Chemokine Ligand and Receptor Expression in the Pregnant Uterus

Reciprocal Patterns in Complementary Cell Subsets Suggest Functional Roles

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During human pregnancy, the uterus is infiltrated by a population of maternal leukocytes that co-exist with fetal cytotrophoblasts occupying the decidua and uterine blood vessels. These immune cells, termed “decidual granulated leukocytes,” are composed predominately (70%) of the CD56^{bright} subset of natural killer cells, accompanied by T cells (15%) and macrophages (15%). The mechanisms underlying the recruitment of these cells are unknown, but by analogy to other systems, chemokines are likely to be involved. We examined the expression patterns of 14 chemokines in the decidualized uterine wall by *in situ* hybridization, and the expression of chemokine receptors on decidual leukocytes by RNase protection. The striking concordance between the expression of chemokines in the uterus and their receptors on decidual leukocytes allowed us to identify numerous receptor-ligand pairs that may recruit the latter cells to the uterus during pregnancy. Additionally, chemokine expression patterns suggested other, nonimmune functions for these molecules, including a role in cytotrophoblast differentiation. Together, our results imply that chemokine networks serve important functions at the maternal-fetal interface. (*Am J Pathol* 2001, 159:2199–2213)

Successful human pregnancy requires that the maternal immune system, in defiance of all known laws of transplantation, tolerates the presence of a conceptus expressing paternal antigens. The mechanisms behind this immunological paradox constitute an area of active investigation. In accord with its biological importance, current evidence points to multiple levels of regulation sup-

porting maintenance of the fetal hemi-allograft.¹ For example, the exclusive expression of the nonclassical class I molecule HLA-G² by extravillous cytotrophoblasts probably plays a role in the down-regulation of natural killer (NK) cell cytotoxicity via interaction with inhibitory receptors on the latter cells.³

Limiting immune cell access to the fetal allograft may be another mechanism involved in maternal tolerance. Accumulating evidence supports this hypothesis. First, a large and specific population of immune cells, termed decidual leukocytes, infiltrates the pregnant uterus. These immune cells account for at least 15% of all cells in the decidualized uterine wall and are identifiable from early pregnancy through term (Yan Zhou, University of California at San Francisco, personal communication). The decidual leukocyte population is unique, being composed predominately (~70%) of an unusual type of NK cell (CD56^{bright}/CD16[–]) with contributions from T cells (~15%) and monocytes (~15%).^{4,5} These cells express the activation markers Fas, CD69, and CD71.^{6–8} However, their cytolytic capacity is limited in comparison to that of peripheral NK cells.⁹ Taken together, these special features of decidual leukocytes imply that maternal tolerance relies, at least in part, on the presence of a selected population of immune cells at the maternal-fetal interface.

Second, by comparison to other systems,¹⁰ it is likely that the specificity of decidual leukocyte composition is governed at the level of cell trafficking. This has been demonstrated in the mouse, where microdomains of differentially expressed adhesion molecules involved in cell homing have been identified within the pregnant uterus.

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This expression is functionally correlated with the distinct localization of neutrophils, monocytes, and NK cells to different portions of the uterus. Accordingly, each leukocyte subset expresses adhesion molecules that interact with the endothelial counterreceptors in its respective microdomain.¹¹

Finally, although the contribution of chemokines to the recruitment of decidual leukocytes has not previously been considered in the human, these molecules are required for effective leukocyte homing in other systems¹² and likely play an analogous role during pregnancy. To date, only interleukin-8, MCP-1, and RANTES have been localized to the pregnant human uterus,^{13,14} and the possible effects of this expression on decidual leukocytes have not been investigated.

Therefore, this report advances the literature in three ways. First, it presents the expression patterns of a panel of 14 chemokines in the decidua. The results show that both fetal and maternal tissues are richly endowed with chemokines. Next, it examines chemokine receptor expression by decidual leukocytes. Taken together, the data identify potential ligand-receptor pairs involved in leukocyte trafficking to the pregnant uterus. Finally, the chemokine expression patterns it describes suggest the possibility of additional, nonimmune roles for these molecules in placentation and cytotrophoblast differentiation.

Materials and Methods

Human Tissue Collection

Informed consent was obtained from all patients from whom tissue or blood was collected. Placental and decidual tissue from elective termination of pregnancy (6 to 22 weeks) or from normal term delivery (34 to 40 weeks) was collected within 1 hour of isolation, washed thoroughly in phosphate-buffered saline (PBS) with antibiotics, and placed on ice. Tissue to be used for *in situ* hybridization was immediately put in 10% buffered formalin. After overnight fixation, tissue was transferred to 70% ethanol and embedded in paraffin for sectioning.

Buffy coats used as a source of peripheral blood mononuclear cells (PBMCs) were obtained from Blood Centers of the Pacific (San Francisco, CA). No information was available regarding the sex, age, or pregnancy status of the blood donors.

Cell Isolation

Cytotrophoblasts

Cells were isolated from pooled first- or second-trimester human placentas by published methods.^{15,16} Briefly, placentas were subjected to a series of enzymatic digests that detached cytotrophoblast stem cells from the underlying stromal core of the chorionic villus. Detached cytotrophoblasts were purified over a Percoll gradient and cultured on Matrigel-coated substrates (Collaborative Biomedical Products, Bedford, MA) for various lengths of time in serum-free medium: Dulbecco's mod-

ified Eagle's medium, 4.5 g/L glucose (Sigma Chemical Co., St. Louis, MO) with 2% Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis, IN), 1% penicillin/streptomycin, 1% sodium pyruvate, 1% Hepes, and 1% gentamicin (UCSF Cell Culture Facility).

