Title
Chemokine expression and function at the maternal-fetal interface

Author
Drake, Penelope Marie

Publication Date
2001

Peer reviewed|Thesis/dissertation

Chemokine Expression and Function at the Maternal-Fetal Interface

by

Penelope Marie Drake

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

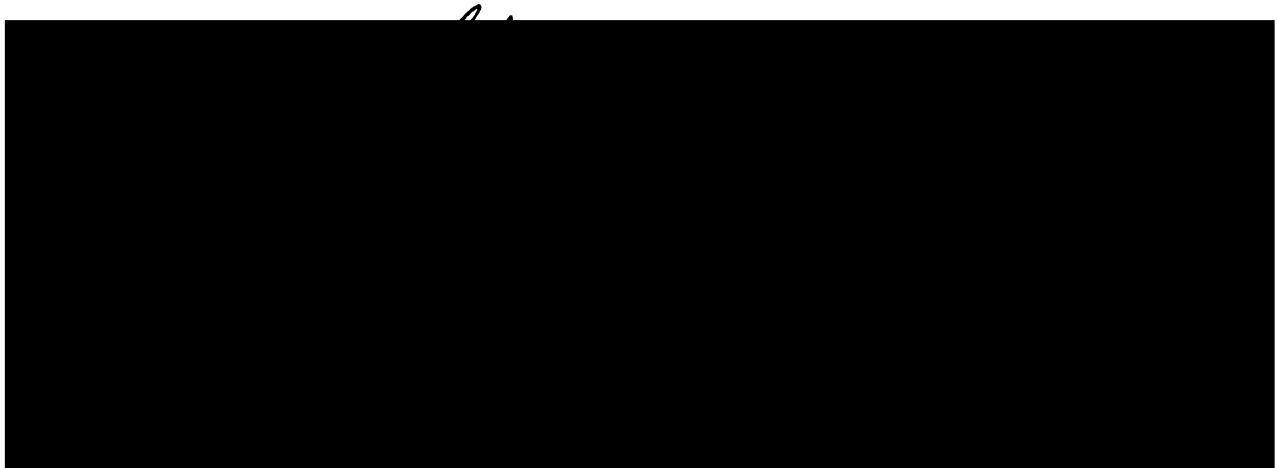
Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO



Date University Librarian

Degree Conferred: .....

For my grandparents:

Yiayia, my lovely hero who inspired me with her independence and grace;
and
Grandma and Granddad Drake, whose hard work, integrity and humility set a standard I aspire to honor.

And to my parents, who loved me even when they didn't understand me.

Acknowledgements

I'd like to begin by thanking Dee Gunn, who gave me the initial guidance on chemokines that was so crucial to getting this project up and running. Over the past few years, I have appreciated his technical advice and moral support. However, I am most deeply indebted to him for facilitating my use of the CVRI in situ core and for introducing me to Carmen Tam, the in situ goddess masquerading as technician, who trained me.

In my pre-chemokine days, I had incredible collaborative support from Joe Phillips and Lewis Lanier, who advised me on decidual leukocytes, HLA-G, and inhibitory receptors. I am especially grateful to them for their kindness to me as a beginning graduate student. Joe and Lewis taught me to do flow cytometry and generously provided antibodies for the characterization of decidual leukocytes.

A warm thank you to Art Weiss, chair of my thesis committee, for his leadership and guidance; and to committee members Nigel Killeen, Mattias Wabl and Joe Phillips for their advice, support, and fruitful discussions.

Thanks also to Paul Dazin, for the many hours on the Facscan that he scheduled seemingly by magic at the last minute, and for his pleasant company and all of the tea.

A standing up, Star-Spangled Banner trumpeting, hand over my heart, teary-eyed thank you to the members of the Fisher Lab who have formed the bulk of my family over the past six years. Especially important to me are my relationships with these past and present members of the lab: Susan Fisher, Aka Prakabpol, Olga Genbacev, Mike McMaster, Mary Janatpour, Van Hoang, Yan Zhou, Kristy Red Horse, Ed Caceres, Leslie Waite, Rebecca Joslin, Anna Chodos, Valerie Bellingard-Dubouchaud and Kirsten Woo.

A Julie Andrews cresting the hill, melody swelling, arms spread wide embracing thank you to these friends who have helped me have Confidence in Me (and in them) during the confidence-testing experience called graduate school. Atop my list of Favorite Things are: Andrea Cupp, Tracy Fulton, Steffi Mandl, Ingrid Mehlhorn, Laura Napolitano, Dana Rohde, Savannah Partridge, Jennifer Frazier, Van Hoang and her husband James Buckman, Mary Janatpour and her entire family— especially her husband Mark and their sons Brenden and Cameron, and finally, my little sister Mayanna Austin.

And a Jose Cuervo tinted Costa Rican sunset full of love to my family—Mom, Dad, Debbie, Sara, Larry, Zack, Leo and Bev.

Finally, if I picked the winning lotto numbers every day for twelve months straight, I still wouldn't be as lucky as I was the day Susan Fisher became my mentor. Like many other faculty members at UCSF, Susan is a top-notch scientist. Studying with her has provided me with incredible opportunities to learn about all aspects of being an academic researcher, and I feel very well grounded as a result of her guidance. However, Susan's non-scientific attributes are what really distinguish her as a fabulous mentor. Her sense of humor has been a godsend, and her seemingly perfect balance of family, science and sanity have served as an inspiration to me. I remember when I first came to the lab being surprised to hear Susan engage in baby talk over the phone with her then four-year old daughter Katherine. It didn't matter who else was in her office or the environs, if Katherine was on the phone, Susan was cooing to her. I was at first shocked, then amazed, then in love. What's more, Susan makes this down-to-earth accessibility open to all of us in the lab. She understands that life exists outside of HSW 604, and she's been a supportive friend through the highs and the lows. Three other wonderful things about Susan are her laugh, her boundless enthusiasm over new results, and her love of parties and champagne. Thinking about how much fun this time with her has been makes me more wiggly than some golden retrievers I could name. Thank you, Susan, thank you, thank you, thank you for the whole shebang.

Chemokine Expression and Function at the Maternal-Fetal Interface

by

Penelope M. Drake

Graduate Advisor: Susan J. Fisher, Ph.D.

ABSTRACT

A long-standing unanswered biological question is how the placental-fetal unit avoids rejection by the maternal immune system. The unusual anatomy of the maternal-fetal interface suggests that the mechanisms must include a regulatory dialog between the mother and fetus that subverts the standard methods of maternal self-identification and immune surveillance. Several unique immunological aspects of pregnancy have been identified; one of the most striking is the unorthodox maternal leukocyte population that homes to the pregnant uterus—decidual granulated leukocytes. Dominated by specialized $CD56^{bright}$ NK cells, these leukocytes are the maternal immune ambassadors that accompany the engrafted fetus through gestation. Presumably they are selected for their ability to tolerate paternal allo-antigens, yet respond to pathogens.

This

migration a

responses in

showed that

Ia that indu

was a major

medium. Ne

and CD56

chemotaxis as

cytotrophobla

the more gener

maternal-fetal i

The analy

suggested that t

expressed in the

identify other co

RNase protectio

their receptors in

This thesis focuses on the influence of chemokines, molecules that control the migration and activation of leukocytes, on the decidual leukocyte population and immune responses in the placental bed. Through a combination of molecular techniques we showed that placental cells (cytotrophoblasts) produced and secreted the chemokine MIP-1α that induced a calcium flux in cells with compatible receptors. Additionally, MIP-1α was a major contributor to the chemotactic activity in cytotrophoblast conditioned medium. Neutralization of MIP-1α in the medium abrogated nearly half of the monocyte and $CD56^{bright}$ NK cell migration towards cytotrophoblast conditioned medium in *in vitro* chemotaxis assays. The results of this study suggest the specific conclusion that cytotrophoblasts can attract monocytes and $CD56^{bright}$ NK cells by producing MIP-1α, and the more general hypothesis that these cells may organize and act on leukocytes at the maternal-fetal interface.

The analysis of MIP-1α expression and function at the maternal-fetal interface suggested that this molecule belonged to a network of chemokines and their receptors expressed in the pregnant uterus. Accordingly, additional experiments were initiated to identify other components of this circuitry. The results of in situ hybridization and RNase protection studies showed abundant and specific expression of chemokines and their receptors in both the placental and uterine compartments. Furthermore, the

reciprocal expression of ligand-receptor pairs by decidual leukocytes and uterine tissues suggested that the expression patterns observed had functional consequences. Other localization patterns suggested that chemokines expressed at the maternal-fetal interface had novel non-immune functions, likely related to vasculogenesis/angiogenesis and autocrine regulation of cytotrophoblast differentiation. Together, the results presented suggest that chemokines are an integral component of the unusual cell-cell interactions that lead to normal placentation and immune tolerance of the hemi-allogeneic fetus.

The provocative results described here suggest numerous models of chemokine action at the maternal-fetal interface, and provide a solid informational foundation for future research that addresses specific functional questions. These include investigations into mechanisms of decidual leukocyte and Hofbauer cell recruitment, chemokine regulation of vasculogenesis/angiogenesis in both maternal and fetal compartments, and chemokine participation in placental development, particularly with regard to cytotrophoblast differentiation. Importantly, the resulting paradigms will give new insights into both normal placentation, and pregnancy complications related to placental abnormalities, such as preterm labor and preeclampsia. The results of these future studies will advance our knowledge of specific processes in human reproduction, and will provide a novel contribution to our understanding of chemokine biology.

Table of Contents

List of Figures and Tables

Chapter One

Chapter Three

Chapter Four

Chapter Six

Chapter Seven

Chapter One – Placentation and Cytotrophoblast Differentiation

Implantation

The initial stages of preimplantation development, from zygote to morula, occur as the embryo encased within the non-adhesive zona pellucida transits the fallopian tube (Figure 1-1). The morula reaches the uterine cavity approximately two to three days postconception. Within 72 hours, the embryo enters the blastocyst stage and hatches from the zona, exposing its outer covering of trophoblasts. The few human embryos that have been recovered after maturation *in vivo* appear to be covered by multinucleate (syncytial) trophoblasts (Hertig and Rock, 1973); whereas at least some embryos that develop *in vitro* are covered by mononuclear cytotrophoblasts. Because of this discrepancy, the trophoblast cell type that mediates adhesion to the uterus remains unknown. Implantation occurs around the sixth to seventh day postconception. By day 10 postconception, the blastocyst is completely embedded in subepithelial, interstitial stromal tissue, the uterine epithelium regrows over the implantation site, and mononuclear cytotrophoblasts stream out of the trophoblastic shell, further invading the uterus (Benirschke and Kaufmann, 1991) (see Fig. 1-1).

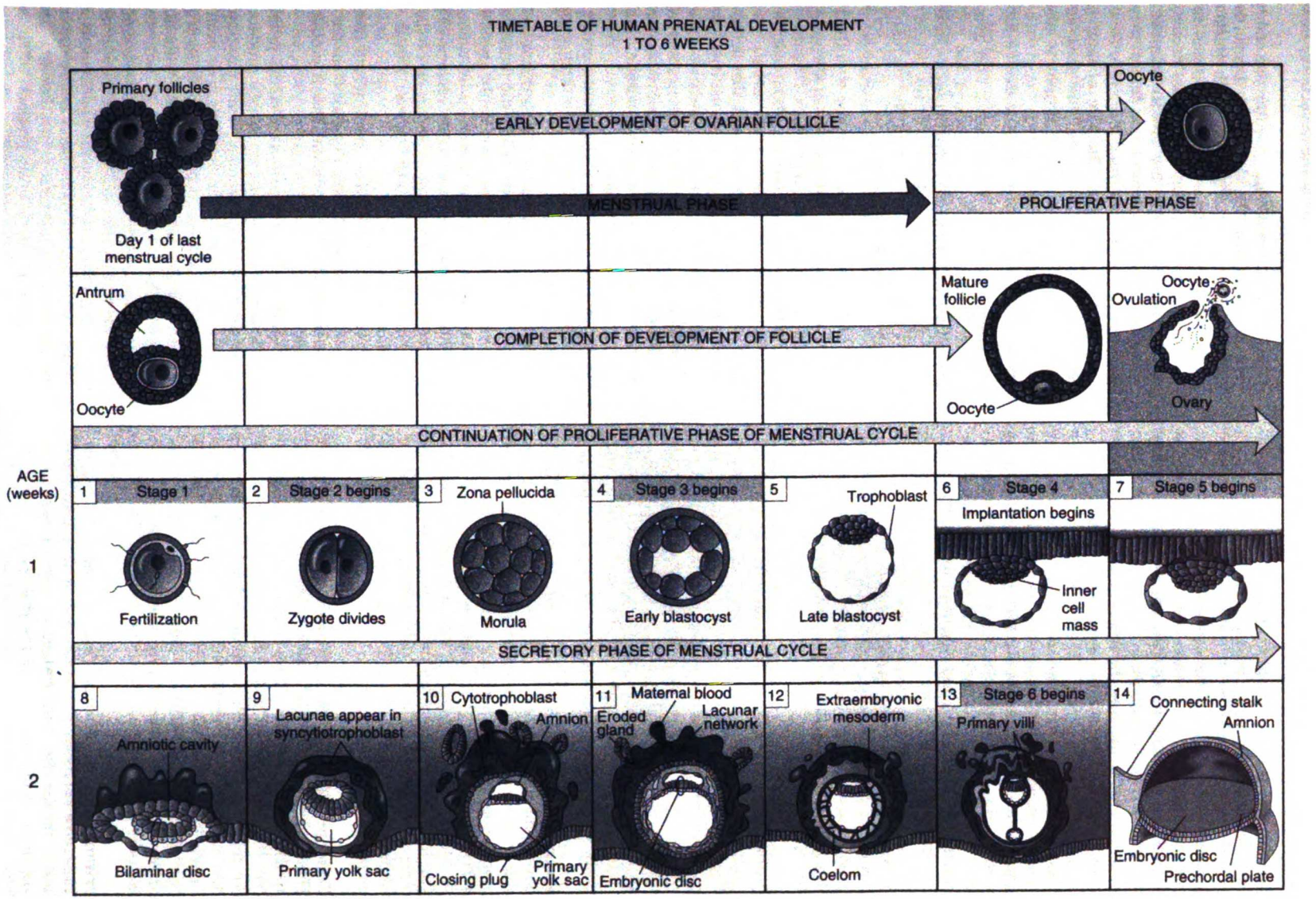

TIMETABLE OF HUMAN PRENATAL DEVELOPMENT
1 TO 6 WEEKS
Primary follicles
Day 1 of last menstrual cycle
EARLY DEVELOPMENT OF OVARIAN FOLLICLE
MENSTRUAL PHASE
PROLIFERATIVE PHASE
Oocyte
Antrum
Oocyte
COMPLETION OF DEVELOPMENT OF FOLLICLE
Mature follicle
Oocyte
Oocyte
Ovulation
Ovary
CONTINUATION OF PROLIFERATIVE PHASE OF MENSTRUAL CYCLE
AGE (weeks)
1
2
1 Stage 1
Fertilization
2 Stage 2 begins
Zygote divides
3 Zona pellucida
Morula
4 Stage 3 begins
Early blastocyst
5 Trophoblast
Late blastocyst
6 Stage 4
Implantation begins
Inner cell mass
7 Stage 5 begins
SECRETORY PHASE OF MENSTRUAL CYCLE
8
Amniotic cavity
Bilaminar disc
9
Lacunae appear in syncytiotrophoblast
Primary yolk sac
10 Cytotrophoblast
Amnion
Closing plug
Primary yolk sac
11 Maternal blood
Eroded gland
Lacunar network
Embryonic disc
12
Extraembryonic mesoderm
Coelom
13 Stage 6 begins
Primary villi
14
Connecting stalk
Amnion
Embryonic disc
Prechordal plate

Figure 1-1

Figure 1-1. Sequence of Events Surrounding Fertilization and Implantation. The early stages of development are illustrated, beginning with fertilization and cleavage of the zygote, which occur in the uterine tube. By the time it reaches the uterine cavity, the conceptus has progressed to the blastocyst stage. Implantation occurs over the next week, and initial placental development begins with the establishment of primary villi and a cytotrophoblastic shell. (Adapted from Moore and Persaud 6th Ed.)

Following implantation, the endometrial stroma around the blastocyst commences a reaction which eventually spreads outward to include the entire endometrium. This process, known as decidualization, consists of a modification of stromal cells and an increase in the number of maternal leukocytes within the tissue, and is completed around day seventeen to eighteen (reviewed in (Hamilton, 1972)). When the stromal cells have fully differentiated to their decidual phenotype, they can assume a number of different morphologies which probably relate to distinct functions within the tissue. Nevertheless, a typical decidual cell is polyhedral in shape and contains vacuoles filled with glycogen and lipids. The three histologically distinct layers characteristic of non-pregnant endometrium (stratum compactum, stratum spongiosum, stratum basale) are retained in the decidualized uterus (Figure 1-2). Similar to the secretory phase endometrium, decidualized uterine glands are very convoluted and tortuous. The glands actively secrete a nutritive mixture of proteins, mucopolysaccharides, glycogens and lipids, which contributes to the maintenance of the early blastocyst before a blood supply is established.

Placental development and structure

The human placenta is comprised of two types of chorionic villi—floating villi and anchoring villi (Figure 1-3). Both are composed of a stromal core encased in a basement membrane overlain by cytotrophoblast stem cells. These stem cells can adopt one of two differentiation fates. In floating villi—so called because they float in a pool of maternal blood—cytotrophoblast stem cells fuse to form a multi-nucleate syncytium (syncytiotrophoblasts) that covers the villus. Gases, nutrients and wastes are exchanged between mother and fetus across this syncytial surface. Similarly, in anchoring villi—so called because they form a physical attachment that anchors the placenta to the uterus—most cytotrophoblast stem cells fuse into a syncytial covering over the villus. However, at selected sites stem cells break through the syncytium and form a column of non-polarized cells. Where these cell columns touch the uterine wall, the cytotrophoblasts invade the maternal tissue, thus physically joining the placenta and uterus. Upon breaching the uterus, cytotrophoblasts leave the column and invade the decidua as single cells, migrating through the endometrium and as far as the first third of the myometrium. A portion of these invasive cytotrophoblasts home specifically to maternal spiral arterioles (Figure 1-2). They remodel these vessels and replace the endothelial cells lining the lumen. A fully remodeled arteriole is a large bore vessel with

fetal cytotrophoblast cells in place of maternal endothelial cells (Figure 1-3). The molecular aspects of the invasive differentiation pathway have been more extensively characterized than those of the fusion pathway, largely because *in vitro* techniques are available to manipulate invasive (extravillous) cytotrophoblast cell differentiation. Cytotrophoblast stem cells can be isolated as a highly pure population of cells and cultured on an extracellular matrix substrate (Fisher et al., 1989; Librach et al., 1991). The isolation and culture conditions force the cells down the invasive differentiation pathway and with time in culture they recapitulate much of the *in vivo* differentiation program. This allows for manipulation of the differentiation process whereby factors can be directly tested for their effect on cytotrophoblasts. Much of the following data has been obtained using this culture system.

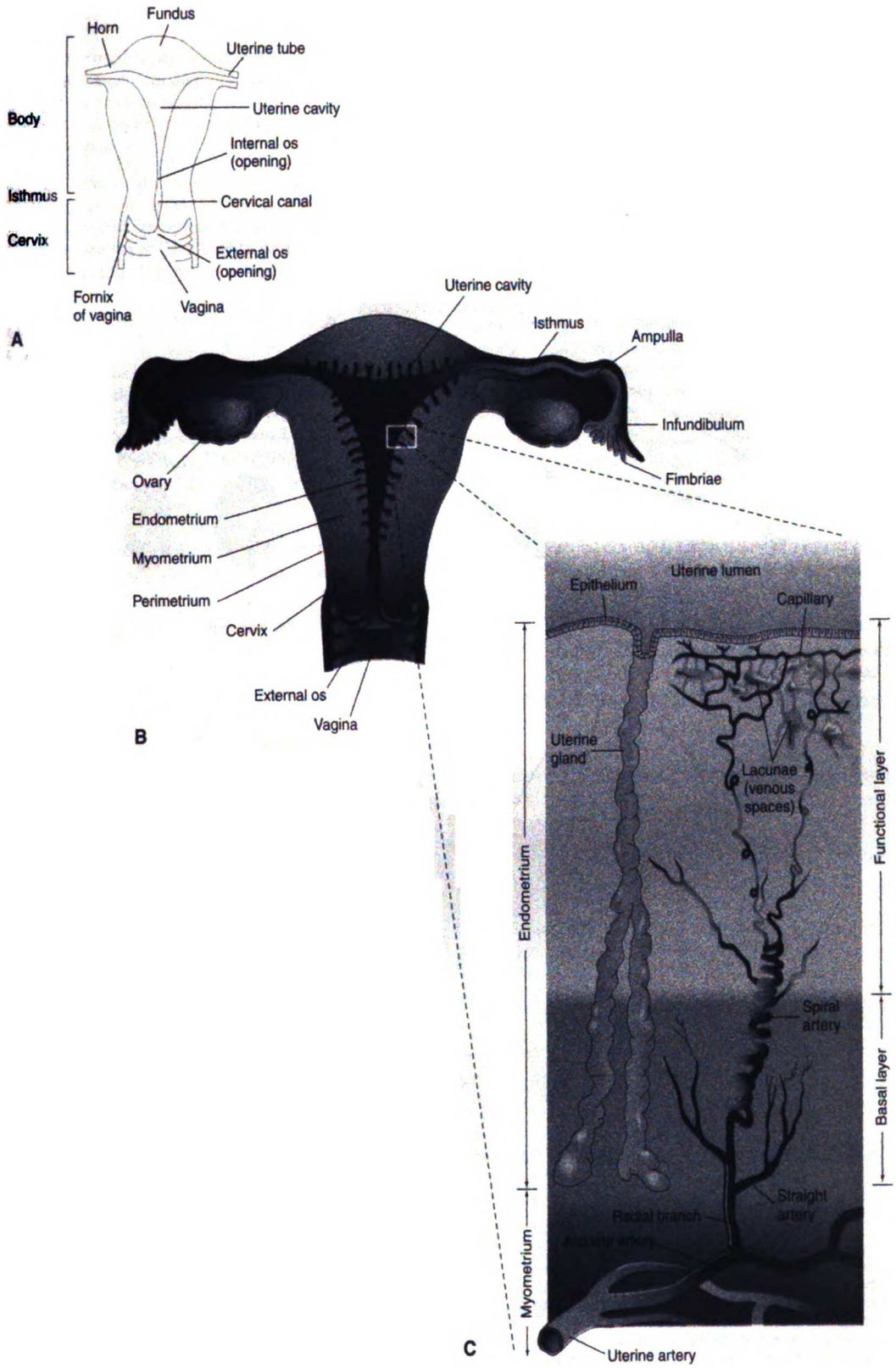

Fundus
Horn
Uterine tube
Body
Uterine cavity
Internal os
(opening)
Isthmus
Cervical canal
Cervix
External os
(opening)
Fornix
of vagina
Vagina
A
Uterine cavity
Isthmus
Ampulla
Infundibulum
Fimbriae
Ovary
Endometrium
Myometrium
Perimetrium
Cervix
External os
Vagina
B
Epithelium
Uterine lumen
Capillary
Uterine
gland
Lacunae
(venous
spaces)
Endometrium
Functional layer
Basal layer
Spiral
artery
Straight
artery
Radial branch
Myometrium
C
Uterine artery

Figure 1-2

Figure 1-2. Strata of the Uterine Wall. The uterine wall is composed of three zones: perimetrium, myometrium and endometrium. The outer region, perimetrium, is a sheet of peritoneum that is firmly attached to the underlying muscular myometrial layer. Endometrium, the lining of the uterine cavity, develops over the course of the menstrual cycle in preparation for implantation. The blastocyst embeds itself into the endometrial lining, and invasive fetal cytotrophoblasts stream from the placenta, infiltrating the endometrium and the first third of the myometrium. Implantation induces a decidualization reaction in the endometrium, a process which includes influx of a specialized population of maternal immune cells termed decidual granulated leukocytes. As they leave the placenta proper to invade the uterus, fetal cytotrophoblasts come into contact with these maternal leukocytes. Many of the invasive fetal cells home to and remodel maternal spiral arterioles within the endometrium and myometrium, removing the vessels' muscular wall and replacing the lining of endothelial cells. This results in a second region of contact between the fetal cells and the maternal immune system—exposure to peripheral blood leukocytes circulating through remodeled vessels. (Adapted from Moore and Persaud 6th Ed.)

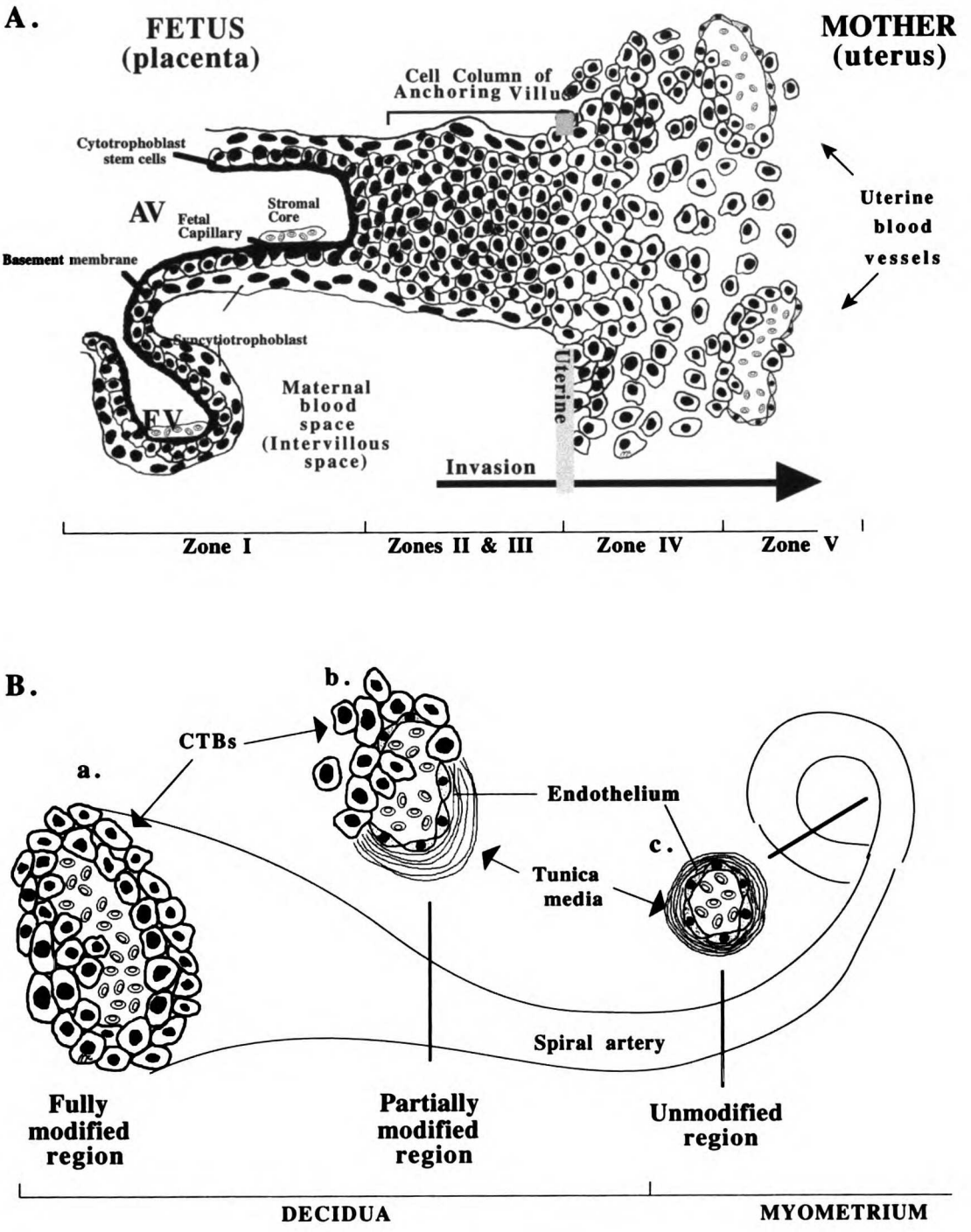

A.
FETUS
(placenta)
MOTHER
(uterus)
Cell Column of
Anchoring Villus
Cytotrophoblast
stem cells
AV
Fetal
Capillary
Stromal
Core
Basement membrane
Syncytiotrophoblast
FV
Maternal
blood
space
(Intervillous
space)
Uterine
Invasion
Uterine
blood
vessels
Zone I
Zones II & III
Zone IV
Zone V
B.
b.
CTBs
a.
Endothelium
c.
Tunica
media
Spiral artery
Fully
modified
region
Partially
modified
region
Unmodified
region
DECIDUA
MYOMETRIUM

Figure 1-3

Figure 1-3. The Cellular Architecture of the Human Fetal-Maternal Interface. The basic structural unit of the placenta is the chorionic villus, composed of a stromal core with blood vessels, wrapped in 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 as depicted in Panel A. The cells either fuse to form the syncytiotrophblast which covers floating villi (FV), or join a column of extravillous CTBs at the tips of anchoring villi (AV). The syncytial covering of floating villi mediates the nutrient, gas and waste exchange between fetal and maternal blood. The anchoring villi, through the attachment of CTB columns, establishes physical connections between the fetus and the mother. Invasive CTBs penetrate the uterine wall up to the first third of the myometrium encountering a population of maternal immune cells, termed decidual granulated leukocytes, resident to the uterine stroma. As depicted in Panel B, a portion of the extravillous CTBs home to uterine spiral arterioles. They remodel these vessels by destroying the muscular wall and replacing the endothelial lining. Thus, CTBs encounter distinct populations of maternal leukocytes in two locations—the decidual granulated leukocytes in the uterine wall, and the peripheral blood leukocytes circulating through remodeled uterine vessels. (Figure by Mike McMaster, Ph.D.)

Differentiation of cytotrophoblasts

A successful pregnancy depends on a functioning placenta, which in turn depends on the proper differentiation of its trophoblast populations. Little is known about the transcriptional regulation of either the fusion or the invasive differentiation pathways, but studies of placental development in the mouse provide clues. Mash2, a mammalian homologue of Drosophila achaete/scute complex genes, is involved in the maintenance of the murine trophoblast stem cell population, a critical determinant of placental growth potential. Commitment to the differentiation pathway that leads to the invasive population of murine trophoblast cells is characterized by the downregulation of the expression of inhibitor of DNA binding proteins (Ids) (Jen et al., 1997), the mSNA zinc finger transcription factor (Nakayama et al., 1998), and OCT-4 (Palmieri et al., 1994). Upregulated expression of the positive regulator Hand1 is required to complete this differentiation process (Firulli et al., 1998; Riley et al., 1998). Although there are numerous differences between the mouse and the human placenta at a morphological level, many of the same transcriptional regulators that control murine placentation are expressed by human trophoblasts (Janatpour et al., 1999). Functional evidence supports the hypothesis that they also play an important role in human placental development. For example, Id-2 regulates aspects of cytotrophoblast differentiation along the invasive

pathway (Janatpour et al., 2000), and OCT-4 overexpression silences βhCG expression (Liu and Roberts, 1996). Finally, the human system has been used to discover novel transcriptional regulators that play critical roles in placental development. For example, Northern blot analyses showed that the human homologue of Drosophila glial cells missing (GCM), a major regulator of neuronal cell fate, is primarily expressed in human cytotrophoblasts (Janatpour et al., 1999). Gene deletion studies in mouse later confirmed that GCM regulates crucial aspects of epithelial-mesencymal interactions during the initial stages of murine placentation (Anson-Cartwright et al., 2000).

Interestingly, retroviral gene products also appear to be important in placental development. The syncytin protein, expressed in human syncytiotrophoblasts, may be involved in the formation of the multi-nucleate syncytium which covers most of the placenta. Evidence for this role comes from *in vitro* studies demonstrating that syncytin expression is sufficient to induce fusion in cell lines (Mi et al., 2000).

Invading cytotrophoblasts extensively modulate their adhesion molecule expression in a stepwise fashion. Particularly striking is the downregulation of adhesion receptors characteristic of cytotrophoblast stem cells (integrin α6β4 and epithelial cadherin) and the onset of expression of adhesion receptors characteristic of endothelium, including

cadherins (vascular endothelial cadherin and cadherin-11), immunoglobulin-family receptors (vascular cell adhesion molecule-1, platelet-endothelial cell adhesion molecule-1 and Mel-CAM), and integrins (αVβ3, α1β1 and α5β1) (Damsky and Fisher, 1998). In addition to allowing cytotrophoblasts that line maternal vessels to masquerade as vascular cells, many of these receptors also play important roles in invasion. Analysis of the effects of adding function-perturbing antibodies to the *in vitro* model of cytotrophoblast invasion reveals a delicate balance. For example, integrins αVβ3 and α1β1 promote cytotrophoblast invasion, but α5β1 restrains it (Damsky et al., 1994). *In vivo*, trophoblast invasion is likely influenced by uterine ECM components (e.g., laminin, fibronectin, and osteopontin) that are ligands for trophoblast integrins (Lessey and Arnold, 1998).

Invading cytotrophoblasts also extensively modulate their proteinase repertoire (Huppertz et al., 1998). Of particular importance is their ability to express and activate matrix metalloproteinase-9 (MMP-9), a major regulator of cytotrophoblast invasiveness *in vitro* (Librach et al., 1991). The simultaneous upregulation of tissue inhibitor of metalloproteinase-3 (TIMP-3) expression is another example of the balancing mechanisms that hold cytotrophoblast invasion in check (Bass et al., 1997). Although trophoblast-associated urokinase-type plasminogen activator is probably not involved in invasion, it, like the aforementioned adhesion molecules, may play a role in vascular

mimicry (Queenan et al., 1987). A subset of these proteinases and inhibitors (e.g., MMP-9, TIMP-3) is produced by human embryos *in vitro*, suggesting that they could function from implantation onward.

