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Human Placental Cytotrophoblasts Attract Monocytes and CD56^{bright} Natural Killer Cells via the Actions of Monocyte Inflammatory Protein 1 α

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Abstract

During human pregnancy, the specialized epithelial cells of the placenta (cytotrophoblasts) come into direct contact with immune cells in several locations. In the fetal compartment of the placenta, cytotrophoblast stem cells lie adjacent to macrophages (Hofbauer cells) that reside within the chorionic villus stroma. At sites of placental attachment to the mother, invasive cytotrophoblasts encounter specialized maternal natural killer (NK) cells (CD56^{bright}), macrophages, and T cells that accumulate within the uterine wall during pregnancy. Here we tested the hypothesis that fetal cytotrophoblasts can direct the migration of these maternal immune cells. First, we assayed the chemotactic activity of cytotrophoblast conditioned medium samples, using human peripheral blood mononuclear cells as targets. The placental samples preferentially attracted NK cells (both CD56^{dim} and CD56^{bright}), monocytes, and T cells, suggesting that our hypothesis was correct. A screen to identify chemokine activity through the induction of a Ca²⁺ flux in cells transfected with individual chemokine receptors suggested that cytotrophoblasts secreted monocyte inflammatory protein (MIP)-1 α . This was confirmed by localizing the corresponding mRNA and protein, both in vitro and in vivo. MIP-1 α protein in conditioned medium was further characterized by immunoblotting and enzyme-linked immunosorbent assay. Immunodepletion of MIP-1 α from cytotrophoblast conditioned medium showed that this chemokine was responsible for a significant portion of the induced monocyte and CD56^{bright} NK cell chemotaxis. These data suggest the specific conclusion that cytotrophoblasts can attract monocytes and CD56^{bright} NK cells by producing MIP-1 α and the more general hypothesis that these cells may organize and act on leukocytes at the maternal–fetal interface.

Key words: chemokine • pregnancy • leukocyte • immune tolerance • trophoblast

Introduction

Early in human pregnancy, as part of a process known as decidualization, maternal leukocytes traffic to the uterus, where the fetal-derived placenta has implanted. The histology of this area is diagrammed in Fig. 1 (see also references 1–4). The placenta is made up of individual units termed chorionic villi. Each villus has a connective tissue core that

contains fetal blood vessels and numerous macrophages, termed Hofbauer cells. The macrophages often lie adjacent to a thick basement membrane, which underlies a layer of cytotrophoblast stem cells that are the progenitors of all the trophoblast lineages.

The differentiation pathway taken by the cytotrophoblast stem cells depends on their location. In floating villi, the cytotrophoblasts fuse to form a multinucleate syncytial covering. These villi are attached at only one end to the tree-like fetal portion of the placenta. The rest of the villus

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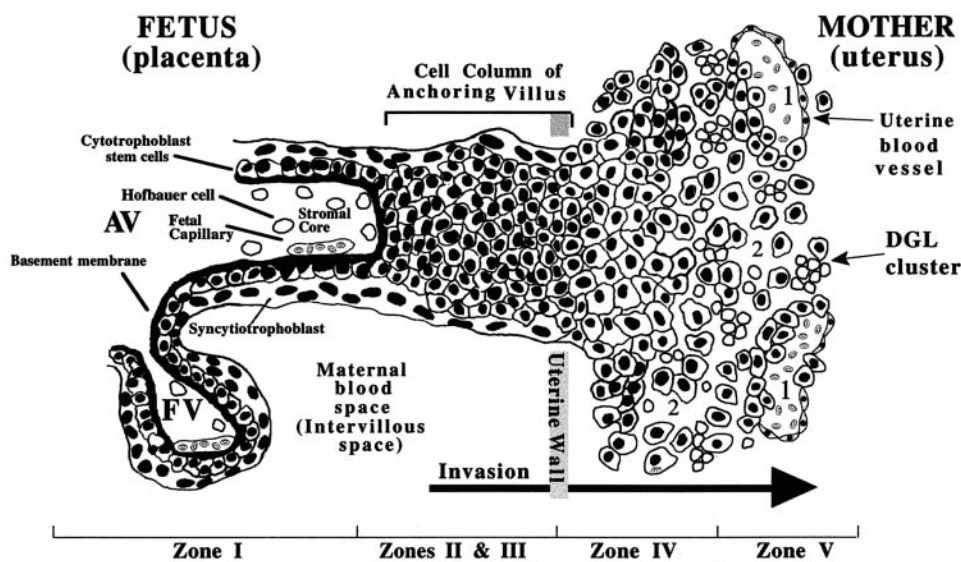


Figure 1. The cellular architecture of the human maternal–fetal interface where fetal placental cells (cytotrophoblasts) are in close proximity to maternal leukocytes that reside within the uterine wall. The chorionic villus is the basic structural unit of the placenta. A subset, termed anchoring chorionic villi (AV), establishes physical connections between the fetus and the mother. Anchoring villi form when cytotrophoblast stem cells detach from their basement membrane and form a column of nonpolarized mononuclear cells that invade the uterus. These invasive cytotrophoblasts rapidly traverse most of the uterine parenchyma, where they encounter numerous maternal immune cells, predominantly CD56^{bright} NK cells, but also macrophages and T cells. Invasive cytotrophoblasts target uterine spiral arterioles for in-

vasion. They remodel these vessels by replacing the endothelial lining and destroying the muscular wall. Thus, cytotrophoblasts are in direct contact with both maternal peripheral blood (sites marked 1) and decidual leukocytes (sites marked 2). FV, floating villi.

floats in a stream of maternal blood, which optimizes exchange of substances between the mother and fetus across the syncytium. In anchoring villi, cytotrophoblast stem cells detach from the basement membrane and form a column of nonpolarized mononuclear cells that invade the uterus. As a result, these villi are attached at one end to the fetal portion of the placenta and at the other end to the uterus. This arrangement anchors the villus to the uterine wall. Invasive cytotrophoblasts rapidly traverse most of the uterine parenchyma (interstitial invasion). They also breach the uterine veins and arteries they encounter (endovascular invasion). Their interactions with veins are confined to the portions of the vessels that lie near the inner surface of the uterus, but beginning in the second trimester, cytotrophoblasts migrate in a retrograde direction along much of the intrauterine course of the arterioles. Eventually, these fetal cells completely replace the maternal endothelial lining and partially disrupt the muscular wall of these vessels. This unusual process diverts uterine blood flow to the floating villi.

Endovascular invasion places fetal cells from the placenta in direct contact with maternal blood in the uterine vessels. Our previous work shows that endovascular cytotrophoblasts modulate their adhesion molecule phenotype to resemble that of endothelial cells, a phenomenon we termed pseudovasculogenesis (4). Interstitial invasion brings cytotrophoblasts into direct contact with maternal immune cells in the uterus, termed decidual granulated leukocytes (DGLs).¹ Both invasive cytotrophoblasts and DGLs have unusual immunological properties. For example, the fetal cells express the nonclassical MHC class Ib molecules HLA-G and HLA-E, rather than the classical molecules

HLA-A and -B (5–8). The expression of HLA-C by cytotrophoblasts has also been reported (9). The maternal DGLs have an unusual composition: ~70% are specialized (CD56^{bright}CD16⁻) NK cells, and the remainder include equal contributions of monocytes (~15%) and T cells (~15%) (10). During the first half of human pregnancy, DGLs account for a significant portion of the maternal cells within the uterus (11). This phenomenon is not unique to humans, as recruitment of uterine NK cell populations during pregnancy has also been described in mice (12–14) and pigs (15, 16). Although the functional consequences of cytotrophoblast interactions with DGLs are not well understood, experimental evidence in mice suggests that the immune cells promote endovascular invasion and placental development (17, 18). This idea is in accord with observations in other tissues that immune cells, primarily macrophages, secrete trophic factors that promote the growth and differentiation of resident cells (19–21).

The mechanisms underlying DGL recruitment to the uterus during pregnancy are not understood, but in the context of their known functions, chemokines are likely to be involved. Chemokines are a family of structurally related molecules that mediate immune cell trafficking to sites of inflammation (22) and to secondary lymphoid organs (23). Here we used an *in vitro* model to test the hypothesis that chemokines derived from fetal cytotrophoblasts play a role in maternal DGL trafficking to the uterus during pregnancy. In this model, samples of human cytotrophoblast conditioned medium (CM) and human peripheral blood leukocytes were placed on opposite sides of a Transwell filter membrane. We found that the medium samples recruited a leukocyte population with a composition that was very similar to that of DGLs. Then, we used this model to show that a cytotrophoblast-derived chemokine, monocyte inflammatory protein 1 α (MIP-

¹Abbreviations used in this paper: CM, conditioned medium; DGLs, decidual granulated leukocytes; MIP-1 α , monocyte inflammatory protein 1 α ; SFM, serum-free medium.

