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 Li

*J Immunol* 2008; 180:2367-2375; doi: 10.4049/jimmunol.180.4.2367

http://www.jimmunol.org/content/180/4/2367

**References**

This article *cites 36 articles*, 15 of which you can access for free at:
http://www.jimmunol.org/content/180/4/2367.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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 fetus-derived 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 s a key cell of human placenta, the fetal cytotrophoblast plays an important role in successful pregnancy. These cytotrophoblast cells differentiate along either the vil-

1 Abbreviations used in this paper: EVCT, extravillous trophoblast; ST, syncytiotrophoblast; DLC, decidual leukocyte; VCT, villous trophoblast; rh, recombinant human.

2 Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, Shanghai Medical College, Fudan University, Shanghai, China; †Department of Gynecology, Affiliated Hospital of Medical College, Qingdao University, Qingdao, China; and ‡Department of Obstetrics and Gynecology, Affiliated Hospital, Hainan Medical College, Haikou, China

Received for publication June 25, 2007. Accepted for publication December 6, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the National Basic Research Program of China (2006CB944009, to D.-J.L.), the Key Project of the National Natural Science Foundation of China (30730087, to D.-J.L.), the National Natural Science Foundation of China (30670787 to D.-J.L. and 30700763 to Y.H.), Shanghai Leading Academic Discipline Project B117 to (D.-J.L.), and the Program for the Outstanding Medical Academic Leader of Shanghai (to D.-J.L.).

2 Address correspondence and reprint requests to Dr. Da-Jin Li, Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, Shanghai Medical College, Fudan University, Shanghai 200011, China. E-mail address: djli@shmu.edu.cn

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/52.00
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 × 10^6/ml and maintained in DMEM/F12 complete medium (2 mM glutamine, 25 mM HEPES, 100 IU/ml penicillin, and 100 µg/ml streptomycin), supplemented with 10% FBS (Invitrogen Life Technologies), seeded on dishes precoated with matrigel, and incubated in 5% CO2 at 37°C. After 72 h in 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 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 cytotkratin-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 normal 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 × 10^5, 5 × 10^5, 1 × 10^6, 5 × 10^6, 1 × 10^7, 5 × 10^7 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 CXCX6 with FITC-conjugated anti-CD14 and PE-conjugated anti-CXCR6 mAbs. For triple-labeling, T cells (CD3+) and γδ T cells (CD3+ γδTCR+) were gated and analyzed for the expression of CXCR6 with allopoxycyanin-conjugated anti-CD3, FITC-conjugated anti-CXCR6 and PE-conjugated anti-CXCL16 mAbs.

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-star 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 TTT AC-3′, reverse primer: 5′-CTG 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 TTC TT-3′, reverse primer: 5′-CTG CTG TCT TTG GGA CCT TGT-3′, and TM: 5′-AGC TCA AAG GAA GAC GCG 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

T CELLS AND MONOCYTES MIGRATING INTO DECIDUA

Downloaded from http://www.jimmunol.org/ by guest on July 29, 2017
anti-\(\gamma\)-\(\delta\) 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 allopocacycin-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-\(\mu\)m pore size. The trophoblast CM of 600 \(\mu\)l, 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 \(\mu\)l of PBMC or DLC suspensions containing 10\(^6\) cells was added to the upper chamber. Where indicated, 30 \(\mu\)g/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, \(\gamma\)-\(\delta\) 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