Chapter Two – The Maternal Immune Response to Pregnancy

Factors involved in maternal tolerance

Mammalian pregnancy necessitates that the maternal immune system, in defiance of all known laws of allo-transplantation, tolerates the presence of an hemi-allogeneic conceptus. This immunological paradox poses a compelling problem for biologists (for a current review see (Thellin et al., 2000)). Evidence suggests that this phenomenon is regulated by many systems acting at multiple levels.

Syncytiotrophoblasts and invasive cytotrophoblasts are presumed to be essential to maternal tolerance because they lie at the maternal-fetal interface (Figure 1-3), where they are in direct contact with cells of the maternal immune system. Several studies suggest that none of the trophoblast subpopulations express major histocomplatibility complex (MHC) class II molecules (Bulmer and Johnson, 1985; Redman, 1983), and that both cytotrophoblast stem cells and syncytiotrophoblasts lack MHC class I expression. In contrast, extravillous cytotrophoblasts increase expression of the nonclassical MHC class I protein HLA-G as they differentiate and invade the uterus (Ellis et al., 1990; Kovats et al., 1990; McMaster et al., 1998). This pattern of expression, together with the fact that HLA-G exhibits limited polymorphism

(Bainbridge et al., 1999), suggests it has an important function in maternal tolerance. However, the exact mechanisms involved remain enigmatic. One obstacle has been the identification of HLA-G receptor(s) on relevant cells, a controversial area (Bainbridge et al., 1999).

Although it is clear that immune recognition of paternally-derived antigens occurs during pregnancy (Tafuri et al., 1995), cytotoxicity against trophoblast must be inhibited. The factors responsible for this localized immunosuppression are unclear, but likely include cytotrophoblast-derived IL-10, a cytokine that inhibits alloresponses in mixed lymphocyte reactions (Roth et al., 1996). Interestingly, steroid hormones including progesterone can have similar effects (Pavia et al., 1979). Progesterone signaling to the thymus is also necessary during pregnancy. Such signals induce thymic involution and a consequent loss of T-cell precursors. If thymic involution is blocked in pregnant mice, fertility is impaired (Tibbetts et al., 1999).

The expression of macrophage migration inhibitory factor (MIF) by placental cytotrophoblasts may be another component contributing to maternal tolerance. MIF is expressed in other areas of immune privilege such as the anterior chamber of the eye (Wistow et al., 1993), where it inhibits the NK-mediated lysis of corneal epithelial cells

(Apte et al., 1998). This capacity to inhibit cytolysis by NK cells could be important at the maternal-fetal interface, where NK cells are the most abundant population of maternal leukocytes. Cytotrophoblast production and secretion of MIF has been demonstrated (Arcuri et al., 1999), but the functional effects of cytotrophoblast-derived MIF on NK cells have not been shown.

Similarly, the placenta is reported to express Fas ligand (Bamberger et al., 1997; Hunt et al., 1997), a molecule which induces apoptosis in target cells bearing the receptor Fas. This system is used by cytotoxic T cells and NK cells to kill targets, and is also maintains leukocyte homeostasis (for reviews see (Depraetere and Golstein, 1997; Ju et al., 1999)). Fas ligand expression has been widely touted as a mechanism for establishing immune privilege, and for this reason it has been studied in the context of maternal tolerance (reviewed in (Guller and LaChapelle, 1999)). Studies in both mouse and human describe Fas ligand production by the placenta (Runic et al., 1996), and the soluble form of this molecule is found in cytotrophoblast conditioned medium (Penelope Drake, unpublished data). Furthermore, mice genetically deficient in Fas ligand are reported to have multiple fertility problems, including leukocytic infiltrates at the site of implantation, and small litter sizes (Hunt et al., 1997).

Other factors that are likely involved in maternal tolerance include the complement system because deletion of the complement regulator, Crry, leads to fetal loss secondary to placental inflammation in mice (Xu et al., 2000). Additionally, pharmacological data, also from work in mice, suggest that trophoblasts express an enzyme, indoleamine 2,3-dioxygenase, that rapidly degrades tryptophan, which is essential for T-cell activation (Munn et al., 1998). Whether this same mechanism operates in humans is not yet known, although syncytiotrophoblasts express indoleamine 2,3-dioxygenase (Kamimura et al., 1991) and systemic tryptophan levels fall during pregnancy (Schrocksnadel et al., 1996).

Effects of the maternal immune system on pregnancy

An intact maternal immune system is not absolutely required for implantation and pregnancy maintenance although certain immune cells or their products may be necessary to achieve an optimal pregnancy. For example, lack of uterine NK cells is associated with placental and decidual defects and small litters of underweight pups in the mouse (Guimond et al., 1997; Guimond et al., 1998). Furthermore, it appears that uterine NK cells are involved in the remodeling of uterine arteries during pregnancy, a

critical requirement for optimal blood flow to the placenta (Croy et al., 2000). However, women with agammaglobulinemia can reproduce, and studies in SCID (severe combined immunodeficient) mice suggest that T lymphocyte-derived cytokines are not necessary for successful pregnancy (Croy and Chapeau, 1990). Moreover, genetically identical mice have been bred for generations refuting the notion that HLA antigen dissimilarity is necessary for reproduction. This notion is further weakened by genetic analyses of these antigens in couples who experience recurrent spontaneous abortions (Jin et al., 1995).

Immunological causes have been implicated in up to 60 percent of women with recurrent pregnancy loss (Hill, 1994). These include abnormalities in both humoral and cellular immunity. Antiphospholipid autoantibodies are associated with recurrent pregnancy loss (Yasuda et al., 1995), but the mechanism responsible for adverse fetal outcome in such women is poorly understood. Although more controversial, an association between antisperm antibodies and recurrent abortion has been noted (Witkin and Chaudhry, 1989). The hypothesis that antitrophoblast antibodies may be involved in reproductive failure has also been proposed, but not substantiated. In a subset of women, recurrent abortion may result from abnormal maternal T_H1-type cellular immune response involving the cytokines, interferon-γ and tumor necrosis factor, which are toxic

Chapter Three – The Immune Compartments of the Placenta

Part One: Decidual granulated leukocytes

Phenotype and distribution

From an immunologic perspective, one of the most interesting populations of uterine cells are decidual granulated leukocytes (DGLs). Although estimates vary, a minimum of 10 to 15 percent of all cells found in the decidua are leukocytes, many of which are found in large lymphoid clusters (Mincheva-Nilsson et al., 1994). DGLs are comprised predominantly (~70%) of specialized ($CD56^{bright}/CD16^{-}$) natural killer (NK) cells, monocytes (~15%) and T cells (~15%), which include equal ratios of CD4 and CD8 cells (Fig. 3-1) (Bulmer et al., 1991b; King et al., 1989b). The pregnant uterus represents one of the only known sites where NK cells preferentially concentrate *in vivo*. However, this phenomenon is not unique to humans; uterine NK cell populations have also been described in mouse (Croy and Kiso, 1993; Croy et al., 1996; Head, 1996), rat and pig (Engelhardt et al., 1997; Yu et al., 1993; Yu et al., 1994).

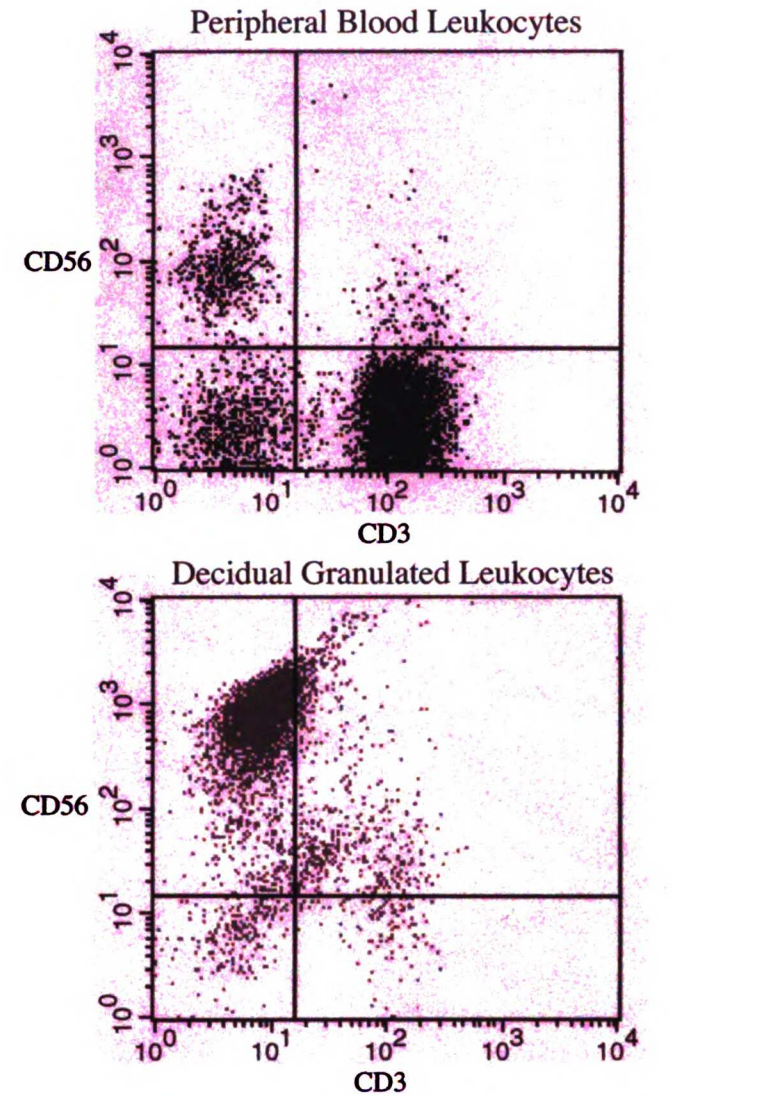

Peripheral Blood Leukocytes
CD56
CD3
Decidual Granulated Leukocytes
CD56
CD3
Decidual stroma
Gland
DGLs

Figure 3-1

Figure 3-1. Comparison of CD3 and CD56 Expression on Peripheral Blood Mononuclear Cells (PBMCs) and Decidual Granulated Leukocytes (DGLs). Isolated PBMCs (Top panel) and DGLs (Middle panel) were labeled with fluorescence-labeled antibodies and analyzed by flow cytometry. Note the shift from a predominant CD3+ T cell population in the blood, to a predominantly CD56+ bright NK cell population in the uterus. The bottom panel shows DGLs *in situ*. As shown, they commonly form aggregates and are frequently found near glands. (Penelope Drake, unpublished data).

Like invasive cytotrophoblasts, these maternal leukocytes have unusual properties. The decidual NK cells are distinct from the average peripheral blood NK cell. Specifically, decidual NK cells express the antigen CD56 at a very high level on the cell surface, and they are thus designated $CD56^{bright}$ cells, along with 10% of peripheral blood NK cells that share this phenotype (Lanier et al., 1986). Conversely, 90% of all peripheral blood NK cells express low cell surface levels of CD56, and are referred to as $CD56^{dim}$. Other phenotypic differences between these populations of NK cells include the expression of CD16 and CD57 on $CD56^{dim}$ cells and their absence on $CD56^{bright}$ cells. In the expression of these three antigens, decidual NK cells are indistinguishable from the peripheral blood $CD56^{bright}$ population (reviewed in (King and Loke, 1991)).

Decidual NK cells and peripheral blood $CD56^{bright}$ cells share other similarities, among them the constitutive expression of the high-affinity IL-2 receptor, and responsiveness to low concentrations of IL-2 (Saito et al., 1996) (Nagler et al., 1989; Nagler et al., 1990; Nishikawa et al., 1991). Both of these cell populations have a high capacity for proliferation and a low capacity for cell killing as compared to $CD56^{dim}$ NK cells (Ferry et al., 1990; Nagler et al., 1989). However, it is not known whether the pool of peripheral blood $CD56^{bright}$ cells represents the source of decidual NK cells, or whether

the decidual NK cells emigrate as a different, perhaps less differentiated cell, and adopt the decidual NK cell phenotype *in situ*. Little is known about the function of the peripheral $CD56^{bright}$ NK cells or their relationship to $CD56^{dim}$ peripheral NK cells, although it is postulated that $CD56^{bright}$ cells are immature precursors to $CD56^{dim}$ cells (Nagler et al., 1989).

Surface antigens expressed by decidual NK cells suggest the cells are activated. They express CD49a (VLA-1), VLA-4, PECAM, increased levels of CD54 (I-CAM) and decreased levels of CD45RA, accompanied by increased CD45RO (Burrows et al., 1993; Burrows et al., 1995; Geiselhart et al., 1995; Haynes et al., 1997; Marzusch et al., 1993; Mincheva-Nilsson et al., 1992; Ruck et al., 1994). Additionally, decidual NK cells and T cells express CD69 (Nishikawa et al., 1991), the transferrin receptor (CD71) and Fas (Penelope Drake, unpublished data. See Figs. 3-2 and 3-3). Furthermore, the cells express an arsenal of cytotoxic molecules that are also useful as markers of activation (Griffiths and Mueller, 1991). Among these, decidual NK cells contain perforin, granzyme A and TIA-1 (T cell intracellular antigen-1) (Gulan et al., 1997; King et al., 1993; Lin et al., 1991; Rukavina et al., 1995). The expression of these factors indicates that the cell is equipped for killing, and raises the obvious question—what prevents a cytolytic attack by maternal leukocytes against fetal trophoblasts?

NK cells will preferentially kill a cell that lacks surface major histocompatibility (MHC) class I proteins, but engagement of an inhibitory receptor on the NK cell by a class I molecule on the target cell prevents this killing (reviewed in (Lanier, 1998; Lanier et al., 1997; Long, 1999)). Inhibitory receptors, widely expressed on peripheral NK cells and CD8+ T cells, are also found on decidual leukocytes. In the human, there are three class of inhibitory receptors, the KIR (killer immunoglobluin receptor) and ILT (immunoglobulin-like transcript) families, which are part of the immunoglobulin superfamily, and the CD94/NKG2 family of lectin-like molecules (reviewed in (López-Botet et al., 2000)). Although the extracellular domains of these inhibitory molecules differ, they all employ MHC class I molecules as ligands, and their intracellular domains contain immunoreceptor tyrosine-based inhibition motifs (ITIMs), which are responsible for propagation of the inhibitory signal within the cell. As is characteristic of the complex and balancing nature of the immune system, inhibitory receptor families also contain activating members whose effect is to enhance cell killing. In place of the ITIM, the intracellular domain of activating receptors contains an ITAM (immunoreceptor tyrosine-based activation motif) sequence that mediates downstream signaling. Decidual NK cells and T cells express members of each class (KIR, ILT and CD94/NKG2) of inhibitory molecules and their activating counterparts (Figure 3-4)

(Biassoni et al., 1999; Hiby et al., 1997; Miki et al., 1998; Verma et al., 1997). The functional implications of this expression will be discussed later in this chapter.

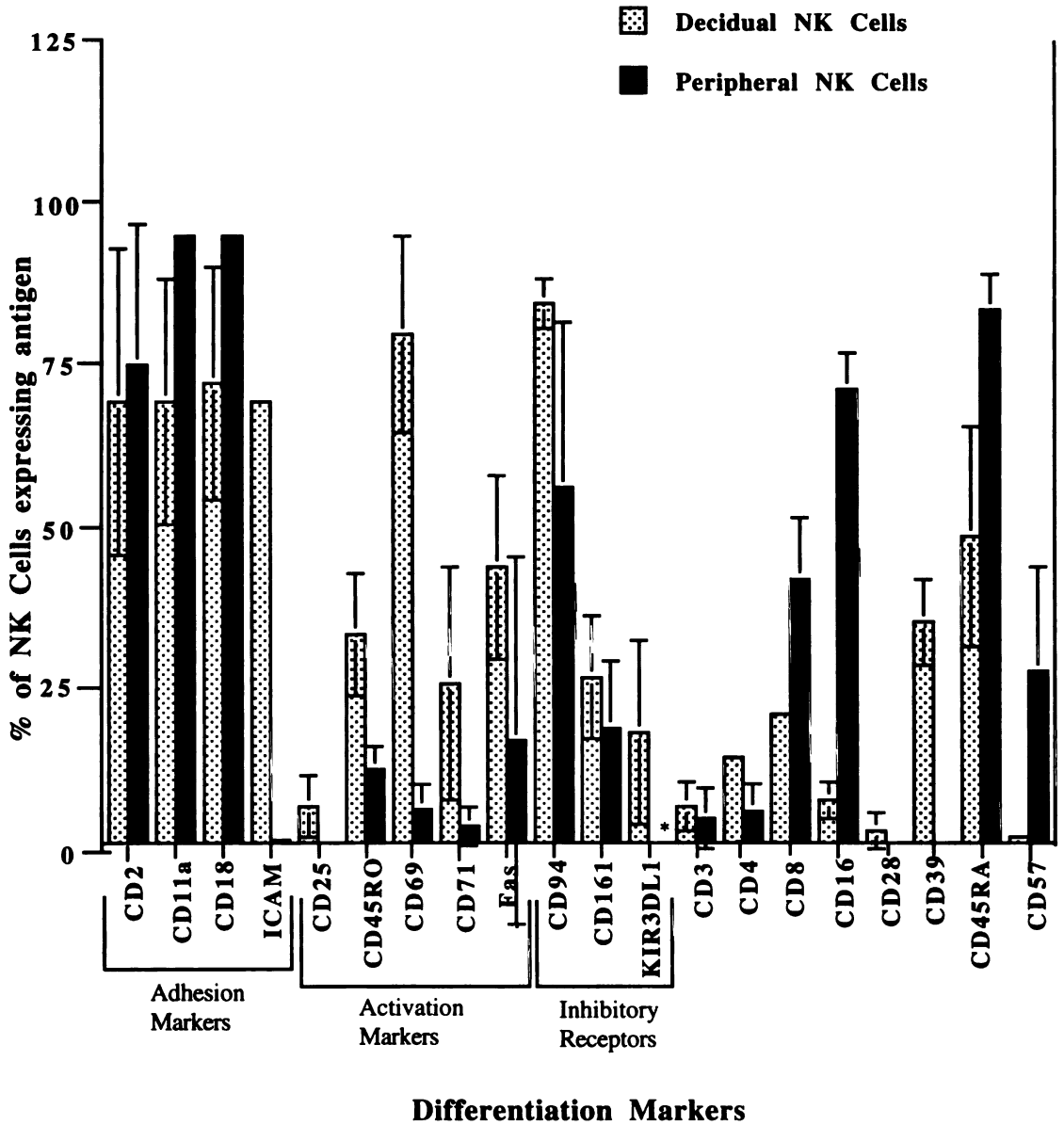

Decidual NK Cells
Peripheral NK Cells
% of NK Cells expressing antigen
125
100
75
50
25
0
CD2
CD11a
CD18
ICAM
CD25
CD45RO
CD69
CD71
Fas
CD94
CD161
KIR3DL1
CD3
CD4
CD8
CD16
CD28
CD39
CD45RA
CD57
Adhesion Markers
Activation Markers
Inhibitory Receptors
Differentiation Markers

Figure 3-2

Figure 3-2. Summary of the Phenotype of CD56+ Decidual NK Cells. Isolated DGLs were labeled with fluorescently-conjugated antibodies and analyzed by flow cytometry. Expression of CD markers was compared to that of peripheral blood NK cells. For DGLs, n = from 1 to 6, depending on the marker, with the mean value of n = 3.4. For PBMCs, n = from 2 to 4, with the mean value of n = 3.7. Error bars represent the standard deviation (Penelope Drake, unpublished data).

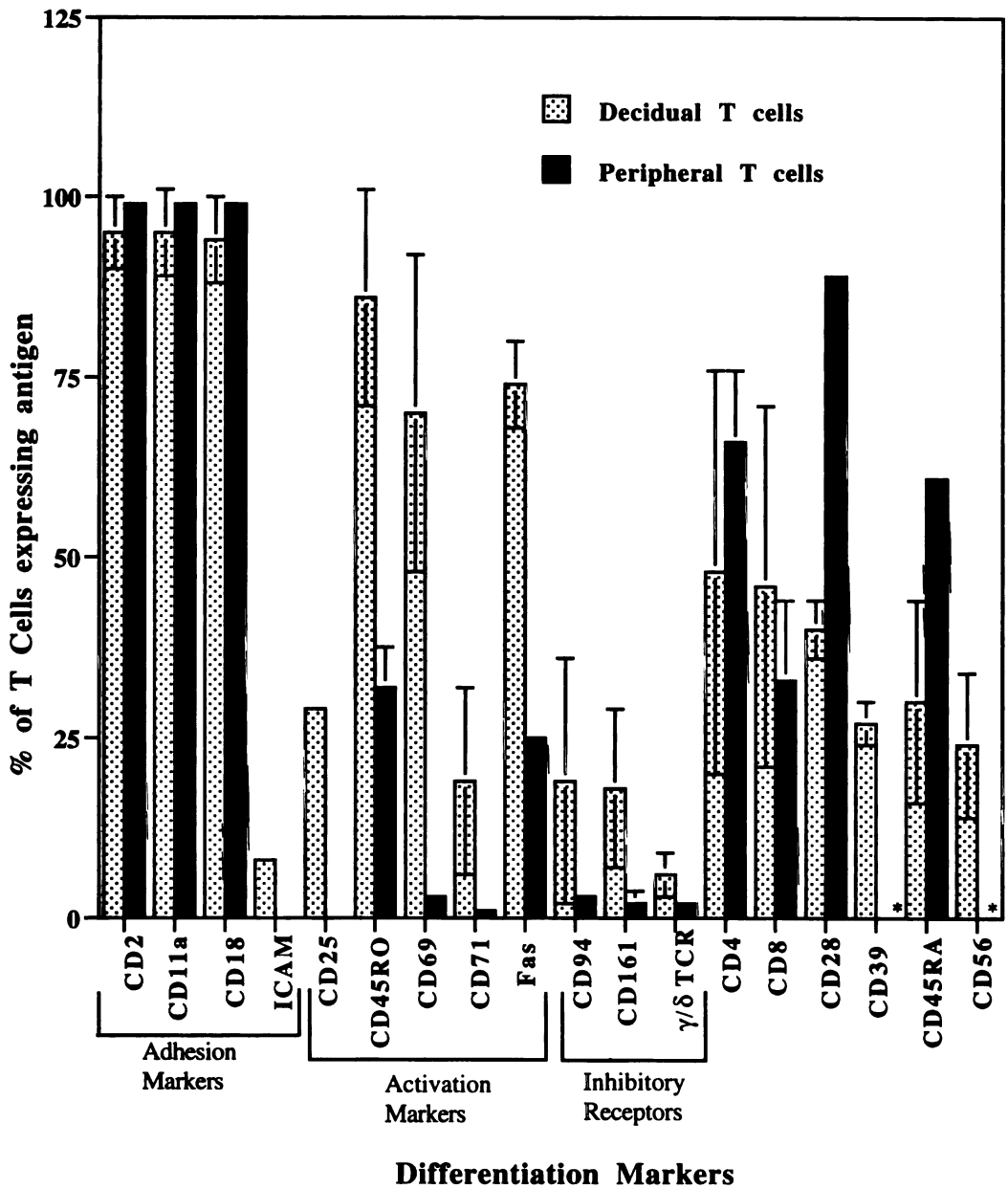

125
100
75
50
25
0
% of T Cells expressing antigen
Decidual T cells
Peripheral T cells
CD2
CD11a
CD18
ICAM
CD25
CD45RO
CD69
CD71
Fas
CD94
CD161
γ/δ TCR
CD4
CD8
CD28
CD39
CD45RA
CD56
Adhesion Markers
Activation Markers
Inhibitory Receptors
Differentiation Markers

Figure 3-3

Figure 3-3. Summary of the Phenotype of CD3+ Decidual T Cells. Isolated DGLs were labeled with fluorescently-conjugated antibodies and analyzed by flow cytometry. Expression of CD markers was compared to that of peripheral blood T cells. For DGLs, n = from 1 to 6, depending on the marker, with the mean value of n = 3. For PBMCs, n = from 1 to 3, with the mean value of n = 1.8. Error bars represent the standard deviation (Penelope Drake, unpublished data).

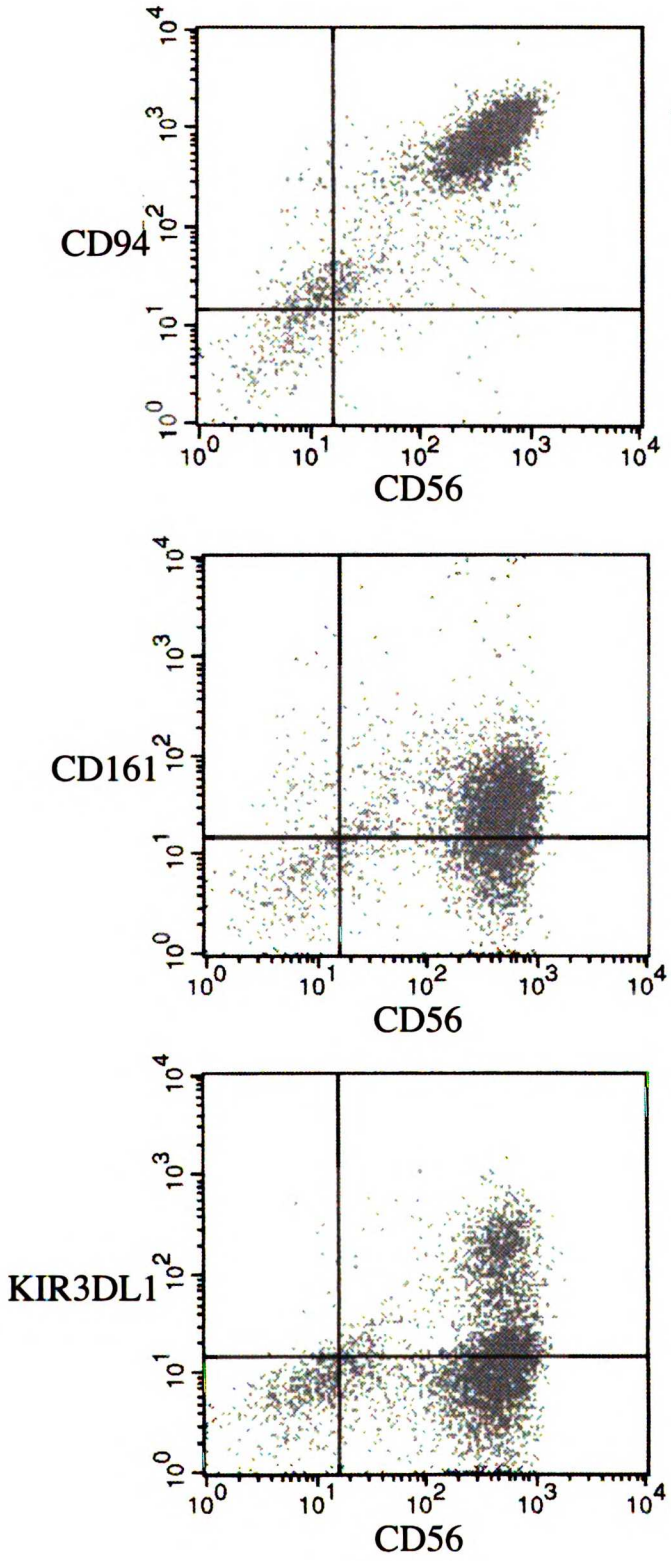


Figure 3-4

Figure 3-4. CD56+ Decidual Leukocytes Express Inhibitory Receptors. Isolated DGLs were labeled with fluorescently-conjugated antibodies recognizing specific inhibitory receptors and analyzed by flow cytometry. CD94 (Top panel) and CD161 (Middle panel) are members of the lectin-like inhibitory receptors. KIR3DL1 (Bottom panel) is a member of the killer immunoglobluin receptor family, part of the immunoglobulin superfamily (Penelope Drake, unpublished data).

Function

The *in vivo* function of human uterine NK cells is unknown, although in mouse, their absence has been associated with the lack of metrial glands (a specialized region of murine decidua), small placentas and small litter sizes (Guimond et al., 1997; Guimond et al., 1998). In the context of the paradoxical phenomenon of maternal immune tolerance, much investigation has been made into the cytotoxic capabilities of decidual leukocytes. . Most of these studies have used *in vitro* chromium release assays to compare the killing of various target cell lines by decidual leukocytes and peripheral blood leukocytes. Of note is the difficulty of interpreting the results of these experiments, due to failure to incorporate appropriate controls. For example, many studies fail to analyze non-trophoblasts adherent targets in parallel.

In general, these studies show that decidual leukocytes are capable of inducing lysis in NK-sensitive target cells, albeit at a lower efficiency than peripheral blood leukocytes achieve against the same targets (Ferry et al., 1990). Pre-incubation with IL-2 increases the cytotoxicity of decidual leukocytes (Ferry et al., 1991; King and Loke, 1990; Manaseki and Searle, 1989; Saito et al., 1993). Target cells that are subject to lysis by the latter cells include the K562 and .221 lymphoblastoid cell lines, which lacks

endogenous MHC class I expression. Transfection of class I expressing plasmids into .221 cells confers protection from lysis by NK cells, including decidual NK cells, by allowing the NK cells' inhibitory receptors to bind their class I ligands.

One important unanswered question concerning maternal tolerance and the function of decidual leukocytes is the role played by HLA-G, a non-classical MHC class I protein (see Figs. 3-5 and 3-6 for MHC structure and processing). HLA-G is a pregnancy-associated class I molecule whose protein expression is limited to fetal cells of the placenta—specifically, to extravillous cytotrophoblasts in the uterus and to both amniocytes and extravillous cytotrophoblasts of the chorion in the amnion-chorion (Chumbley et al., 1993; Ellis et al., 1990; Kovats et al., 1990; McMaster et al., 1998; McMaster et al., 1995; Yelavarthi et al., 1991). The only other known site of HLA-G protein expression in normal tissues is in a subpopulation of thymic epithelial cells (Crisa et al., 1997). Reports of extravillous cytotrophoblasts expressing HLA-C and HLA-E remain controversial(King et al., 2000a; King et al., 1996a; King et al., 2000b; Pröll et al., 1999). Given this conspicuous expression of HLA-G, accompanied by the lack of other placental HLA proteins an attractive model for maternal tolerance invokes the interaction of HLA-G with inhibitory receptors to prevent cytolytic attack by NK cells. Simultaneously, the limited polymorphism of HLA-G suggests the possibility that it acts

as a universally recognized self molecule that would not trigger a T-cell mediated attack. In this regard, the possibility that certain tumor cells express HLA-G is under intense investigation. Thus far, expression of this class Ib molecule has been detected in human melanoma cells (Paul et al., 1998).

Although pieces of this puzzle are in place, the big picture remains obscure. Transfection of .221 cells with HLA-G can protect the target from NK lysis, by both direct and indirect means (Allan et al., 1999; King et al., 1993) reviewed in (López-Botet et al., 2000). The indirect protection (demonstrated in .221 cells) is rendered through HLA-E, another non-classical class I protein. This molecule, which is constitutively expressed, binds the HLA-G leader sequence and is transported to the cell surface. In this location, HLA-E interacts with the inhibitory receptor CD94/NKG2a, which is expressed on nearly all NK cells, including the decidual population.

HLA-G can also protect a target cell from lysis by directly interacting with inhibitory receptors. Among the receptors currently known to directly engage HLA-G are ILT2 (LIR-1), ILT4 (LIR-2) and KIR2DL4. Through interaction with these receptors, the placental specific class I molecule is able to decrease the cytotoxicity of effector cells in *in vitro* assays. Much has been made of the possibility that it plays a

similar protective role *in vivo*, downregulating the hostile advances of maternal leukocytes against fetal tissues. The spatial and temporal distribution of HLA-G supports this *in vivo* role, as does the distribution of HLA-G compatible inhibitory receptors (reviewed in (Lanier, 2000)). ILT2 (LIR1) is expressed on subsets of NK cells (including decidual NK cells), T cells, B cells, monocytes and macrophages, while ILT4 is expressed only on monocytes, tissue macrophages and dendritic cells (reviewed in (López-Botet et al., 2000)).