1 α), is one of the factors in the CM that plays a major role in attracting monocytes and NK cells in vitro. Since we also detected expression of MIP-1 α mRNA and protein in cytotrophoblasts in situ at the human maternal–fetal interface, we hypothesize that this chemokine has similar effects during pregnancy.

Materials and Methods

Collection of Human Placentas and Leukocytes. Studies involving placental tissues were approved by the University of California at San Francisco (UCSF) Institutional Review Board. Placentas were from patients who had elective pregnancy terminations (6–22 wk) or normal term deliveries. The tissue was immediately collected into PBS with antibiotics (1% penicillin/streptomycin and 1% gentamicin), washed in the same solution, and briefly stored on ice before processing. Human leukocytes 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.

Isolation and Culture of Placental Cells. Cytotrophoblasts were isolated from pools of first or second trimester placentas or from individual third trimester placentas as described previously (24–26). Briefly, placentas were subjected to a series of enzymatic digestions, which detached cytotrophoblast stem cells from the underlying stromal cores of the chorionic villi. Detached cytotrophoblasts were isolated on a Percoll gradient, and remaining leukocytes were extracted by virtue of their interactions with CD45-coated beads, which were then removed with a magnetic device (Dako). The final purity of the cells was 90–95%, with CD45⁺ cell contamination <5%. The cells were cultured on Matrigel-coated substrates (Collaborative Biomedical Products) in serum-free medium (SFM): DMEM 4.5 g/liter glucose (Sigma-Aldrich), supplemented with 2% Nutridoma (Boehringer Mannheim), 1% penicillin/streptomycin, 1% sodium pyruvate, 1% Hepes, and 1% gentamicin (UCSF Cell Culture Facility). After 12, 24, 36, and 48 h in culture, the CM was removed, passed through a 0.2- μ m filter, and stored at –20°C before use. To prepare control SFM (SFM/MG), Matrigel-coated plates were incubated without cells in medium for the times indicated above. Placental fibroblasts were isolated from denuded connective tissue villus cores as described previously (25). The cells were maintained in DMEM H-21 containing 10% FBS, 5% glutamine, 1% penicillin/streptomycin, and 1% gentamicin. The JAR human choriocarcinoma cell line was maintained as described previously (25).

Isolation of Human PBMCs. Human PBMCs were prepared from a buffy coat by erythrocyte sedimentation with 6% dextran T500 (Amersham Pharmacia Biotech) followed by Histopaque-1077 (Sigma-Aldrich) separation. PBMCs were washed in PBS and resuspended at 10⁷ cells/ml in DME-H21 (UCSF Cell Culture Facility) containing 1.0% BSA (Sigma-Aldrich).

Chemotaxis Assay. Chemotaxis assays were performed according to published methods (27). CM with initial MIP-1 α concentrations of between 50 and 100 ng/ml as determined by ELISA (see below) were used in the assay. In some cases, up to seven cytotrophoblast CM samples were pooled according to gestational age in trimesters and concentrated with a Centriprep-3 centrifugal concentrator (Amicon Inc.). The CM was diluted with DME-H21 (UCSF Cell Culture Facility) containing 1.0% BSA (Sigma-Aldrich), hereafter referred to as blank medium, to produce samples with MIP-1 α concentrations ranging from 100

ng/ml to 10 pg/ml. Transwell inserts (6.5 mm in diameter, 5- μ m pore; Corning Inc.) were placed in 24-well plates. 600 μ l of cytotrophoblast CM, blank medium, or blank medium supplemented with recombinant human MIP-1 α (R&D Systems) was added to the bottom compartment of each insert, and 100 μ l of a human PBMC cell suspension containing 10⁶ cells was added to the top. Where indicated, 5 μ g/ml of control goat IgG (R&D Systems) or anti-MIP-1 α IgG that neutralized activity (R&D Systems) was added to the cell suspension. Negative controls included blank medium and SFM/MG. Positive controls included blank medium to which recombinant MIP-1 α was added at concentrations ranging from 100 ng/ml to 10 pg/ml. After incubation for 3 h at 37°C in a standard tissue culture incubator, the Transwell inserts were removed and a known number of 15- μ m microsphere beads (Bangs Laboratories) was added to the wells before the medium and cells in the bottom compartment were transferred to polystyrene tubes. Cells and beads were pelleted by centrifugation (300 g; 10 min). All but 100–200 μ l of the supernatant was removed, and fluorochrome-conjugated antibodies recognizing CD14 (which labels monocytes), CD56 (which labels NK cells), and CD3 (which labels T cells) (Becton Dickinson) were added to the tubes, which were then incubated at 4°C in the dark for 1 h. Cells were washed twice in PBS containing 2% FCS. The mixture of cells and beads was analyzed by flow cytometry on a FACScan™ (Becton Dickinson) with CELLQuest™ software (Becton Dickinson). The absolute number of cells that migrated was extrapolated from the percent recovery of the beads. All assays were done in triplicate.

Ca²⁺ Flux Assays. Reporter cell lines were generated as described previously (28–30). HEK-293 parental cells were transfected with human CCR1 (31–33) and CCR2 (28, 34, 35), and 300-19 parental cells were transfected with CCR3 (36–39), CCR5 (40, 41), and CX3CR1 (42, 43). The nontransfected parent lines served as negative controls. Prior to analyses, medium samples were concentrated 5–10-fold (vol/vol) over a heparan sulfate column according to the manufacturer's directions (Amersham Pharmacia Biotech), after which they were dialyzed against PBS overnight at 4°C. Intracellular Ca²⁺ release was monitored as described previously (28). Briefly, the target cells were preloaded with the Ca²⁺-sensitive dye calcein AM (Molecular Probes Inc.), washed, and resuspended in 1 μ l of RPMI 1640. The experiment started with the addition of 20 μ l of concentrated cytotrophoblast CM, 20 μ l of concentrated SFM/MG as a negative control, or 5 μ l of a stock solution (10 mM) of the relevant recombinant chemokine as a positive control (MIP-1 α , MCP-1, eotaxin, RANTES [regulated on activation, normal T cell expressed and secreted], or fractalkine). The magnitude of the Ca²⁺ flux was determined spectrophotometrically.

In Situ Hybridization. In situ hybridization was performed using published methods (44). Isolated cytotrophoblasts were cultured (as described above) on a 200- μ l plug of Matrigel in a Millicell-CM culture insert, 12 mm in diameter, 0.4- μ m pore (Millipore). After 24 h in culture, the cells, together with the Matrigel substrate, were dissected from the inserts and fixed in 3% paraformaldehyde for 30 min at 4°C. Then the cytotrophoblasts and Matrigel were subjected to an increasing gradient of sucrose in PBS. After 20 min each in 5, 10, and 15% sucrose, the cells were incubated overnight in a 1:1 (vol/vol) mixture of optimum cutting temperature (OCT) compound and 15% sucrose. The next day, the cell plug was embedded in OCT and frozen in liquid nitrogen. 5- μ m sections were cut, mounted on poly-L-lysine-coated slides, and stored at –20°C until use.

Tissue specimens ($n = 9$) for in situ hybridization 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 paraffin embedded. 5- μm sections were cut and mounted on poly-L-lysine-coated slides, deparaffinized, and rehydrated before use.

To produce labeled probes, the EST plasmid clone ID no. 154848, which contains an 837-bp insert of the human MIP-1 α cDNA, was cut with either NotI or EcoRI (GIBCO BRL). The linearized vectors were used as templates for the synthesis of ^{35}S -labeled cRNA probes using T3 (antisense) or T7 (sense) RNA polymerases (45).