**Table I. The monoclonal fluorescent-conjugated Abs and isotypes used in this study**

<table>
<thead>
<tr>
<th>Labeling Ab</th>
<th>Dosage, in (\mu)l</th>
<th>Isotype</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human CXCR6-PE</td>
<td>20</td>
<td>Mouse IgG2b</td>
<td>CXCR6</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Anti-human CD3-AP</td>
<td>20</td>
<td>Mouse IgG2a, k</td>
<td>TCR associated</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Anti-human CD14-FITC</td>
<td>20</td>
<td>Mouse IgG2a, k</td>
<td>Phenotype of myelomonocytic lineage</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Anti-human CD56-FITC</td>
<td>20</td>
<td>Mouse IgG2a, k</td>
<td>Receptor of NK, NKT cells</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Anti-human CD16-PE/Cy5</td>
<td>20</td>
<td>Mouse IgG1, k</td>
<td>FcR of NK cells</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Anti-human (\gamma)(\delta) TCR-FITC</td>
<td>20</td>
<td>Mouse IgG1, k</td>
<td>(\gamma)-(\delta) TCR associated</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Isotype-matched control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse IgG2a, k-allopocacycin</td>
<td>20</td>
<td></td>
<td>for CD3</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Mouse IgG2a, k-FITC</td>
<td>20</td>
<td></td>
<td>for CD14, CD56</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Mouse IgG1, k-PE/Cy5</td>
<td>20</td>
<td></td>
<td>for CD16</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Mouse IgG1, k-FITC</td>
<td>20</td>
<td></td>
<td>for (\gamma)-(\delta) TCR</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Mouse IgG2b, k-PE</td>
<td>20</td>
<td></td>
<td>for CXCR6</td>
<td>BioLegend</td>
</tr>
</tbody>
</table>

**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 (CyB) 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 cytrophoblasts (B), and extravillous cytrophoblasts (C) by immunocytochemistry, and the positive stainings were recognized by villous cytrophoblasts and ST (E) and by extravillous cytrophoblasts (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 $3.45 \times 10^7$ CXCL16 and $3.78 \times 10^8$ 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 1 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 \( \frac{1}{1000} \times 10^6 \) cells/ml, the accumulated concentration of CXCL16 was 2.690 ± 0.180 ng/ml after culture for 100 h. The 100-h accumulated levels of CXCL16 were 0.190 ± 0.014 and 0.170 ± 0.04 ng/ml, respectively, when seeded in \( 5 \times 10^4 \) and \( 1 \times 10^5 \) cells/ml. When seeded in \( 5 \times 10^3 \) 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 took count of 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 as 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 addition of the isotypic IgG control (Fig. 4, A, 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 increased migration of CD56+/CD16+ NK cells and 5.18-fold increase 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. However, we did not find the migration of NKT cells in our experimental system (Fig. 4, B–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. 4, C and D). CXCL16 at 100 ng/ml could also be enriched by a 92% increase of peripheral T lymphocytes (Fig. 4C). Moreover, CD56−CD16−NK, but not CD56−CD16+, 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−CD16−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. 5, A–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...
and CXCL16 than PBMC. Also, we observed that addition of control; DLC appeared to have stronger responses to the CM or CXCL16 to the DLC. Both the CM from cytotrophoblasts expressed CXCR6 moderately but was not recruited by CXCL16. Having shown that CXCL16 in cytotrophoblast CM induces the migration of DLC sub-populations, we then assayed the chemotactic activity of the CM and 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^bright^CD16^-^NK, CD56^bright^CD16^-^NK, T, γδ T, and NKt cells, and monocyes. 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 rhCXCL16 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 NKt 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 sub-types 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 periphery 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 maternofetal interface, especially in γδ T lymphocytes and CD14^+^ monocytes. The expression of CXCR6 on T lymphocytes and NKT 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 secret plenty of CXCL16 at the maternofetal interface, it is possible that CXCR6 up-regulation is needed for T lymphocyte (especially γδ T lymphocyte) and NK cell retention in decidua. But it seems that CXCL16/CXCR6 is important for decidual monocyte recruitment rather than for its residence. Although CD56^bright^CD16^-^ 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 DLC, we propose that CXCL16, as one of the plasma membrane chemokines expressed on trophoblasts, plays a role in retention of special compositions of the DLC.

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 decidual T lymphocytes and monocytes. It should be noted that DLC 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 DLC 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 decidual tissue (7, 32–34). In the present study, it is interesting that neither peripheral nor decidual 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 chemotractant 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 EVCT and cytotoxic trophoblast express CXCR6 at a high level, and the EVCTs invade into uterine decidua with a chance to direct contact with maternal leukocytes in placentaion, CXCR6/CXCL16 interactions happen easily between decidual 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 DLC 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 DLC 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.

References