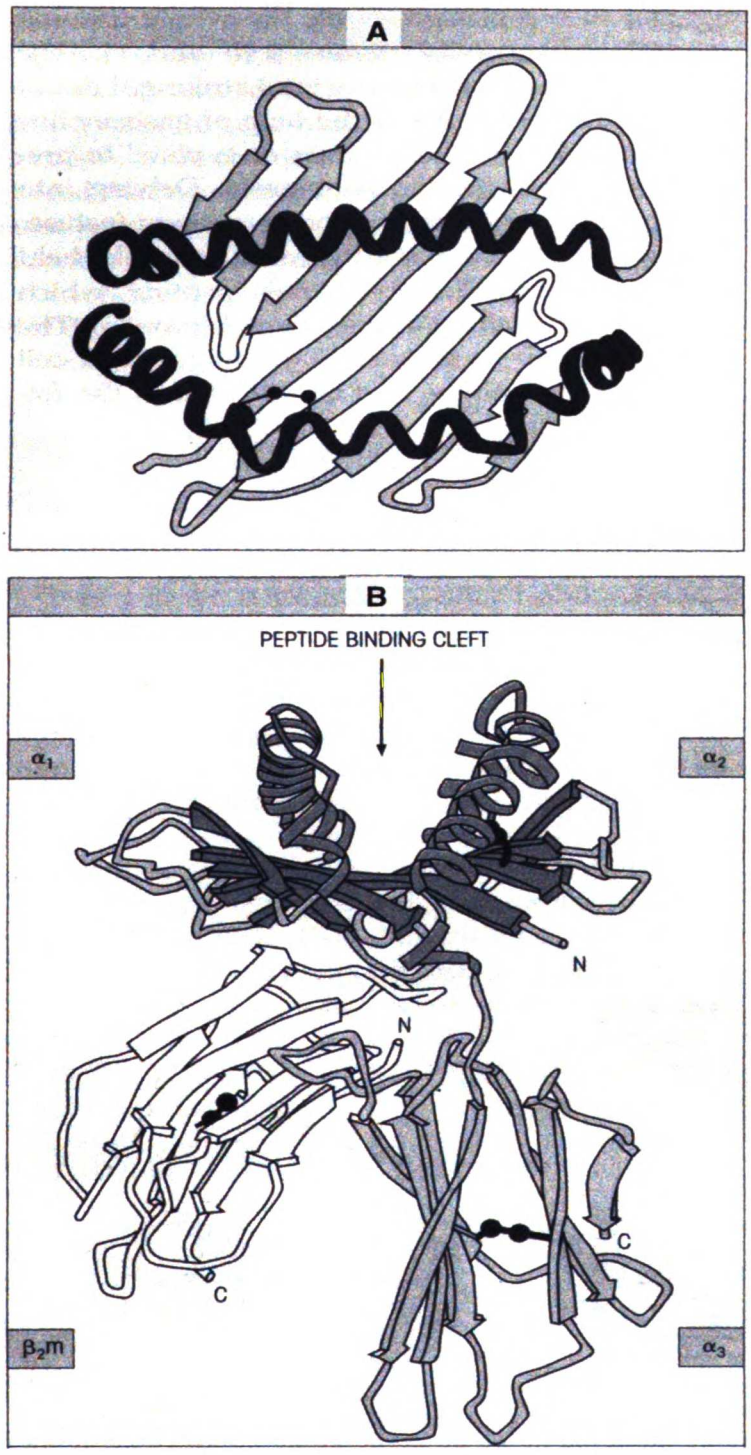


Figure 3-5

Figure 3-5. Structure of an MHC Class I Molecule. The molecule is composed of a heavy chain with three globular domains (α_1, α_2, α_3), non-covalently complexed with a smaller globular protein, β_2 microglobulin. The membrane-proximal portions (α_3 and β_2 microglobulin) are classic Ig domains, while the distal globular segments (α_1, α_2) come together as α-helices on a β-pleated sheet. A groove is formed between the α-helices, and the molecule complexes with short (~9 residues) peptides, which bind this pocket. Peptide binding is required to stabilize the molecules before transport to the cell surface. The peptide binding groove is demonstrated from the superior view (A) and the lateral view (B). (Adapted from Roitt 8th Ed.)

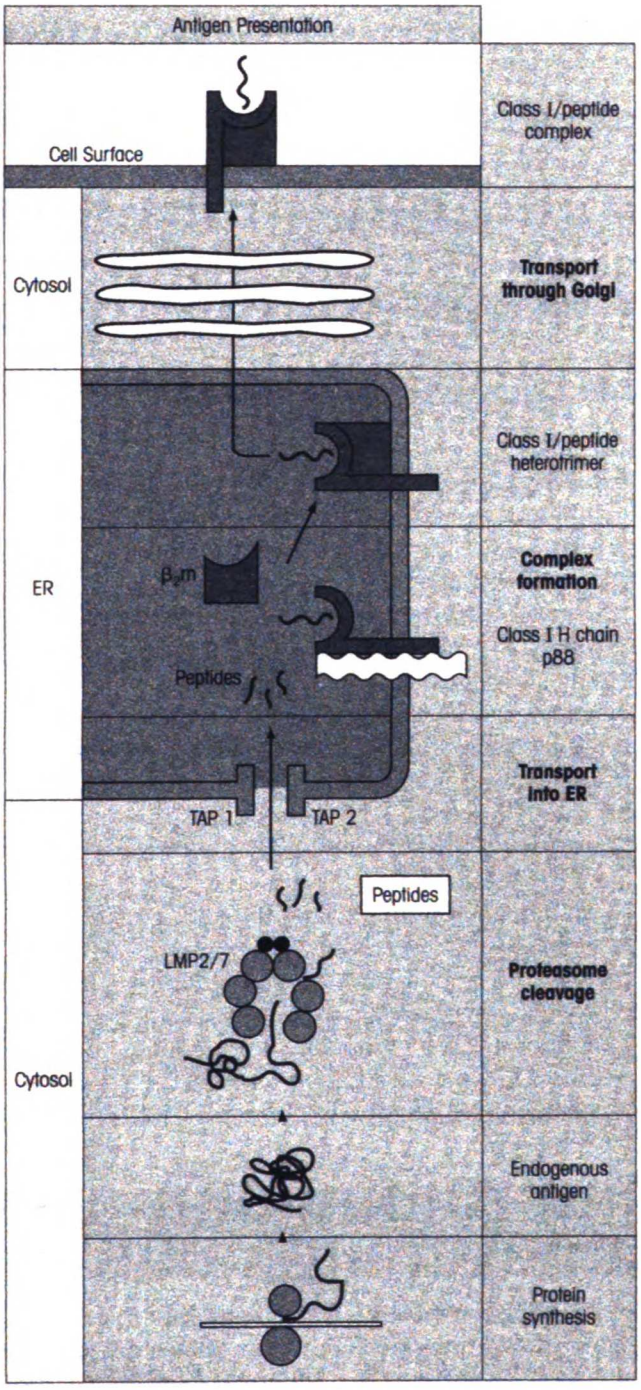

Antigen Presentation
Cell Surface
Class I/peptide complex
Cytosol
Transport through Golgi
Class I/peptide heterotrimer
ER
β_2m
Complex formation
Class I H chain p88
Peptides
Transport into ER
TAP 1
TAP 2
Peptides
LMP2/7
Proteasome cleavage
Cytosol
Endogenous antigen
Protein synthesis

Figure 3-6

Figure 3-6. Processing and Peptide Loading of an MHC Class I Molecule. Proteins in the cytoplasm are degraded into peptides by the proteasome. The peptides are then translocated into the endoplasmic reticulum (ER) by TAP1/TAP2 membrane transporters. In the ER, the association of MHC class I heavy chain with β_2 microglobulin and peptide is coordinated by multiple chaperone proteins. After the MHC is fully assembled, it is transported to the membrane for antigen presentation on the cell surface. (Adapted from Roitt 8th Ed.)

The distribution of KIR2DL4 is somewhat controversial, with one report that it is exclusively expressed on decidual NK cells and another that it is expressed on all NK cells (reviewed in (López-Botet et al., 2000)). The functional importance of both KIR2DL4 and ILT2 expression remain in question, as HLA-G tetramers bind to peripheral blood monocytes (predominately via ILT4) but not NK cells (Allan et al., 1999). However, as these tetramers were made using HLA-G expressed in *E. coli* they lack carbohydrate structures that may be relevant to binding *in vivo.* Primary cytotrophoblasts modify HLA-G by adding a large polylactosamine chain (McMaster et al., 1998). This is in contrast to simple biantennary structures carried on most class I molecules, including HLA-G produced by cell lines. The unusual glycosylation on endogenously produced HLA-G may change its binding properties—one explanation for the failure to detect interactions between inappropriately glycoslyated tetramers and NK cells. Additionally, the effect of polylactosaminylation on peptide loading has yet to be considered.

Furthermore, expression of KIR2DL4 in some, but not all, NK cell lines inhibits lysis of HLA-G bearing target cells (Rajagopalan and Long, 1999). Thus, the cellular context in which the HLA-G/KIR2DL4 interaction occurs is important. This phenomenon could be related to the unusually short intracytoplasmic domain of the KIR,

which contains only one, as opposed to the usual two ITIM motifs. KIR2DL4 also contains a charged residue within its transmembrane domain, a characteristic feature of the activating members of the KIR family (reviewed in (López-Botet et al., 2000)). As the activating receptors lack intrinsic signaling domains, this charged residue facilitates interactions with other membrane proteins that effect downstream signaling. An interaction of this sort between KIR2DL4 and a second signaling molecule could have profound implications for the function of HLA-G and decidual NK cells. These pathways, if they exist, have yet to be elucidated (Biassoni et al., 1999; Colonna et al., 1998).

In conclusion, the alluring hypothesis that HLA-G interactions with inhibitory receptors on decidual leukocytes results in maternal tolerance is still only a theory. Among the remaining questions to be directly addressed is the protective role of HLA-G expressed on cytotrophoblast cells. In vitro, cytotrophoblasts are resistant to NK-cell lysis (Ferry et al., 1991; King et al., 1989a; King and Loke, 1990), but thus far HLA-G has not been directly linked to this protection.

Recruitment

The recruitment of DGLs to the uterus has long been a black box, although by analogy to other systems, chemokines—molecules involved in leukocyte trafficking—are likely to be important. To our knowledge, this thesis is the first study to identify both a panel of chemokines expressed in the decidual stroma and a concurrent array of chemokine receptors expressed by DGLs (Chapter Seven). Cognate chemokine receptor-ligand pairs suggest functional roles for these molecules in leukocyte homing to the pregnant uterus. Implications of these studies and of the role of chemokines in DGL recruitment are further discussed in Chapters Four and Seven.

Leukocyte trafficking to the uterus is not limited to pregnancy, for example, the composition and abundance of leukocytes in the non-pregnant uterus changes over the course of the menstrual cycle (Bulmer et al., 1991b; Kamat and Isaacson, 1987; King et al., 1989b).(See Fig. 3-7 for a summary of the cycle). In terms of abundance, during the proliferative phase, leukocytes account for ~8% of all endometrial cells. This number increases to ~23% in late secretory endometrium (Bulmer et al., 1991b). In terms of composition, the leukocytes that populate non-pregnant endometrium are distinct from the decidual leukocyte population (Fig. 3-8). In proliferative and early secretory

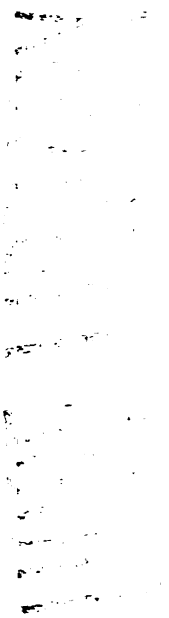
endometrium, CD3+ T cells comprise nearly half and CD14+ macrophages one-third of the total leukocyte population. CD56+ NK cells account for less than half of the total endometrial leukocytes.

During the late secretory phase (and continuing into the first trimester of pregnancy, if pregnancy is established) the proportion of leukocytes in the stroma increases. Concomitant with this increase in total cell number is a rise in the number of CD56+ NK cells, such that these cells comprise ~57% of uterine leukocytes in the late secretory phase and ~75% of decidual leukocytes during early pregnancy (Bulmer et al., 1991b). The total number of T cells remains the same during the expansion of the leukocyte compartment; thus reducing the contribution of T cells to ~20% of all leukocytes in the late secretory phase and ~15% of decidual leukocytes in the first trimester of pregnancy. Similarly, the macrophage contribution diminishes to ~26% of cells in the late secretory phase and ~15% of decidual leukocytes (Bulmer et al., 1991b). If pregnancy is not established, neutrophils infiltrate the uterus at the onset of menses, presumably to act as scavengers and prevent infection (Poropatich et al., 1987). Of note is the fact that both the leukocytes and endometrial cells are lost during menstruation, thus each ensuing endometrial proliferation entails leukocyte recruitment.

Several mechanisms likely account for the recruitment and retention of specific leukocyte populations within the uterine wall. With regard to menstruation, founder cells have to be recruited to the endometrium and replaced. Expansion of particular sub-populations is also likely to play a role. Evidence suggests that at least some of the increase in uterine NK cells during the menstrual cycle and early pregnancy is due to proliferation of these cells *in situ;* they express the proliferation marker Ki67 (King et al., 1991; Pace et al., 1989). However, the extent to which mitosis accounts for this increase in cell number is not known.

The tight coordination of the endometrial/decidual leukocyte cell populations with hormonal status suggests that the latter molecules play a role in regulating leukocyte recruitment to the uterus. This hypothesis is supported by several observations. First, recruitment of decidual leukocytes may occur normally with an ectopic pregnancy (Stewart-Akers et al., 1997; Vassiliadou and Bulmer, 1998). This implies that systemic signals of pregnancy are sufficient to induce leukocyte infiltration and proliferation. Second, studies of ovariectomized women demonstrated that exogenous administration of both estrogen and progesterone are required for CD56+ leukocytes to localize to the decidua (Loke, 1995). Additionally, withdrawal of progesterone appears to be involved

in the influx of neutrophils and monocytes that precedes menses (Critchley et al., 1996; Staples, 1983).



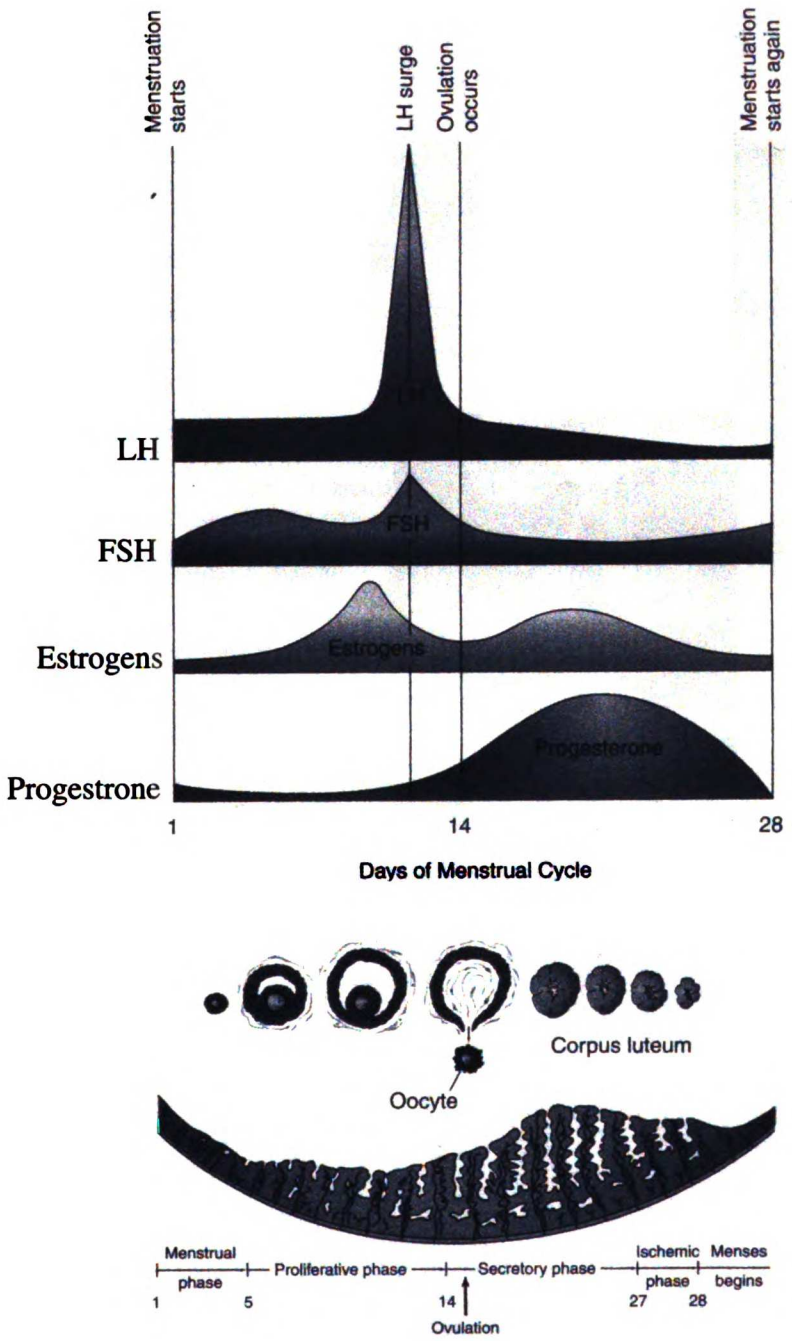


Figure 3-7

Figure 3-7. Summary of Events in the Menstrual Cycle. The fluctuations in blood levels of various hormones are correlated with the development of the ooycte and the maturation of the uterine lining in preparation for fertilization and implantation. Note that estrogens rise in the early (proliferative) phase of the cycle, followed by an LH surge which signals ovulation. Subsequently, during the late (secretory) phase of the cycle progesterone levels rise. If fertilization does not occur, estrogen and progesterone levels fall, leading to the sloughing of the uterine lining and menstruation. (Adapted from Moore and Persaud 6th Ed.)

Leukocyte subsets as a proportion of the total leukocyte population

Antigen	Mean % of CD45-positive population			
	Proliferative	Early secretory	Late secretory	Early pregnancy
CD38	52.3	45.6	61.3	75.4
CD56	48.2	47.4	57.2	79.4
CD2	54.8	56.7	52.4	57.2
CD3	45.4	37.5	21.5	18.9
CD8	28.5	24.0	14.9	15.7
CD16	3.4	2.6	1.9	1.4
CD14	33.6	27.2	26.4	37.9
Class II MHC	50.1	41.9	40.2	46.7

Figure 3-8

Figure 3-8. Changes in the Composition of Uterine Leukocytes Throughout the Menstrual Cycle. Decidual NK cells, demonstrated in this figure as CD56+ and CD38+ cells, dominate the CD45+ decidual granulated leukocyte compartment at the beginning of pregnancy. The decidual NK cell prominence begins during the late secretory phase of the menstrual cycle, when NK cell numbers rise concomitant with a drop in T cells and macrophages. The resulting decidual leukocyte population is very distinct from that occuring earlier in the menstrual cycle, during the proliferative and early secretory phases, which is characterized by large T cell and macrophage components. (Adapted from Bulmer, et al. 1991)

As leukocytes themselves lack hormone receptors (Bulmer et al., 1991a; King et al., 1996b; Loke, 1995), their responses to estrogen and progesterone levels must be mediated indirectly. The most likely intermediaries to coordinate leukocyte recruitment with the hormonal cycle are chemokines. Chemokines are known regulators of leukocyte trafficking, and their expression is influenced by a number of factors. Although we are only beginning to explore the role of chemokines in the uterus and placental bed, initial results already link chemokine expression to the menstrual cycle. In particular, the expression of IL-8 and MCP-1 in the endometrium is regulated during the menstrual cycle (reviewed in (García-Velasco and Arici, 1999)). IL-8 is expressed in smooth muscle cells surrounding blood vessels, in glands and in lumenal epithelia; levels peak during the early to mid-proliferative phase and again during the late secretory phase (Jones et al., 1997; Milne et al., 1999). MCP-1 has a similar expression pattern, but levels increase in the premenstrual phase and peak in the menstrual endometrium. Evidence that these changes in MCP-1 and IL-8 expression are due to fluctuations in hormone levels is provided by studies demonstrating that *in vivo* withdrawal of progesterone by administration of mifepristone (RU486) to women prior to vacuum aspiration increases the expression of IL-8 and MCP-1 in the decidua. *In vitro* studies on

endometrial explants and cell lines show similar effects (Critchley et al., 1999; Critchley et al., 1996; Kelly et al., 1997; Kelly et al., 1994).

It should be noted that the cycling endometrium also modulates the expression of adhesion molecules used by leukocytes to effect cell trafficking. The expression patterns of I-CAM-1, PECAM, VCAM-1 and E-selectin vary throughout the menstrual cycle (Tabibzadeh et al., 1994; Tawia et al., 1993). Thus, the means of leukocyte recruitment to the uterus are modulated both at the level of chemotactic factors and adhesion receptors, implying coordination of signals that regulate this process. Comparing the expression patterns of these factors in the decidua to the few other tissues in which NK cells are known to concentrate—most notably the murine liver in response to mCMV infection, and early in allografted tissues (Nemlander et al., 1983; Salazar-Mather et al., 2000) —might reveal commonalities in chemokine and adhesion molecule regulation that could be linked to NK cell recruitment.

This thesis greatly expands what is known about the expression of chemokines in the pregnant uterus (Chapter Seven). In addition, it provides the first evidence that placental interactions with the uterus influence the recruitment and retention of decidual leukocytes. Specifically, the chemokine MIP-1α is produced by isolated cytotrophoblasts

in vitro and by extravillous cytotrophoblasts *in vivo* (Chapter Six). MIP-1α can effect the migration of NK cells, monocytes and T cells *in vitro*, and cytotrophoblasts may use it to regulate the movement of leukocytes on a local level. Together, the results of these studies suggest that integration of chemokine and chemokine receptor networks at the maternal-fetal interface are a key component of the immunological mechanisms that operate in this unique environment.

Part Two: Hofbauer cells

The second population of immune cells in the basal plate is a cohort of macrophages designated Hofbauer cells (reviewed in (Benirschke, 2000)). These cells are confined to the fetal compartment of the placenta, residing in the stroma of placental villi (Figure 6-1). Their arrangement and number is similar to the specialized macrophage population found in the liver, namely Kupffer's cells. As the dominant population of leukocytes within this tissue (mast cells are also seen on occasion), the function of Hofbauer cells is intriguing and still unknown. Hofbauer cells are first seen in the villi on day 18 post-coitus and remain until the end of pregnancy (Boyd, 1970). They are pleomorphic cells with many vacuoles and a granulated cytoplasm. The origin of Hofbauer cells is not known, though it is generally believed that they are fetal-derived cells perhaps arising from stromal cells within the villus core. It has also been proposed that Hofbauer cells may have different origins throughout gestation and thus may actually represent a heterogenous population of cells (reviewed in (Benirschke, 2000)).

Although nothing is known about the role of Hofbauer cells in pregnancy, descriptions of their capabilities and antigen expression shed light on their possible *in vivo* actions. Hofbauer cells can divide both *in vivo* and *in vitro* (Castellucci et al., 1987;

Frauli and Ludwig, 1987). They express CD4 (Goldstein et al., 1988; Lairmore et al., 1993; Nakamura and Ohta, 1990), CD14 (Bulmer, 1984; Zaccheo et al., 1989), Fc receptors (Goldstein et al., 1988; Oliveira et al., 1986), and MHC class I and II on their cell surface (Bulmer et al., 1988; Sutton et al., 1986; Uren and Boyle, 1985), and secrete the cytokines IFN-α, IFN-β and IL-1 (Bulmer et al., 1990; Flynn et al., 1985; Glover et al., 1987; Howatson et al., 1988; Toth et al., 1991). They are phagocytic cells, capable *in vivo* of ingesting material surrounding them in the villus stroma. Together, these qualities suggest the potential for Hofbauer cells to monitor their environment and to initiate an immune response against pathogens.

The migration patterns of Hobauer cells further support this idea (reviewed in (Benirschke, 2000)). During the first half of pregnancy Hofbauer cells reside predominantly inside collagen-free intercommunicating stromal channels within the villus core (Castellucci, 1984; Castellucci, 1980; Castellucci, 1982). These channels, which have been likened to a lymphatic system, run parallel to the long axis of the villus and communicate with channels from neighboring villi. Evidence that Hofbauer cells move from channel to channel, and between channels and the surrounding stroma, led to the hypothesis that these macrophages might participate in host defense, or perhaps in remodeling of the villus core during placental development (Castellucci, 1980). Finally,

an additional role proposed for Hofbauer cells is the regulation of cytotrophoblast growth and differentiation along the syncytial pathway, as suggested by *in vitro* experiments combining Hofbauer cell conditioned medium and isolated cytotrophoblasts (Khan et al., 2000).

Unfortunately, little else is known about these interesting cells. The work presented here further underscores their importance, as many of the chemokines expressed within the villus stroma act on macrophages, and may be involved in recruiting and regulating the actions of these cells. Furthermore, as macrophages also produce many of the chemokines localized to the villus stroma, Hofbauer cells are a likely source of this abundant expression.

Chapter Four – Chemokines

Chemokines as chemoattractants

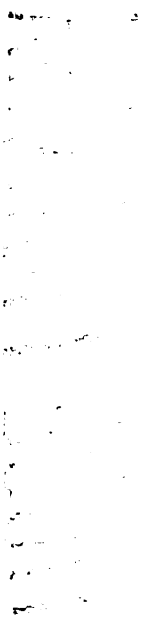
Members of the cytokine superfamily, chemokines (short for chemoattractant cytokine) are small, secreted proteins. As their name suggests, chemokines are capable of inducing the directed migration of cells bearing the appropriate target, one of a family of seven transmembrane-spanning G-protein coupled receptors. Although their sphere of influence is probably broader, chemokines are best understood for their role in leukocyte trafficking—in situations of inflammation and in homeostatic migration throughout the body.

Among the first chemokines to be described were protoypes of three of the major subclasses of this group, IP-10 (Luster et al., 1985), IL-8 (Yoshimura et al., 1987) and MCP-1 (Matsushima et al., 1989). Distinctions between these molecules and the subsets they represent are based upon sequence homology that has corresponding functional consequences. Now comprising some 30 members and still growing (reviewed in (Zlotnik et al., 1999)), the chemokine field has exploded in the past eight years as bioinformatics and expressed sequence tag databases (dbEST) have made it possible to

rapidly identify and clone new ligands and receptors according to their sequence homology to known molecules. There are at least 14 known chemokine receptors, most of which promiscuously recognize multiple ligands. Similarly, a given chemokine ligand generally interacts with more than one receptor. A chart of chemokines and their receptors is given in Figure 4-1. This complex web of ligand-receptor interactions complicates the biology of chemokine actions. Consequently, we are only beginning to understand the function and *in vivo* relevance of most chemokines. In situ hybridization has been particularly useful in elucidating function, as expression patterns suggest molecular actions. Definitive proof comes from the generation of mice carrying null mutations in either the ligands or receptors. Again, the promiscuity of family members sometimes complicates interpretation of resulting phenotypes.

The chemokine family can be subdivided into four classes, designated according to the arrangement of the first two of four conserved cysteine residues (reviewed in (Zlotnik et al., 1999) and (Schall and Bacon, 1994)). The α-chemokines, also called the CXC family, have one amino acid residue between the first and second conserved cysteines. This class can be further subdivided based on the presence or absence of a three-amino acid sequence, Glu-Leu-Arg, immediately preceding the CXC residues. This sequence, called the ELR motif, confers many functional properties upon the chemokine, including

the ability to bind the receptor CXCR2 which is expressed on neutrophils. ELR-containing CXC chemokines such as IL-8 are generally neutrophil chemoattractants, while CXC chemokines lacking the ELR, such as IP-10, primarily bind other CXC receptors and act as lymphocyte chemoattractants.



Chemokine Receptors	**Chemokines**
CC Receptors	Compatible Ligands
CCR1	MIP-1α, RANTES, MCP-3
CCR2	MCP-1, MCP-2, MCP-3, MCP-4
CCR3	Eotaxin, Eotaxin-2, RANTES, MCP-2, -3, -4
CCR4	TARC, MDC
CCR5	RANTES, MIP-1α, MIP-1β
CCR6	MIP-3α/LARC/Exodus
CCR7	MIP-3β/ELC, 6Ckine/SLC, Exodus 2
CCR8	I-309,TARC, MIP-1β
CCR9	MCP-1, -2, -4, MIP-1α, -1β, RANTES, Eotaxin, HCC
CCR10	MCP-1
CXC Receptors	
CXCR1	IL-8, GCP-2
CXCR2	ELR, C-X-C
CXCR3	IP10, Mig, I-TAC
CXCR4	SDF-1
CXCR5	BCA-1/BLC
C Receptors	
XCR1	Lymphotactin
CX3C Receptors	
CX3CR1	Fractalkine/Neurotactin
Promiscuous Receptors	
DARC	Many CC and CXC Chemokines
Viral Receptors	
CMV US28	CC Chemokines

Figure 4-1

Figure 4-1. Chemokines and Their Cognate Receptors. This list depicts the four classes of chemokines, determined by the conservation and spacing of specific cysteine residues. The promiscuity of chemokine receptors and some ligands is evident. (Adapted from Kunkel, 1999)

In the β-chemokines, also called the CC chemokines, the first two cysteine residues are adjacent to one another. CC chemokines such as MCP-1 generally act on monocytes, lymphocytes, basophils and eosinophils. Together, the CXC and CC chemokines represent the majority of known chemokines, however two other classes exist that contain a total of three genes. The C class consists of lymphotactin and its highly homologous sister SCM-1β, and the CX_3C class is fractalkine. Lymphotactin has lost the first of its conserved cysteines and fractalkine has three amino acids separating its first and second cysteine residues. Both of these unusual chemokines are chemotactic for lymphocytes—including T cells and NK cells. Fractalkine also attracts monocytes ((Bazan et al., 1997; Imai et al., 1997), reviewed in (Zlotnik et al., 1999)).

Chemokines are secreted, highly basic proteins capable of binding "presentation molecules" in the extracellular space—sulfated proteins and proteoglycans on cell surfaces and in the extracellular matrix. They can also exist and function as soluble molecules, an attribute perhaps more important *in vitro* than *in vivo* (reviewed in (Schall and Bacon, 1994)). The binding capacity of chemokines allows for the establishment of a concentration gradient along which cells can migrate (Butcher and Picker, 1996). The discovery that chemokine expression can be induced in a range of tissues was the first

indication that these molecules could be produced as part of an immune response. This and related observations quickly led to the revelation of chemokines' first accredited biological function—their role in leukocyte recruitment to areas of inflammation. Subsequently, chemokines were also shown to be mediators of the homeostatic migration of leukocytes throughout the body (reviewed in (Cyster, 1999; Schall and Bacon, 1994; Zlotnik et al., 1999)). It is now established that chemokines regulate the cyclic trafficking of immune cells (from blood to lymph to blood, or to sites of infection) throughout the body.

Chemokine function beyond chemotaxis

The homing of a leukocyte to a particular location requires more than a chemotactic gradient. Generally, the leukocyte is passing through a tissue in a blood vessel and must escape from this compartment and extravasate into the tissue before following the chemotactic trail. Extravasation has been extensively studied (Figure 4-2); four steps have been identified: i) leukocyte rolling along the endothelium; ii) activation of integrin expression on the leukocyte; iii) tight adhesion of the leukocyte to the endothelium; iv) diapedesis of

Circulating lymphocyte enters the high endothelial venule in the lymph node
Binding of L-selectin to GlyCAM-1 and CD34 allows rolling interaction
LFA-1 is activated by chemokines bound to extracellular matrix
Activated LFA-1 binds tightly to ICAM-1
Diapedesis — lymphocyte emigrates into the lymph node
LFA-1
L-selectin
GlyCAM-1
ICAM-I
CD34
IL-8

Figure 4-2

Figure 4-2. Leukocyte Exit From the Bloodstream Involves Chemokines. As depicted in the panels above, leukocyte emigration to tissues is a multi-step process. It begins with an adhesive interaction between the leukocytes and endothelium, mediated by selectins and specific carbohydrate moieties, which causes circulating leukocytes to slow by rolling along the vessel wall. Next, chemokines in the local environment cause the leukocyte to activate its integrins such that it is now able to bind counter-receptors on the endothelial wall. This third step yields tight binding of the leukocyte to the vessel wall, preparing it for the final step of extravasation—diapedesis, the physical act of crawling between endothelial cells and exiting the lumen. Chemokines may also be involved in this final step, directing the leukocyte in its migration. (Adapted from Janeway 3rd Ed.)

the leukocyte across the endothelium into the tissue (reviewed in (Butcher and Picker, 1996; Springer, 1995)). In addition to the chemotactic gradient that aids cells undergoing step iv (see next section and (Nieto et al., 1998)), chemokines also function at step ii of the extravasation pathway (Campbell et al., 1998). In this case, certain chemokines induce integrin activation, conformational changes that render the cells ready to bind (Campbell et al., 1998). The interaction of these integrins with counter-receptors expressed on the endothelium confers tight binding, halting the rolling leukocyte at the appropriate place for diapedesis.

As discussed above, chemokine function encompasses more than the attractive activities for which the molecules are named. Many chemokine effects can be described using the broad term activation. In general, recognition of a chemokine primes a cell to act—either to extravasate, to engage another cell, to kill a target cell, or to produce cytokines (Loetscher et al., 1996; Maghazachi et al., 1996; Nieto et al., 1998; Taub et al., 1995). One particularly interesting property of the CXC chemokines is their ability to promote or inhibit angiogenesis. CXC chemokines containing an ELR motif are angiogenic, while those lacking the motif are angiostatic. This functional distinction is attributable to the presence or absence of the ELR motif (Strieter et al., 1995). Chemokines also have mitogenic or anti-mitogenic effects on hematopoietic cells (as well

as endothelial cells), and certain chemokines protect against death by apoptosis (reviewed in (Zlotnik et al., 1999)). The CXC chemokine SDF-1 is necessary for normal brain development, demonstrating that chemokines play as yet undefined roles in non-immune contexts (Ma et al., 1998).

Chemokines and NK cells

The interplay between chemokines and NK cells is not yet well understood, although the groundwork has been laid. As suggested by the preceding section, chemokines have multiple affects on NK cells. Chemoattractants of these cells include lymphotactin, fractalkine, SLC, CKβ-ll, MIP-1α, MIP-1β, RANTES, MCP-1, MCP-2, MCP-3, SDF-1, MDC and IP-10 (Allavena et al., 1994; Giancarlo et al., 1996; Godiska et al., 1997; Loetscher et al., 1996; Maghazachi, 1997; Maghazachi et al., 1994; Maghazachi et al., 1997; Taub et al., 1995) (Maghazachi et al., 1996) (Bazan et al., 1997; Hedrick et al., 1997; Imai et al., 1997). Most of these factors also induce granule exocytosis and increase the cytotoxicity of the NK cell.