On day 1 of the in situ hybridization, slides were allowed to sit at room temperature for 5 min. They were then placed in PBS for 5 min, before incubation at room temperature in 4% paraformaldehyde in PBS for 10 min, followed by a 5-min wash in $0.5\times$ SSC ($1\times = 150\text{ mM NaCl}$, $15\text{ mM sodium citrate}$, $\text{pH } 7.4$). Sections were deproteinized with $1\ \mu\text{g/ml}$ proteinase K for 10 min at room temperature and then washed for 10 min in $0.5\times$ SSC. They were fixed again in 4% paraformaldehyde for 3 min, followed by a final wash in $0.5\times$ SSC for 5 min. After a 3-h prehybridization at $55\text{--}60^\circ\text{C}$ in rHB2 buffer (50% formamide, 0.3 M NaCl , 20 mM Tris , $\text{pH } 8.0$, 5 mM EDTA , $1\times$ Denhardt's [0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA], 10% dextran sulfate, $10\text{ mM dithiothreitol}$), the slides were incubated overnight at $55\text{--}60^\circ\text{C}$ in a humidified chamber with $200\ \mu\text{l}$ of hybridization buffer: rHB2 buffer containing $500\ \mu\text{g/ml}$ yeast tRNA and $1,200,000\text{ cpm}$ of ^{35}S -labeled antisense (experimental) or sense (negative control) cRNA probes. After hybridization, sections were washed twice, 10 min each, in $2\times$ SSC (300 mM NaCl , $30\text{ mM sodium citrate}$) with $10\text{ mM }\beta\text{-mercaptoethanol}$ and 1 mM EDTA . Slides were then immersed in an RNaseA solution (500 mM NaCl , 10 mM Tris , $\text{pH } 8.0$, and $10\ \mu\text{g/ml}$ RNaseA) for 30 min at room temperature and washed twice, 10 min each, in $2\times$ SSC/ β -mercaptoethanol/EDTA. The sections were then subjected to a high stringency wash ($0.1\times$ SSC, $10\text{ mM }\beta\text{-mercaptoethanol}$, 1 mM EDTA) for 2–3 h at 60°C before they were washed twice, 10 min each, in $0.5\times$ SSC without β -mercaptoethanol or EDTA. Finally, the sections were dehydrated (2 min per step) in a series of graded (30, 60, 80, 95, and 100%) ethanol solutions that contained $0.3\text{ M NH}_4\text{Ac}$. The slides were dried for 2 h in a fume hood before being dipped in Kodak NTB-2 liquid emulsion. Afterwards 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 before they were examined in both dark and bright field using a Zeiss Axiophot microscope.

Immunohistochemistry. Antibodies included anticytokeratin, 7D3 rat antihuman (46); anti-CD45Rb, PD7/26 mouse antihuman (Dako); anti-MIP-1 α , C-16 goat antihuman (Santa Cruz Biotechnology); FITC-conjugated donkey antimouse; and rhodamine-conjugated donkey antirat (Jackson ImmunoResearch Laboratories). For staining of in vitro cultures, sections of cell aggregates were prepared as described above, and then antibody binding was detected using VECTASTAIN[®] ABC kits (PK-6102 and PK-6104; Vector Laboratories) according to the manufacturer's instructions. Signal was detected with a DAB peroxidase substrate kit (Vector Laboratories). As a negative control, primary antibody was omitted on some sections. Slides were counterstained with hematoxylin and eosin before analysis. For staining of intact tissues, specimens were fixed in 3% paraformaldehyde for 5 min, flash frozen in liquid nitrogen, and sectioned (5 μm). Slides were immersed in ice cold methanol for 10 min, washed in

PBS, and exposed to primary antibody (1:25 dilution) for 4 h. Slides were then washed in PBS and incubated with secondary antibody (1:100 dilution) for 1 h, washed in PBS, and mounted with VECTASHIELD[®] mounting medium (Vector Laboratories). Negative controls included specimen incubation with normal goat serum.

Northern Hybridization. Total RNA was extracted from cytotrophoblasts immediately after the cells were isolated or after 12, 24, or 36 h in culture, according to published methods (47). Blots were prepared as follows. $10\ \mu\text{g}$ of total RNA was separated by formaldehyde-agarose gel electrophoresis, transferred to Nytran membranes (Schleicher and Schuell, Inc.), and analyzed by Northern blot hybridization as described previously (48–50). Gels were stained with acridine orange before transfer to ensure integrity of the RNA samples and to confirm that equal amounts of RNA had been loaded in each lane. The MIP-1 α -specific probe (see above) was generated by using standard methods (51). [^{32}P]CTP random oligonucleotide primers (Amersham Life Science, Inc.) and the Klenow fragment of DNA polymerase I (GIBCO BRL) were used to label the probe, which had a specific activity of $2 \times 10^9\text{ dpm}/\mu\text{g}$.

Immunoblotting. Samples of cytotrophoblast CM were concentrated by heparin affinity chromatography as described above. Proteins were separated by SDS-PAGE on 10% gels and transferred to nitrocellulose membranes (Schleicher and Schuell, Inc.). Nonspecific antibody binding was blocked by incubating the membranes for 1 h at room temperature in MT-PBS: PBS containing 5% nonfat dried milk (Carnation Nestle USA Inc.), and 0.1% Tween-20 (Sigma-Aldrich). The membranes were incubated overnight at 4°C with the primary antibody, anti-MIP-1 α goat polyclonal IgG (C-16; Santa Cruz Biotechnology) diluted 1:20 (vol/vol) in MT-PBS. The membranes were washed three times, 10 min each, in PBS containing 0.1% Tween-20 (T-PBS) before they were incubated for 1 h at room temperature in the secondary antibody, horseradish peroxidase-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Labs Inc.) diluted 1:3,000 (vol/vol) in MT-PBS. Finally, the membranes were washed three times, for 10 min each, in T-PBS and processed for detection of bound antibody by chemiluminescence according to the manufacturer's instructions (ECL; Amersham Pharmacia Biotech).

ELISA. ELISA kits for quantifying human MIP-1 α (catalog no. DMA00) were purchased from R&D Systems, and the assays were performed according to the manufacturer's instructions.

Endotoxin Detection. The Endotoxin Detection Kit (catalog no. 3070000) from ICN Pharmaceuticals, Inc. was used according to the manufacturer's instructions.

Statistical Analysis. To describe the general effects of cytotrophoblast CM on cell migration, data from all chemotaxis experiments were analyzed together. Because the data were nonparametrically distributed, the results were reported as the median and interquartile range. To determine the significance of the effect of neutralizing the MIP-1 α in the chemotaxis assays, each experiment was considered individually. Data within individual experiments were normally distributed and were analyzed with a two-tailed Student's t test. Subsequently, the mean percentage change (and standard deviation) in migration due to addition of a neutralizing antibody was calculated for all experiments. For ELISA, analysis of variance, followed by a Student-Newman-Keuls test, was used to compare the concentration of MIP-1 α in medium from first trimester, second trimester, and term cytotrophoblasts. The Spearman Rank Order Correlation was used to test for a trend between presence of endotoxin and levels of MIP-1 α in cytotrophoblast CM.

Results

Cytotrophoblast CM Attracts NK Cells, Monocytes, and T Cells. As an initial test of our hypothesis that placental cells can direct the migration of decidual leukocytes, we characterized the chemoattractant properties of CM harvested from cultures of primary cytotrophoblasts, using human PBMCs as targets. Freshly isolated cytotrophoblasts are a mixture of stem cells and cells in the initial stages of differentiation. After 24–48 h in culture, the cytotrophoblasts have fully differentiated along the pathway that leads to invasion of the uterine wall, a process that places them in direct contact with resident maternal cells, including leukocytes (for review see reference 4). Thus, CM samples were harvested after the cells had been in culture for >12 h. Preliminary experiments showed that cytotrophoblast CM isolated from first, second, and third trimester placentas induced the chemotaxis of monocytes, NK cells, and T cells from peripheral blood. The results suggested that cytotrophoblasts produce soluble factors that can direct the migration of leukocytes found at the maternal–fetal interface during pregnancy.

A Chemokine in Cytotrophoblast CM Signals through MIP-1 α Receptors. Next, we investigated whether the chemotactic factors in the CM included chemokines. Cell lines transfected with individual chemokine receptors were challenged with cytotrophoblast CM that was concentrated over a heparan sulfate column. Positive controls included exposure of each cell line to a ligand that activated the relevant receptor. Cells expressing one of the following receptors were exposed to the ligand indicated: CCR1/RANTES, CCR2/MCP-1, CCR3/eotaxin, CCR5/MIP-1 α , and CX3CR1/fractalkine. In all cases, addition of the recombinant chemokine induced a robust Ca²⁺ influx in the transfected line (data not shown). Negative controls included exposure of the transfected cells to serum-free control medium incubated on Matrigel in the absence of cells (SFM/MG) and addition of cytotrophoblast CM to the corresponding untransfected parental cell line.

Results typical of these experiments are summarized in Fig. 2. Concentrated cytotrophoblast CM, but not concentrated SFM/MG, induced a Ca²⁺ flux in cells expressing CCR1 (Fig. 2 A; $n = 4$) and CCR5 (Fig. 2 B; $n = 5$), but not CCR2, CCR3, or CX3CR1 (data not shown; $n = 4$, $n = 4$, and $n = 3$, respectively). CM samples from all three trimesters had the same pattern of activity, although the magnitude of the response varied among samples. As MIP-1 α is the only chemokine that is a ligand for both CCR1 and CCR5 but not CCR3, we hypothesized that MIP-1 α was present in the CM.

