cytometry. Because the DLC are recruited from the peripheral blood rather than by self-renewal in the decidua, chemokines have been implicated as pivotal players in trafficking of immune cells to multiple target organs, which hints that some chemokines expressed highly at the maternal-fetal interface are required for effective homing of the leukocytes. Because human trophoblasts during early pregnancy yield chemokine CXCL16 continuously and abundantly, we hypothesized that CXCL16 acted as one of the fetal contributions to DLC enrichment in the decidua. To validate this possibility, we then examined whether CXCR6, the only receptor of CXCL16, was expressed in the DLC populations.

In humans, the chemokine receptor CXCR6 is expressed preferentially on Th1 and T-cytotoxic 1 polarized memory CD4⁺ and CD8⁺ lymphocytes, and has been detected on large proportions of the tissue-infiltrating lymphocytes from patients with inflammatory disorders (27). We found that CXCR6 was expressed selectively in the leukocytes, except NK cells at the human maternal-fetal interface, especially in γδ T lymphocytes and CD14⁺ monocytes. The expression of CXCR6 on T lymphocytes and NK T cells is up-regulated when they are recruited into decidua from peripheral blood, but the expression of CXCR6 on the decidual monocyte is down-regulated. Recent reports suggested that some of leukocytes present in the peripheral blood should be equipped with a complete repertoire of chemokine receptors that will enable their rapid migration to various organs; once these cells reach their target organ the chemokine receptors were down-regulated, except from those needed for their retention to escape further trafficking (19, 25). As trophoblasts can secrete plenty of CXCL16 at the maternofetal interface, it is possible that CXCR6 up-regulation is needed for T lymphocyte (especially γδ T lymphocyte) and NKT cell retention in decidua. But it seems that CXCL16/CXCR6 is important for decidual monocyte recruitment rather than for its residence. Although CD56brightCD16⁻ NK cells constitute the vast majority of the DLC population, both peripheral and decidual NK cells hardly expressed CXCR6, which suggests CXCL16/CXCR6 are apart from NK cell migration; we and others have demonstrated that decidual NK cell migration is directed by CXCL12/CXCR4 interaction (18, 19).

We have demonstrated that T lymphocytes and monocytes migrate into the decidua by expressing CXCR6. In addition, the CM from human trophoblast cells showed robust chemotactic activity to the peripheral NK cells, T lymphocytes, and monocytes. The unavailability of the neutralizing Ab to CXCR6 restricted us from confirming our result through blocking CXCR6 of these immune cells. But the migration of T cells and monocytes was significantly decreased when CXCL16 was absent or the anti-CXCL16-neutralizing Ab was added in the medium, which clearly indicates that the chemotaxis of the trophoblast CM on these cells is attributable to the presence of CXCL16. In the same way, CXCR6 was expressed selectively on the decidual T cells, γδ T cells, and monocytes, and the chemotaxis of CXCL16 in trophoblast CM on
these cells was demonstrated in the present study, which suggests that CXCL16 not only recruits peripheral T cells and monocytes into decidua but also attracts these cells to stay there. Although we did not measure the adhesion activity of CXCL16 to these DCL, we propose that CXCL16, as one of the plasma membrane chemokines expressed on trophoblasts, plays a role in retention of special compositions of the DCL.

As we have seen, the homing of leukocytes from the blood into the tissues involves at least three consecutive steps: tethering and rolling mediated by primary adhesion molecules, exposure to a chemotactic stimulus provided by chemokines and chemokine receptors, and arrest mediated by activated integrins (28–31). Each of these steps is necessary for the accumulation of leukocytes at the site of decidua. The highly specific combinatorial expression of adhesion molecules, endothelial counterreceptors, chemokines, and chemokine receptors in pregnant uterine blood vessels means that there are intricate homing processes of the three-step combination (1). The present report has demonstrated that CXCL16 may be capable of the recruitment of the decidua T lymphocytes and monocytes. It should be noted that DCL infiltration is also regulated by other molecules, as is suggested by the known chemokines and receptors in previous reports. It is possible that human trophoblasts use distinct sets of these molecules to influence DCL trafficking.

NKT cells constitute a lineage of hemopoietic cells that share both phenotypic and functional characteristics with NK cells and effector T lymphocytes. In addition to peripheral blood, human NKT cells have been cloned from bone marrow, liver, and decidua tissue (7, 32–34). In the present study, it is interesting that neither peripheral nor decidua NKT cells respond to CXCL16 even though nearly half of the population also expressed CXCR6. As we have known that CXCL16, besides functioning as a soluble chemoattractant to recruit different target cells, also serves as a surface-bound transmembrane molecule and mediates stable adhesion to CXCR6-expressing cells (20, 21). Both because the EVCTs and cytotrophoblast express CXCL16 at a high level, and the EVCTs invade into uterine decidua with a chance to direct contact with maternal leukocytes in placentation, CXCR6/CXCL16 interactions happen easily between decidua NKT cells and fetal trophoblasts, and play a role in mediating cell-cell contact and/or signaling (35). Therefore, the interactions might also be present between other DCL and trophoblasts. It is possible at this stage that CXCR6 plays a role in modulating the development of these cells or activation in decidua.

In this study, we have demonstrated that human T lymphocytes, γδ T lymphocytes, and monocytes are attracted during pregnancy from the peripheral blood into the decidua via the interactions between CXCR6 on their surface and CXCL16 secreted by the embryo-derived trophoblasts. The interaction between CXCR6 and CXCL16 might also lead to the retention of these cells that have already migrated into the decidua, which comprises the DCL population pattern with which the placenta coexists. Furthermore, transmembrane-type CXCL16 expressed on trophoblasts may also be important for firm adhesion to T lymphocytes, γδ T lymphocytes, NKT cells, and monocytes, which will endow the fetal trophoblasts with the capacity to regulate maternal immune cells displaying different functional behavior at the maternalfetal interface, which affords them an additional level of control in modulating the response of maternal immune cells in this sensitive location so as to keep the pregnancy going smoothly (36).

Disclosures
The authors have no financial conflict of interest.

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