Interestingly, chemokines can induce the polarization of NK cells (and T cells) bound to endothelial cells or to extracellular matrix (Nieto et al., 1997; Nieto et al.,

1998). Polarization of cells is required for many functions associated with chemokine action—cell migration, degranulation, target cell recognition and conjugate formation between target and effector cells (reviewed in (Nieto et al., 1998)). Presumably, the mechanism by which chemokines increase the cytotoxicity of NK cells is through the polarization of the NK cell, which precedes the aforementioned actions. Chemokine-induced polarization results in two separate areas of membrane specialization on the NK cell (Nieto et al., 1998). Chemokine receptors are localized to the leading edge of the migrating cell, which allows it to follow a chemotactic gradient. Opposite the leading edge, on the trailing side of the NK, cell another specialized region develops called the uropod, which bears a concentration of cell adhesion receptors such as ICAM-1 and ICAM-3. The NK cell engages its target at the leading edge, but it interacts with other NK cells through the adhesion molecules on its uropod. Thus, the uropod functions as a recruitment device for concentrating lymphocytes near cellular targets.

NK cells not only respond to chemokines, but also produce them, for example lymphotactin, MIP-1α and IL-8 (Bluman et al., 1996; Hedrick et al., 1997; Somersalo et al., 1994). The fact that NK cells both produce and respond to lymphotactin and MIP-1α suggests the possibility of a self-regulating amplification system that allows the small number of NK cells that initially respond to a pathogen or a tumor to recruit additional

NK cells and other effector cells. This model is strengthened by the observation that NK cells increase their chemokine production when conjugated to a target cell (Nieto et al., 1998).

Chemokines and leukocyte trafficking in the placental bed

The concentration of $CD56^{bright}$ NK cells in the pregnant uterus is an unusual phenomenon, and the mechanisms underlying the specific recruitment of these cells are not well understood. One confounding issue is that no single chemokine has been identified that attracts NK cells but not other leukocytes. The most specific NK cell attractants also act on T cells (i.e., lymphotactin, fractalkine) (Hedrick et al., 1997; Imai et al., 1997) and most chemokines that attract NK cells affect a wide spectrum of leukocytes (e.g. MIP-1α) (Schall et al., 1993). Another difficulty when considering $CD56^{bright}$ cell trafficking is that very few descriptions of chemokine action on NK cells distinguish between $CD56^{dim}$ and $CD56^{bright}$ populations. Therefore, we know almost nothing about differential effects of chemokines on the latter cells. One exception is the observation that SLC and CKβ-11, both ligands for the CCR7 receptor, preferentially attract $CD56^{bright}$ cells over $CD56^{dim}$ cells (Kim et al., 1999). However, both of these

molecules are also chemotactic for T cells, B cells and dendritic cells (reviewed in (Kim et al., 1999)).

Molecular mechanisms that regulate leukocyte trafficking in other locations may provide a model for NK cell concentration in the decidua (Butcher and Picker, 1996). It is likely that the homing of specific leukocyte populations to the uterus is the end result of the combinatorial actions of molecules that influence individual steps in the extravasation process (Figure 4-2). For example, a cell might use the following receptor/ligand pairs to extravasate at a site of inflammation: step i, rolling (L-selectin: GlyCAM-1); step ii., integrin activation (SLC: CCR7); step iii., firm adhesion (ICAM-1: $\alpha L\beta 2$ integrin) and step iv., cell polarization and directed migration (MIP-1α: CCR5). It is established that each of the four stages of extravasation is reversible and independent; each requires a signal generated from the appropriate ligand-receptor interaction before passing on to the next stage. In the absence of the appropriate signal, the cell eventually detaches from the endothelium and moves on. Therefore, each event of extravasation becomes a checkpoint, which admits only cells that express the proper molecules. Given the diversity of molecules that could potentially regulate each step, it would be easy to generate unique combinations that could be used to achieve specific homing of leukocyte

subsets. For a thorough discussion of this model see Butcher and Picker, 1996 or Springer, 1995.

Can unique combinatorial subsets of adhesion molecules and chemokines be identified in the decidua and on decidual leukocytes? In terms of chemokine expression in the decidua, very little is known at present. Out of more than 30 identified chemokines, only three (MCP-1, IL-8 and RANTES) have been previously described in the decidua or cycling endometrium. Published studies on chemokines in the uterus have focused largely on two molecules involved in neutrophil and monocyte recruitment, IL-8 and MCP-1, respectively, because the neutrophil influx that immediately precedes menstruation serves as a well-defined cell trafficking event. Both IL-8 and MCP-1 are produced in the endometrium (reviewed in García-Velasco and Arici, 1999), and their expression, which is coordinated with the menstrual cycle, is probably regulated at some level by hormones (see Chapter Three). RANTES expression in the endometrium has also been described (Hornung et al., 1997). Additionally, a few studies have shown that cultured decidual cells can be stimulated with cytokines or bacteria to produce MIP-1α (Dudley et al., 1995). This thesis significantly extends the above information by defining the expression patterns of ten chemokines at the maternal-fetal interface, i.e., placental and uterine tissues (Chapter Seven). Additionally, it delineates a set of chemokine

receptors that are expressed by decidual granulated leukocytes (Chapter Seven). Together, this data establishes new ligand-receptor pairs that could be involved in recruitment of these cells at two stages (ii and iv) of the extravasation process.

In terms of adhesion molecules expressed in the placental bed, immunohistochemical studies reveal an abundance of functionally relevant molecules on uterine endothelium, including NCAM (Yan Zhou/Susan Fisher, personal communication), ICAM-1, VCAM-1, PECAM, $\alpha 6\beta 4$, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha 6\beta 4$ and VLA-1 through –6 (Haynes et al., 1997; Ruck et al., 1994). Although some of these factors may be involved in anchoring the endothelium to its underlying basement membrane, others are likely to participate in the recruitment of decidual leukocytes. In support of this hypothesis, the latter cells express counter-receptors such as CD56 (NCAM), ICAM-1, PECAM, LFA-1, and VLA-1, -3, -4 and –5. Additionally, *in vitro* studies of peripheral NK cell adhesion to endothelium under static conditions indicate that in the absence of stimulation, LFA-1/ICAM-1 is the predominant adhesion pair used by NK cells. In the presence of activated endothelium, the interactions of VLA-4 (on the NK cell) and VCAM-1 (on the endothelium) also produce significant adhesion(Allavena et al., 1991). Subsequent to extravasation, decidual leukocyte expression of integrins such as $\alpha V\beta 1$,

αMβ2, αVβ3, αEβ7 may help their migration through decidual stroma via adhesion to extracellular matrix components(Ruck et al., 1994).

It should be emphasized that the highly specific expression of NCAM on decidual endothelium, accompanied by the dense expression of NCAM on decidual NK cells (which are identified by their $CD56^{bright}$ phenotype—see Chapter Three) suggests a homing mechanism that exploits the homophillic binding properties of this molecule. This model is easily testable, although to our knowledge has not been studied to date .

In summary, a great deal remains to be learned about leukocyte trafficking to the uterus in both the pregnant and non-pregnant states. The following addresses the former question and sets the stage for additional work addressing the latter.

Chapter Five - 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 (6-22 wk) or from normal term delivery (34-40 wk) was collected within one hour of isolation, washed thoroughly in PBS with antibiotics and placed on ice. Tissue that was to be used for immunohistochemistry was immediately fixed for 5 minutes in 3% paraformaldehyde (PFA), washed thoroughly in PBS, then embedded in OCT and frozen in liquid nitrogen. Tissue that was 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.

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 and Conditioned Medium (CM) Preparation

Cytotrophoblast and Villous Stroma

CTBs were isolated from pooled first- or second-trimester (Fisher et al., 1989; Librach et al., 1991) and individual third-trimester (Kliman et al., 1986) human placentas by published methods. Briefly, placentas were subjected to a series of enzymatic digests, which detached CTB stem cells from the underlying stromal core of the chorionic villus. Detached CTBs were purified over a Percoll gradient, and were cultured on Matrigel-coated substrates (Collaborative Biomedical Products, Bedford, MA) for varying lengths of time in serum-free medium [DMEM 4.5 g/L glucose (Sigma Chemical Co., St. Louis, MO), with 2% Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis, IN), 1% penicillin/strepomycin, 1% sodium pyruvate, 1% Hepes, 1% gentamycin (UCSF Cell Culture Facility)]. The connective tissue villus cores which remained after enzymatic removal of CTBs were further trypsinized, then cultured in serum-free medium (SFM) for varying lengths of time to obtain stromal conditioned medium (CM). As a control for all CTB CM, SFM was cultured on Matrigel-coated plates with no cells (SFM/MG) for varying lengths of time.

Placental Fibroblasts and JAR Cells

Placental fibroblasts were isolated from first-trimester placentas as previously described (Fisher et al., 1989). Cells were cultured in DMEM H-21 with 10% fetal bovine serum (FBS), 5% glutamine, 1% penicillin/strepomycin, 1% gentamycin. CM was collected from the cells every 72 hours. The JAR choriocarcinoma cell line was maintained as previously described (Fisher et al., 1989).

Preparation of Decidual Granulated Leukocytes

Decidua was minced with a razor blade, then incubated at 10ml/g of tissue in RPMI-1640 (UCSF Cell Culture Facility) containing 10% FBS, 0.1% collagenase (Sigma Chemical Co., St. Louis, MO), 0.02% DNAse I (Boehringer Mannheim) and 0.02% EDTA (Sigma) for one hour at room temperature. Following this digestion, the supernatant was removed and centrifuged. The cell pellet was resuspended in Hank's buffered saline solution (HBSS), layered over Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden and centrifuged for 30 minutes 900 x g. The band of DGLs at the interface was removed, washed 3 times in PBS and cultured in SFM for 24 to 72 hours.

Isolation of Human Peripheral Blood Mononuclear Cells

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 Chemical Co.) separation. PBMCs were washed in PBS and resuspended at 1 x10^7 cells/ml in DME-H21 (UCSF Cell Culture Facility) containing 1.0% BSA (Sigma Chemical Co.).

Chemotaxis Assay

Chemotaxis assays were carried out by published methods (Morales et al., 1999). CM with initial MIP-1α concentrations of between 50 and 100 ng/ml as determined by ELISA (see below) were used in the assay. In some cases, up to 7 cytotrophoblast CM samples were pooled according to gestational age in trimesters and concentrated with a Centriprep-3 centrifugal concentrator (Amicon Inc., Beverly, MA). The CM was diluted with in DME-H21 (UCSF Cell Culture Facility) containing 1.0% BSA (Sigma Chemical Co.), hereafter referred to as blank medium, to produce samples with MIP-1α concentrations ranging from 100 ng/ml to 10 pg/ml. Transwell inserts (6.5 mm in diameter, 5-μm pore; Corning Inc., Corning, NY) were placed in 24-well plates. 600 μl of cytotrophoblast CM, blank medium, or blank medium supplemented with recombinant human MIP-1α (R&D Systems Inc., Minneapolis, MN) was added to the bottom

compartment of each insert, and 100 µl of a human PBMC cell suspension containing 1 x 10^6 cells was added to the top. Where indicated, 5 µg/ml of control goat IgG (R&D Systems) or anti-MIP-1α 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-1α was added. 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-µm microsphere beads (Bangs Laboratories, Fishers, IN) 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 µl 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; San Jose, CA) 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 (Beckton Dickinson). The absolute number of cells that migrated was extrapolated from the percent recovery of the beads. All assays were done in triplicate.

Ca^{++} Flux Assays

Reporter cell lines were generated as previously described (Gosling et al., 1997; Myers et al., 1995; Tsou et al., 1998). HEK-293 parental cells were transfected with human CCR1 (Ben-Baruch et al., 1995; Gao et al., 1993; Neote et al., 1993) and CCR2 (Charo et al., 1994; Franci et al., 1995; Myers et al., 1995), and 300-19 parental cells were transfected with CCR3 (Combadiere et al., 1995; Daugherty et al., 1996; Kitaura et al., 1996; Ponath et al., 1996), CCR5 (Combadiere et al., 1996; Raport et al., 1996) and CX3CR1 (Combadiere et al., 1998; Imai et al., 1997). The non-transfected parent lines served as negative controls. Prior to analyses, medium samples were concentrated 5-10 fold (v/v) over a heparan sulfate column according to the manufacturer's directions (Amersham Pharmacia Biotech AB), after which they were dialyzed against PBS overnight at 4°C. Intracellular Ca^{++} release was monitored as previously described (Myers et al., 1995). Briefly, the target cells were preloaded with the Ca^{++}-sensitive dye calcein AM (Molecular Probes Inc., Eugene, OR), washed, and resuspended in 1 µl of RPMI-1640. The experiment started with the addition of 20 µl of concentrated cytotrophoblast CM, 20 µl of concentrated SFM/MG as a negative control, or 5 µl of a stock solution (10 mM) of the relevant recombinant chemokine as a positive control (MIP-1α, MCP-1, eotaxin, RANTES or fractalkine). The magnitude of the Ca^{++} flux was determined spectrofluorometrically.

In Situ Hybridization

In situ hybridization was carried out using published methods (44). Isolated cytotrophoblasts were cultured (as described above) on a 200-µl plug of Matrigel in a Millicell-CM culture insert, 12 mm in diameter, 0.4-µm 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 (v/v) 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. Sections (5 µm) were cut, mounted on poly-L-lysine-coated slides, and stored at -20°C until use.

Placental villi, basal plate and membrane specimens to be used in *in situ* hybridization were fixed with 10% formalin in phosphate-buffered-saline (PBS) at room temperature overnight, washed twice in PBS, dehydrated through a graded ethanol series, cleared in xylene, and paraffin embedded. Sections (5 µm) were cut and mounted on poly-L-lysine coated slides, deparaffinized and rehydrated before use.

To produce labeled probes, the EST plasmid clone ID# 154848, which contains an 837-bp insert of the human MIP-1α cDNA, was cut with either Not1 or EcoR1 (Gibco

BRL, Rockville, MD). The linearized vectors were used as templates for the synthesis of ^{35}S-cRNA probes using T3 (antisense) or T7 (sense) RNA polymerases (Melton et al., 1984).

On day 1 of the in situ hybridization, slides were allowed to sit at room temperature for 5 min. Then they were 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 0.5X SSC (1X =150 mM NaCl, 15 mM sodium citrate, pH 7.4). Sections were deproteinated with 1 µg/ml proteinase K for 10 min at room temperature, then washed for 10 min in 0.5X SSC. Then they were fixed again in 4% paraformaldehyde for 3 min, followed by a final wash in 0.5X SSC for 5 min. After a 3-h prehybridization at 55-60°C in rHB2 buffer (50% formamide, 0.3 M NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1X Denhardt's [0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA], 10% dextran sulfate, 10 mM dithiothreitol), the slides were incubated overnight at 55-60°C in a humidified chamber with 200 µl of hybridization buffer: rHB2 buffer containing 500 µg/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 min each, in 2X SSC (300 mM NaCl, 30 mM Na citrate) with 10 mM ß-mercaptoethanol and 1mM EDTA. Slides were then immersed in an RNase A solution (500 mM NaCl, 10 mM Tris, pH 8.0, and 10 µg/ml RNase A) for 30 min at room temperature and washed

twice, 10 min each, in 2X SSC/ß-mercaptoethanol/EDTA. Then the sections were subjected to a high stringency wash (0.1X SSC, 10 mM ß-mercaptoethanol, 1 mM EDTA) for 2-3 h at 60° C before they were washed twice, 10 min each, in 0.5X SSC without ß-mercaptoethanol or EDTA. Finally, the sections were dehydrated (2 min/step) in a series of graded (30%, 60%, 80%, 95% and 100%) ethanol solutions that contained 0.3 M NH_4Ac. 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 anti-cytokeratin, 7D3 rat anti-human (Damsky et al., 1992) and anti-CD45Rb, PD7/26 mouse anti-human (Dako). Sections of cell aggregates were prepared as described above and then antibody binding was detected using Vectastain ABC kits (PK-6102 and PK-6104) according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). 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 prior to analysis.

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 (Chomczynski and Sacchi, 1987). Blots were prepared as follows. 10 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 (De et al., 1990; Lehrach et al., 1977; McMaster et al., 1995). 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-1α-specific probe (see above) was generated by using standard methods (Tabor, 1993). [^{32}P]CTP random oligonucleotide primers (Amersham Life Science, Inc.; Piscataway, NJ) and the Klenow fragment of DNA polymerase I (Gibco BRL, Rockville, MD) were used to label the probe, which had a specific activity of 2 x 10^9 dpm/μg.

Immunoblotting

Samples of cytotrophoblast CM were concentrated by heparin affinity chromatography as described above. Then 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., Solon, OH) and 0.1% Tween-20 (Sigma Chemical Co.). Then the membranes were incubated overnight at 4°C with the primary antibody—anti-MIP-1α goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:20 (v/v) 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., West Grove, PA) diluted 1:3000 (v/v) 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, Buckinghamshire, UK).

ELISA

Quantikine human MIP-1α ELISA kits (catalog # DMA00) were purchased from R&D Systems and were used according to manufacturer's instructions.

Endotoxin Detection

The Endotoxin Detection Kit (catalog # 3070000) from ICN Pharmaceuticals, Inc. (Costa Mesa, CA) 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-1α 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-1α 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-1α in cytotrophoblast conditioned medium.

RNase Protection Assay

Total RNA from decidual granulated leukocytes was prepared as described above (see Northern Hybridization). RiboQuant multi-probe RNase protection assay kits were purchased from Pharmingen and were used according to manufacturer's instructions. Briefly, three probe sets (hCR5, hCR6 and hCR8), encompassing a total of seventeen 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 comprised additional controls for expression.

Chapter Six – Human Placental Cytotrophoblasts Attract Monocytes and CD56^{bright} NK Cells Via the Actions of MIP-1α

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 villous stroma. At sites of placental attachment to the mother, invasive cytotrophoblasts encounter specialized maternal 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 PBMCs 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 Ca^{++} flux in cells transfected with individual chemokine receptors suggested that cytotrophoblasts secreted MIP-1α. This was confirmed at the mRNA level, both in vitro and in vivo, by in situ hybridization. MIP-1α protein in conditioned medium was further characterized by immunoblotting and ELISA. Immunodepletion of MIP-1α 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-1α, and the more general hypothesis that these cells may organize and act on leukocytes at the maternal-fetal interface.

Introduction

During human pregnancy, leukocytes traffic to areas where placental cytotrophoblasts reside. The histology of these areas is diagrammed in Fig. 1 (see also (Boyd, 1970; Brosens and Dixon, 1966; Cross et al., 1994; Damsky and Fisher, 1998)). The fetal portion of 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 the cytotrophoblast stem cells take 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 *floats* in a stream of maternal blood, which optimizes exchange, across the syncytium, of substances between the mother and fetus. In *anchoring villi*, cytotrophoblast stem cells detach from the basement membrane and form a column of non-polarized 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 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 (Damsky and Fisher, 1998). Interstitial invasion brings cytotrophoblasts into direct contact with maternal immune cells in the uterus, termed decidual granulated leukocytes (DGLs). 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 (Copeman et al., 2000; Ellis et al., 1990; Kovats et al., 1990). The expression of HLA-C by cytotrophoblasts has also been reported(King et al., 2000). 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%) (Bulmer et al., 1991). During the first half of human pregnancy, DGLs account for more than half of the maternal cells within the uterus (Starkey et al., 1988). This phenomenon is not unique to humans, as recruitment of uterine NK cell populations during pregnancy has also been described in mouse (Croy and Kiso, 1993; Croy et al., 1996; Head, 1996) and pig (Engelhardt et al., 1997; Yu et al., 1993). Although the functional consequences of cytotrophoblast interactions with DGLs are not well understood, experimental evidence in the mouse suggests that the immune cells promote endovascular invasion and placental development (Croy et al., 2000; Guimond et al., 1998). 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 (Rappolee and Werb, 1988; Rappolee and Werb, 1992).

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 (Schall and Bacon, 1994) and to secondary lymphoid organs (Cyster, 1999). 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 DGLs. Then, we used this model to show that a cytotrophoblast-derived chemokine, monocyte inflammatory protein-1α (MIP-1α), 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 MIP-1α mRNA expression in cytotrophoblasts in situ at the human maternal-fetal interface, we hypothesize that this chemokine has similar effects during pregnancy.

Results

Cytotrophoblast Conditioned Medium 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 (reviewed in ref. 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 Conditioned Medium Signals Through MIP-1α 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-1α, and CX3CR1/fractalkine. In all cases, addition of the recombinant chemokine induced a robust Ca^{++} 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 Ca^{++} flux in cells expressing CCR1 (Fig. 2A; n = 4) and CCR5 (Fig. 2B; 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. Since MIP-1α is the only chemokine that is a ligand for both CCR1 and CCR5, but not CCR3, we hypothesized that MIP-1α was present in the CM.

Cytotrophoblasts Express MIP-1α mRNA in vitro. To confirm that cytotrophoblasts secreted MIP-1α, we first examined production of MIP-1α 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 prior to hybridization (see Material and Methods). Fig. 3A is a micrograph 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 (Librach et al., 1991). 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. 3B) and antisense probes (Fig. 3C) showed that cytotrophoblasts synthesize MIP-1α mRNA. Immuno-histochemistry on adjacent sections was performed to demonstrate that the cell aggregate was comprised of cytotrophoblasts, particularly to exclude the possibility of macrophage contamination. The staining revealed the presence of cytokeratin, a cytotrophoblasts 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-1α 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-1α. 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 approximately 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-1α 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-1α mRNA.

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

We used an ELISA to quantify MIP-1α in cytotrophoblast CM from first trimester (n=17), second trimester (n=18), and term (n=7) cells that were cultured for 12-48 h. Levels of this chemokine detected in individual samples ranged from 1.2 - 82 ng/ml (Fig. 5B). CM from five preparations of first trimester and one preparation of second trimester cytotrophoblasts contained amounts of MIP-1α protein that were below the <7 pg/ml lower detection limit of the assay. Analysis of variance, followed by the Student-Newman-Keuls test, demonstrated that term cytotrophoblasts produced significantly more ($P < .05$) MIP-1α than cytotrophoblasts isolated from first and second trimester placentas. As expected, MIP-1α was not detected in control SFM/MG or placental fibroblast CM (data not shown). The five pairs of connected data points in Fig. 5B show MIP-1α levels in medium samples from the same culture over time. In accord with the results of the Northern blot hybridization

analyses of MIP-1α mRNA levels, the corresponding protein accumulated in the medium during the course of the experiment.

To ensure that the MIP-1α 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-1α in the medium with the presence or absence of endotoxin, a factor that stimulates chemokine production by monocytes but not cytotrophoblasts. Twenty samples of cytotrophoblast CM were simultaneously assayed for endotoxin by the Limulus Amebocyte Lysate assay and for MIP-1α protein concentration by ELISA. There was no correlation between the two variables, as determined by the Spearman Rank Order Correlation test (Figure 5C). This result shows that cytotrophoblasts are the source of MIP-1α protein in the medium.

Cytotrophoblasts Express MIP-1α mRNA in vivo. As a final step in assessing cytotrophoblast production of MIP-1α, we used in situ hybridization to study mRNA expression by cells in situ at the maternal-fetal interface (n=9). Fig. 6A shows a micrograph 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. Hybridization of these tissue sections with ^{35}S-labeled antisense probe (Fig. 6B) revealed MIP-1α mRNA expression by cells within anchoring villi and by extravillous cytotrophoblasts within the uterine wall. As a negative control, adjacent sections were hybridized with sense probe (Fig. 6C), which yielded no signal.

MIP-1α in Cytotrophoblast CM Induces the Migration of Monocytes and CD56^{bright} NK cells. Having shown that cytotrophoblasts produce MIP-1α, 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-1α in our system, using recombinant chemokine with unstimulated PBMCs as targets (Fig. 7). 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-1α antibody which was used in subsequent experiments.

The results showed that the response to recombinant MIP-1α varied among the various PBMC populations we analyzed. As reported by other investigators (Wang et al., 1993) large numbers of monocytes migrated in every experiment (n = 8). The results of a typical analysis are shown in Fig. 7A. Monocyte chemotaxis peaked at a MIP-1α concentration of 10 ng/ml, addition of anti-MIP-1α 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-1α, although somewhat less consistently. In 6 out of 9 experiments $CD56^{bright}$ and $CD56^{dim}$ NK cells migrated towards recombinant MIP-1α (Fig. 7B and C, respectively). Again, chemotaxis was not observed in the presence of anti-MIP-1α. Finally, the effects of MIP-1α on T cells were highly variable. In three of nine experiments we observed chemotaxis that was reversed by the addition of anti-MIP-1α (data not shown).

Next we assayed the chemotactic activity of cytotrophoblast CM, using PBMCs as targets. In these experiments, MIP-1α in cytotrophoblast CM was quantified by ELISA, 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. 8A.

Monocyte migration toward cytotrophoblast CM typically peaked at 10 ng/ml of MIP-1α (six of eight times) as did the migration of these cells in response to the recombinant chemokine (compare Fig. 7A and Fig. 8A). At the peak, 16% (3% to 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% to 5%) of the cells migrated. Taken together, these data showed that cytotrophoblast CM stimulated a 7-fold (3.7-fold 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-1α) concentration that was tested (Fig. 8A). At this point 4% (0.6% to 5%) of all $CD56^{dim}$ cells and 5.4% (1.8% to 10.5%) of all $CD56^{bright}$ cells migrated. Basal migration stimulated by control medium was similar for both NK subtypes: 0.4% (0.1% to 0.9%) for $CD56^{dim}$ cells and 0.6% (0.2% to 1%) for $CD56^{bright}$ cells. Thus, cytotrophoblast CM induced a 7.7-fold increase (5-fold to 12-fold) in the migration of $CD56^{dim}$ cells and a 5.5-fold increase (2.8-fold 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-1α) concentrations tested (Fig. 8A). At the highest concentration, 1.7% (1.3% to 3.5%; n = 9) of T cells migrated as compared to basal migration, which was 0.5% (0.4% to 1.22%).

Accordingly, cytotrophoblast CM increased T cell migration by 2-fold (1.7-fold to 4-fold) over basal levels, a relatively modest response compared to that of monocytes and NK cells.

Finally, we analyzed the effects of adding function-perturbing anti-MIP-1α on cytotrophoblast CM-induced PBMC chemotaxis. In five experiments, neutralizing MIP-1α activity reduced monocyte migration at its peak by 44.2% ± 10.0% (mean ± SD) as compared to the addition of a control IgG. Fig. 8B 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 ($P < .05$). Data from the other two experiments were less significant ($P < .075$ and $P < .20$). In four experiments, abolishing MIP-1α activity in the CM reduced migration of $CD56^{bright}$ NK cells by 66.8% ± 20.0% as compared to the control IgG. An example of these data is shown in Fig. 8C. 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 was chemotactic for both $CD56^{dim}$ NK cells and T cells, antibody neutralization showed that this effect did not depend on MIP-1α activity, suggesting a more complex phenomenon (data not shown).

Discussion

The mechanisms whereby the human hemi-allogeneic 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 (Roth et al., 1996), TGFß (Dungy et al., 1991; Kauma et al., 1990) and MIF (Arcuri et al., 1999), that downregulate immunological responses in other settings (Apte et al., 1998; Bejarano et al., 1992; Letterio and Roberts, 1998). 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 (Munn et al., 1998). Finally, several novel mechanisms of immune regulation are used at the maternal-fetal interface, including high local levels of progesterone (Pavia et al., 1979), and expression of the non-classical MHC class I molecule, HLA-G (Kovats et al., 1990), 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.

Now, we 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 peripheral blood leukocytes. 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 (Baggiolini et al., 1994; Loetscher et al., 1996; Premack and Schall, 1996). 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-1α, 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-1α 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 (see Fig. 1). In situ hybridization analyses of tissues that contained the maternal-fetal interface confirmed that cytotrophoblasts inside the uterine wall express MIP-1α mRNA in vivo. Although the production of MIP-1α by cytotrophoblasts was highly reproducible, the concentration of secreted chemokine varied among cell preparations, ranging from < 7 pg/ml to 82 ng/ml in the CM. This finding correlates with similar observations of variability in cytokine (e.g., IL-10) and hormone (e.g., placental lactogen) production among different placentas (Paradowska et al., 1997; Roth et al., 1996; Suwa and Friesen, 1969). As 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-1α, since 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-1α in the cytotrophoblast medium strongly attracted monocytes, well-established targets for this molecule (Wang et al., 1993). The fact that the dose-response curves for monocyte chemotaxis towards cytotrophoblast CM and recombinant MIP-1α 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 approximately 15% of the target monocytes, and addition of anti-MIP-1α to cytotrophoblast CM reduced monocyte migration by approximately 45%. Together, these data indicate that cytotrophoblasts rely heavily on MIP-1α in the CM to regulate monocyte chemotaxis. Other cytotrophoblast-derived

chemokines, such as RANTES, may also contribute to this phenomenon (Hornung et al., 1997).

Cytotrophoblast CM was also highly chemotactic for NK cells, another established target of MIP-1α (Loetscher et al., 1996; Taub et al., 1995). 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. Since $CD56^{bright}$ 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 SLC and CKß-11, both ligands for the CCR7 receptor, preferentially attract $CD56^{bright}$ over $CD56^{dim}$ cells (Kim et al., 1999). Here, we report that MIP-1α 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-1α has differential effects on these two NK subsets. Thus, via MIP-1α, 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-1α is a reported attractant for activated T cells, it was not responsible for the migration of these cells towards cytotrophoblast CM. In accord with previously published data, the unstimulated T cells within our PBMC target population did not migrate towards recombinant MIP-1α (Taub et al., 1993). Similarly, the migration of unstimulated T cells towards cytotrophoblast CM was not affected by addition of anti-MIP-

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

To our knowledge, this report is the first demonstration 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 (Bulmer et al., 1991). 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 (Vassiliadou and Bulmer, 1998). 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 (Kruse et al., 1999). These adhesion molecules are intergral to a multi-step homing process that also requires chemokines. In mouse the absence of MIP-1α or one of its receptors (CCR5) did not affect NK cell infiltration of the pregnant uterus (Chantakru et al., 2001). Given the number of known chemokines and receptors, it is likely that mouse and human employ distinct sets of these molecules to influence decidual leukocyte chemotaxis.

Here, we demonstrate that the human cytotrophoblasts produce MIP-1α, which endows them with the capacity to direct the migration of NK cells, monocytes and T cells—cells that compose the decidual granulated leukocyte population with which the placenta co-exists. This activity could contribute to the recruitment of decidual leukocytes, or may be used at a local level to manipulate the micro-immune 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.

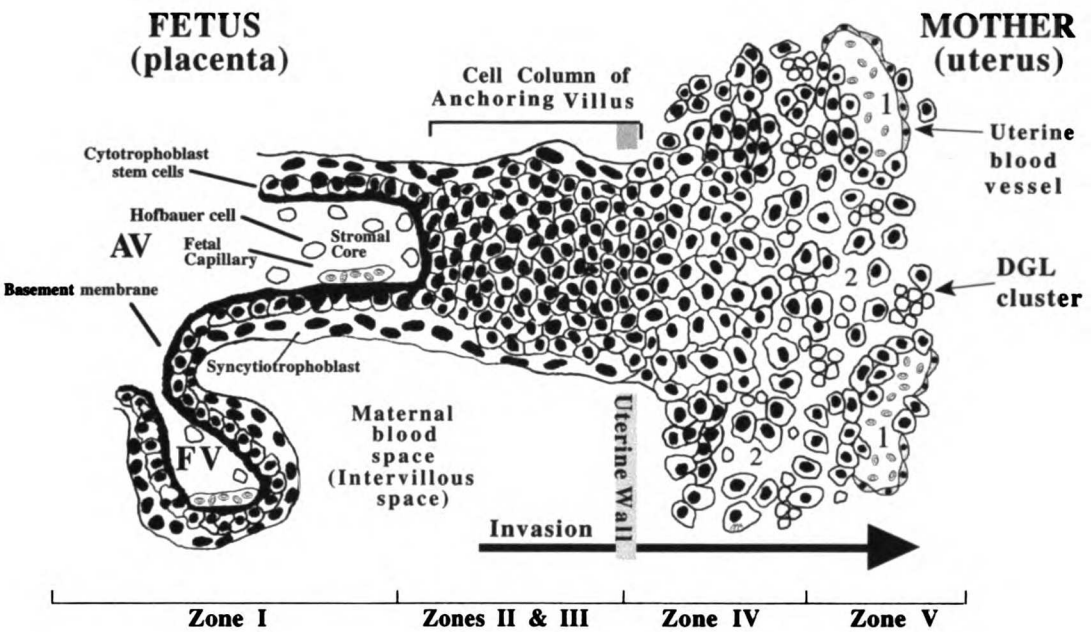

FETUS
(placenta)
MOTHER
(uterus)
Cell Column of
Anchoring Villus
Cytotrophoblast
stem cells
Hofbauer cell
AV
Fetal
Capillary
Stromal
Core
Basement membrane
Syncytiotrophoblast
FV
Maternal
blood
space
(Intervillous
space)
Uterine Wall
Invasion
Uterine
blood
vessel
DGL
cluster
1
2
2
1
Zone I
Zones II & III
Zone IV
Zone V

Figure 6-1

Figure 6-1. Anatomy of the Placental Bed. At the fetal-maternal interface, 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), establish 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 non-polarized 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 invasion. 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).