Cytotrophoblasts Express MIP-1 α mRNA In Vitro. To confirm that cytotrophoblasts secreted MIP-1 α , we first examined production of MIP-1 α mRNA by isolated cells. Initially, we used in situ hybridization for this purpose. The cytotrophoblasts were cultured for 24 h on Matrigel, and then the cells together with the substrate were fixed and sectioned before hybridization (see Materials and Methods). Fig. 3 A is a photomicrograph of a histological section

of the cultured cells that was stained with hematoxylin and eosin. The morphology is typical of cytotrophoblasts cultured under these conditions (26). The cells rapidly aggregate, and those in direct contact with the matrix invade the substrate, which is no longer visible due to solubilization during the hybridization procedure. In situ hybridization with sense (Fig. 3 B) and antisense probes (Fig. 3 C)

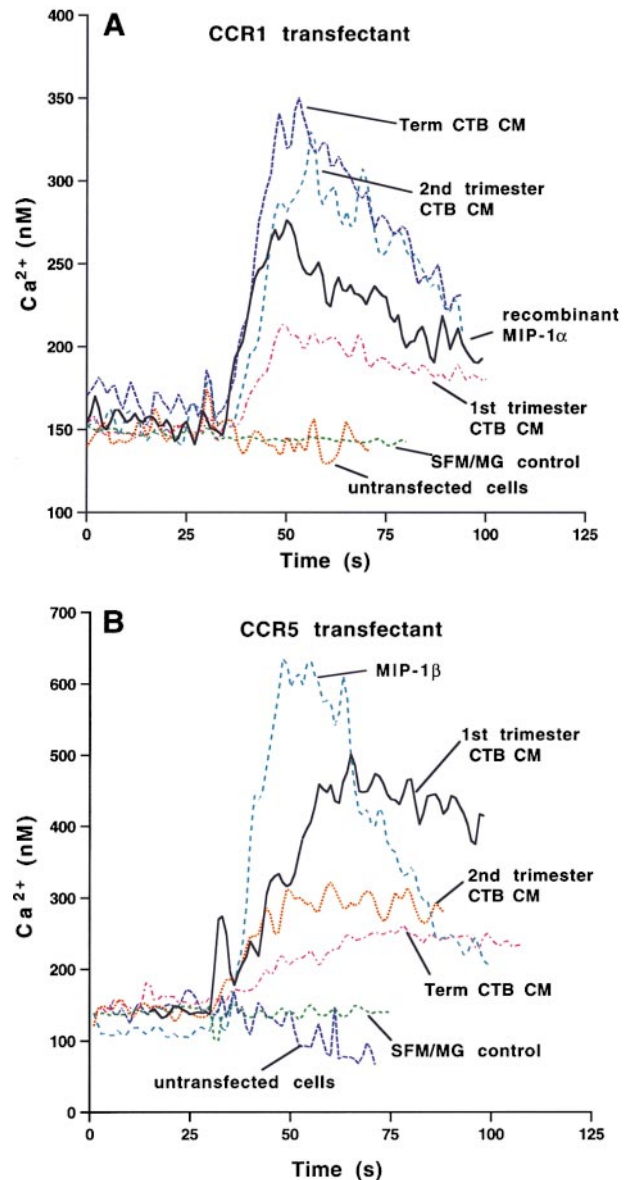


Figure 2. Cytotrophoblast CM signals through MIP-1 α receptors. Cytotrophoblast (CTB) CM was screened for its ability to induce a calcium flux in reporter cells transfected with individual chemokine receptors. Negative controls included exposure of the transfected cells to serum-free control medium incubated with Matrigel in the absence of cells (SFM/MG) and addition of cytotrophoblast CM to the corresponding untransfected parental cell line. Recombinant chemokines were used as positive controls. Concentrated cytotrophoblast CM induced a Ca²⁺ flux in cells expressing CCR1 (A) and CCR5 (B), but not CCR2, CCR3, or CX3CR1 (data not shown).

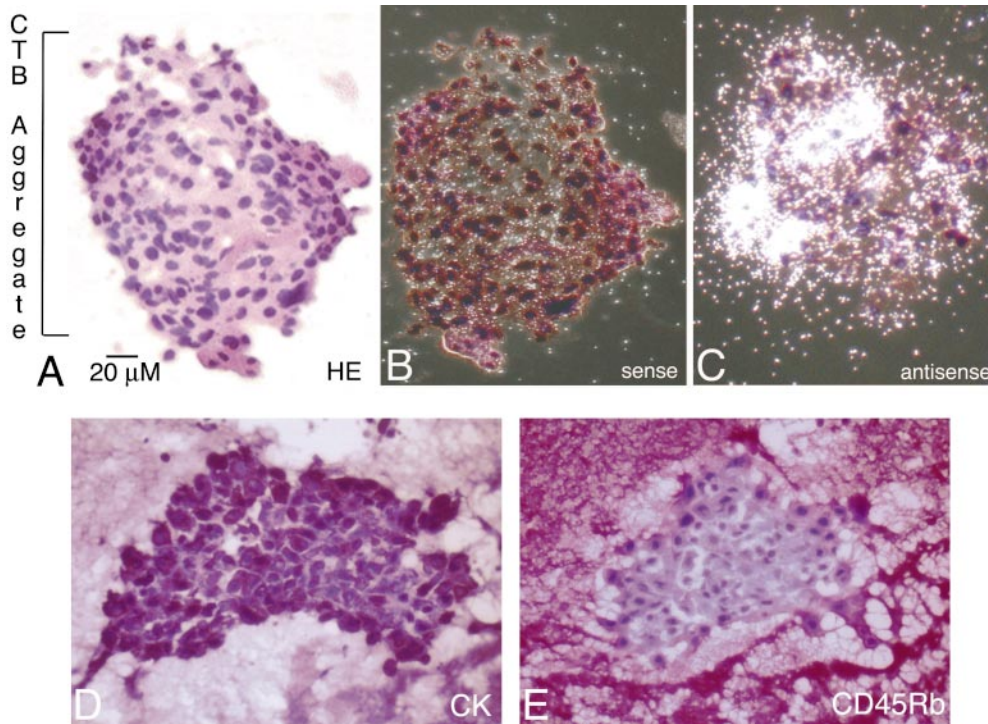


Figure 3. In situ hybridization demonstrates that cultured cytotrophoblasts synthesize MIP-1 α mRNA in vitro. (A) Bright field micrograph of a histological section of cultured cytotrophoblast cells that was stained with hematoxylin and eosin (HE). Dark field micrographs of control sense probes (B) were negative, while experimental antisense probes (C) revealed cytotrophoblast expression of MIP-1 α mRNA, detected as white dots (4 wk exposure). Immunohistochemistry on adjacent sections demonstrated positive staining for the cytotrophoblast marker cytokeratin (CK) (D) and the absence of staining for the macrophage marker CD45Rb (E).

showed that cytotrophoblasts synthesize MIP-1 α mRNA. Immunohistochemistry on adjacent sections was performed to demonstrate that the cell aggregate was composed of cytotrophoblasts, particularly to exclude the possibility of macrophage contamination. The staining revealed the presence of cytokeratin, a cytotrophoblast marker (Fig. 3 D), and the absence of CD45Rb, a marker for bone marrow-derived cells (Fig. 3 E).

We used Northern blot hybridization to quantify cytotrophoblast expression of MIP-1 α mRNA as a function of differentiation/invasion in vitro. Membranes with RNA samples isolated from first trimester cytotrophoblasts were hybridized with a 32 P-labeled cDNA probe to MIP-1 α . The results are shown in Fig. 4. Freshly isolated cytotrophoblasts expressed very low levels of the 800-bp mRNA that encodes this chemokine. Expression of an RNA of the expected size was dramatically upregulated by \sim 30-fold after 12 h in culture. Levels remained high at the 24-h time point and decreased after 36 h in culture. RNA samples that were isolated from second trimester cells showed the same upregulation of MIP-1 α mRNA expression during the first 12 h of culture. Neither placental fibroblasts nor the choriocarcinoma (malignant cytotrophoblast) JAR cell line expressed detectable levels of MIP-1 α mRNA.