Placental Fibroblasts

Placental fibroblasts were isolated from first-trimester placentas as previously described.¹⁵ Cells were cultured in Dulbecco's modified Eagle's medium H-21 with 10% fetal bovine serum, 5% glutamine, 1% penicillin/streptomycin, and 1% gentamicin.

Isolation of Human PBMCs

Human PBMCs were prepared from a buffy coat by erythrocyte sedimentation with 6% dextran T500 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) followed by Histopaque-1077 (Sigma) separation. PBMCs were washed in PBS and extracted for RNA.

Preparation of Decidual Granulated Leukocytes

Decidua was minced with a razor blade, then incubated at 10 ml/g of tissue in RPMI-1640 (UCSF Cell Culture Facility) containing 10% fetal bovine serum, 0.1% collagenase (Sigma), 0.02% DNase I (Boehringer Mannheim), and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma) for 1 hour at room temperature. After this digestion, the supernatant was removed and centrifuged. The cell pellet was resuspended in Hanks' buffered saline solution, layered over Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) and centrifuged for 30 minutes at $900 \times g$. The band of decidual leukocytes at the interface was removed, washed three times in PBS, and extracted for RNA.

In Situ Hybridization

In situ hybridization was performed using published methods.¹⁷ Tissue specimens to be used were fixed with 10% formalin in PBS at room temperature overnight, washed twice in PBS, dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin. Sections (5 μ m) were cut and mounted on poly-L-lysine-coated slides, deparaffinized, and rehydrated before use.

The EST plasmid clones with the identification numbers 490222 (BRAK), 140701 (fractalkine), 505324 (GCP-2), 1147321 (GRO α), 209366 (HCC-1), 491243 (IP-10), 1058310 (ITAC), 488534 (MCP-1), 322873 (MCP-3), 484740 (MIG), 154848 (MIP1 α), 183073 (SDF-1), and 503192 (SLC) were used as templates for probe synthesis. Because these plasmids were constructed in the same way, identical methods could be used in preparing each probe. Plasmids were linearized on one side of the gene insert with either *Eco*RI or *Not*I (Life Technologies, Inc., Rockville, MD). The linearized vectors were used as

templates for the synthesis of ^{35}S -RNA probes using T3 (antisense) or T7 (sense) RNA polymerase.¹⁸

On day 1 of the *in situ* hybridization, slides were allowed to sit at room temperature for 5 minutes. Then they were placed in PBS for 5 minutes before incubation at room temperature in 4% paraformaldehyde in PBS for 10 minutes, followed by a 5-minute wash in 0.5× standard saline citrate (SSC) (1× = 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.4). Sections were deproteinized with 1 $\mu\text{g}/\text{ml}$ proteinase K for 10 minutes at room temperature, then washed for 10 minutes in 0.5× SSC. Then they were fixed again in 4% paraformaldehyde for 3 minutes, followed by a final wash in 0.5× SSC for 5 minutes. After a 3-hour prehybridization at 55 to 60°C in rHB2 buffer [50% formamide, 0.3 mol/L NaCl, 20 mmol/L Tris, pH 8.0, 5 mmol/L EDTA, 1× Denhardt solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulfate, 10 mmol/L dithiothreitol], the slides were incubated overnight at 55 to 60°C in a humidified chamber with 200 μl of hybridization buffer: rHB2 buffer containing 500 $\mu\text{g}/\text{ml}$ yeast tRNA and 1,200,000 cpm of ^{35}S -labeled antisense (experimental) or sense (negative control) cRNA probes. After hybridization, sections were washed twice, 10 minutes each, in 2× SSC with 10 mmol/L of β -mercaptoethanol and 1 mmol/L of EDTA. Slides were then immersed in an RNase A solution (500 mmol/L NaCl, 10 mmol/L Tris, pH 8.0, and 10 $\mu\text{g}/\text{ml}$ RNase A) for 30 minutes at room temperature and washed twice, 10 minutes each, in 2× SSC/ β -mercaptoethanol/EDTA. Then the sections were subjected to a high stringency wash (0.1× SSC, 10 mmol/L β -mercaptoethanol, 1 mmol/L EDTA) for 2 to 3 hours at 60°C before they were washed twice, 10 minutes each, in 0.5× SSC without β -mercaptoethanol or EDTA. Finally, the sections were dehydrated (2 minutes/step) in a series of graded (30, 60, 80, 95, and 100%) ethanol solutions that contained 0.3 mol/L NH_4Ac . The slides were dried for 2 hours in a fume hood before being dipped in Kodak NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY). Afterward they were dried overnight in the dark, boxed, and stored at 4°C until they were developed. The sections were stained with hematoxylin and eosin (H&E) before they were examined in both dark and bright field using a Zeiss Axiophot microscope. To report the data in a tabular format, results were translated into a scoring system based on the density of white dots that composed the signal. Designations were as follows: ++, very intense to intense signal; +, moderately intense to weak signal; −, extremely weak or absent signal. Various human tissues (Novagen Inc., Madison, WI) served as positive controls: BRAK, colon adenocarcinoma (catalog no. 70337-3); GCP-2, endocrine tissue set (catalog no. 70320-3); IP-10, ovary serous tumor (catalog no. 70392-3); ITAC, lung bronchoalveolar carcinoma (catalog no. 70367-3); MCP-1, hematal and immune tissue set (catalog no. 70321-3); MCP-3, lung bronchoalveolar carcinoma (catalog no. 70367-3); MIG, malignant melanoma (catalog no. 70417-3); SLC, lymph node tumor (catalog no. 70375-3); SDF-1, human tissue set 1 (catalog no. 70312-3); and GRO α , malignant melanoma (catalog no. 70417-3).