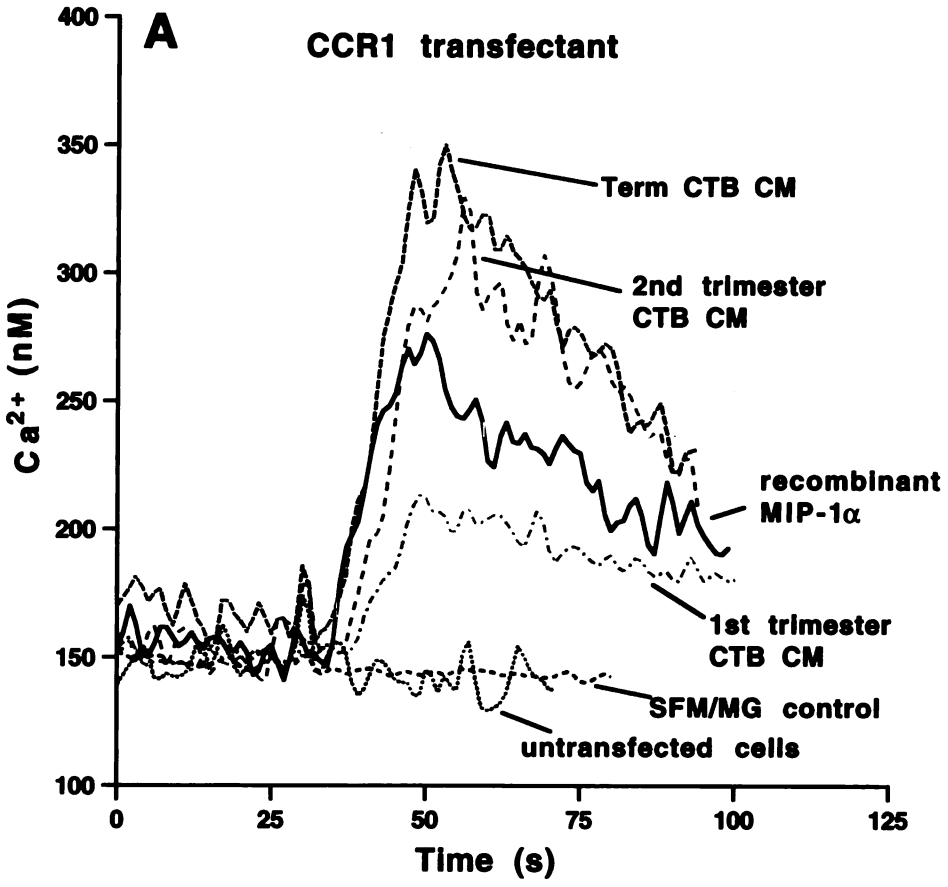

A
CCR1 transfectant
Term CTB CM
2nd trimester
CTB CM
recombinant
MIP-1α
1st trimester
CTB CM
SFM/MG control
untransfected cells
Ca2+ (nM)
Time (s)
400
350
300
250
200
150
100
0
25
50
75
100
125

Figure 6-2

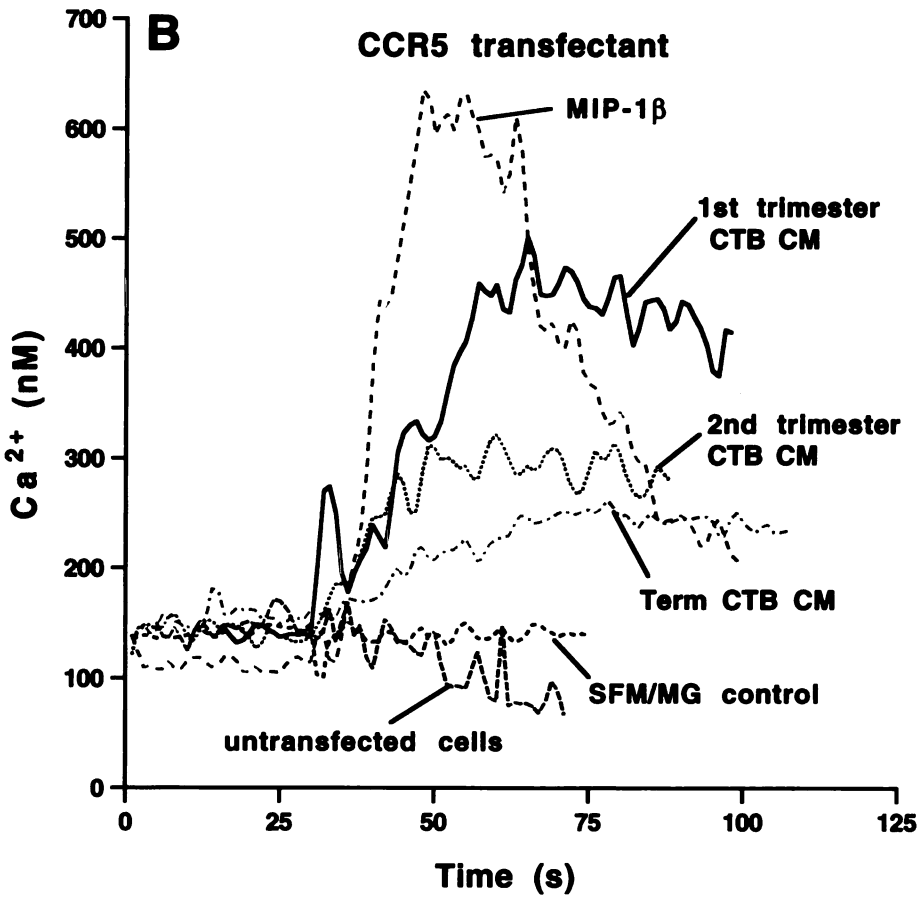

B
CCR5 transfectant
MIP-1β
1st trimester
CTB CM
2nd trimester
CTB CM
Term CTB CM
SFM/MG control
untransfected cells
Ca 2+ (nM)
Time (s)
700
600
500
400
300
200
100
0
0
25
50
75
100
125

Figure 6-2

Figure 6-2. Cytotrophoblast Conditioned Medium Signals Through MIP-1α Receptors. Cytotrophoblast (CTB) conditioned medium (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 Ca^{++} flux in cells expressing CCR1 (A) and CCR5 (B), but not CCR2, CCR3 or CX3CR1 (data not shown).

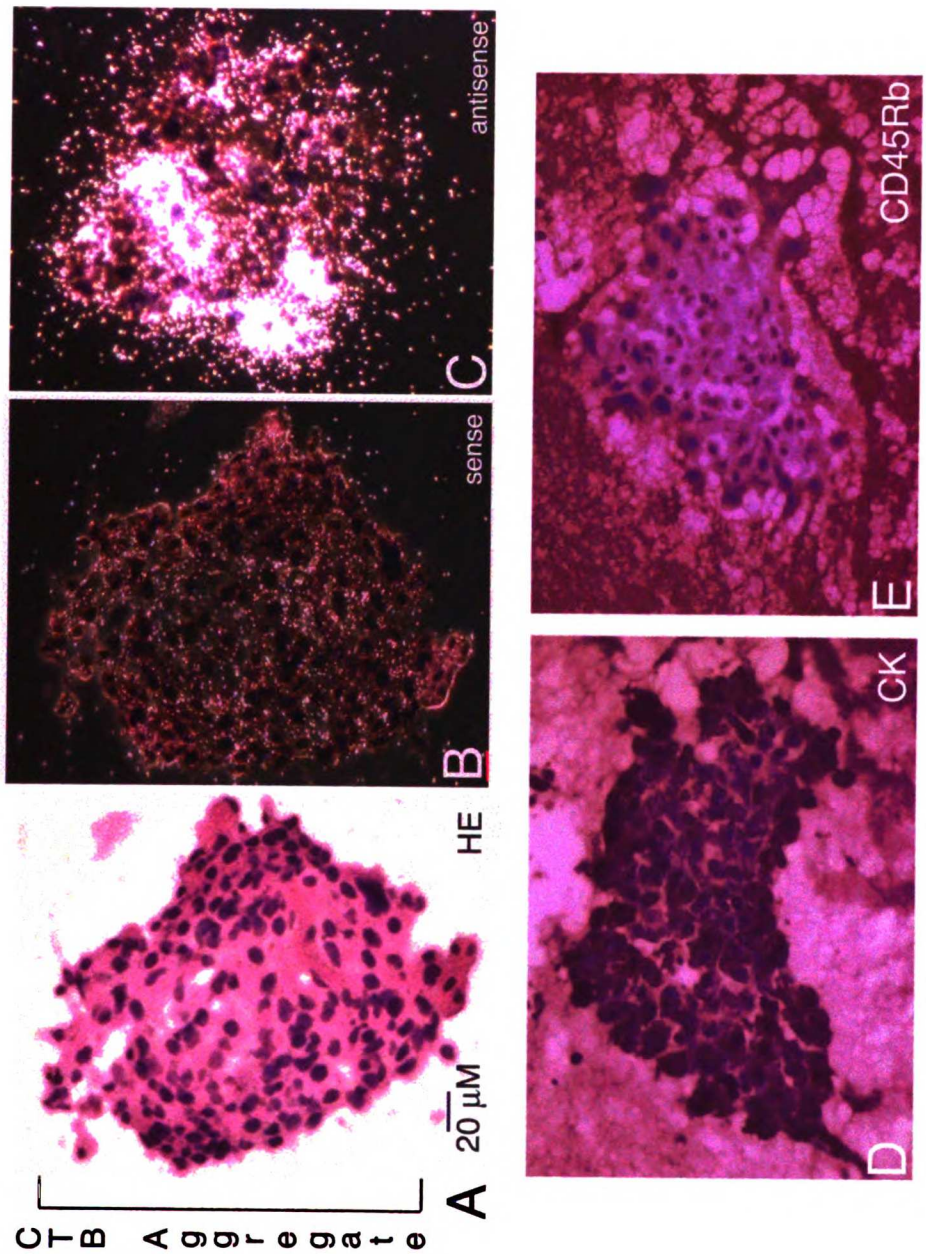


Figure 6-3

Figure 6-3. In Situ Hybridization Demonstrates That Cultured Cytotrophoblasts Synthesize MIP-1α 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-1α mRNA, detected as white dots (4 wk exposure). Immunohistochemistry on adjacent sections demonstrated positive staining for the cytotrophoblast marker cytokeratin (D) and the absence of staining for the macrophage marker CD45Rb (E).

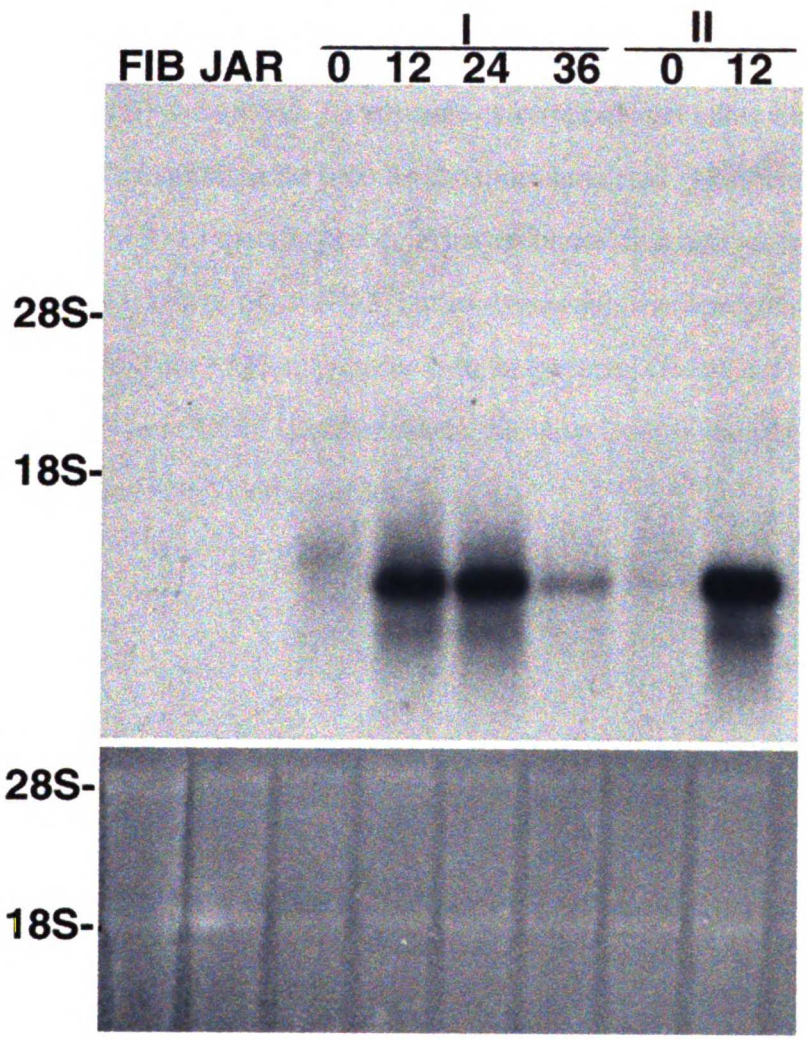


Figure 6-4

Figure 6-4. Northern Blot Hybridization Shows That Cytotrophoblasts Modulate MIP-1α mRNA Expression in Culture. (Top panel) Cytotrophoblast expression of MIP-1α 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 times indicated. Northern blot analysis was performed using a MIP-1α specific probe. Prior to culture, first and second trimester cells expressed very low levels of this mRNA, but its expression was upregulated by approximately 30-fold after 12 h in culture. Neither placental fibroblasts nor the JAR cell line expressed MIP-1α mRNA. (Bottom panel) Acridine Orange staining of the gel prior to transfer demonstrated equal loading of RNA.

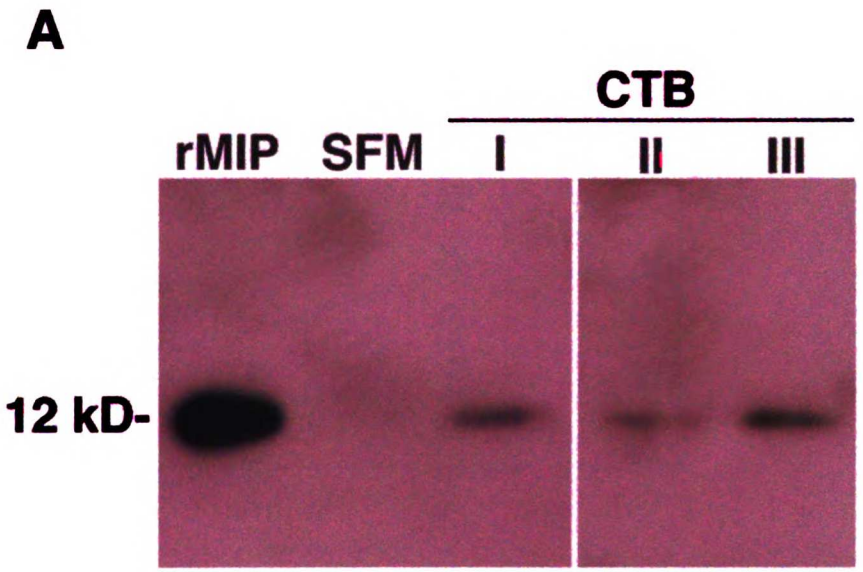


Figure 6-5

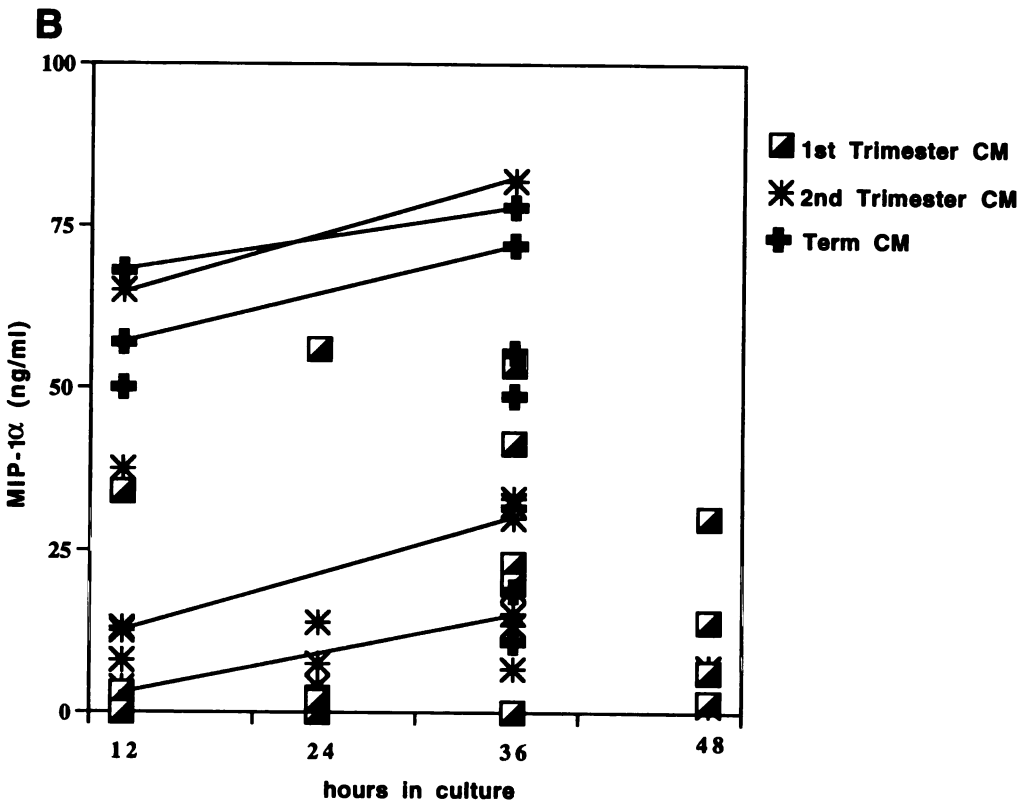

B
100
75
50
25
0
MIP-1α (ng/ml)
12
24
36
48
hours in culture
1st Trimester CM
2nd Trimester CM
Term CM

Figure 6-5

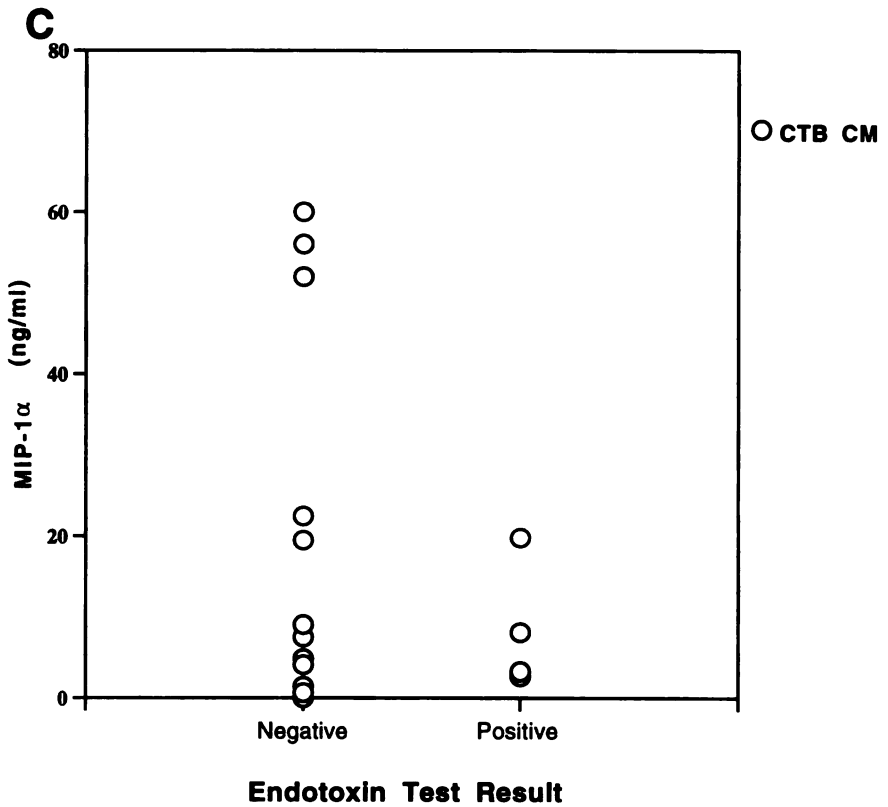
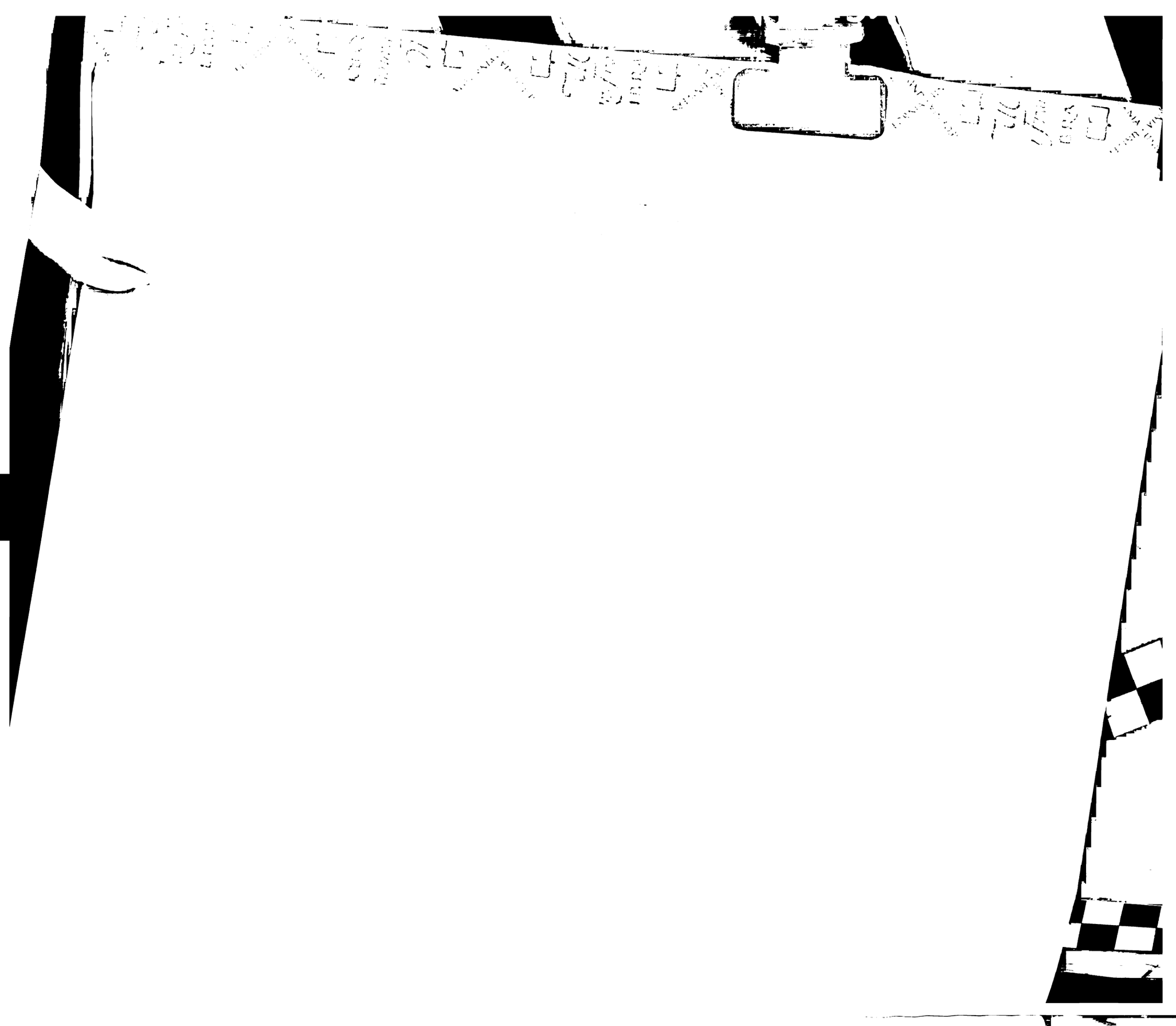

C
80
60
40
20
0
MIP-1α (ng/ml)
CTB CM
Negative
Positive
Endotoxin Test Result

Figure 6-5

Figure 6-5. Cytotrophoblasts Secrete MIP-1α Protein In Vitro. (A) Immunoblotting with a MIP-1α-specific polyclonal IgG. This antibody reacted with a single band of the expected size (12 kD) in a sample of recombinant MIP-1α (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 serum-free medium (SFM). (B) MIP-1α 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-1α 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-1α was not detected in control SFM/MG or placental fibroblast CM (data not shown). (C) The presence or absence of endotoxin was compared to the concentration of MIP-1α protein in cytotrophoblast CM. Twenty medium samples were assayed, four of which were positive for endotoxin. Chemokine concentrations in the samples ranged from 0.6 to 60 ng/ml. The Spearman Rank Order Correlation, which tests for trends between data sets, failed to show a correlation between the two variables.



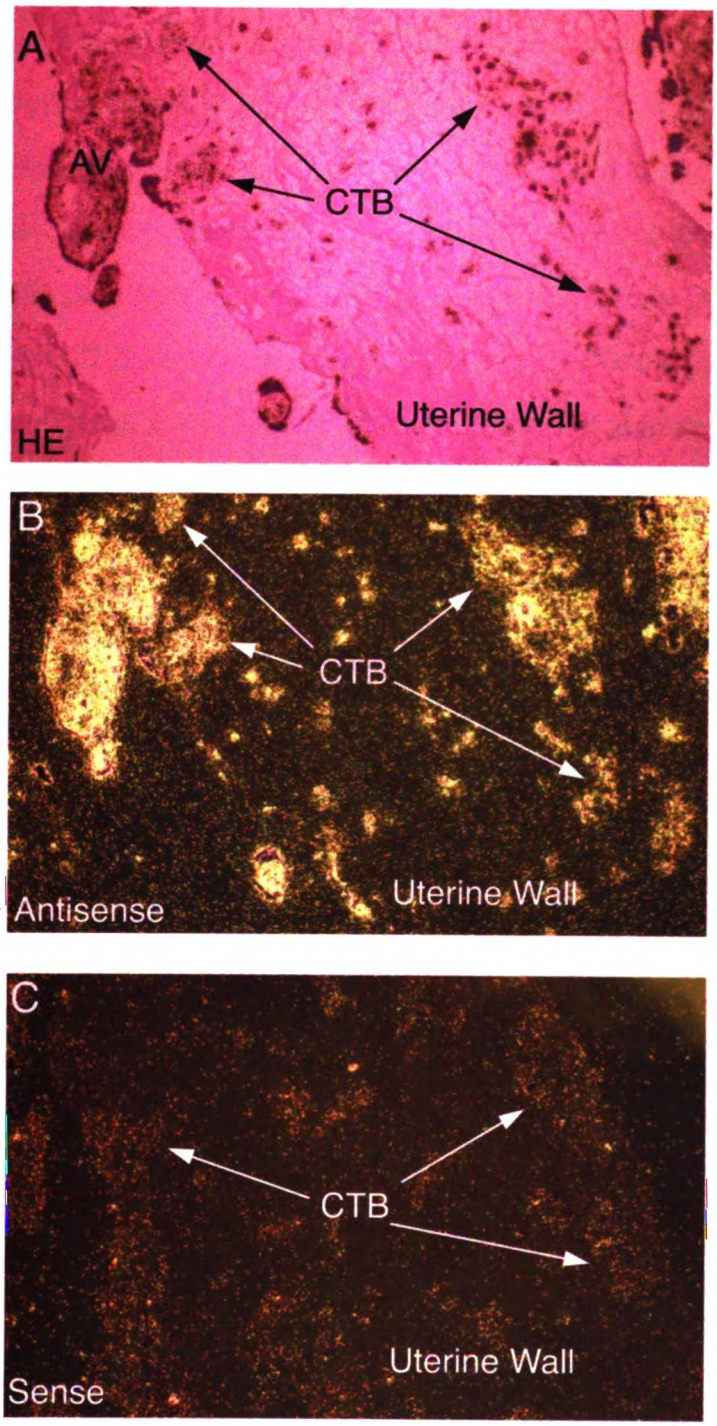

A
AV
CTB
Uterine Wall
HE
B
CTB
Antisense
Uterine Wall
C
CTB
Uterine Wall
Sense

Figure 6-6

Figure 6-6. Invasive Cytotrophoblasts Express MIP-1α mRNA In Vivo. In situ hybridization on tissue sections of the maternal-fetal interface demonstrates the presence of MIP-1α 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-1α antisense probe (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.

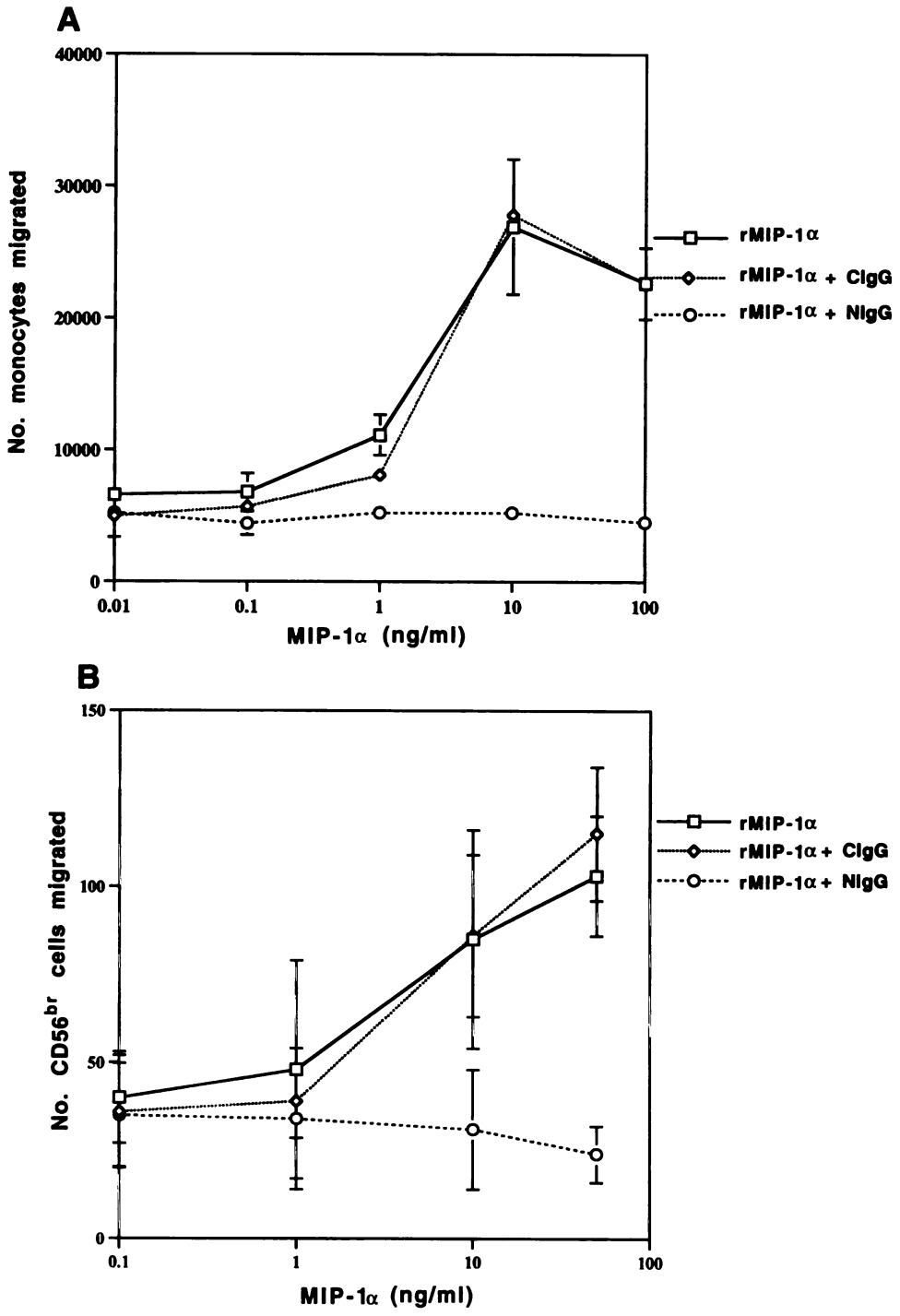

A
40000
30000
20000
10000
0
No. monocytes migrated
0.01
0.1
1
10
100
MIP-1α (ng/ml)
rMIP-1α
rMIP-1α + CIgG
rMIP-1α + NIgG
B
150
100
50
0
No. CD56br cells migrated
0.1
1
10
100
MIP-1α (ng/ml)
rMIP-1α
rMIP-1α + CIgG
rMIP-1α + NIgG

Figure 6-7

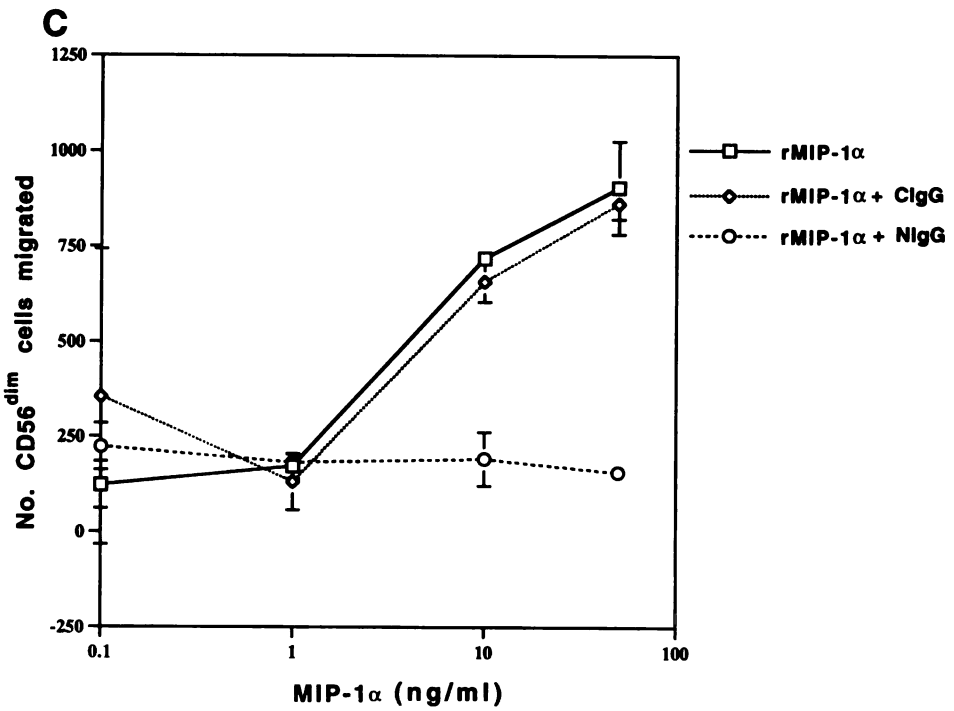

C
1250
1000
750
500
250
0
-250
No. CD56dim cells migrated
0.1
1
10
100
MIP-1α (ng/ml)
rMIP-1α
rMIP-1α + CIgG
rMIP-1α + NIgG

Figure 6-7

Figure 6-7. The Effects of Recombinant MIP-1α on PBMC Chemotaxis. (A-C) Results of a representative experiment. (A) Monocyte chemotaxis towards a dilution series of recombinant MIP-1α (rMIP-1α) peaked at a concentration of 10 ng/ml. Addition of a neutralizing anti-MIP-1α IgG (NIgG) returned migration to baseline levels, and a control IgG antibody (CIgG) added at the same concentration had no effect. (B) $CD56^{bright}$ and (C) and $CD56^{dim}$ NK cells also responded to recombinant MIP-1α, but their migration did not peak within the range of concentrations tested. Addition of anti-MIP-1α reduced NK cell migration to baseline.