Cytotrophoblasts Express MIP-1 α Protein In Vitro. We also examined the cells' expression of MIP-1 α at the protein level, using two methods. In the first, immunoblot analyses were performed using an MIP-1 α -specific IgG. The results are shown in Fig. 5 A. This antibody reacted with a single band of the expected size (12 kD) in a sample of recombinant MIP-1 α . Immunoreactive bands of the same estimated molecular mass were present in concen-

trated CM samples of cytotrophoblasts isolated from first, second, and third trimester placentas. Neither the control SFM/MG medium nor placental fibroblast CM (data not shown) contained proteins that reacted with anti-MIP-1 α .

We used an ELISA to quantify MIP-1 α in cytotrophoblast CM from first trimester ($n = 27$), second trimester ($n = 20$), and term ($n = 13$) cells that were cultured for 12–48 h. Levels of this chemokine detected in individual samples ranged from 0.6 to 82 ng/ml (Fig. 5 B). CM from eight preparations of first trimester and one preparation of second trimester cytotrophoblasts contained amounts of MIP-1 α protein that were below the 175 pg/ml lower de-

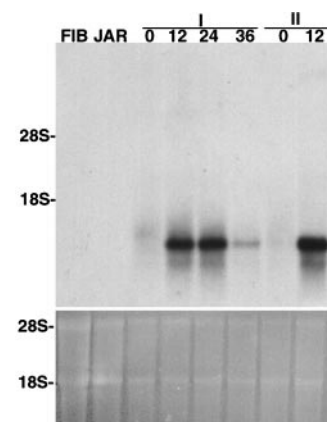


Figure 4. Northern blot hybridization shows that cytotrophoblasts modulate MIP-1 α mRNA expression in culture. Top panel: Cytotrophoblast expression of MIP-1 α mRNA was quantified as a function of differentiation/invasion in vitro. Total RNA was isolated from first trimester placental fibroblasts (FIB) from the choriocarcinoma cell line JAR, and from first (I) and second (II) trimester cytotrophoblasts either immediately after isolation (0 h) or after culturing the cells for the hours indicated. Northern blot analysis was performed using an MIP-1 α -specific probe. Prior to culture, first and second trimester cells expressed very low levels of this mRNA, but its expression was upregulated by \sim 30-fold after 12 h in culture. Neither placental fibroblasts nor the JAR cell line expressed MIP-1 α mRNA. Bottom panel: Acridine orange staining of the gel before transfer demonstrated equal loading of RNA.

tection limit of the assay. Analysis of variance, followed by the Student-Newman-Keuls test, demonstrated that term cytotrophoblasts produced significantly more MIP-1 α ($P < 0.05$) than cytotrophoblasts isolated from first and second trimester placentas. As expected, MIP-1 α was not detected in control SFM/MG or placental fibroblast CM (data not shown). The five pairs of connected data points in Fig. 5 B show MIP-1 α levels in medium samples from the same culture over time. In accord with the results of the Northern blot hybridization analyses of MIP-1 α mRNA levels, the corresponding protein accumulated in the medium during the course of the experiment.

To ensure that the MIP-1 α detected in the CM samples represented cytotrophoblast-derived chemokine and not chemokine produced by contaminating monocytes within the primary culture, we compared the levels of MIP-1 α in

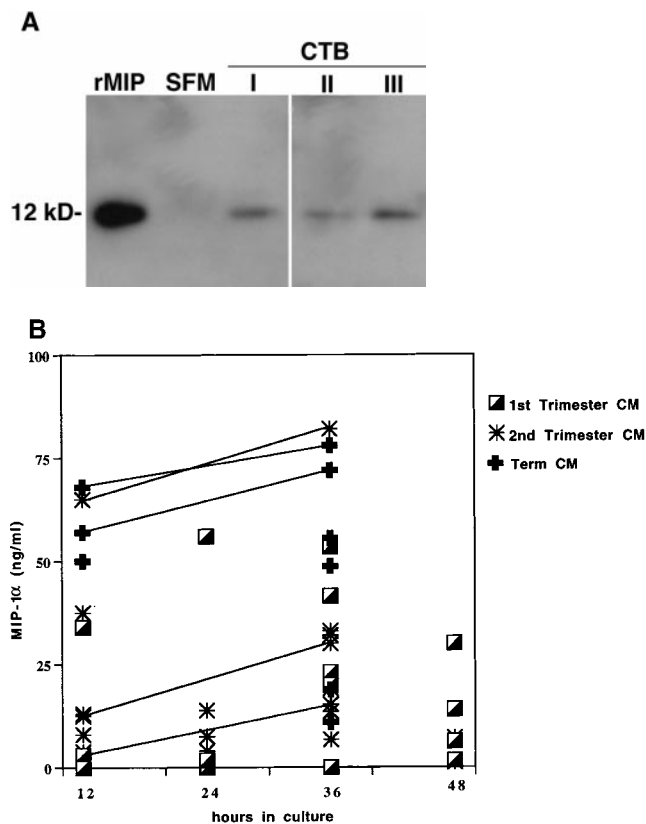


Figure 5. Cytotrophoblasts secrete MIP-1 α protein in vitro. (A) Immunoblotting with an MIP-1 α -specific polyclonal IgG. This antibody reacted with a single band of the expected size (12 kD) in a sample of recombinant MIP-1 α (rMIP). Immunoreactive bands of the same estimated molecular mass were present in concentrated CM samples of cytotrophoblasts isolated from first (I), second (II), and third (III) trimester placentas, but not the control SFM. (B) MIP-1 α concentrations in cytotrophoblast CM as assessed by ELISA. CM was harvested from first trimester, second trimester, and term cells that were cultured for 12–48 h. Levels of this chemokine detected in individual samples ranged from 1.2–82 ng/ml. The five sets of connected data points show MIP-1 α levels in medium samples from the same culture over time, demonstrating that the protein accumulated in the medium during the course of the experiment. As expected, MIP-1 α was not detected in control SFM/MG or placental fibroblast CM (data not shown).

the medium with the presence or absence of endotoxin, a factor that stimulates chemokine production by monocytes but not cytotrophoblasts. 20 samples of cytotrophoblast CM were simultaneously assayed for endotoxin by the Limulus Amebocyte Lysate assay and for MIP-1 α protein

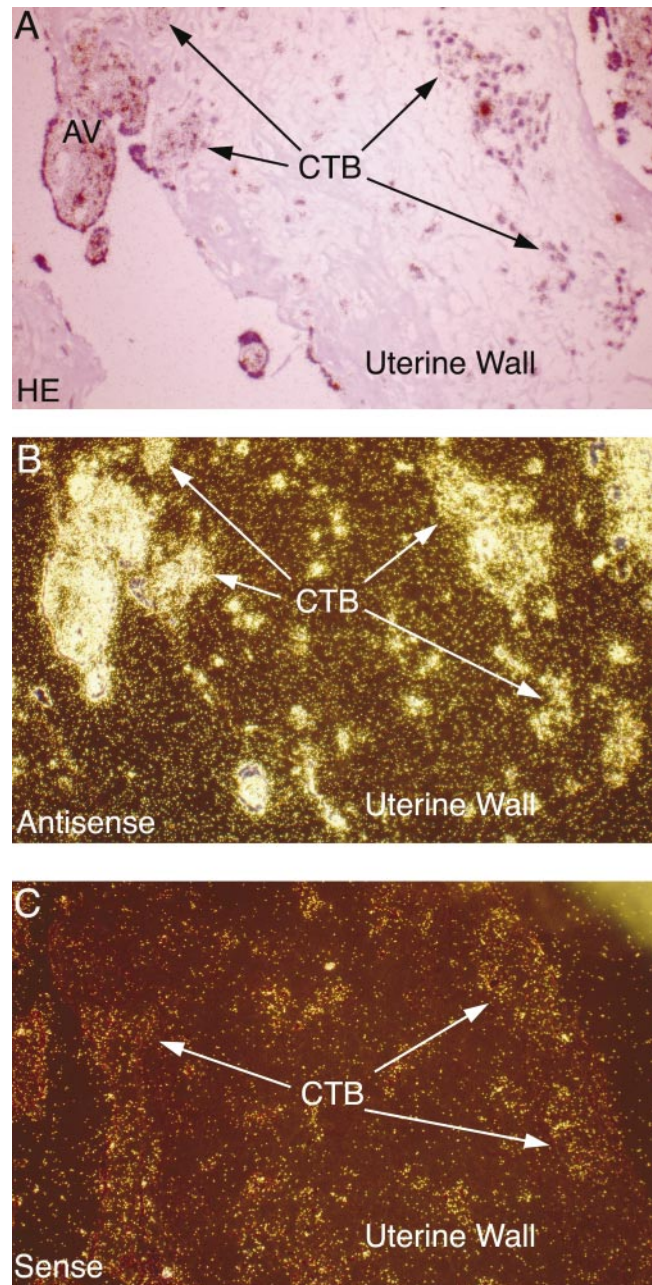


Figure 6. Invasive cytotrophoblasts express MIP-1 α mRNA in vivo. In situ hybridization on tissue sections of the maternal–fetal interface at term demonstrates the presence of MIP-1 α mRNA in cytotrophoblasts within the uterine wall. (A) Bright field micrograph of a histological section that was stained with hematoxylin and eosin (HE). Clusters of cytotrophoblasts are easily seen within a loose meshwork of extracellular matrix components. (B) Dark field micrograph of the same section. White dots indicate signal from ^{35}S -labeled MIP-1 α mRNA in cytotrophoblasts (8-wk exposure). This signal is absent in an adjacent section (C) that was incubated with sense probe as a negative control. AV, anchoring villus.

concentration by ELISA. There was no correlation between the two variables, as determined by the Spearman Rank Order Correlation test (data not shown). This result shows that cytotrophoblasts are the source of MIP-1 α protein in the medium.