Northern Hybridization

Total RNA was extracted from cytotrophoblasts immediately after the cells were isolated or after 12, 24, or 36 hours in culture, according to published methods.¹⁹ RNA was also collected from whole placental villi, and fibroblasts were collected as described above. Ten μg of total RNA was separated by formaldehyde-agarose gel electrophoresis, transferred to Nytran membranes (Schleicher and Schuell, Inc., Keene NH), and analyzed by Northern blot hybridization as previously described.^{20–22} Gels were stained with acridine orange before transfer to ensure integrity of the RNA samples and to confirm equal loading. Probes for the chemokines BRAK, GCP-2, and SLC were generated using standard methods.²³ [^{32}P]CTP, random oligonucleotide primers (Amersham Life Science, Inc., Piscataway, NJ) and the Klenow fragment of DNA polymerase I (Life Technologies, Inc.) were used to label the probes, which had a specific activity of 2×10^9 dpm/ μg .

RNase Protection Assay

Total RNA from decidual granulated leukocytes was prepared as described above. RiboQuant multiprobe RNase protection assay kits were purchased from Pharmingen and used according to the manufacturer's instructions. Briefly, three probe sets (hCR5, hCR6, and hCR8), encompassing a total of 17 receptors, were labeled with ^{32}P and hybridized to RNA (from 2 to 20 μg) from decidual leukocytes. After RNase digestion, protected bands were resolved on sequencing gels and identified by size, using undigested probe as a reference size marker. Positive controls included probes for L32 and GAPDH transcripts, to confirm RNA integrity and loading. Yeast and PBMC RNA were additional controls for expression.

Results

Studying Chemokine Expression in the Context of the Maternal-Fetal Interface

Figure 1 depicts the anatomy of the region where the maternal and fetal compartments come together. With regard to the fetal compartment, the placenta is composed of two classes of chorionic villi—floating and anchoring—that share many of the same histological features. Both have mesenchymal villus cores that contain fetal blood vessels, surrounded by a layer of cytotrophoblast stem cells that fuse to form a syncytium covering the villus surface. But only anchoring villi form specialized structures termed columns—aggregates of mononuclear cytotrophoblasts that bridge the gap between the maternal and fetal compartments by attaching to, and then invading, the uterus. Initially, this invasion is primarily interstitial, ie, through the uterine parenchyma. Numerous fetal cytotrophoblasts are thus distributed throughout the maternal

Figure 1. Anatomy of the maternal-fetal interface, where the fetal-derived placenta attaches to the mother's uterus. This region is depicted on a gross level at the **top (lavender box)**; the histology is shown at the **bottom**. The basic structural unit of the placenta is the chorionic villus, composed of a stromal core with blood vessels, surrounded by a basement membrane, and overlain by cytotrophoblast (CTB) stem cells. As part of their differentiation pathway, these stem cells detach from the basement membrane and adopt one of two lineage fates. They either fuse to form the syncytiotrophoblast, which covers floating villi (FV), or join a column of extravillous cytotrophoblasts at the tips of anchoring villi (AV). The syncytiotrophoblast mediates nutrient, gas, and waste exchange between fetal and maternal blood. The anchoring villi, through the attachment of cytotrophoblast columns, establish physical connections between the fetus and the mother. Invasive cytotrophoblasts penetrate the uterine wall up to the first third of the myometrium, encountering a population of maternal immune cells, termed decidual granulated leukocytes (DGLs), resident to the uterine stroma. A portion of the extravillous cytotrophoblasts home to uterine spiral arterioles and remodel these vessels by destroying the muscular wall and replacing the endothelial lining.

compartment as isolated cells or cell clusters. As invasion continues, a subset of cytotrophoblasts targets uterine arteries, remodeling these vessels and replacing the maternal endothelial lining. This process happens to a lesser degree in uterine veins as well.

We narrowed the list of candidate chemokine molecules by selecting those known to be expressed in the appropriate tissues. Information in a database of expressed-sequence-tagged clones (<http://cytokine.medic.kumamoto-u.ac.jp/CFC/CK/Chemokine.html>) allowed us to select chemokine cDNAs in expression libraries of

either pregnant uterus or placenta. At the time this work was begun, 14 chemokines fit the established criteria and thus were chosen for in-depth analysis by *in situ* hybridization studies to localize mRNA expression within tissue sections of the maternal-fetal interface. The tissues examined included portions of floating villi, anchoring villi, and the placental bed (Figure 1). This article focuses on chemokine expression and action in the uterine wall, ie, decidual stroma, decidual leukocytes, uterine vessels, and invasive fetal cytotrophoblasts. Another paper (P. Drake, K Red-Horse, MD Gunn, SJ Fisher, in preparation)

Figure 2. Chemokines are diffusely expressed throughout decidual stroma. *In situ* hybridization on tissue sections of the maternal-fetal interface demonstrates the presence of chemokine mRNA in decidual stroma. **A, C, E:** Bright-field micrographs of histological sections stained with H&E. **B, D, F:** Dark-field micrographs of the same sections. **White dots** indicate signal from a 35 S-labeled antisense probe to the chemokine fractalkine (**B**), HCC-1 (**D**), or ITAC (**F**) that was hybridized to a section of second trimester tissue (8-week exposure). In **A**, the fetal compartment, represented by floating villi (FV) and the intervillous space (IVS), is on the **left** and the maternal compartment is on the **right**. Invasion occurs from **left to right** as anchoring villi (AV) attach to the uterine wall and cytotrophoblasts (CTB) migrate into the decidual stroma (Dec). In contrast, **C** and **E** show only the maternal portion, ie, the decidua. Uterine glands can also be identified in the sections.

will detail chemokine expression in the fetal compartment, ie, the placenta proper.