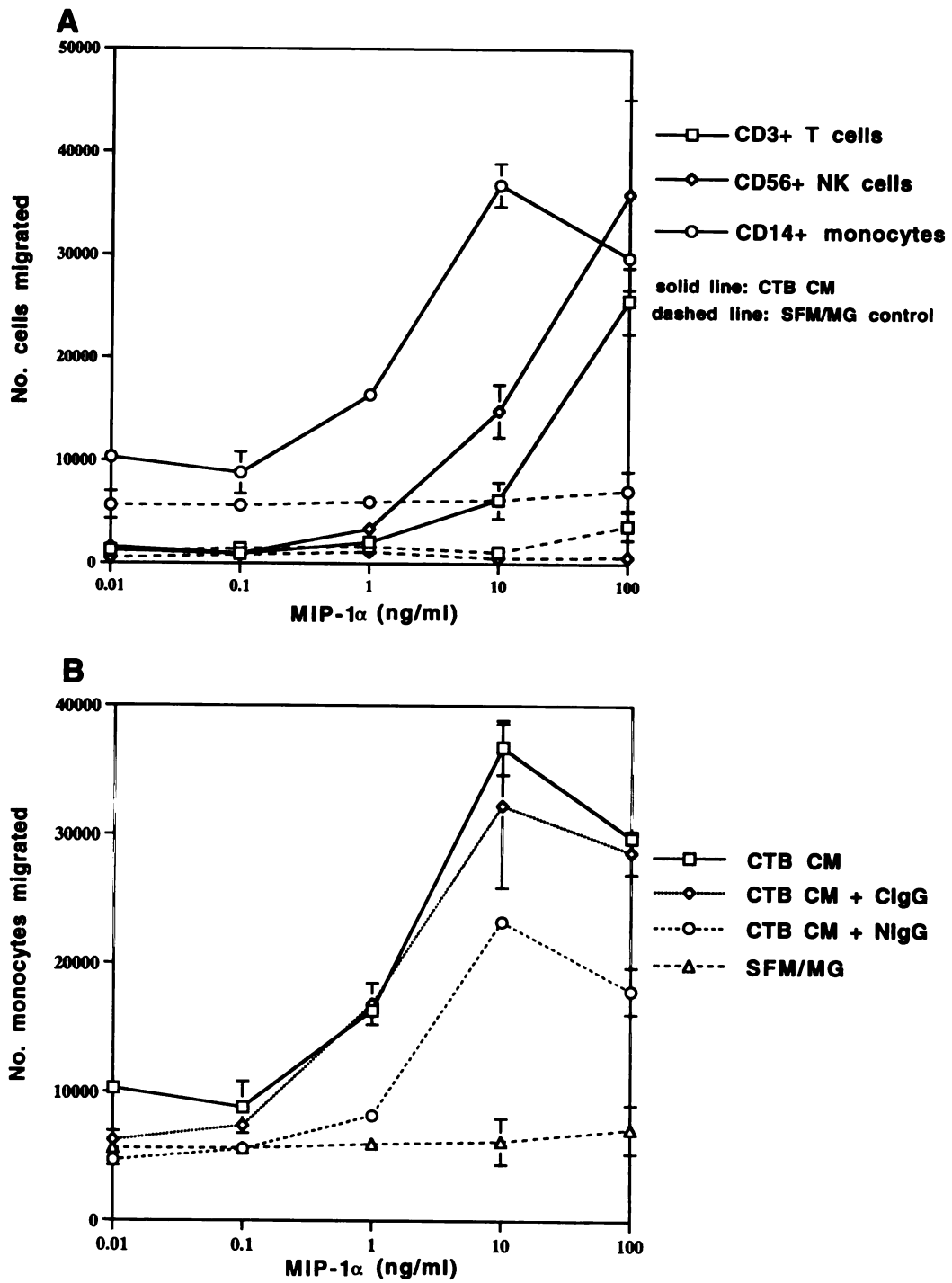

A
50000
40000
30000
20000
10000
0
No. cells migrated
0.01
0.1
1
10
100
MIP-1α (ng/ml)
CD3+ T cells
CD56+ NK cells
CD14+ monocytes
solid line: CTB CM
dashed line: SFM/MG control
B
40000
30000
20000
10000
0
No. monocytes migrated
0.01
0.1
1
10
100
MIP-1α (ng/ml)
CTB CM
CTB CM + CIgG
CTB CM + NIgG
SFM/MG

Figure 6-8

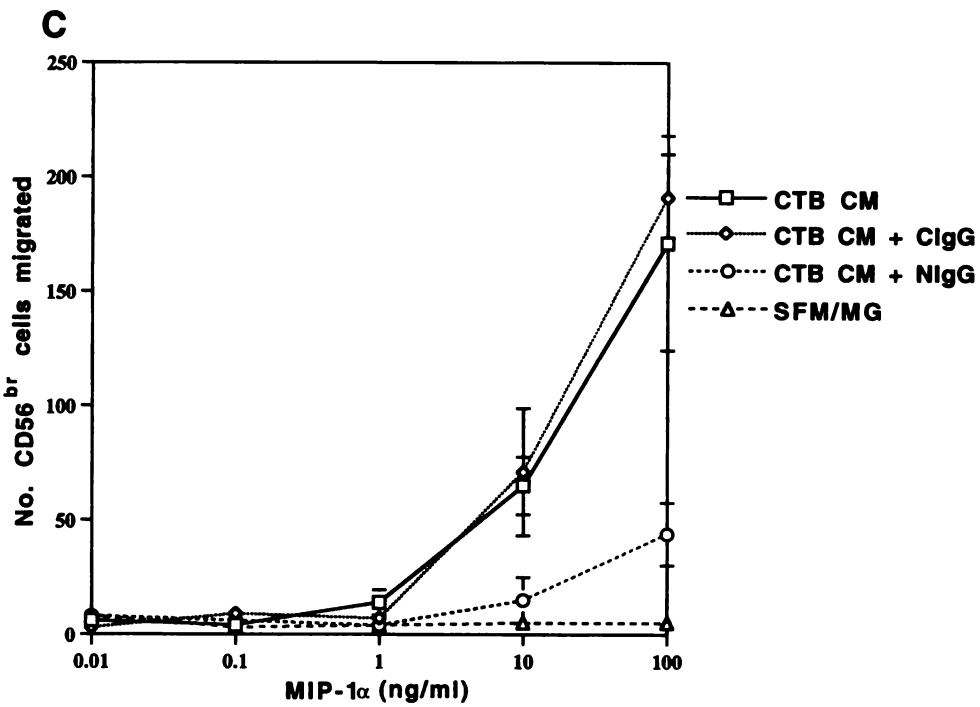

C
250
200
150
100
50
0
No. CD56br cells migrated
0.01
0.1
1
10
100
MIP-1α (ng/ml)
CTB CM
CTB CM + CIgG
CTB CM + NIgG
SFM/MG

Figure 6-8

Figure 6-8. Cytotrophoblast CM Attracts Monocytes and CD56^{bright} NK Cells Via the Action of MIP-1α. Panels A-C depict the results of a representative experiment. MIP-1α 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-1α is summarized in (A). In all experiments the migration of cells toward serum-free medium that was incubated with Matrigel alone (SFM/MG) was used to assess basal migration. Monocyte movement towards the CM peaked at 10 ng/ml of MIP-1α, 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-1α IgG (NIgG) significantly reduced the migration of PBMCs towards cytotrophoblast CM. (B) At its peak, monocyte migration was reduced by 44.2% ± 10.0% (mean ± SD) as compared to the addition of a control IgG (CIgG). (C) Chemotaxis of CD56^{bright} NK cells was diminished by 66.8% ± 20.0% as compared to the control IgG. Neither CD56^{dim} NK-cell nor T-cell migration was affected by neutralization of MIP-1α activity (data not shown).

Chapter Seven – Chemokine Expression in the Placental Bed

Abstract

Although first identified as chemoattractants involved in inflammation, subsequent studies uncovered numerous other processes in which chemokines participate (Chapter Four). Chapter Six described the expression of a particular chemokine, MIP-1α, by cytotrophoblasts, and the effect of its activity on the *in vitro* chemotaxis of leukocytes. The immune subsets that migrated towards cytotrophoblast CM represent the same cells found in the pregnant uterus, suggesting the possibility of a similar *in vivo* effect. We reasoned that this paradigm might involve other chemokines. Therefore, we screened the chemokine database for molecules that were expressed in placenta and pregnant uterus. These factors were then localized at a transcriptional level by in situ hybridization on uterine and placental tissues. Simultaneously, expression of chemokine recepetors by decidual leukocytes was studied by RNAse protection. Together, these results suggest that a number of receptor-ligand pairs are involved in decidual leukocyte recruitment. The expression patterns suggest chemokine involvement in other processes at the maternal-fetal interface such as angiogenesis and cytotrophoblast differentiation.

As shown in Figure 1-3, the maternal-fetal interface covers essentially two compartments, the uterus and the placenta. Specific chemokine expression patterns in these two compartments raised the possibility of regulatory networks acting in cis and trans. The functional ramifications of these expression patterns will require years to elucidate. Nevertheless, these data strengthen the hypothesis that chemokines play a critical role in placentation.

The maternal compartment: chemokine expression in the uterine wall

Successful human pregnancy requires that the maternal immune system, in defiance of all known laws of transplantation, tolerate 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 supporting maintenance of the fetal hemi-allograft (Thellin et al., 2000). For example, the exclusive expression of the non-classical class I molecule HLA-G (Kovats et al., 1990) by extravillous cytotrophoblasts probably plays a role in the downregulation of NK cell cytotoxicity via interaction with inhibitory receptors on the latter cells.

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. Accounting for at least 15% of all cells in the decidualized uterine wall, these immune cells are identifiable from early pregnancy through term (Yan Zhou, Susan Fisher, personal communication), although their presence at the latter time is controversial (Dallenbach-Hellweg, 1981; Starkey, 1993). As discussed in Chapter Three, the decidual leukocyte population is unique: comprised predominately (~70%) of an unusual type of NK cell ($CD56^{bright}$/ CD16-) with contributions from T cells (~15%) and monocytes (~%15) (Bulmer et al., 1991; King et al., 1989). Based upon the expression of markers such as Fas, CD69 and CD71 (Figure 3-2), these cells are activated. However, their cytolytic capacity is limited in comparison to that of peripheral NK cells (Ferry et al., 1990). 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 (Cyster, 1999), 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. 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 counter-receptors in its respective microdomain (Kruse et al., 1999).

Finally, although the contribution of chemokines to the recruitment of decidual leukocytes has not been previously considered, these molecules are required for effective leukocyte homing in other systems (Butcher and Picker, 1996) and likely play an analogous role during pregnancy. To date, only IL-8, MCP-1 and RANTES have been localized to the pregnant human uterus (García-Velasco and Arici, 1999; Hornung et al., 1997), and the possible effects of this expression on decidual leukocytes have not been investigated. Therefore, this thesis advances the literature in two important ways. First, it presents the expression patterns of a panel of nine chemokines in the decidua. Next, it examines chemokine receptor expression by decidual leukocytes. When analyzed together, these data identify potential ligand-receptor pairs involved in leukocyte trafficking to the pregnant uterus.

The fetal compartment: chemokine expression in the placenta

Placental development entails the growth of a fibroblastic villous core encased within a basement membrane covered by a layer of cytotrophoblast stem cells (Figure 1-3). The latter cells can adopt one of two differentiation fates, either fusing to from a multi-nucleate syncytium covering most of the villous surface, or detaching from the basement membrane to join a column of mononuclear cells that migrate into the uterine wall. These invasive cells firmly anchor the placenta to the uterine wall, remodel maternal arteries and divert blood flow to the intervillous space (reviewed in (Cross et al., 1994; Damsky et al., 1993)). In the latter location, nutrients, gases and wastes are transported between the syncytium and fetal blood vessels that course through the stromal villous cores.

Early in placental development, a special population of immune cells takes up residence in the villous stroma. These cells are macrophages of unknown origin and function, termed Hofbauer cells. They are numerous throughout the stroma, but are especially prominent along the basement membrane, just deep to the cytotrophoblast stem cells and syncytium. Based upon their expression of several markers, Hofbauer cells appear to be capable of capturing and presenting antigens (reviewed in (Benirschke,

2000)). However, the significance of this observation is unclear, as classical partners for antigen presentation, T cells and B cells, are not typically found in the villous stroma (Benirschke, 2000).

By analogy to other systems, many of these developmental events could be regulated by chemokines. Four broad areas of potential influence can be identified: proliferation, cell fate, angiogenesis, and leukocyte trafficking. With regard to mitosis, chemokines commonly stimulate proliferation in target cells (Schall and Bacon, 1994; Zlotnik et al., 1999). The link to cell fate is less clear, however the receptor CXCR4 and its ligand SDF1 are expressed in complementary patterns in the developing neuronal, cardiac, vascular, hematopoietic systems, as well as the craniofacial region of the mouse (reviewed in (McGrath et al., 1999)). Null mutations in either of these genes yield defects in these systems and locations. Even more intriguingly, receptor and cognate ligand are also expressed in a reciprocal fashion during gastrulation, when migrating cells (mesoderm and definitive endoderm) express CXCR4 mRNA while embryonic ectoderm cells express SDF1 message (McGrath et al., 1999). Finally, the role of chemokines in angiogenesis and cell trafficking has been well-established (Chapter Four).

To our knowledge, the role of chemokines has not been considered in the context of placental development. This thesis, which describes the mRNA expression of the aforementioned panel of nine chemokines in the placenta, is the first study to address this issue. Importantly, the results identify molecules that may participate in the developmental processes described above, and set the stage for functional experiments to confirm the hypotheses suggested by the data.

Results

Multiple chemokines are expressed at the maternal-fetal interface. As a first approach to our studies of chemokine expression in the placental bed, we narrowed the list of candidate molecules by selecting chemokines 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 chemokines that had been cloned from expression libraries of pregnant uterus or placenta. At the time this work was inititated, fifteen chemokines fit the established criteria. Since then the list has grown to include twenty-one members (Table 7-1). Based on initial results, we chose nine of the original chemokines for in depth analysis by in situ

hybridization studies localizing mRNA expression within tissue sections of the maternal-fetal interface. The tissues examined included portions of floating villi, anchoring villi, and the placental bed—decidualized uterus, with accompanying decidual leukocytes and invasive cytotrophoblasts (Figure 6-1). Positive controls included in situ hybridization on other tissues of known chemokine expression; negative controls consisted of sense probes hybridized to adjacent sections. The results show that both fetal and maternal tissues are richly endowed with chemokines.

Chemokines Cloned from Human Pregnant Uterus or Placenta

CC Family	CXC Family	CX3C Family
CCK1	BRAK	Fractalkine*
Eotaxin	GCP-2	
HCC-1*	GCP-2-like*	
MCP-1*	GRO1	
MCP-3	IL-8	
MIP-1 alpha*	IP-10*	
MIP-4	ITAC*	
RANTES	MIG*	
SLC	PBP	
	SDF1*	

Source: Chemokine website
http://cytokine.medic.kumamoto-u.ac.jp/CFC/CK/Chemokine.html Last updated 4/6/01

Table 7-1

Table 7-1. Numerous chemokines have been cloned from expression libraries of placenta or pregnant uterus. Chemokines thus identified are listed by class. Molecules selected for expression studies are designated with an asterisk. Source: chemokine website (http://cytokine.medic.kumamoto-u.ac.jp/CFC/CK/Chemokine.html). Last updated 4/6/01.

Figures 7-1 through 7-19 present representative expression patterns for fractalkine, GCP-2-like, HCC-1, IP-10, ITAC, MCP-1, MIG, MIP-1α, SDF1 and MIP-4. The results are summarized in Table 7-2. Several patterns emerged. In general, chemokines were abundant; mRNA for a variety of molecules was intensely expressed and widely distributed among cell types in both the maternal and fetal compartments . With the exception of SDF1, expression was remarkably stable across gestation, and in general, expression patterns were highly reproducible (n = from 5 to 19). Interestingly, several of the chemokines shared a common expression pattern. Many factors were broadly distributed throughout the stroma. In the fetal compartment, expression was detected in the villous cores. In the maternal compartment, expression was detected in the decidua. Additionally, individual chemokines were also produced by resident immune cells and by extravillous cytotrophoblasts.

Fractalkine, HCC-1 and ITAC had very similar expression patterns. Both villous cores (Figures 7-1 and 7-2, B and D; Figure 7-3, B, D and F) and decidual stroma (Figures 7-1 and 7-2, D; Figure 7-3, F) were strongly positive with an evenly distributed signal derived predominately from fibroblasts. GCP-2-like mRNA was expressed in a similar pattern (Figure 7-4, B and D) with less consistency and intensity (n=3 positive out

of 8 total samples). Occasional samples did not fit the dominant pattern. For example, ITAC expression by decidual leukocytes was observed in only one sample (data not shown).

Table 7-2

Chemokine Expression in the Placental Bed

Chemokine	Site of Expression							
	Fetal Compartment				Maternal Compartment			
	n =	*Villous fibroblasts*	*Hofbauer cells*	*CTBs in cell column*	n =	*CTBs in uterine wall*	*Decidual stroma*	*Decidual leukocytes*
Fractalkine	8	++ (n=8)	-	-	8	-	++ (n=8)	-
GCP-2-like	8	++ (n=3), - (n=5)	-	-	8	-	++ (n=3), - (n=5)	-
HCC-1	8	++ (n=8)	-	-	8	-	++ (n=8)	-
IP-10	9	+ (n=2), - (n=7)	-	-	10	-	Patches near clusters of leukocytes + (n=10)	-
ITAC	6	++ (n=6)	-	-	7	-	++ (n=7)	+ (n=1), - (n=6)
MCP-1	5	++ (n=3), - (n=2)	++ (n=3), - (n=2)	-	5	-	++ (n=4), - (n=1)	++ (n=4), - (n=1)
MIG	9	+ (n=2), - (n=7)	-	-	5	-	Patchy expression + (n=1), - (n=4)	-
MIP-1α	16	++ (n=14), - (n=2)	++ (n=14), - (n=2)	++ (n=9)**	13	++ (n=9)**	++ (n=13)	++ (n=13)
SDF-1	5	+ (n=2 both 2T), - (n=3 all 1T)	-	+ (n=1, 2T), - (n=4)	4	+ (n=2, 2T), also expression in endothelial cells lining uterine vessels (n=1, 2T)	-	+ (n=1, 1T), - (n=3)

Table 7-2. Summary of chemokine expression in the placental bed. Results of in situ hybridization studies on tissues from the maternal-fetal interface are shown. Expression was assessed in both the fetal and maternal compartments. "n" = number of individual placentas analysed. "++" indicates strong expression, "+" intermediate expression, and "-" the absence of expression. Cases in which non-uniform expression was detected are noted. ** Invasive cytotrophoblasts were only present in 9 samples hybridized to MIP-1α. 1T: first trimester, 2T: second trimester.

MCP-1 and MIP-1α yielded a strong punctate signal in both villous and decidual stroma (Figure 7-5, B, D and F; Figure 7-6, B and D). This characteristic distribution pattern suggested expression by the resident immune populations, Hofbauer cells in the placenta and decidual leukocytes in the uterine wall. Both of these factors were also broadly expressed at a lower level throughout the decidual and villous stroma (e.g., Figure 7-6, B and D). Of special note was the highly consistent expression of MIP-1α by extravillous cytotrophoblasts (Figure 7-6, F), both in cell columns and in the uterine wall (n = 17 of 19). As described in Chapter Six, this expression contributes to the ability of cytotrophoblasts to influence the migration of NK cells, monocytes, and T cells.

IP-10 displayed a distinct expression pattern in patches associated with leukocyte clusters in the decidual stroma (Figure 7-7, B and F). Although expression by leukocytes could not be excluded, at least a portion of the signal was derived from the intercalated stromal cells. Infrequently, IP-10 expression was observed (n = 2 of 9) in the villous stroma (Figure 7-7, D).

SDF1 also generated a unique signal. Unlike the other eight chemokines in the study, production of this molecule appeared to be regulated with gestation. Of the

specimens examined, four of five first trimester samples were negative, while two second trimester samples revealed interesting patterns. In the latter cases, specific expression was seen in several regions. For example, some extravillous cytotrophoblasts were positive for SDF1 (Figure 7-8, F and H). Strikingly, expression of SDF1 mRNA was detected in association with the cells that lined uterine blood vessels (Figure 7-8, D and F). These cells likely included both endothelial and invasive cytotrophoblast populations. Additionally, a broad signal in the villous stroma suggested SDF1 production by fibroblasts (Figure 7-8, B). The lone positive first trimester sample demonstrated a punctate signal consistent with decidual leukocyte expression (data not shown).

MIG was rarely expressed in the placental and decidual tissues examined. With respect to the maternal compartment, one out of five specimens showed patchy expression in decidual stroma (Figure 7-9, B). Similarly, in the fetal compartment, the villous stroma was positive in only two of nine samples (Figure 7-9, D).

Decidual leukocytes express chemokine receptors that bind ligands produced by cells 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 seventeen receptors, were used.

RNA corresponding to multiple receptors, capable of binding a spectrum of CXC, CC and CX_3C chemokines, was expressed by the immune cells. Given that the decidual leukocyte mRNA was isolated from a heterogenous mixture of NK cells, monocytes and T cells, the data did not give information about receptor expression in specific cell types. Nevertheless, at the population level, the results revealed several interesting findings. The data are shown in Figure 7-11 and are summarized in Table 7-3. Receptor expression was remarkably consistent among samples. Accordingly, CCR1, CCR5, CCR7, CXCR3, CXCR4, CX_3CR1 and the orphan receptor STRL33 were highly expressed. CCR2a, CCR2b and CCR4 expression was consistent, though slightly weaker. Occasional expression of CCR8, CXCR1, CXCR2, CXCR5 and the orphan receptor GPR1 was noted. A few samples containing the minimum amount of RNA

(2μg) yielded fewer bands, providing possible clues about the relative expression levels of these factors in decidual leukocytes.

Decidual Leukocyte Expression of Chemokine Receptors as Assessed by RNAse Protection Assay

Probe Set hCR5	*Sample:* µg RNA/sample:	8 wk A 20	9 wk A 20	20 wk 20
CCR1		++	++	++
CCR2a		+	+	++
CCR2b♦		+	+	++
CCR3©		-	-	-
CCR4		+	+	++
CCR5✱		++	++	++
CCR8♦		-	-	+

Probe Set hCR6	*Sample:* µg RNA/sample:	8 wk B 2	8 wk C 17.5	9 wk A 3.7	15 wk 8.1	9+16 wk 20
CXCR1		-	+	-	+	-
CXCR2		-	+	-	+	-
CXCR3		-	++	++	++	++
CXCR4■		++	++	++	++	++
CXCR5		-	+	-	-	-
CCR7		-	++	++	++	++
V28/CX3CR1▲		-	++	++	++	++

Probe Set hCR8	*Sample:* µg RNA/sample:	8 wk A 2.5	16wk A 5.1	20 wk 20	7.5+15 wk 20
STRL33		++	++	++	++
US28		-	-	-	-
CCR3©		-	-	-	-
CCR5✱		++	++	++	++
CXCR4■		++	++	++	++
CCR8♦		-	-	-	-
GPR15		-	-	-	-
GPR1		-	-	-	++
V28/CX3CR1▲		-	++	++	++
CCR2b♦		-	++	++	++

Table 7-3

Table 7-3. Decidual leukocytes express a panel of chemokine receptors. The expression of the former molecules was assessed by RNAse protection assay using three probe sets (hCR5, hCR6 and hCR8) that represented a total of seventeen receptors. Expression was remarkably consistent between nine RNA samples from first and second trimester decidual leukocytes. The results from each probe set are depicted in separate boxes within the table. The gestational age of the samples, and the quantity of RNA hybridized per sample are listed across the top of each box. Gestational age is given in weeks. Inclusion of a letter distinguishes multiple samples of the same gestational age. Receptors that were assayed in more than one probe set are designated with a superscript symbol (♦, ●, ✖, ◆, ■, ♦).

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 described in this chapter strongly supports the latter theory. In situ hybridization showed widespread expression of chemokine mRNA in the chorionic villous stroma and decidua. Very specific expression patterns in other cell types, including cytotrophoblasts and decidual leukocytes, were also noted. The fact that decidual leukocytes express a broad repertoire of chemokine receptors suggests that they can respond to the complex chemokine milieu at the maternal-fetal interface. Together, these data strongly suggest that chemokines are an integral part of placentation, playing important roles in both immune, and non-immune functions.

The maternal compartment

Decidual leukocyte recruitment In the uterine wall, the paramount interest in chemokine function is their role in the recruitment of decidual leukocytes, part of the

holy grail of understanding maternal tolerance. As this thesis describes the distribution of chemokine ligands in the decidua, and the expression of chemokine receptors by decidual leukocytes, it is possible to establish receptor-ligand pairs that might be involved in leukocyte recruitment (Table 7-4) including: CCR1/HCC-1, CCR2/MCP-1, CCR5/MIP-1α, CXCR1/GCP-2-like, CXCR3/ITAC, CXCR4/SDF1 and CX_3CR1/Fractalkine. It is interesting to note that all of the chemokines expressed in the uterus, a partner receptor is expressed by the decidual leukocytes.

In general, the observed chemokine receptor expression reflects what is known about the decidual leukocyte population. For example, 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. Conversely, chemokine receptors characteristic of NK cells, T cells and monocytes were abundant (e.g., CX_3CR1, CXCR3, CCR5). The two decidual leukocyte samples that exhibited CXCR1 and CXCR2 expression, typical of neutrophils, may have contained the latter cells, which can infiltrate the placental bed in pathological processes.

A few other correlations between chemokine receptor expression by decidual leukocytes and other immune populations are noteworthy. CCR7 is commonly expressed

on naïve and Th1-polarized T cells, and on resting but not activated leukocytes. Likewise, CXCR4 is expressed on CD34+ progenitor cells, as well as on numerous mature leukocytes. In contrast, CCR4 expression is not normally found on naïve cells, but is characteristic of memory T cells, especially among subsets that home to the skin.

In terms of receptor expression, the decidual leukocytes resemble both Th1 and Th2 cohorts, although the stronger expression is towards the Th1 group. CCR5 and CCR7, common on Th1-polarized cells, are both highly expressed, while 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 (Roth et al., 1996). In summary, the majority of decidual leukocyte chemokine receptor expression correlates with a Th1-biased population of activated T and NK cells. However, the presence of resting or naïve cells is also suggested (e.g., CCR7 and CXCR4 expression). A flow cytometry-based investigation of chemokine receptor expression on subsets of decidual leukocytes would be most informative (Chapter Eight). Such a study could provide clues to cell function, by analogy to other systems, and simultaneously yield information about the mechanisms of leukocyte recruitment.

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. Specifically, chemokine and chemokine receptors have been identified in rejected cardiac and renal transplants. Rejected murine cardiac tissue expresses fractalkine, IP-10, MIG and ITAC, which are absent in non-rejected grafts. The activated T cell infiltrate expresses CXCR3 (and presumably CX_3CR1). CXCR3 expression has also been noted in rejected human cardiac transplants. In mouse, this receptor is required for acute graft rejection; blocking its function significantly prolongs graft survival. Finally, CXCR4 is expressed on the abundant leukocyte infiltrate in rejected human renal transplants, while normal kidneys lack detectable CXCR4.

Given the reciprocal chemokine receptor and ligand expression described above, how might the identified receptor-ligand pairs help cells traffic appropriately through the decidua? Two models help us understand how decidual leukocytes may interact with their complex molecular environment. First, the multi-step combinatorial model of cell homing (Butcher, 1991; Butcher and Picker, 1996) (see Chapter Four) describes four discrete steps that must occur for a cell to extravasate into a tissue. These steps predominately involve the actions of two classes of factors—chemokines, which provide directional cues, and adhesion molecules, which provide the cell with purchase on its

surroundings. In the combinatorial model, specifity of a tissue-bound leukocyte population is achieved by the requirement of a particular set of chemokine receptors and adhesion molecules to gain entry to the tissue. In this scenario, only a defined and limited subset of all circulating leukocytes possesses the necessary combination of factors to access a confined area. Thus specifity is accomplished. This model has been borne out by numerous examples in the mouse where distinct compartmentalization of leukocyte subsets occurs on the basis of their expression of chemokine receptors (Cyster, 1999). Presumably, such a system applies to the homing of decidual leukocytes, although we are only beginning to describe molecules that might be involved. Supporting evidence again comes from the mouse, in which specific adhesion molecule pairs are reciprocally expressed by discrete immune cell subsets and vascular endothelium in particular microenvironments within the pregnant uterus (Kruse et al., 1999).

The second model describes how decidual leukocytes might interact sequentially with the numerous, overlapping chemokines expressed in the uterine wall. This hypothesis, based on experiments testing the effects of conflicting chemotactic signals on neutrophil migration in agarose, holds that cells retain memory of their recent environment (Foxman et al., 1999). This quality allows them to 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 fashion to its destination within a tissue. For example, a possible combination of factors acting on the migration of decidual leukocytes would be: SDF1/CXCR4, to extravasate; HCC-1/CCR1, to move within the decidual stroma; IP-10/CXCR3, to cluster the cells near glands.

Chemokine Receptor and Ligand Pairs: Candidates for Decidual Leukocyte Homing Effectors

Decidual leukocyte receptor	Uterine wall chemokine
CCR1	HCC-1, MIP-1α,
CCR2a + b	MCP-1
CCR5	MIP-1α
CXCR1	GCP-2-like
CXCR3	ITAC, MIG, IP-10
CXCR4	SDF1
CX3CR1	Fractalkine

Table 7-4

Table 7-4. Compatible chemokine receptor and ligand expression in the placental bed identifies candidate molecules as decidual leukocyte recruitment effectors. The receptor-ligand pairs listed were deduced by combining in situ hybridization data on chemokine expression in the uterine wall, and RNAse protection data on chemokine receptor production by decidual leukocytes.

Given the vital nature of reproduction, it is likely that redundancies are built into its critical components. Our data suggests 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 (e.g., HCC-1 and MIP-1α 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 (Chantakru et al., 2001) does not affect leukocyte composition in the pregnant uterus.

Regulation of angiogenesis The need to establish maternal blood flow to the placenta requires remodeling of maternal arterioles and increased uterine blood flow (Figure 1-3). Accordingly, factors that regulate critical aspects of vasculogenesis/angiogenesis are likely involved, a hypothesis that is substantiated by recent findings from the Fisher laboratory concerning the role of VEGF family members (Yan et al, manusp in prep). However, as elsewhere, vasculogenesis/angiogenesis at the maternal-fetal interface must be tightly regulated. An interesting role for chemokines is emerging. Certain of these molecules have modulatory effects, either promoting or restraining blood vessel growth, activites that depending on the presence or absence,

respectively, of a three amino acid sequence dubbed the ELR motif (Strieter et al., 1995). In this thesis, the majority of chemokines identified in the uterine wall are angiostatic factors lacking this motif (Zlotnik et al., 1999). An example is SDF1, which among the factors localized in our study had a striking and unique pattern of expression by endothelium and endovascular cytotrophoblasts within a subset of uterine vessels. Similarly, ITAC, another angiostatic molecule, is broadly produced within the decidual stroma. However, parallel studies in our laboratory found expression of angiogenic chemokines in patterns overlapping those of the angiostatic molecules described here (Kristy Red Horse, personal communication). Thus, as in most biological processes, including conventional situations in which vasculogenesis/angiogenesis occurs, there is a delicate balance between pro and counteractive forces.

Cytotrophoblast differentiation Part of the invasive cytotrophoblast differentiation pathway involves the localization of a subset of these cells to maternal spiral arterioles. These endovascular cytotrophoblasts are integral to pregnancy success, as they remodel the arterioles to increase blood flow to the placenta. When these vessels are not adequately remodeled, the fetus suffers from intrauterine growth restriction—a condition that has recently been related to numerous adult health problems including cardiovascular disease and diabetes (Barker, 1999; Barker, 2000). Although the

molecular explanation for this connection is enigmatic, the data are incontrovertible. Additionally, faulty remodeling of the uterine arteries also puts the mother at risk for a potentially catastropic pregnancy complication termed preeclampsia, which is characterized by the sudden onset of high blood pressure and proteinurea, signs of widespread systemic vascular damage.

The mechanisms that allow cytotrophoblasts to occupy maternal vessels and function as an endothelium are not understood. The observation that uterine veins are only rarely breached by fetal cells led to the hypothesis that oxygen tension targets the invasive cells preferentially towards arteries (Genbacev et al., 1996; Genbacev et al., 1997). The specific expression of SDF1 by endothelial cells and invasive 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 upregulate the expression of chemokine receptors, and this expression is involved in the preferential seeding of particular tumor cells to specific secondary organs (Muller et al., 2001). 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 (Chapter Eight).