Cytotrophoblasts Express MIP-1 α mRNA and Protein In Vivo. As a final step in assessing cytotrophoblast production of MIP-1 α , we examined cytotrophoblasts in tissue sections of the maternal–fetal interface using in situ hybridization to detect mRNA (Fig. 6) and immunofluorescence to detect protein (Fig. 7). Fig. 6 A shows a photomicrograph of a histological section that was stained with hematoxylin and eosin. The morphology is typical of the uterine wall at term—the cytotrophoblasts lie within a loose meshwork of extracellular matrix components. In situ hybridization of these tissue sections with ³⁵S-labeled antisense probe (Fig. 6 B) revealed MIP-1 α mRNA expression by cells within anchoring villi and by extravillous cytotrophoblasts within the uterine wall ($n = 9$). As a negative control, adjacent sections were hybridized with sense probe (Fig. 6 C), which yielded no signal. Complementary evidence was obtained by immunofluorescence localization ($n = 4$). Cytokeratin expression was used to identify cytotrophoblasts within the uterine wall (Fig. 7 A). Adjacent sections demonstrated that these cells stain with anti-MIP-1 α (Fig. 7 B), suggesting their production of this protein in vivo. The specificity of this pattern was confirmed by the absence of staining when specimens were incubated with normal goat serum as a negative control (Fig. 7 D).

MIP-1 α in Cytotrophoblast CM Induces the Migration of Monocytes and CD56^{bright} NK Cells. Having shown that cytotrophoblasts produce MIP-1 α , we assessed the contribution of this chemokine to the CM activity originally observed: induction of monocyte, NK cell, and T cell chemotaxis. First, we performed control experiments to

characterize the chemotactic activity of MIP-1 α in our system, using recombinant chemokine with unstimulated PBMCs as targets (Fig. 8). A range of concentrations that encompassed those found in cytotrophoblast CM was tested. This control system also allowed us to demonstrate the efficacy of the function-perturbing anti-MIP-1 α antibody that was used in subsequent experiments.

The results showed that the response to recombinant MIP-1 α varied among the various PBMC populations we analyzed. As reported by other investigators (52), large numbers of monocytes migrated in every experiment ($n = 8$). The results of a typical analysis are shown in Fig. 8 A. Monocyte chemotaxis peaked at a MIP-1 α concentration of 10 ng/ml, addition of anti-MIP-1 α returned migration to baseline levels, and a control IgG antibody added at the same concentration had no effect. NK cells also responded to recombinant MIP-1 α , although somewhat less consistently. In six out of nine experiments, CD56^{bright} and CD56^{dim} NK cells migrated toward recombinant MIP-1 α (Fig. 8, B and C, respectively). Again, chemotaxis was not observed in the presence of anti-MIP-1 α . Finally, the effects of MIP-1 α on T cells were highly variable. In three of nine experiments, we observed chemotaxis that was reversed by the addition of anti-MIP-1 α (data not shown).

Next we assayed the chemotactic activity of cytotrophoblast CM, using PBMCs as targets. In these experiments, MIP-1 α in cytotrophoblast CM was quantified by ELISA, and then the medium was diluted to produce CM samples that contained from 100 ng/ml to 10 pg/ml of this chemokine. The chemoattractant activity of these CM samples is summarized in Fig. 9 A.

Monocyte migration toward cytotrophoblast CM typically peaked at 10 ng/ml of MIP-1 α (six of eight times), as did the migration of these cells in response to the recombinant chemokine (compare Fig. 8 A and Fig. 9 A). At the

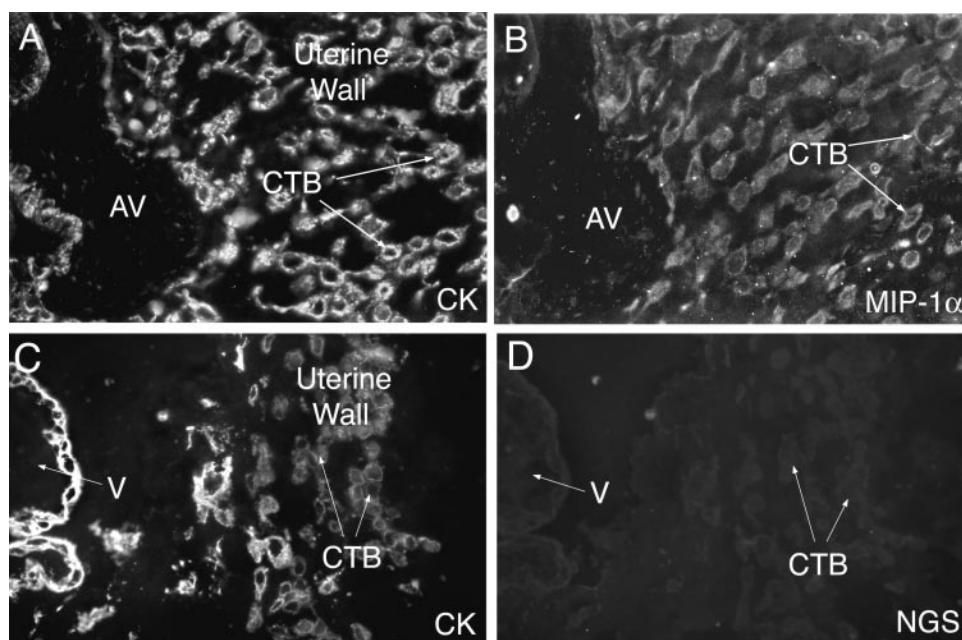


Figure 7. Invasive cytotrophoblasts express protein MIP-1 α in vivo. Immunohistochemistry on tissue sections of the maternal–fetal interface identifies cytochrome (CK)-positive invasive cytotrophoblasts within the uterine wall (A). Staining of an adjacent section reveals that these invasive cytotrophoblasts express MIP-1 α protein (B). The specificity of the MIP-1 α pattern was established by staining with normal goat serum as a negative control. Other specimens were exposed to (C) an anticytokeratin antibody, which demonstrated the cytotrophoblasts within the tissue, and (D) to the control serum, which did not yield a signal. NGS, normal goat serum; V, chorionic villus.