We first addressed the issue of chemokine expression by localizing mRNA production in tissue sections by *in situ* hybridization. Figures 2 through 6 present representative hybridization patterns with antisense probes for the 14 chemokines assessed in this study. On the left of each figure are bright-field micrographs of histological sections stained with H&E to show morphology, and on the right are dark-field micrographs of the same sections to show areas of probe hybridization, visualized by white dots. The results are summarized in Table 1. In general,

chemokines were abundant and intense mRNA signals were widely distributed among cell types. With the exception of BRAK, which was regulated with gestation, expression was remarkably stable throughout pregnancy, and in general the patterns were highly reproducible. When not stated otherwise, the expression pattern was found in the majority of samples examined and was consistent throughout gestation. A few hybridization patterns were shared by several chemokines; in these instances, the relevant figure shows a representative example typical of all molecules within that group. In every case, sense controls yielded no signal. For space con-

Table 1. Chemokine Expression at the Fetal-Maternal Interface

Chemokine	No. placentas*			Expression†			
	1T	2T	Term	Decidual stroma	Decidual leukocytes	CTBs in uterine wall	Blood vessels
CXC							
GCP-2	—	6	6	++ (n = 7), — (n = 5)	—	++ (n = 3)	—
Gro α	—	3	4	+	—	—	—
IP-10	3	4	3	++ (n = 8)‡	—	—	—
ITAC	2	3	2	++	+	—	—
MIG	1	2	1	+	—	—	—
SDF-1	3	2	1	—	+	++ (n = 2)	++ (n = 1)
BRAK§	—	4	4	++ (n = 4), — (n = 4)	—	—	—
CC							
HCC-1	—	3	2	++	—	++ (n = 3)	—
MCP-1	1	2	2	++ (n = 2), — (n = 3)	++ (n = 4), — (n = 1)	—	—
MCP-3	—	3	4	+	—	—	—
MIP-1 α	3	2	8	++	++	++ (n = 10)	—
SLC	—	4	4	—	—	—	++ (n = 4)
CX3C							
Fractalkine	—	3	3	++	—	—	—

*Tissue sections were analyzed by *in situ* hybridization. 1T, first trimester; 2T, second trimester.

†Expression patterns are described by cells positive for mRNA. ++, strong expression; +, intermediate expression; —, no expression.

‡Patches near leukocytes.

§Expression seen in all 2T samples; term samples were negative.

siderations, micrographs of these negative controls are shown in only a portion of the figures.

Numerous Chemokines Are Diffusely Expressed throughout the Decidua

A set of chemokines shared a strong, evenly distributed expression pattern, derived predominately from decidual stroma (Figure 2). Fractalkine, HCC-1, ITAC, and GCP-2 were widely expressed throughout the decidua. As the latter molecule was also produced by other cell types, it is discussed below. For the remaining chemokines in this group, decidual expression was not enhanced in areas of glandular epithelial cells, isolated cytotrophoblasts, or leukocyte clusters, although expression in these cell types could not be excluded. GRO α and MCP-3 (data not shown) were expressed in a similar pattern, but with a weaker signal. Expression of MCP-3 was less consistent than that of the other molecules (Table 1). Occasional samples did not fit the dominant pattern. For example, ITAC expression specifically by decidual leukocytes in addition to diffuse stromal production was observed in only one of seven samples.

Foci of Chemokine Expression in the Decidua

As in Figure 2, the panels in Figure 3 show second trimester decidua only. Uterine blood vessels and leukocytes can be identified in some sections. Antisense probes for MCP-1, MIP-1 α , and BRAK yielded a strong punctate signal. This distribution pattern suggested expression by the resident decidual leukocytes, particularly the macrophage subset (MCP-1 and MIP-1 α), or by isolated patches of stromal cells (Figure 3, BRAK, arrowhead) near clusters of decidual leukocytes (Figure 3, arrow). BRAK expression seemed to be gestationally regulated, as all second trimester samples assessed were

positive whereas term samples were negative. MCP-1 and MIP-1 α were also broadly expressed at a lower level throughout the decidual stroma (data not shown). Additionally, in one sample each, the chemokines MIG and SDF-1 were expressed focally in the decidua.

The chemokine IP-10 displayed a distinctive expression pattern within the maternal compartment. Figure 4 A shows a section of first trimester decidua containing a cluster of decidual leukocytes. These cell clusters are commonly associated with uterine glands and were nearly always accompanied by a strong IP-10 signal, as visualized by *in situ* hybridization (Figure 4B, arrow). Although expression by leukocytes could not be excluded, at least a portion of the signal was derived from the intercalated stromal cells. This expression pattern was highly consistent throughout gestation, as demonstrated by an example of hybridization signals at term (Figure 4D). This sample includes numerous floating villi within the fetal compartment (Figure 4, left) and superficial decidua from the maternal compartment (Figure 4, right). Sections hybridized with the sense probe lacked signal, as is shown on a second trimester decidua sample (Figure 4F).