The Fetal Compartment

Hofbauer Cell Recruitment and Function Early in placental development, a specialized population of macrophages, termed Hofbauer cells, takes up residence in the villous stroma. The mechanisms underlying recruitment of these cells are not known. Furthermore, their source has not been elucidated. The target population could reside in either the fetal or maternal circulation (Figure 1-3). This question is an area of active investigation in our laboratory. Regardless of their origin, the fundamental processes involved in leukocyte trafficking are probably conserved in Hofbauer cell recruitment. Of the chemokines expressed in the villous stroma, many act on monocytes and macrophages. MCP-1, MIP-1α, HCC-1 and fractalkine are ideal candidates for molecules regulating the homing of these cells to the fetal villous stroma.

Additionally, Hofbauer cells themselves may be a source of chemokines. In correlation with chemokine production by macrophages in other tissues, Hofbauer cells in the villous stroma appear to produce both MCP-1 and MIP-1α. Interestingly, their localization within the villi, predominately near the basement membrane, just deep to the

cytotrophoblast stem cells and the overlying syncytium, is an ideal position for sampling factors crossing the syncytium, one possible entry point for invading pathogens. Another entry point, the amnio-chorion, also contains a resident macrophage population that might serve a similar function, although even less is known about these cells.

Indeed, evidence suggests that Hofbauer cells are capable of antigen uptake and presentation (Chapter Two). In the event of an infection, these macrophages, which as a class are potent producers of chemokines and other cytokines, could recruit leukocyte effectors in response to aberrant chemokine expression. In an ongoing study of placental specimens obtained from patients who gave birth prematurely after premature rupture of the membranes and infection, we have noted several examples of abnormal immune cell infiltrates (Yan Zhou, Ling Huang, personal communication). Of these, the single specimen examined for chemokine production thus far demonstrated an irregular expression pattern: IP-10 was produced in the villous stroma.

Other examples of aberrant chemokine expression noted in this study (Table 7-2) may also be due to underlying pathologies. In a separate study, we found that placentas from mothers that smoked during pregnancy (> 20 cigarettes/day) sometimes exhibited anomalous chemokine expression patterns (Kristy Red Horse and Penelope Drake,

unpublished data). Future studies will examine in depth the relationship between chemokine expression and pathology (Chapter Eight).

Development of Fetal Blood Vessels in the Villous Stroma In general, fetal development is an explosive process. Rapid angiogenesis within the placenta proper must occur in advance of fetal growth. Accordingly, this process must be tightly controlled to avoid developmental problems. As described above, CXC chemokines can have both angiogeneic or anti-angiogeneic activities, depending on the presence or absence, respectively, of the ELR motif. In parallel with the results obtained in the uterine wall, the chemokine expression described in the villous stroma consists predominantly of angiostatic factors, largely because most of the molecules selected for study lacked the ELR motif and were thus angiostatic. The one angiogeneic molecule described, GCP-2-like, was broadly expressed in the villous stroma, albeit at a lower frequency than most other chemokines studied. However, concurrent work in our laboratory demonstrated the expression of ELR-containing chemokines in the villous stroma (Kristy Red Horse, personal communication). Thus, as in the uterine wall, it appears that chemokines contribute both positively and negatively to angiogensis in the placenta.

Cell Fate and Placental Development A transitory organ, the placenta undergoes its entire lifespan in nine months, during which time many important processes, including proliferation, patterning and differentiation occur. Evidence points to a role for chemokines in similar events during organogenesis in the fetus (Ma et al., 1998). With this in mind, possible chemokine actions in the placenta are numerous. Particularly intriguing is the potential for these molecules to regulate cell fate decisions. Expression patterns of GCP-2-like and fractalkine often revealed a signal gradient in the villous stroma that peaked at the base of a cell column. Parallel work on other chemokines in our laboratory has demonstrated even more specific patterns, whereby these factors are produced only by the cells immediately opposite the basement membrane from sites of cell column initiation (Kristy Red Horse, personal communication). These patterns suggest the hypothesis that chemokines influence the differentiation of cytotrophoblast stem cells opposite the basement membrane, inducing them to adopt the invasive, rather than the syncytial, phenotype. Although little is known about the role of chemokines in directing cell fate, the expression patterns of SDF1 and CXCR4 in the mouse embryo and blastocyst (McGrath et al., 1999) along with the phenotypes of mice carrying deletions in the respective genes, suggest that this ligand/receptor pair may play a role in developmental decisions. Our work supports this hypothesis and suggests that this is a fertile area for new research.

Finally, chemokines can also exert broad effects such as the induction of proliferation in target cells (Schall and Bacon, 1994; Zlotnik et al., 1999). As the placenta must rapidly grow and develop, apace with the fetus, chemokine action on mitosis of placental targets—villous fibroblasts and cytotrophoblast stem cells—may also be very important. Functional experiments addressing the effects of chemokines on these target cells are important and technically feasible (Chapter Eight).

Summary Through the description of nine chemokine expression patterns at the maternal-fetal interface, this thesis significantly advances our understanding of the importance of these molecules in pregnancy. Normal expression patterns have been established, and variations on these patterns may be used in the future as pathological markers (Chapter Eight). Furthermore, through the concurrent identification of chemokine receptor expression by decidual leukocytes, this thesis sets forward a set of receptor-ligand pairs as candidates for decidual leukocyte homing. We speculate that a subset of these mechanisms are also used in Hofbauer cell recruitment. In conclusion, this work provides a foundation from which to launch future investigations into the biology and function of chemokines at the maternal-fetal interface.

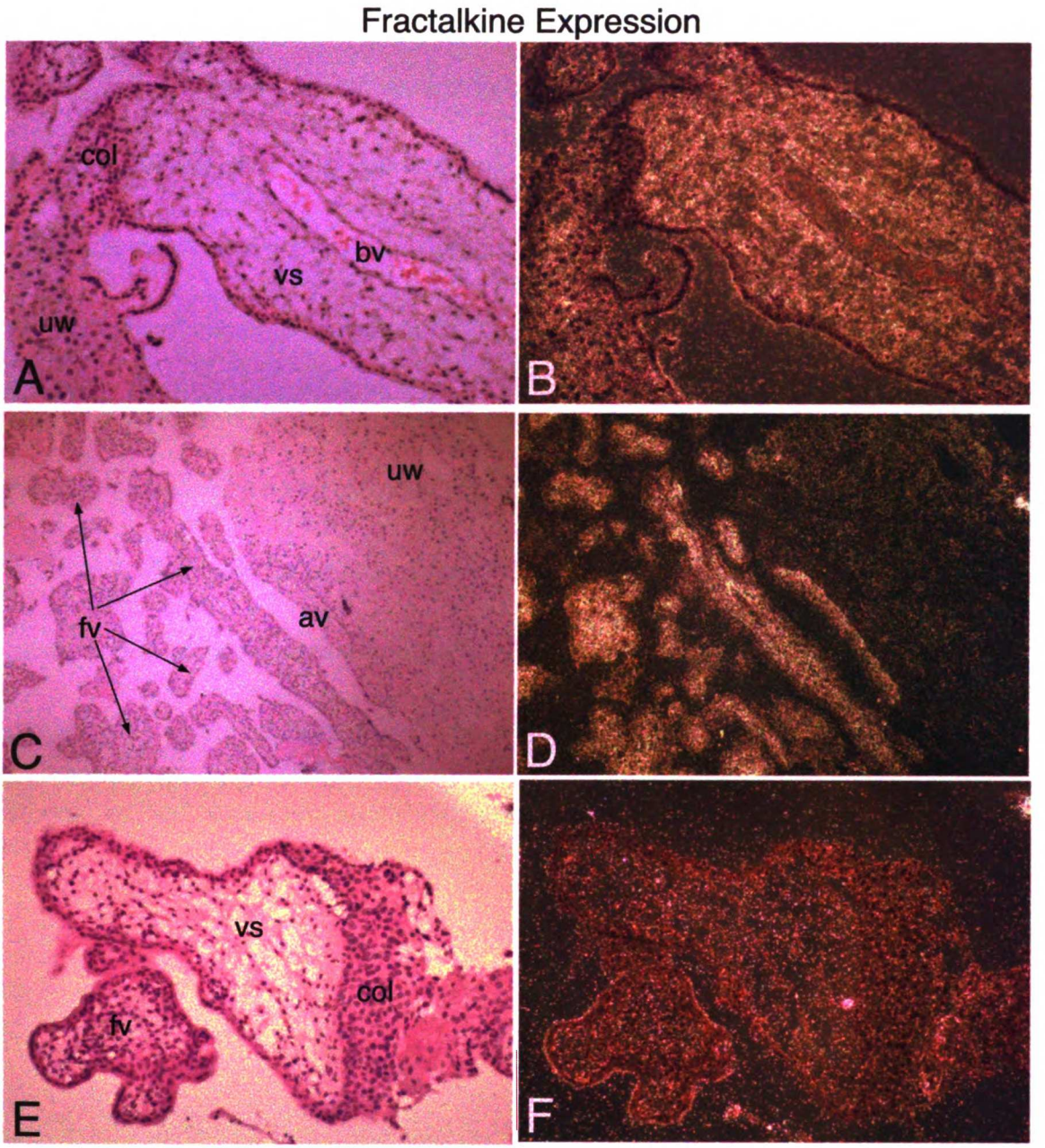


Figure 7-1

Figure 7-1. Fractalkine is expressed in villous and decidual stroma. In situ hybridization on tissue sections of the maternal-fetal interface demonstrates the presence of fractalkine mRNA in the stromal compartments of villi and decidua. (A, C, E) Bright field micrographs of histological sections stained with hematoxylin and eosin (H&E); (B, D, F) dark field micrographs of the same sections. White dots (B) indicate signal from ^{35}S-labeled antisense probe that was hybridized to a section of first trimester tissue (5 wk exposure). A similar pattern was observed when a fractalkine antisense probe was hybridized to second trimester tissue (D). In contrast, no signal was observed when tissue sections were hybridized with a sense probe (e.g., F). vs: villous stroma, uw: uterine wall, col: cytotrophoblast cell column, bv: blood vessel, fv: floating villi.

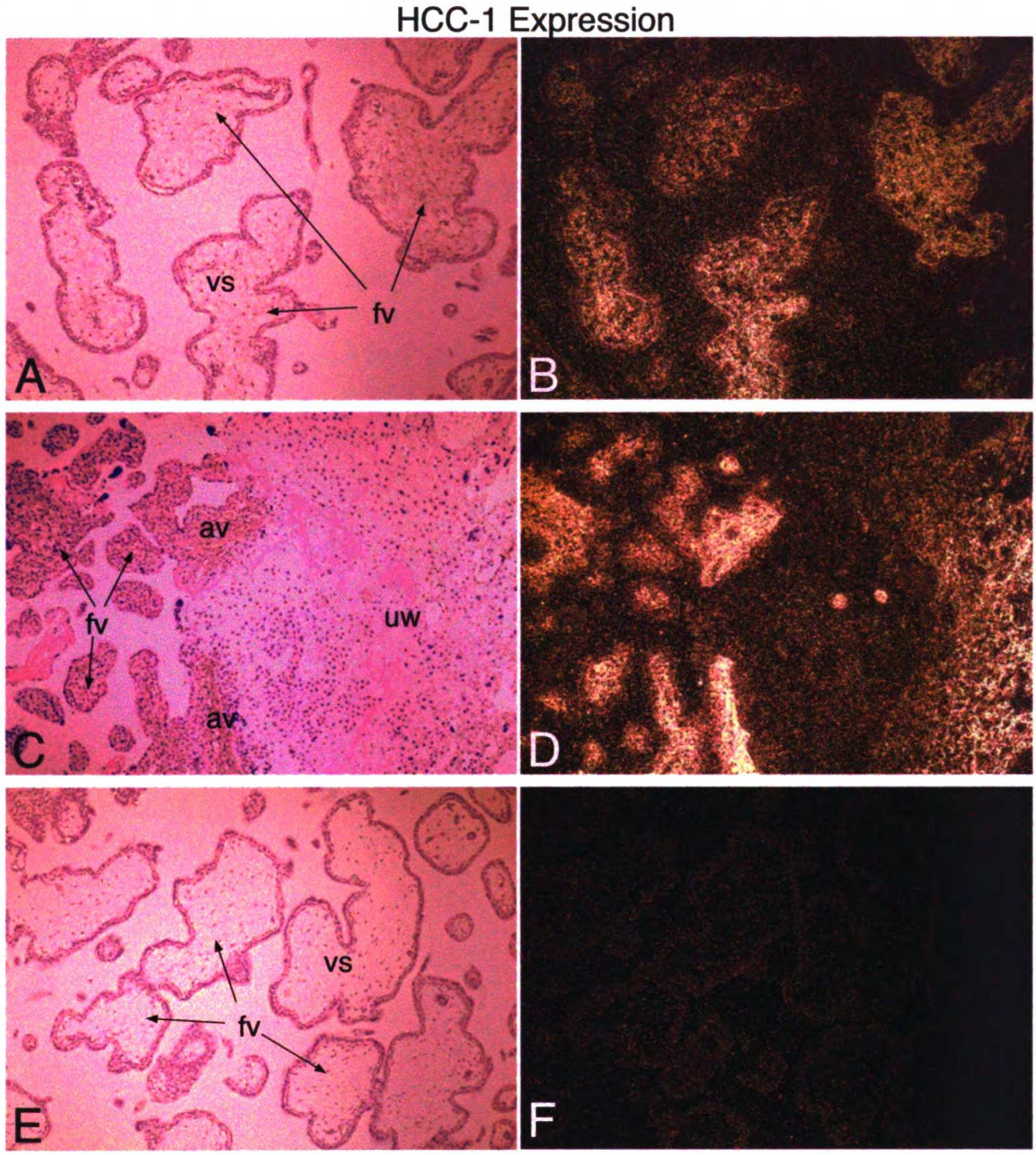


Figure 7-2

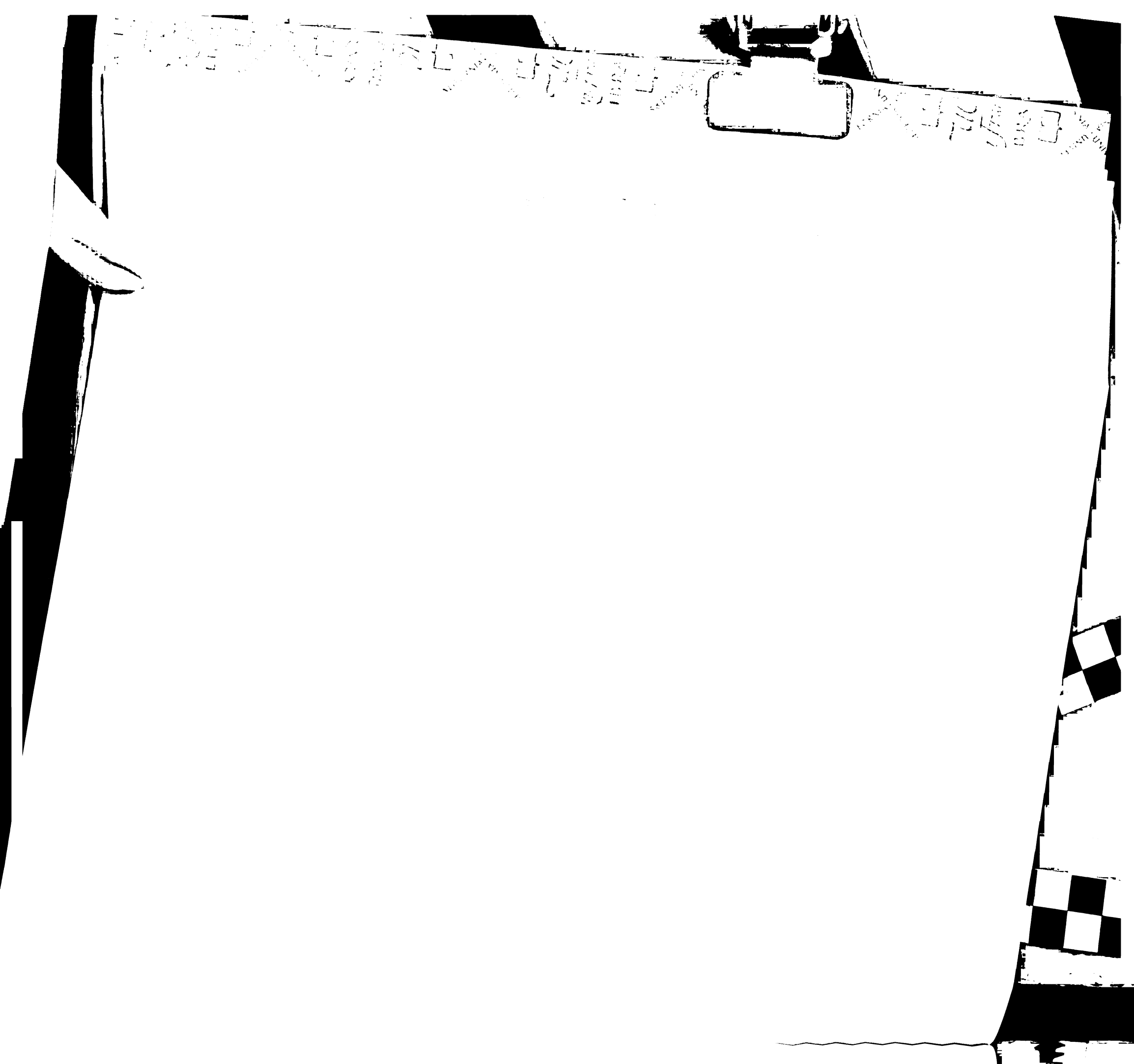


Figure 7-2. HCC-1 is produced by villous and decidual stroma. Panels A, C and E are bright field micrographs of histological sections stained with H&E; Panels B, D and F are dark field micrographs of the same sections. In situ hybridization on first trimester (B) and second trimester (D) tissues revealed abundant HCC-1 expression in the stromal compartments of villi and decidua. White dots indicate signal (8 wk exposure) from an ^{35}S-labeled antisense probe. There was no signal in sections that were hybridized to a sense probe as a negative control (F). av: anchoring villus.

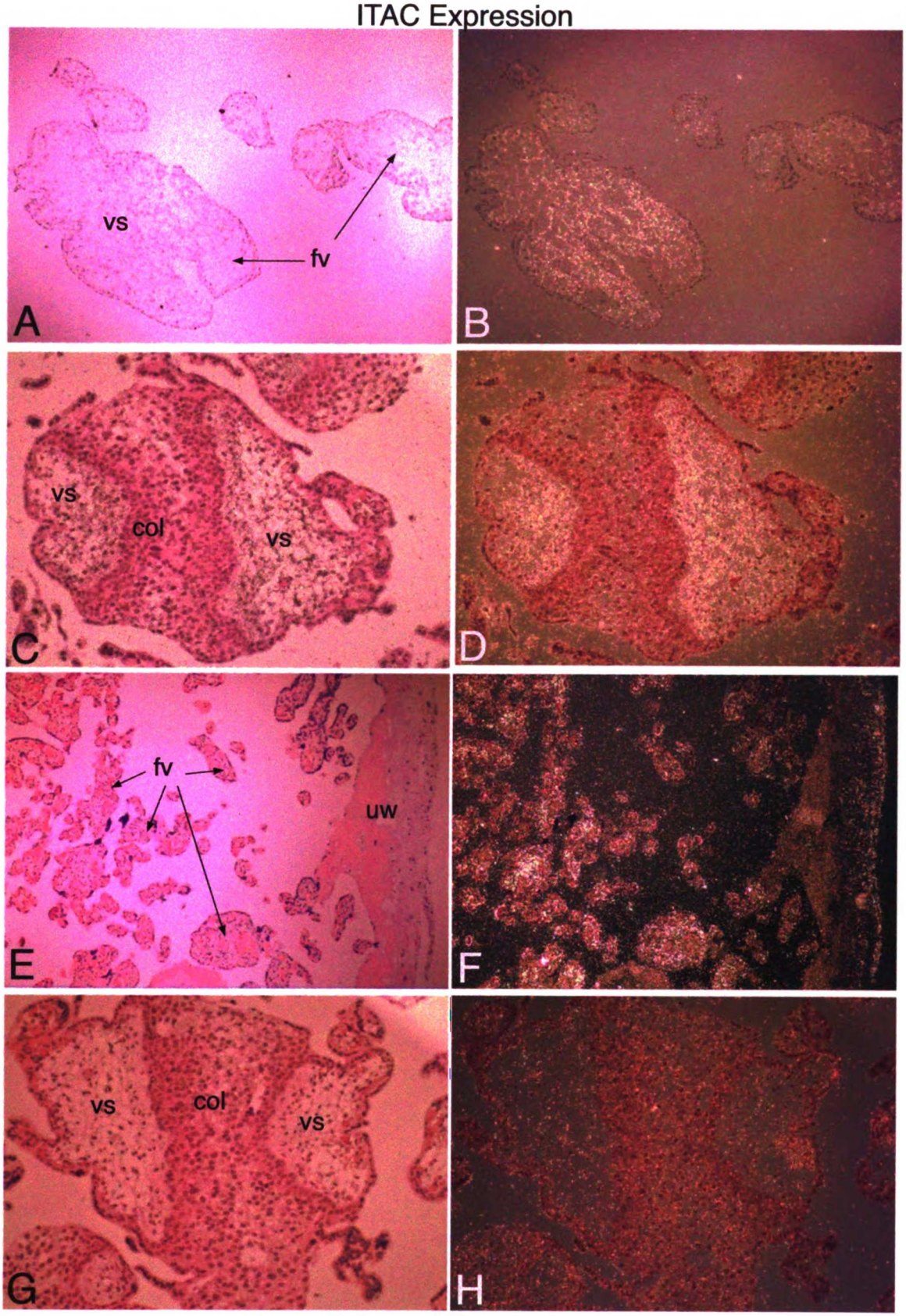


Figure 7-3 164

Figure 7-3. ITAC is produced by villous and decidual stroma. In situ hybridization on first trimester (B), second trimester (D) and term (F) tissues revealed ITAC expression in the stromal compartments of villi and decidua. Panels A, C, E and G are bright field micrographs of histological sections stained with H&E; Panels B, D, F and H are dark field micrographs of the same sections. White dots indicate signal (8 wk expression) from an ^{35}S-labeled antisense probe (B, D and F). Sense probe hybridized to adjacent sections as a negative control yielded no signal (H).

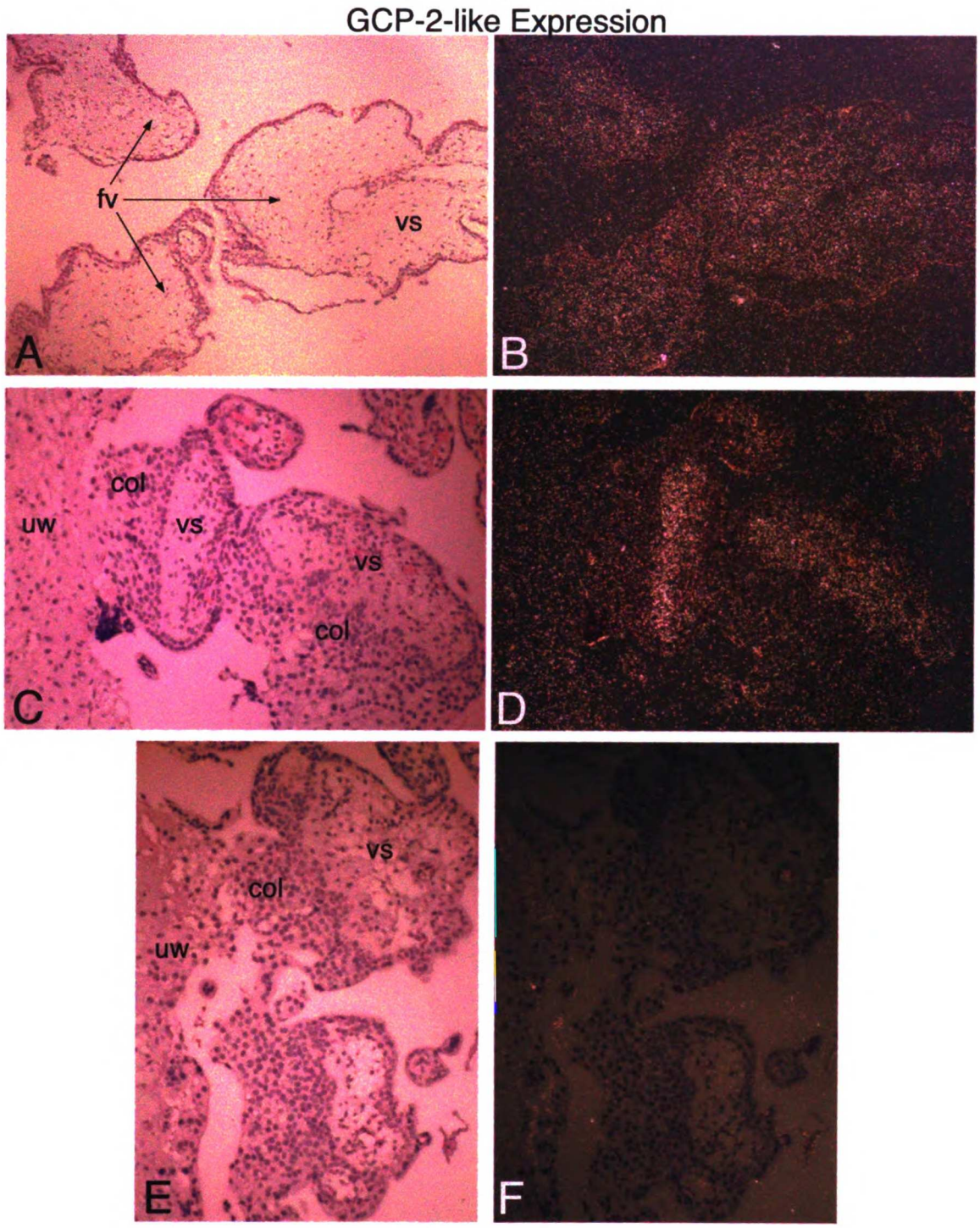


Figure 7-4

Figure 7-4. GCP-2-like is expressed in villous and decidual stroma. In situ hybridization on first trimester (B) and second trimester (D) tissues revealed GCP-2-like signal in the stromal compartments of villi and decidua. Panels A, C and E are bright field micrographs of histological sections stained with H&E; Panels B, D and F are dark field micrographs of the same sections. White dots indicate signal (9 wk exposure) from an ^{35}S-labeled antisense probe (B, D). Sense control was negative (F).

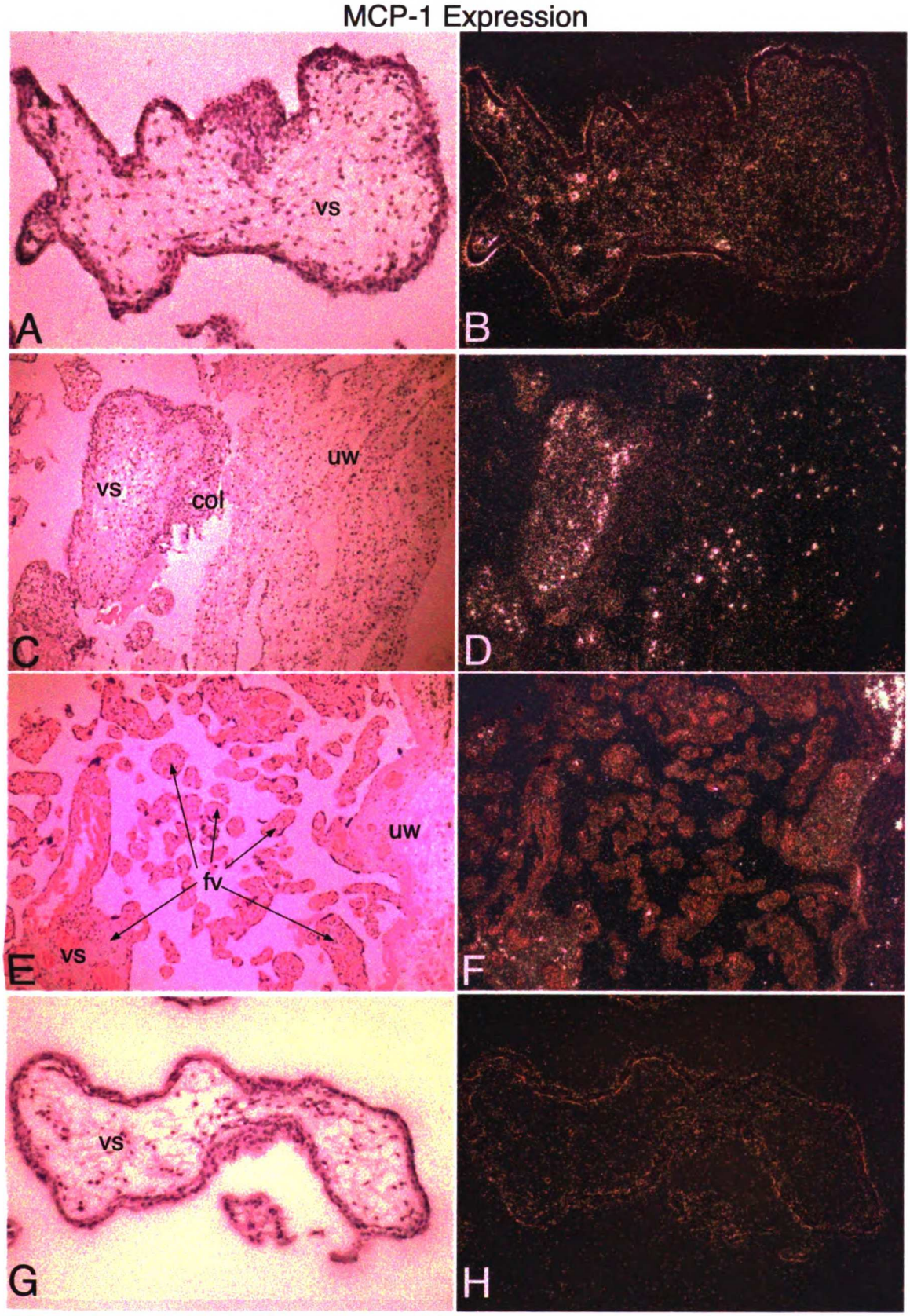


Figure 7-5

Figure 7-5. MCP-1 is expressed by Hofbauer cells and decidual leukocytes. In situ hybridization on first trimester (B), second trimester (D) and term (F) tissue sections of the maternal-fetal interface demonstrated a punctacte expression pattern in both decidual and villous stroma. Panels A, C, E and G are bright field micrographs of histological sections stained with H&E; Panels B, D, F and H are dark field micrographs of the same sections. White dots indicate signal (8 wk exposure) from an ^{35}S-labeled antisense probe (B, D and F). The distribution of this signal was consistent with production by decidual leukocytes and Hofbauer cells in their respective compartments. Signal was absent on tissue sections incubated with sense probe as a negative control (H).

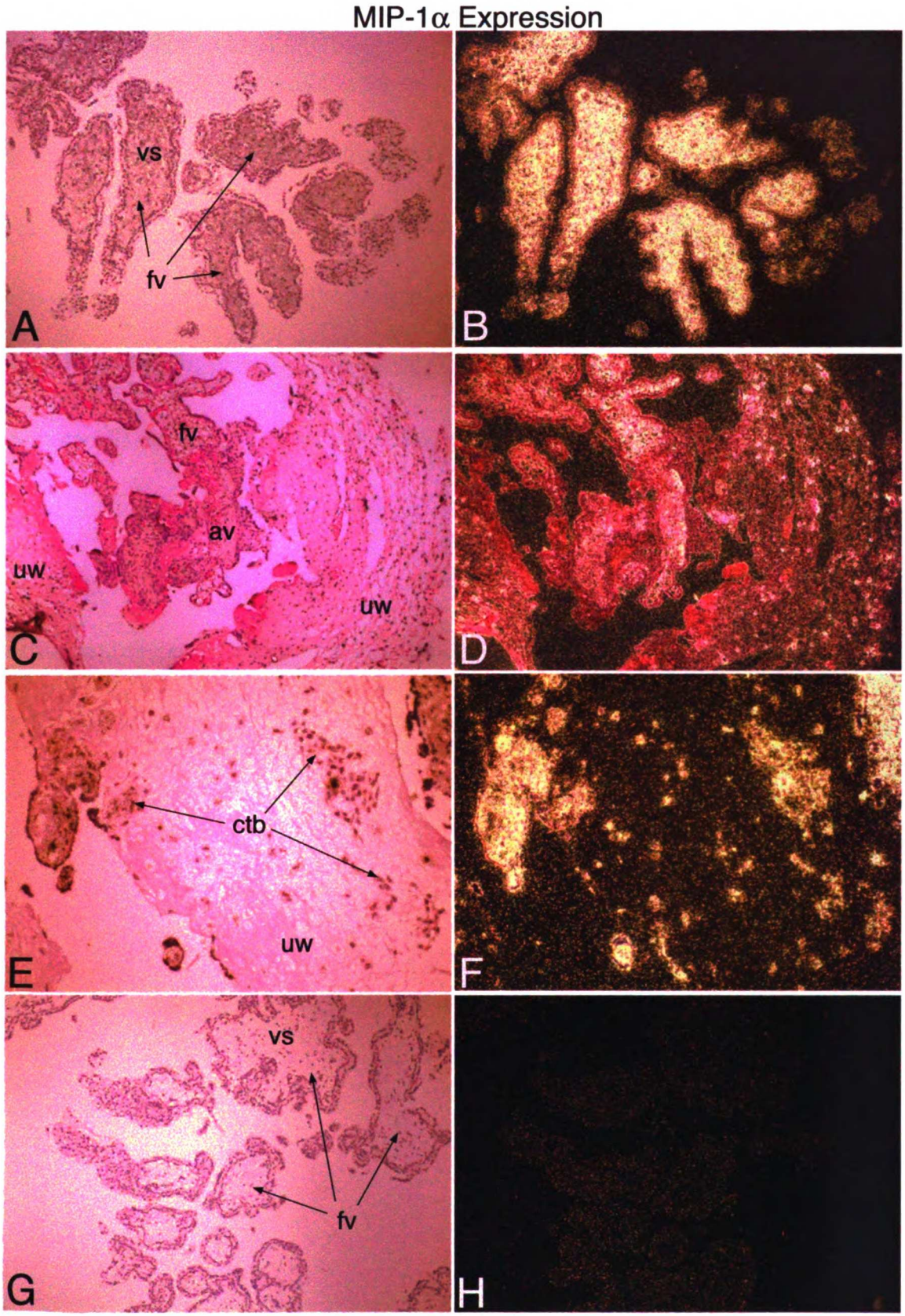


Figure 7-6

Figure 7-6. MIP-1α is expressed by villous stroma, decidua and extravillous cytotrophoblasts. In situ hybridization on first trimester (B), second trimester (D), and term (F) tissue sections of the maternal-fetal interface revealed abundant MIP-1a mRNA expressed in the villous and decidual stroma. In these compartments, signal was composed of even background stromal expression, overlain by a punctate pattern indicative of production by Hofbauer cells and decidual leukocytes (B and D). Additionally, extravillous cytotrophoblasts expressed MIP-1α (F). Panels A, C, E and G are bright field micrographs of histological sections stained with H&E; Panels B, D, F and H are dark field micrographs of the same sections. White dots indicate signal (8 wk exposure) from an ^{35}S-labeled antisense probe (B, D and F). Tissue sections hybridized with sense probe were negative (H).