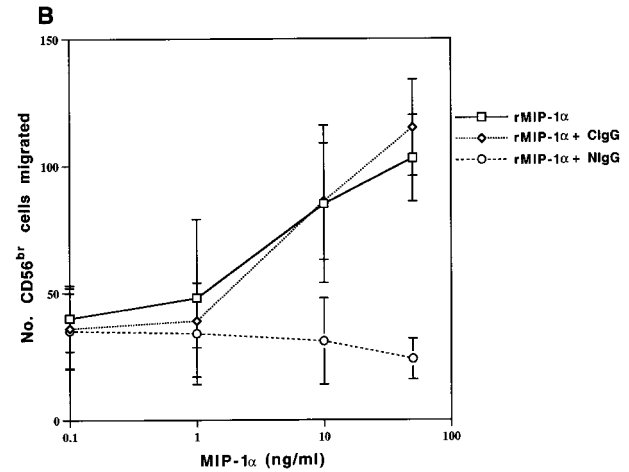
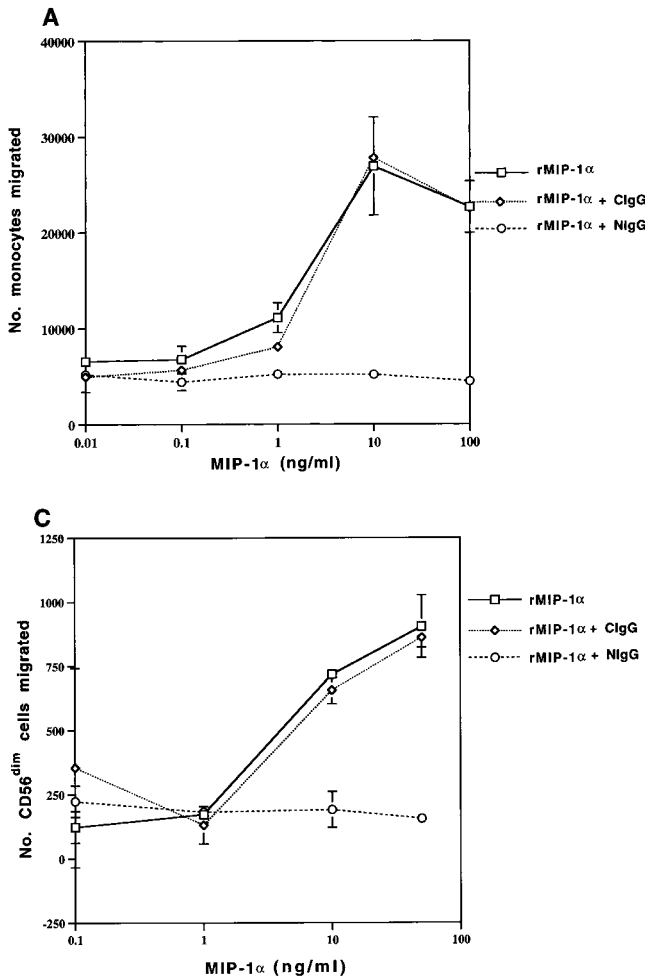


Figure 8. The effects of recombinant MIP-1 α on PBMC chemotaxis. (A–C) Results of a representative experiment. (A) Monocyte chemotaxis toward a dilution series of recombinant MIP-1 α (rMIP-1 α) peaked at a concentration of 10 ng/ml. Addition of a neutralizing anti-MIP-1 α IgG (NIgG) returned migration to baseline levels, and a control IgG antibody (ClgG) added at the same concentration had no effect. (B) CD56^{bright} and (C) CD56^{dim} NK cells also responded to recombinant MIP-1 α , but their migration did not peak within the range of concentrations tested. Addition of anti-MIP-1 α reduced NK cell migration to baseline.

peak, 16% (3–53%) of all monocytes migrated (median and interquartile range; $n = 8$). Since the Matrigel extracellular matrix preparation is a potential source of chemoattractants, basal migration toward medium that was incubated with the matrix substrate in the absence of cells (SFM/MG) was also tested. In this control situation, 1.5% (0.5–5%) of the cells migrated. Taken together, these data showed that cytotrophoblast CM stimulated a sevenfold (3.7- to 16.6-fold) increase in migration over control levels.

Cytotrophoblast CM also attracted CD56^{dim} and CD56^{bright} NK cells. In all but one instance (eight out of nine times), NK cell migration was still increasing at the highest CM (MIP-1 α) concentration that was tested (Fig. 9 A). At this point 4% (0.6–5%) of all CD56^{dim} cells and 5.4% (1.8–10.5%) of all CD56^{bright} cells migrated. Basal migration stimulated by control medium was similar for both NK subtypes: 0.4% (0.1–0.9%) for CD56^{dim} cells and 0.6% (0.2–1%) for CD56^{bright} cells. Thus, cytotrophoblast CM induced a 7.7-fold increase (5- to 12-fold) in the migration of CD56^{dim} cells and a 5.5-fold increase (2.8- to 6.5-fold) in the migration of CD56^{bright} cells.

The pattern of T cell migration in response to cytotrophoblast CM followed that of NK cells, i.e., a peak was not observed within the range of cytotrophoblast CM (MIP-

1 α) concentrations tested (Fig. 9 A). At the highest concentration, 1.7% (1.3–3.5%; $n = 9$) of T cells migrated as compared with basal migration, which was 0.5% (0.4–1.22%). Accordingly, cytotrophoblast CM increased T cell migration by twofold (1.7- to 4-fold) over basal levels, a relatively modest response compared with that of monocytes and NK cells.

Finally, we analyzed the effects of adding function-perturbing anti-MIP-1 α on cytotrophoblast CM-induced PBMC chemotaxis. In five experiments, neutralizing MIP-1 α activity reduced monocyte migration at its peak by $44.2 \pm 10.0\%$ (mean \pm SD) as compared with the addition of a control IgG. Fig. 9 B shows the results of a typical analysis. Here and subsequently we used a two-tailed Student's t test to further analyze the data from individual experiments. The results of three experiments achieved statistical significance ($P < 0.05$). Data from the other two experiments were less significant ($P < 0.075$ and $P < 0.20$). In four experiments, abolishing MIP-1 α activity in the CM reduced migration of CD56^{bright} NK cells by $66.8 \pm 20.0\%$ as compared with the control IgG. An example of these data is shown in Fig. 9 C. The results of three experiments were highly significant ($P < 0.005$). In one case, the data were less significant ($P < 0.1$). Although cytotrophoblast CM