Cytotrophoblast Expression of Chemokines

Recently, we showed that cytotrophoblasts express MIP-1 α .²⁴ Here we report that invasive cytotrophoblasts in the decidua also expressed the chemokines GCP-2, HCC-1, and SDF-1 (Figure 5). This expression pattern was somewhat less consistent than others, in part because of the difficulty in discriminating between cytotrophoblast- and stroma-derived signals. Where decidual fibroblasts were replaced by deposits of extracellular matrix, a common modification in late gestation, chemokine production by cytotrophoblasts was unequivocal. Figure 5A shows the morphology of a section of second trimester decidua. In

Figure 3. Chemokines are expressed in foci within decidua. *In situ* hybridization on tissue sections of the uterine wall demonstrates punctate expression of chemokine mRNA in decidua. **A, C, E:** Bright-field micrographs of histological sections stained with H&E. **B, D, F:** Dark-field micrographs of the same sections. **White dots** indicate signal from a ³⁵S-labeled antisense probe to the chemokine MCP-1 (**B**), MIP-1α (**D**), or BRAK (**F**) that was hybridized to a section of second trimester tissue (8-week exposure). Uterine blood vessels (BV) and leukocytes can be identified in some sections.

the upper part of the section, stromal cells remain and GCP-2 expression can be seen throughout this area (Figure 5B), as described above (Figure 2). Note the additional production of this chemokine by cytotrophoblasts (Figure 5, A and B, arrows). In contrast, the nearby decidua leukocytes do not seem to be a source of GCP-2 (Figure 5, A and B, arrowheads). HCC-1 had a similar pattern of expression (Figure 5, C and D). The tissue section focuses on an area within a second trimester decidua sample. Stromal cells remain in the upper right side of the panel, but have been replaced by extracellular matrix in the lower left side. Invasive cytotrophoblasts can be seen within this matrix. Again, HCC-1 expression is

apparent in the decidua stroma as described above (Figure 2) and in the fetal cytotrophoblast population (Figure 5D, arrows). Figure 5, E and F, show a second trimester tissue section that includes the fetal compartment (bottom). Groups of invasive cytotrophoblasts at the decidua border are positive for the chemokine SDF-1.

Chemokine Expression in Uterine Blood Vessels

Cells that lined a subset of vessels, which tended to be arteries, also expressed the chemokine SDF-1. Figure 6A shows two remodeled blood vessels within second tri-

Figure 4. Chemokine expression at sites of leukocyte clustering. *In situ* hybridization on tissue sections of the uterine wall demonstrates punctate expression of IP-10 mRNA in decidual stroma. **A, C, E:** Bright-field micrographs of histological sections stained with H&E. **B, D, F:** Dark-field micrographs of the same sections. **White dots (B and D)** indicate signal from a ^{35}S -labeled antisense probe hybridized to tissue sections (8-week exposure). **A:** First trimester decidua with accompanying glands and a cluster of decidual leukocytes. **B:** IP-10 mRNA expression associated with leukocyte cluster. **Inset** reveals that signal is derived in part from stromal cells intercalated with leukocytes; scale bar, 10 μm . **White box** indicates location of magnified **inset**. **C:** Term sample with fetal (**left**) and maternal (**right**) compartments. **F:** Sections that were hybridized to a sense probe as a negative control yielded no signal.

mester decidua that were breached by fetal cytotrophoblasts. The latter cells are clearly identifiable by morphological criteria in their walls (arrows). *In situ* hybridization demonstrated that these fetal cells strongly expressed SDF-1 mRNA (Figure 6B); expression by resident maternal cells could not be excluded.

mRNA for the chemokine SLC was also found in cells lining a subset of vessels. In contrast to SDF-1, this signal, which was associated with a thin layer of cells that composed the lumen, tended to localize to veins (Figure 6, C and D). Although expression by endovascular cytotrophoblasts could not be excluded, clusters of these

cells within the walls of arteries did not hybridize with the antisense probe (Figure 6D, arrow). Sense probes yielded no signal; Figure 6F shows a representative example in second trimester tissue.

Cultured Cytotrophoblasts Regulate Chemokine Expression with Differentiation

We confirmed the specificity of a subset of the *in situ* hybridization probes by using them concurrently for Northern blot hybridization. We compared expression by

Figure 5. Invasive cytotrophoblasts express chemokines. *In situ* hybridization on tissue sections of the uterine wall demonstrates punctate expression of chemokine mRNA in fetal cytotrophoblasts within the decidua. **A, C, E:** Bright-field micrographs of histological sections stained with H&E. **B, D, F:** Dark-field micrographs of the same sections. **White dots** indicate signal from a ^{35}S -labeled antisense probe to the chemokine GCP-2 (**B**), HCC-1 (**D**), or SDF-1 (**F**) that was hybridized to a section of second trimester tissue (8-week exposure). In **A** the fetal compartment is toward the **bottom** of the panel, and cytotrophoblasts have invaded the uterine wall from this direction. In the region surrounding these invasive cells, much of the decidual stroma has been replaced with extracellular matrix, allowing easy visualization of the encapsulated cytotrophoblasts (**A** and **B**, **arrows**). At the **top** part of the section, the stroma is intact, and decidual leukocytes can be identified (**A** and **B**, **arrowheads**). **C** and **D** are similar; here the tissue section focuses on an area within a second trimester decidual sample. Stromal cells remain at the **top right** side of the section, but have been replaced by extracellular matrix in the **bottom left** side. Invasive cytotrophoblasts can be seen within this matrix (**arrows**). **E** and **F:** The second trimester tissue section includes the fetal compartment (**bottom**). Groups of invasive cytotrophoblasts can be seen at the decidual border (**arrows**).

either first or second trimester cytotrophoblasts with that observed in chorionic villi and placental fibroblasts. For these studies, we took advantage of an *in vitro* system to model cytotrophoblast differentiation in which isolated purified cytotrophoblast stem cells recapitulate the invasive differentiation pathway with time in culture.²⁵ The Northern blot results dovetailed with the *in situ* hybridization studies (Figure 7). Specificity of the probes was further indicated as bands of the appropriate size were

detected. BRAK mRNA was detected in chorionic villi, a result in agreement with *in situ* analyses in this portion of the placenta (P. Drake et al, manuscript in preparation). In cytotrophoblasts, a very faint signal was detected in isolated cells before culture, but at no other time points ($n = 2$). In contrast, GCP-2 mRNA was not produced by chorionic villi, but was expressed in cytotrophoblasts after 12 hours in culture ($n = 2$). This suggested that *in vivo* these cells increase GCP-2 production as they in-

Figure 6. Chemokines are expressed in uterine vessels. *In situ* hybridization demonstrates expression of chemokine mRNA in blood vessels in the uterine wall. **A, C, E:** Bright-field micrographs of histological sections stained with H&E. **B, D, F:** Dark-field micrographs of the same sections. **White dots** indicate signal from a ^{35}S -labeled antisense probe to the chemokine SDF-1 (**B**) or SLC (**D**) that was hybridized to a section of second trimester tissue (8-week exposure). **A:** Two remodeled vessels within decidua. Fetal cytotrophoblasts in their walls can be clearly identified by morphological criteria (**arrows**). **B:** *In situ* hybridization demonstrated that these fetal cells strongly expressed SDF-1 mRNA. **C:** A section of second trimester decidua in which three blood vessels can be identified. **D:** Two of these, on the **left** and the **top right**, are positive for SLC expression. On the **bottom right**, a remodeled vessel containing cytotrophoblasts (**C, arrow**) is negative for SLC (**D, arrow**). Sense probes yielded no signal; **F** shows a representative example in second trimester tissue.

vade the uterine wall, an idea supported by the *in situ* hybridization data (Figure 5B).

Decidual Leukocytes Express Chemokine Receptors that Recognize Ligands Produced in the Uterine Wall

As a second step in assessing the contribution of chemokines to decidual leukocyte recruitment, we examined the expression of chemokine receptors by these cells at

the transcriptional level. Decidual leukocyte RNA ($n = 9$) was analyzed for evidence of chemokine receptor expression, using RNase protection assays. Three probe sets, encompassing a total of 17 receptors, were used (Figure 8). Each probe set is shown as a panel with the results of a representative experiment: hCR5 (Figure 8A), hCR6 (Figure 8B), and hCR8 (Figure 8C). Along the left side of each panel is a list of the chemokine receptors identified by each set. Probes for individual receptors are distinguished from each other by size, which decreases

Figure 7. Northern blot hybridization confirms the specificity of *in situ* hybridization studies. Five lanes represent RNA isolated from chorionic villi (villi), placental fibroblasts (fib), or cytotrophoblasts (CTB) after 0, 12, or 24 hours in culture. Expression of BRAK or GCP-2 is indicated by the presence of a band, the size of which is shown at the right. The bottom row depicts acridine orange staining of the gel before transfer to demonstrate equal loading.

from top to bottom. Across the top of each panel the source of analyzed RNA is listed: yeast, PBMCs, and decidual leukocytes from the gestational week indicated. Bands are indicative of chemokine receptor mRNA. Protected bands have a slightly higher mobility than the undigested probes run as size markers in the left-hand column. Lines connect pairs of undigested and digested probes.

RNA corresponding to multiple receptors, capable of binding a spectrum of CC, CXC, and CX₃C chemokines, was expressed by the immune cells. Given that the decidual leukocyte mRNA was isolated from a heterogeneous mixture of NK cells, monocytes, and T cells, the data did not give information about receptor expression by specific cell types. Nevertheless, at the population level, the results revealed several interesting findings that are summarized in Table 2. Receptor expression was remarkably consistent among samples. Accordingly,

Figure 8. Decidual leukocytes express a panel of chemokine receptors. RNase protection assays were used to determine the production of these molecules. Three probe sets (**A**: hCR5; **B**: hCR6; and **C**: hCR8) were labeled with ³²P and hybridized to RNA isolated from first and second trimester decidual leukocytes (*n* = 9). After digestion and separation on a sequencing gel, the presence of mRNA transcripts was detected by protected bands of the appropriate size. Undigested probe sets were used as reference size markers (left lanes). Protected bands run slightly faster than the undigested probes. Lines connect pairs of undigested and digested probes. Positive controls included probes for L32 and GAPDH transcripts, to confirm RNA integrity and loading. PBMC and yeast RNA served, respectively, as positive and negative controls for expression. **B: Inset** is a shorter exposure (2 days) than the rest of the panel (1 week) to clearly demonstrate strongly expressed bands in that region. **White box** indicates the portion shown in **inset**. **A** and **C** were exposed for 4 and 2 days, respectively.

Table 2. Decidual Leukocyte Expression of Chemokine Receptors as Assessed by RNase Protection Assay

Probe set	Expression*													
	CCR1	CCR2a	CCR2b	CCR3	CCR4	CCR5	CCR7	CCR8	CXCR1	CXCR2	CXCR3	CXCR4	CXCR5	V28/CX3CR1
hCR5														
8wkA (20) [†]	++	+	+	-	-	++		-						
9wkA (20)	++	+	+	-	-	++		-						
20wk (20)	++	++	++	-	+	++		+						
hCR6														
8wkB (2)							-		-	-	-	++	-	-
8wkC (7.5)							+		+	+	++	++	+	++
9wkA (3.7)							+		-	-	++	++	-	++
15wk (8.6)							+		+	+	++	++	-	++
9+16wk (20)							+		-	-	++	++	-	++
hCR8														
8wkA (2.5)			-	-		++		-				++		-
16wkA (5.1)			++	-		++		-				++	++	-
20wk (20)			++	-		++		-				++	++	-
7.5+15wk (20)			++	-		++		-				++	++	-

*As in Table 1.