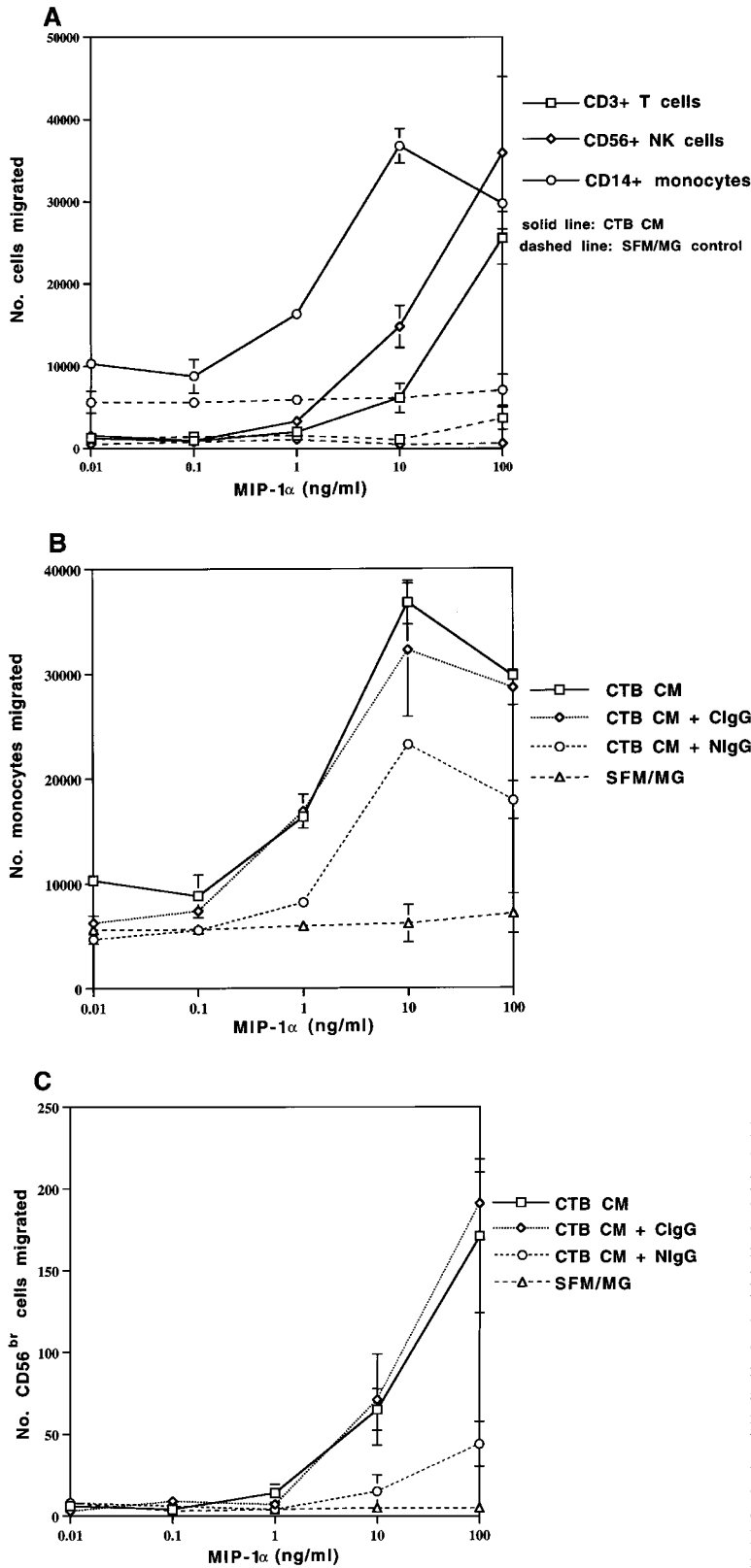


Figure 9. Cytotrophoblast CM attracts monocytes and CD56^{bright} NK cells via the action of MIP-1 α . Panels A–C depict the results of a representative experiment. MIP-1 α levels in cytotrophoblast CM samples were determined by ELISA. The chemoattractant activity of cytotrophoblast CM that contained from 0.01 to 100 ng/ml MIP-1 α is summarized in A. In all experiments, the migration of cells toward SFM that was incubated with Matrigel alone (SFM/MG) was used to assess basal migration. Monocyte movement toward the CM peaked at 10 ng/ml of MIP-1 α , while NK cell and T cell migration was still increasing at the highest concentration of CM (chemokine) tested. (B and C) Addition of a neutralizing anti-MIP-1 α IgG (N1gG) significantly reduced the migration of PBMCs toward cytotrophoblast CM. (B) At its peak, monocyte migration was reduced by 44.2 \pm 10.0% (mean \pm SD) as compared to the addition of a control IgG (C1gG). (C) Chemotaxis of CD56^{bright} NK cells was diminished by 66.8 \pm 20.0% as compared to the control IgG. Neither CD56^{dim} NK cell nor T cell migration was affected by neutralization of MIP-1 α activity (data not shown).

was chemotactic for both CD56^{dim} NK cells and T cells, antibody neutralization showed that this effect did not depend on MIP-1 α activity, suggesting a more complex phenomenon (data not shown).

Discussion

The mechanisms whereby the human hemiallogeneic placenta avoids rejection by the maternal immune system are under intense investigation. In accord with the central role of placentation in pregnancy maintenance, current evidence suggests multiple levels of regulation. There are several notable examples in which fetal trophoblasts produce soluble factors with activities that have the potential to modify maternal immune functions. These molecules include cytokines and growth factors, such as IL-10 (53), TGF- β (54, 55), and MIF (56), that downregulate immunological responses in other settings (57–59). Interestingly, the placenta has also adopted strategies to regulate the immune system at a metabolic level by producing the enzyme indoleamine 2,3-dioxygenase, which catabolizes tryptophan needed by activated T cells (60). Finally, several novel mechanisms of immune regulation are used at the maternal–fetal interface, including high local levels of progesterone (61) and expression of the nonclassical MHC class I molecule HLA-G (7), which also has a soluble form. All of these products downregulate the immune response, particularly the activities of T cells and NK cells, the primary mediators of allogeneic graft rejection.

We now add chemokines, regulators of leukocyte trafficking, to the list of placenta-derived immunomodulators. In this report, we demonstrate that cytotrophoblasts express chemokines that can direct the migration of leukocytes similar to those found at the maternal–fetal interface. In vitro chemotaxis assays characterized the effects of cytotrophoblast CM on PBMCs. The chemotactic responses of the target cells to the CM resembled those observed when leukocytes are challenged with chemokines, in terms of concentration dependence, time course, and efficacy with regard to cell number (62–64). Accordingly, we used reporter cells transfected with specific receptors to test for the presence of chemokines in the medium. This assay revealed the presence of MIP-1 α , a result we confirmed by complementary analyses at both the RNA and protein levels. Freshly isolated cytotrophoblast stem cells expressed very low amounts of MIP-1 α mRNA, although expression increased dramatically as the cells differentiated in culture. This suggested that in vivo, cytotrophoblasts upregulate the expression of this chemokine as they differentiate/invade the uterine wall (Fig. 1). In situ hybridization and immunofluorescence analyses of tissues that contained the maternal–fetal interface confirmed that cytotrophoblasts inside the uterine wall express MIP-1 α mRNA and protein in vivo. Although the production of MIP-1 α by cytotrophoblasts in vitro was highly reproducible, the concentration of secreted chemokine varied among cell preparations, ranging from <175 pg/ml to 82 ng/ml in the CM. This finding correlates with similar observations of variability in cyto-

kine (e.g., IL-10) and hormone (e.g., placental lactogen) production among different placentas (53, 65, 66). Because each individual is the product of a unique genetic, hormonal, and immunological environment, it follows that production of downstream effector molecules by tissues from individual donors reflects these differences.

In spite of this variability in chemokine production, specific aspects of the chemotactic properties of cytotrophoblast CM were clearly attributable to the presence of MIP-1 α , as eliminating its activity from the cytotrophoblast CM altered the chemotaxis of target cells in our in vitro assay. In general, these effects reflected the previously published actions of this chemokine on leukocyte subsets. For example, MIP-1 α in the cytotrophoblast medium strongly attracted monocytes, well established targets for this molecule (52). The fact that the dose–response curves for monocyte chemotaxis toward cytotrophoblast CM and recombinant MIP-1 α were essentially superimposable suggested that this chemokine was a dominant cytotrophoblast-derived factor regulating monocyte migration. Additionally, in terms of absolute cell numbers, cytotrophoblast CM stimulated migration of \sim 15% of the target monocytes, and addition of anti-MIP-1 α to cytotrophoblast CM reduced monocyte migration by \sim 45%. Together, these data indicate that cytotrophoblasts rely heavily on MIP-1 α in the CM to regulate monocyte chemotaxis. Other cytotrophoblast-derived chemokines, such as RANTES, may also contribute to this phenomenon (67).

Cytotrophoblast CM was also highly chemotactic for NK cells, another established target of MIP-1 α (63, 68). As the NK cells that reside in the uterus during pregnancy are of the CD56^{bright} rather than the CD56^{dim} phenotype, we analyzed the chemotaxis of each population of cells separately. As CD56^{bright} cells comprise only 10% of all circulating NK cells, most studies investigating chemokine activity have not distinguished between the two subtypes. One exception is the report that secondary lymphoid chemokine and CK β -11, both ligands for the CCR7 receptor, preferentially attract CD56^{bright} over CD56^{dim} cells (69). Here, we report that MIP-1 α activity in cytotrophoblast CM is responsible for nearly 70% of the migration of CD56^{bright} cells we observed. In contrast, the contribution of this chemokine to the migration of CD56^{dim} cells is less clear, suggesting that other factors in cytotrophoblast CM may be responsible for this activity. Therefore, in this system MIP-1 α has differential effects on these two NK subsets. Thus, via MIP-1 α , cytotrophoblasts are equipped with a potent mechanism that may allow them to direct the movement of the two major populations of leukocytes, monocytes and CD56^{bright} NK cells, with which they interact at the maternal–fetal interface.

Finally, although MIP-1 α is a reported attractant for activated T cells, it was not responsible for the migration of these cells toward cytotrophoblast CM. In accord with previously published data, the unstimulated T cells within our PBMC target population did not migrate toward recombinant MIP-1 α (70). Similarly, the migration of unstimulated T cells toward cytotrophoblast CM was not affected by ad-

dition of anti-MIP-1 α to the medium. These findings suggest the presence in the CM of T cell chemoattractants whose identity we have not yet established.

This report demonstrates that cytotrophoblasts may be capable of contributing to the recruitment of decidual leukocytes. It should be noted that the uterine stroma itself is capable of attracting circulating leukocytes, as distinct populations of immune cells migrate through the uterus during the course of the menstrual cycle (71). Furthermore, decidual leukocytes can be found in the decidua in the event of an ectopic pregnancy, when the placenta has implanted outside of the uterus (72). Therefore, decidual leukocyte infiltration is also regulated by the decidual vasculature, as is suggested by the highly specific combinatorial expression of adhesion molecules in pregnant mouse uterine blood vessels (73). These adhesion molecules are integral to a multi-step homing process that also requires chemokines. In mice, the absence of MIP-1 α or one of its receptors (CCR5) did not affect NK cell infiltration of the pregnant uterus (74). Given the number of known chemokines and receptors, it is likely that mice and humans employ distinct sets of these molecules to influence decidual leukocyte chemotaxis.

Here, we demonstrate that the human cytotrophoblasts produce MIP-1 α , which endows them with the capacity to direct the migration of NK cells, monocytes, and T cells, cells that comprise the DGL population with which the placenta coexists. This activity could contribute to the recruitment of decidual leukocytes or may be used at a local level to manipulate the microimmune environment at the maternal-fetal interface. Either scenario affords fetal cytotrophoblasts an additional level of control in regulating the immune activity of maternal cells in this sensitive location.

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