[†]Gestational age in weeks (quantity of RNA, in micrograms, hybridized per sample).

CCR1 and CCR5 (Figure 8A), CXCR3, CXCR4, and V28/CX₃CR1 (Figure 8B), and the orphan receptor STRL33 (Figure 8C, along with additional examples of CCR5, CXCR4, and V28/CX₃CR1), were highly expressed. CCR2a, CCR2b, CCR4 (Figure 8A), and CCR7 (Figure 8B) expression was consistent, although slightly weaker. Occasional expression of CXCR1, CXCR2, CXCR5 (Figure 8B), and CCR8 (Figure 8A) was noted. A few samples containing the minimum amount of RNA (2 μ g) yielded fewer bands, providing possible clues about the relative expression levels of these factors in decidual leukocytes.

Discussion

Given the immunologically sensitive aspect of pregnancy in which the conceptus avoids rejection by the maternal immune system, one might predict the relative absence in the placenta and uterus of chemokines, molecules that attract leukocytes to tissues. However, the unusual recruitment of specific subsets of leukocytes to the uterus during pregnancy suggests an alternative hypothesis under which chemokines are expressed in a tightly controlled manner. The data in this manuscript strongly support the latter idea. *In situ* hybridization showed widespread expression of chemokine mRNA in the de-

cidal stroma. Specific expression patterns in other cell types, including cytotrophoblasts and decidual leukocytes, were also noted. The broad repertoire of chemokine receptors expressed by decidual leukocytes suggests that they can respond to the complex chemokine milieu at the maternal-fetal interface. Taken together, these data identify receptor-ligand pairs that might be involved in leukocyte recruitment, summarized in Table 3. These include CCR1/HCC-1, CCR2/MCP-1, CCR5/MIP-1 α , CCR7/SLC, CXCR3/ITAC, CXCR4/SDF-1, and CX₃CR1/fractalkine. It is interesting to note that all of the chemokines expressed in the uterus find a partner receptor on decidual leukocytes.

In general, the observed chemokine receptor expression reflects what is known about the decidual leukocyte population. For example, chemokine receptors characteristic of NK cells, T cells, and monocytes were abundant (eg, CX₃CR1, CXCR3, CCR1, CCR2, CCR5, and CCR7), as were their ligands within the decidua (fractalkine, IP-10, MIP-1 α , MCP-1, HCC-1, and SLC) (see Table 3). Conversely, receptors that are expressed only on cell subsets not normally found in the pregnant uterus, such as CCR3 on eosinophils, were not identified in our screen. CXCR1 and CXCR2, typical of neutrophils, were either not expressed ($n = 3$) or present at very low levels

Table 3. Chemokine Receptor and Ligand Pairs: Candidates for Decidual Leukocyte Homing Effectors*

Decidual leukocyte receptor	Uterine wall chemokine	Leukocytes with receptors [†]
CCR1	HCC-1, MIP-1 α	NK, T, Mono., Neut.
CCR2 a+b	MCP-1	NK, act. T, Mono.
CCR5	MIP-1 α	CD56 ^{bright} NK, T (Th1), Mono.
CCR7	SLC	CD56 ^{bright} NK, T, Mono.
CXCR1 [‡]	GCP-2-like, GCP-2	Neutrophils
CXCR2 [‡]	Gro α , GCP-2	Neutrophils
CXCR3	ITAC, IP-10	CD56 ^{bright} NK, T (Th1)
CXCR4	SDF-1	Widely expressed
CX3CR1	Fractalkine	NK, T, Mono.

*Pairs were deduced by combining *in situ* hybridization data on chemokine expression in the uterine wall with RNase protection data on chemokine receptor production by decidual leukocytes.

[†]Cell subsets that express the designated receptors.^{31,40}

[‡]Low level of expression, $n = 2$.

($n = 2$). The two positive samples may have contained neutrophils that can infiltrate the placental bed in pathological processes.²⁶

Through a combination of models describing cell homing by a multistep combinatorial extravasation process,¹² followed by a stepwise navigation of chemokine gradients,²⁷ one can envision a series of molecules with sequential actions that recruit decidual leukocytes from the circulation and localize them within the uterus. Because the first step in leukocyte recruitment is extravasation from the blood stream, the chemokines expressed in the lumen of uterine vessels are particularly important for identifying the initial population of immune immigrants. Interestingly, these molecules, SLC and SDF-1, were previously characterized by their involvement in the homeostatic migration of cells through secondary lymphoid organs (SLC) and the localization of immature cells during development (SDF-1). Particularly striking, considering that CD56^{bright} NK cells comprise 70% of decidual leukocytes, is the observation that SLC preferentially attracts these cells over the CD56^{dim} subset. Expression of SLC on cells that compose the lumen of uterine vessels may therefore serve as a selective first step, allowing CD56^{bright} cells to dominate in the decidua.

In the context of leukocyte extravasation, it is important to note that early in pregnancy, before the utero-placental circulation is established, blood flow at the maternal-fetal interface is distinct from elsewhere in the body. Before 10 weeks of gestation, uterine arteries are occluded with aggregates of invading cytotrophoblasts, blocking direct blood flow. Thus, the maternal blood reaching the placenta is restricted to capillary flow or plasma filtrate.²⁸ Interestingly, decidual leukocytes are thought to be recruited during this period of low shear stress, but it is not known how these unusual conditions affect their extravasation. Previously defined rolling and tethering mechanisms may be altered in response to the different requirements in this location. Conversely, blood flow at the maternal-fetal interface becomes forceful later in gestation, measured in liters per minute at term.²⁶ In response to the gestational alterations in circulation through the intervillous space, local chemokine actions may also change. For example, SLC and SDF-1 expressed on large-bore second trimester vessels may serve to target leukocytes for emigration and return to the maternal circulation.

Once extravasation is completed, cell migration through the web of chemokines expressed in the decidua may occur as described in other systems.²⁷ Here, cells retain memory of their recent environment and can prioritize a new, weaker chemotactic signal over an older, stronger one. Accordingly, the cell can follow one attractant after another, using combinations of molecules to guide it in a step-by-step manner to its destination within a tissue. For example, a possible combination of factors acting on the migration of decidual leukocytes is: SLC/CCR7 to extravasate, HCC-1/CCR1 to move within the decidual stroma, and IP-10/CXCR3 to cluster the cells near glands. The mouse provides corollary evidence, whereby specific adhesion molecules that coordinate with chemokines to effect cell targeting are reciprocally

expressed in pairs on discrete immune cell subsets and vascular endothelium in particular microenvironments within the pregnant uterus.¹¹ This expression parallels the localization of these immune subsets to these microenvironments.

In terms of receptor expression, the decidual leukocytes resemble both Th1 and Th2 cohorts, although the stronger expression is toward the Th1 group. CCR5 and CCR7, common on Th1-polarized cells, are both highly expressed, whereas CCR4 and CCR8, preferentially expressed by Th2-polarized cells, are produced at lower levels. This finding is in agreement with previous work from our laboratory that reported the production of both Th1 and Th2 cytokines at the maternal-fetal interface.^{29,30} In summary, the majority of chemokine receptor expression by decidual leukocytes correlates with a Th1-biased population of activated T and NK cells. However, the presence of resting or naïve cells is also suggested (eg, CCR7 and CXCR4 expression). A flow cytometry-based investigation of chemokine receptor expression on subsets of decidual leukocytes would be most informative; by analogy to other systems, it could provide clues to cell function, as well as yield information about the mechanisms of leukocyte recruitment. Recently CD56^{bright} NK cells from the peripheral blood were found to have a chemokine receptor repertoire distinct from that of CD56^{dim} NK cells and NK-T cells.³¹ These data support our results, as the former cells express high levels of CCR5, CCR7, CXCR3, and CXCR4—molecules also expressed by decidual leukocytes, which are predominantly composed of CD56^{bright} NK cells. Accordingly, these receptors interact with ligands that are expressed in the decidua.

On a teleological note, it is interesting that many of the same receptor-ligand pairs expressed at the maternal-fetal interface are also expressed in rejected allogeneic organ grafts. For instance, rejected murine cardiac tissue expresses fractalkine, IP-10, MIG, and ITAC, which are absent in nonrejected grafts. The activated T-cell infiltrate expresses the receptors for these chemokines, CX3CR1 and CXCR3. When either of these receptors is blocked with a function-perturbing antibody, graft survival is significantly prolonged.^{32,33} Although we do not understand the exact meaning of these data, they suggest that novel immune mechanisms are entailed in the special case of fetal allo-transplantation during pregnancy, as a subset of chemokines and/or receptors that are indicative of transplant rejection is also expressed at the maternal-fetal interface.

Given the vital nature of reproduction, it is likely that redundancies are built into its critical components. Our data suggest that this principle applies to chemokine function. For example, several of these molecules shared identical expression patterns at the maternal-fetal interface (HCC-1, ITAC, fractalkine). Additionally, some of the molecules expressed in similar locations share the same receptors (eg, HCC-1 and MIP-1 α each bind CCR1), suggesting overlapping functions. Finally, our results are in agreement with gene deletion studies in mice that show that the loss of individual chemokines or receptors

does not affect leukocyte composition in the pregnant uterus.³⁴

In addition to the recruitment of decidual leukocytes, chemokine expression patterns in the uterine wall suggest that these molecules are involved in nonimmune aspects of placentation and cytotrophoblast differentiation. One intriguing possibility is the role of chemokines in cytotrophoblast targeting to maternal spiral arterioles. Part of the invasive differentiation pathway involves the localization of a subset of these fetal cells to maternal vessels. The mechanisms underlying this homing phenomenon are not well understood. The observation that uterine veins are only rarely breached by fetal cells led to the hypothesis that oxygen gradients help target the invasive cells preferentially toward arteries.^{35,36} The specific expression of SDF-1 by cells that occupy uterine vessels, including cytotrophoblasts, suggests that chemokines also regulate this process. Although traditionally considered to be regulators of leukocyte trafficking, the role of chemokines in the directed migration of other cells is gaining increasing acceptance as more examples are described. For instance, metastatic tumors up-regulate the expression of chemokine receptors, and this expression is involved in the preferential seeding of particular tumor cells to specific secondary organs.³⁷ In this context, it will be interesting to identify chemokine receptors expressed by cytotrophoblasts, and to test the effects of exogenous chemokine application on their differentiation, which includes a novel switch of their adhesion phenotype such that these epithelial cells take on vascular- and tumor-like properties.³⁸

Finally, these data give information about chemokine networks that function at the maternal-fetal interface during normal pregnancy. The fact that expression of chemokines and their receptors tended to be constant throughout pregnancy suggests important roles from the earliest time points onward. We hypothesize that deviations are indicative of underlying pathologies, and that understanding how chemokine expression changes in specific pregnancy complications will give insights regarding their etiology. Good candidates for initial studies are complications associated with inflammatory leukocytic infiltrates, such as preterm labor with infection of the chorioamniotic membranes. Work in the mouse has already shown that the placenta produces the chemokines KC and MIP-2 in response to *Listeria monocytogenes* infection.³⁹ Future investigations combining disease models with other approaches will assess at multiple levels the biology and function of chemokines at the maternal-fetal interface.

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