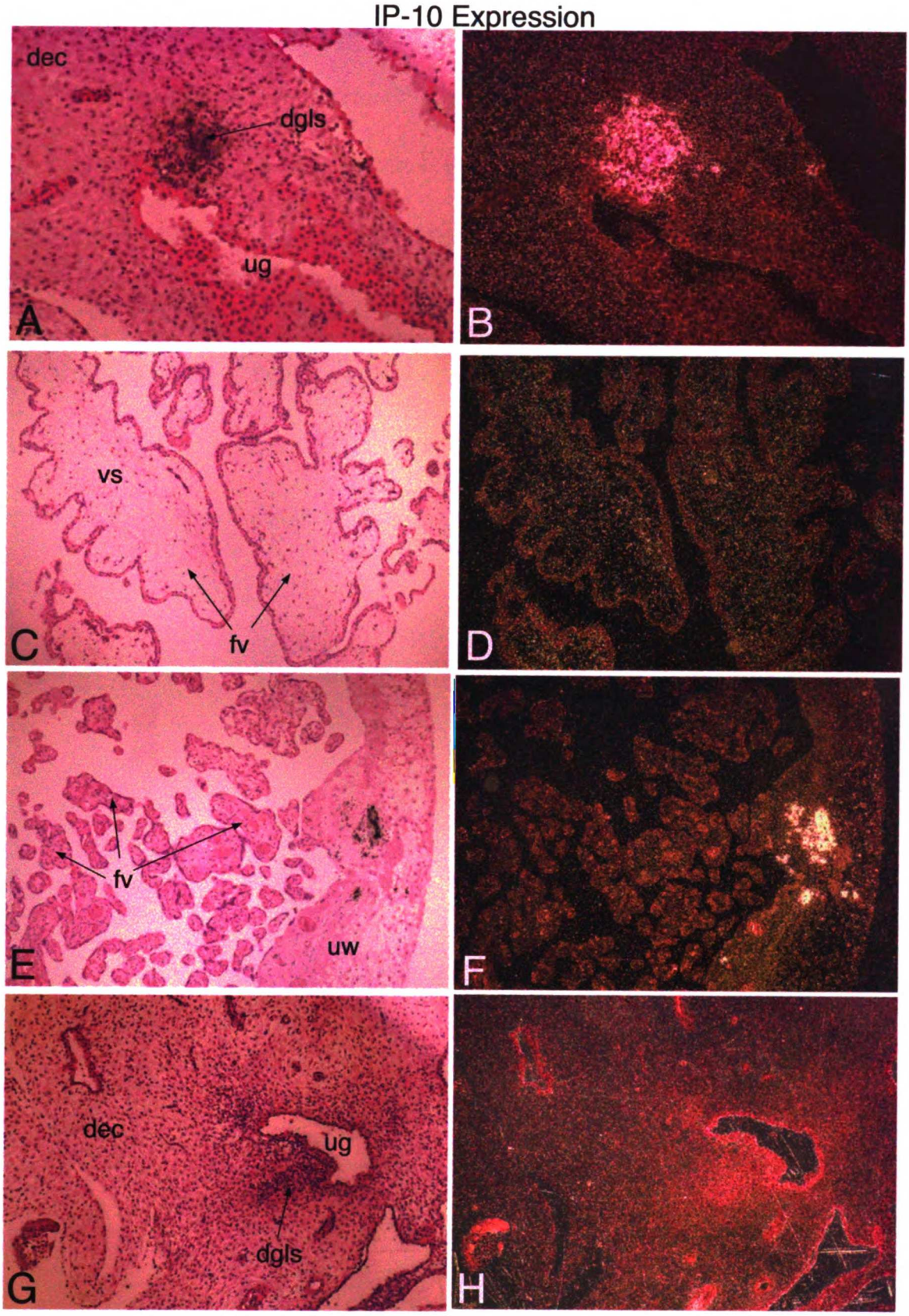
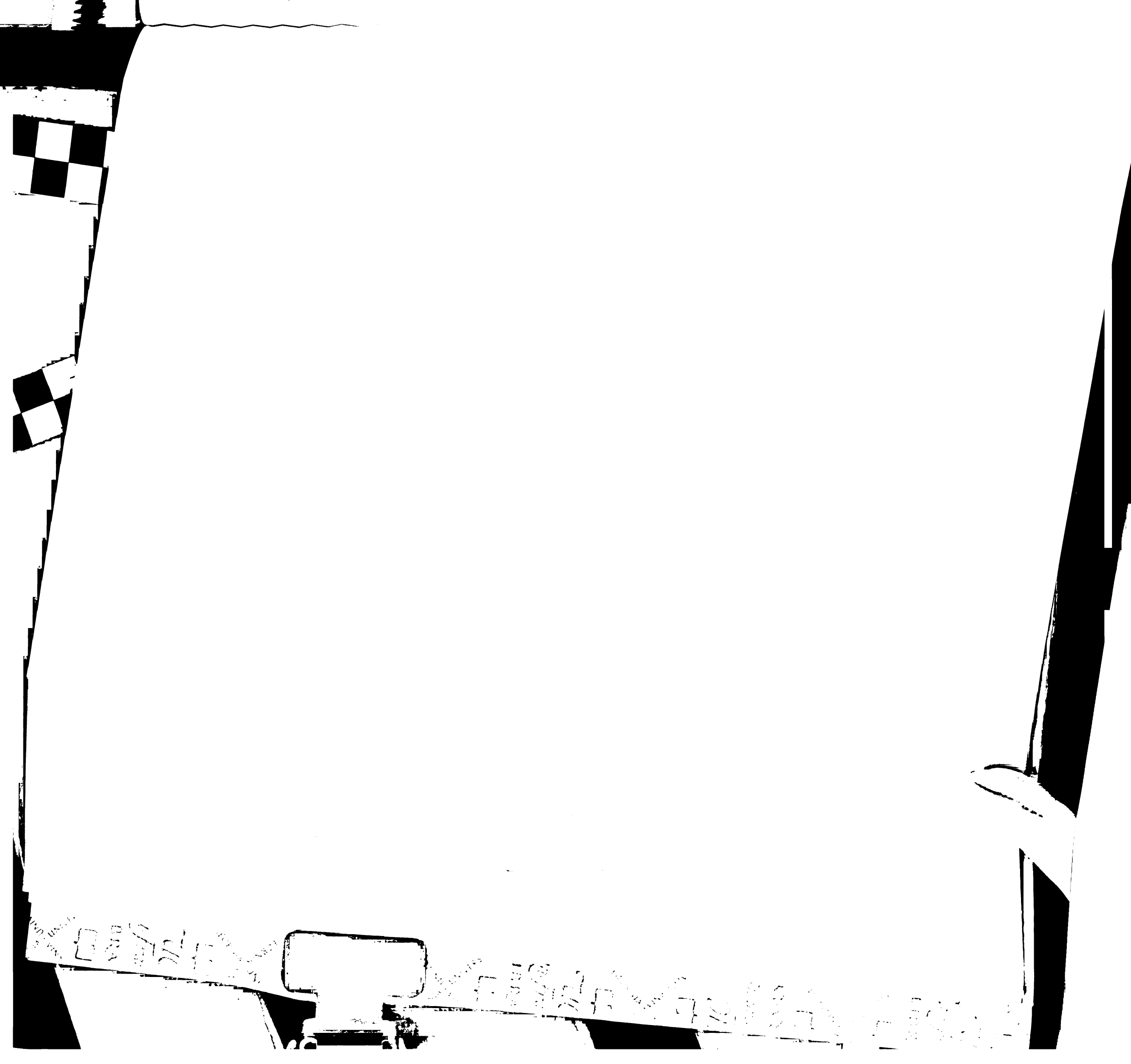


Figure 7-7

Figure 7-7. IP-10 is expressed near clusters of decidual leukocytes. In situ hybridization on first trimester (B and D) and term (F) tissues demonstrated IP-10 expression in patches of decidual stroma associated with clusters of leukocytes (B and F). Occasionally, weaker expression was also seen in the villous stroma (D). Panels A, C, E and G are bright field micrographs of histological sections stained with H&E; Panels B, D, F and H are dark field micrographs of the same sections. White dots indicate signal (8 wk exposure) from an ^{35}S-labeled antisense probe (B, D and F). Sections hybridized with sense probe as a negative control yielded no signal (H). dec: decidua, dgls: decidual leukocytes, ug: uterine gland.



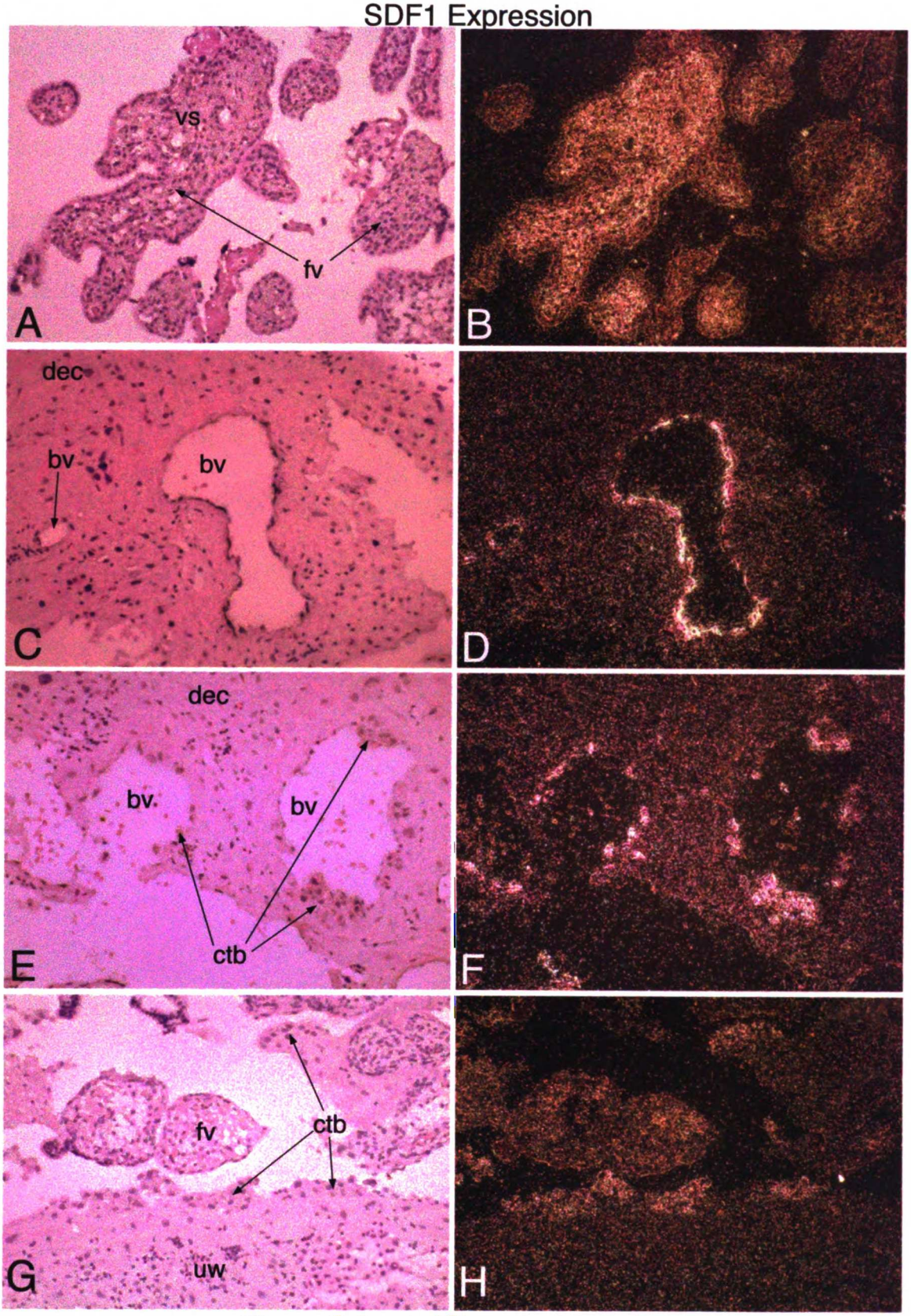


Figure 7-8 174

Figure 7-8. SDF1 is expressed in uterine vessels—by endothelium and endovascular cytotrophoblasts. In situ hybridization of second trimester tissue sections of the maternal-fetal interface demonstrated SDF1 mRNA in villous stroma (B), and in the endothelium (D) and endovascular cytotrophoblasts (F) lining some uterine blood vessels. Non-endovascular extravillous cytotrophoblasts were also positive for SDF1 (H). Panels A, C, E and G are bright field micrographs of histological sections stained with H&E; Panels B, D, F and H are dark field micrographs of the same sections. White dots indicate signal (8 wk exposure) from from an ^{35}S-labeled antisense probe (B, D, F and H). Adjacent sections hybridized with sense probe as a negative control yielded no signal (data not shown). ctb: cytotrophoblasts.

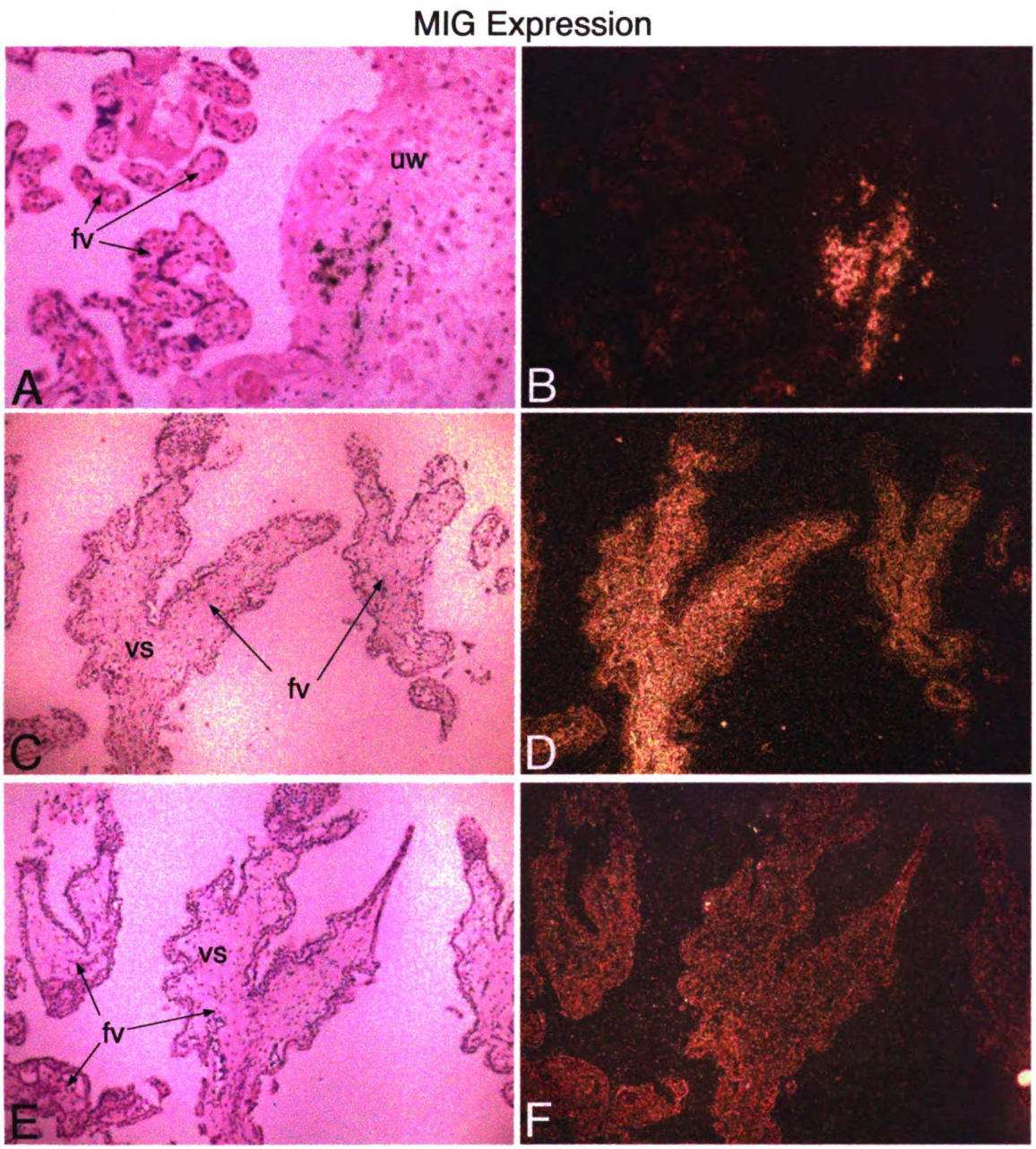


Figure 7-9

Figure 7-9. MIG expression is occasionally seen in villous stroma and decidua. Although the majority of samples analyzed did not express MIG, in situ hybridization on some first trimester (D) and term (B) specimens revealed expression in villous stroma (D) or in patches in the decidua (B). Panels A, C and E are bright field micrographs of histological sections stained with H&E; Panels B, D and F are dark field micrographs of the same sections. White dots indicate signal (8 wk exposure) from an ^{35}S-labeled antisense probe (B and D). Adjacent sections hybridized with sense probe as a negative control yielded no signal (F).

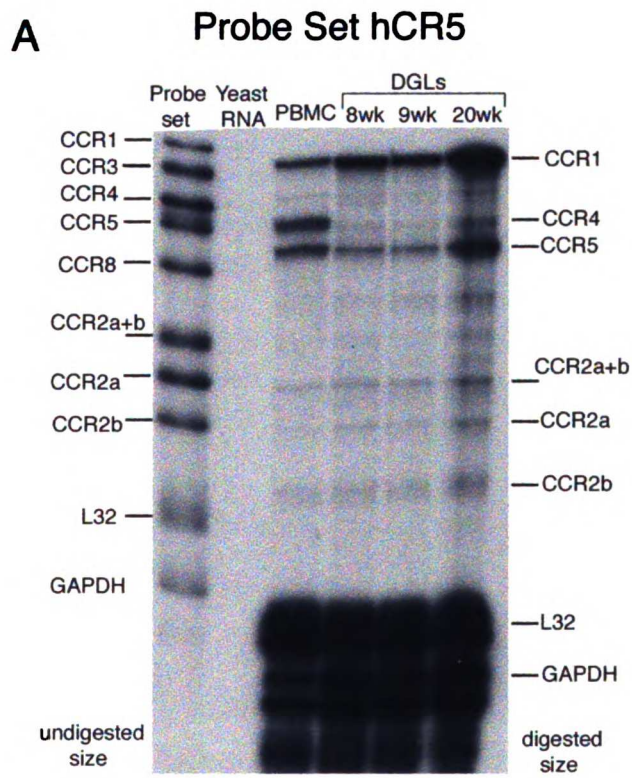

A
Probe Set hCR5
Probe set
Yeast RNA
PBMC
DGLs
8wk
9wk
20wk
CCR1
CCR3
CCR4
CCR5
CCR8
CCR2a+b
CCR2a
CCR2b
L32
GAPDH
undigested size
CCR1
CCR4
CCR5
CCR2a+b
CCR2a
CCR2b
L32
GAPDH
digested size

Figure 7-10

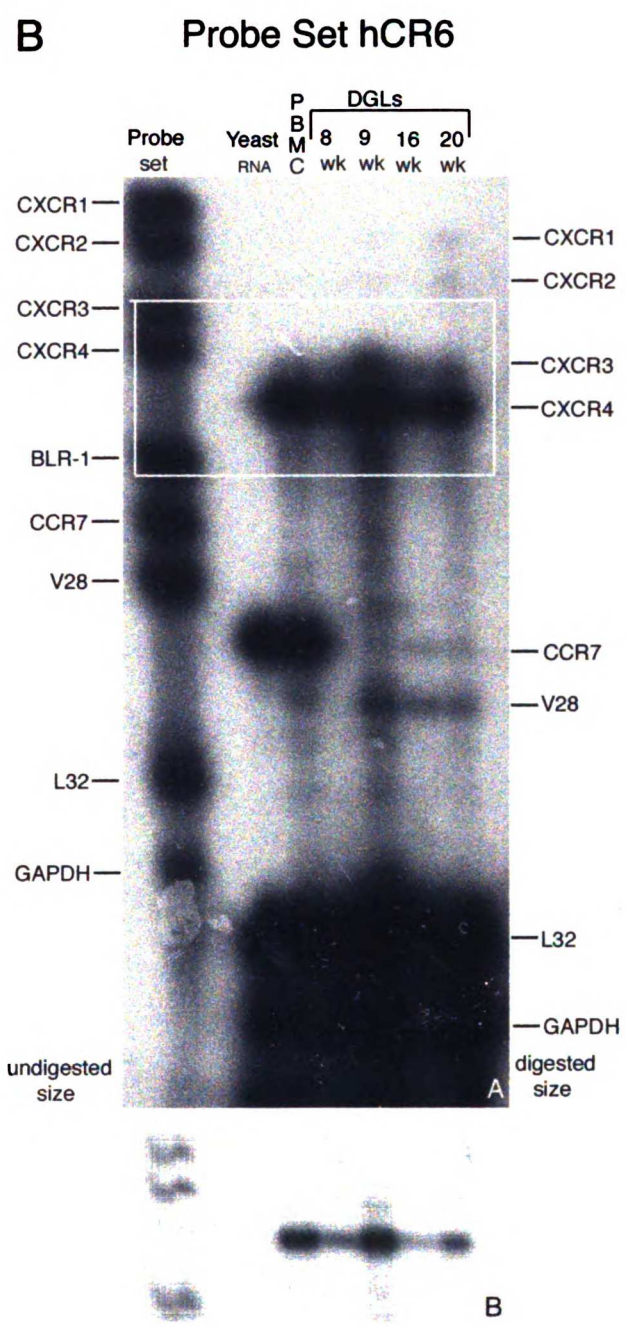

B
Probe Set hCR6
Probe set
Yeast RNA
PBMC
DGLs
8 wk
9 wk
16 wk
20 wk
CXCR1
CXCR2
CXCR3
CXCR4
BLR-1
CCR7
V28
L32
GAPDH
undigested size
digested size
A
B

Figure 7-10

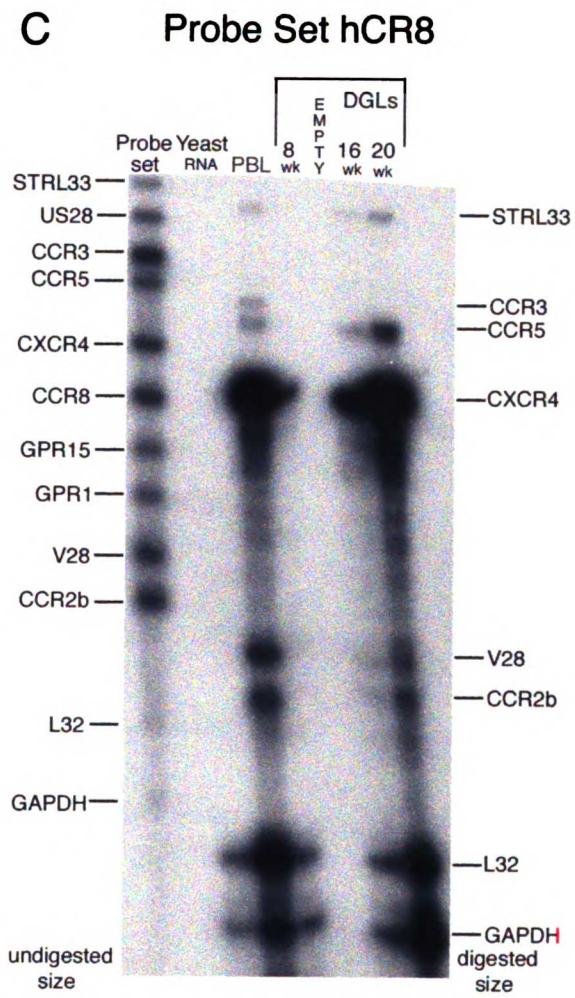

C
Probe Set hCR8
DGLs
EMPTY
Probe set
Yeast RNA
PBL
8 wk
16 wk
20 wk
STRL33
US28
CCR3
CCR5
CXCR4
CCR8
GPR15
GPR1
V28
CCR2b
L32
GAPDH
undigested size
STRL33
CCR3
CCR5
CXCR4
V28
CCR2b
L32
GAPDH
digested size

Figure 7-10

Figure 7-10. Decidual leukocytes express a panel of chemokine receptors. RNAse protection assays were used to determine the production of the former molecules. Three probe sets (hCR5:Panel A, hCR6:Panel B and hCR8:Panel C) were labeled with ^{32}P 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). 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. Panel B contains an inset (B) that is a shorter exposure (2 days) than the rest of the panel (1 wk) to clearly demonstrate strongly expressed bands in that region. The region outlined with a white box indicates the portion shown in inset B. Panels A and C were exposed for 4 and 2 days, respectively.

Chapter Eight – Future Directions

An ever-increasing body of literature supports the principle that chemokines are important contributors to a myriad of biological processes. Originally identified as mediators of inflammation, their role in homeostatic leukocyte trafficking is now well defined. Their participation in the development of the nervous, cardiovascular and hematopoietic systems has also been observed. Now, we add the maternal-fetal interface to the growing list of sites where both conventional and novel chemokine functions are important.

This thesis introduces the novel concept that fetal cytotrophoblasts can influence the trafficking of immune cells in their environment. In situ hybridization and other assays demonstrated that the former cells express chemokines, such as SDF1 and MIP-1α. Functional in vitro chemotaxis assays using cytotrophoblast CM as an attractant for target PBMCs showed that the fetal cells secrete factors that act on the same immune subsets that are found in the pregnant uterus. Immunodepletion of MIP-1α from the CM revealed that this molecule was responsible for a significant portion of NK cell and monocyte chemotaxis.

Additionally, this thesis describes the expression patterns of nine chemokines in the placenta and decidua, and chemokine receptor expression by decidual leukocytes. In so doing, it identifies a set of candidate molecules for paracrine control of leukocyte homing to the pregnant uterus. Furthermore, chemokine expression patterns suggest that the placenta also uses these molecules in other aspects of its own novel autocrine differentiation programs. The information herein sets the stage for experiments examining chemokine actions at the maternal-fetal interface.

Future studies will investigate these effects on two levels. At the cellular level, we will pursue three lines of inquiry. First, we will assess the expression of chemokine receptors on individual decidual leukocytes by flow cytometry. Fluorescently-labeled antibodies recognizing human chemokine receptors (R&D Systems) will be used in combination with fluorescently-conjugated anti-CD3, anti-CD56, anti-CD14 and anti-CD45 antibodies, so that expression of specific receptors can be assigned to particular immune subsets. The results will give us a clearer picture of the mechanisms underlying decidual leukocyte recruitment, and will also refine the phenotype of these cells. This information, through comparison with both normal and pathological immune trafficking in other systems, will provide insights into decidual leukocyte function, allowing us to develop testable hypotheses.

Second, the expression patterns revealed by in situ hybridization suggest that chemokines are involved in invasive cytotrophoblast targeting to maternal arterioles. To address this possibility, we will use two model systems of cytotrophoblast differentiation currently in place in our laboratory: cultures of primary cells and villous explants (Fisher et al., 1985; Genbacev et al., 1992). Highly purified cytotrophoblast stem cells can be cultured on an extracellular matrix (ECM) substrate. These cells recapitulate *in vitro* the processes they undergo *in vivo* as part of the invasive differentiation pathway, including migration and invasion of the ECM. Anchoring villous explants are essentially organ cultures. Intact pieces of placenta that include cell columns are cultured on an extracellular matrix plug. The cytotrophoblasts in the cell columns attach to and invade the plug of matrix, mimicking their *in vivo* invasion of the uterine wall. Chemokine effects on the migration and chemotaxis of cytotrophoblasts will be assessed by addition of exogenous chemokines to the culture systems, and, where indicated, the use of function-perturbing antibodies to block chemokine activity. Alternatively, delivery of antisense oligonucleotide using adenoviral vectors could be used for the latter purpose. Assay design will incorporate elements from elegant three-dimensional chemotaxis experiments described by Butcher and colleagues (Foxman et al., 1997). Simultaneously, cytotrophoblast expression of chemokine receptors will be examined at the transcriptional

level by RNAse protection assays (RPA) on RNA from isolated cells (Chapter Seven). Expression of the receptor CCR10, not included in the RPA probe sets but of particular interest because it is present at high levels in the placenta, will be examined by Northern blot and in situ hybridization. At the completion of these studies we will understand whether chemokines contribute to the cytotrophoblast targeting to maternal arterioles and the molecules involved in this phenomenon including adhesion molecules and proteases which are likely downstream targets.

Third, general effects of chemokines on placental growth and development (Chapter Seven) will be investigated using the two models systems described above. Placental fibroblasts will also be isolated and cultured for study. In each culture system, chemokines will be supplied to the cells, either as soluble factors in the culture medium, or mixed into the ECM substrate which supports them. Effects of these factors on proliferation, invasion, and differentiation will be assessed. Proliferation will be monitored by BrDU incorporation, and if warranted, by analyzing the expression of the specific set of mitotic regulators expressed in cytotrophoblasts (Genbacev et al., 2000). Invasion will be assessed as the cells' ability to traverse ECM-coated filters. Migration, perhaps a more useful endpoint, will be evaluated using a newly acquired computer set-up. Differentiation will be monitored using a panel of stage-specific antigens that include

adhesion molecules and the trophoblast class I molecule HLA-G (Damsky and Fisher, 1998; McMaster et al., 1998). In general, chemokine activity will be abrogated using function-perturbing antibodies and/or the expression of antisense oligonucleotides. Taken together, the results of these experiments will broaden our understanding of the role of chemokines in placental development, an area about which nothing is known.

Chemokine effects on pregnancy will also be addressed at the level of the uterus. In this case, we will use in situ hybridization to compare chemokine expression in normal placentas to expression in pathological placentas from pregnancy complications. This work will be part of a large scale study our laboratory is conducting in collaboration with Dr. Roberto Romero [Chief, Perinatology Research Branch (Intramural Division), National Institute of Child Health and Human Development]. The first known attempt to take an in-depth look at pregnancy complications at the cellular level, this study was established to thoroughly characterize several common pathologies of pregnancy from multiple angles, including assessing the contribution of chromosomal abnormalities, aberrant apoptosis, and irregular expression of molecular differentiation markers. Accordingly, chemokine expression patterns will be assessed as part of the latter aim. The pregnancy complications selected for analysis are preeclampsia (PE), intrauterine growth retardation, preterm labor with chorioamnionitis, preterm labor without

chorioamnionitis, and cytomegalovirus (CMV) transmission to the fetus. We are particularly interested in how chemokine expression is modulated with infection (e.g., chorioamnionitis, CMV). However, chemokine expression may also be aberrant in other conditions. For instance, a hallmark of PE is shallow invasion and reduced remodeling of maternal arterioles by cytotrophoblasts. If chemokines help to regulate arteriole targeting of these fetal cells (see above), then dysregulation of their expression could contribute to PE. In this way, pathologies of pregnancy can serve as useful indirect in vivo tests of hypotheses.

At the conclusion of these experiments we will have described the expression patterns of chemokines in several common pregnancy complications. These patterns will be useful in many ways. First, they will contribute to the fingerprint of molecular expression left by individual pathologies. Eventually, we hope to be able to distinguish different pregnancy complications through the assessment of a panel of molecular markers, and ultimately to develop a diagnostic test for these conditions. With regard to the latter goal, circulating chemokines are interesting targets. In addition, changes in the expression of chemokines associated with pathologies should suggest functional roles for these molecules, especially when considered in the histological context in which they are presented. For instance, we have seen many examples of aberrant leukocytic infiltration

in the villous stroma and decidua. We anticipate that these specimens will display irregular chemokine expression, and the molecules identified will be implicated in the abnormal recruitment of the immune cells. From a long-term perspective, an understanding of chemokines involved in such "inflammatory" pregnancy complications might lead to the development of therapeutics that could block chemokine function and prevent unwanted leukocyte accumulation at the maternal-fetal interface. Although not a current focus of our laboratory, chemokine expression patterns in unexplained cases of recurrent abortion could give important clues to this frustrating and enigmatic condition.

In summary, the data presented in this thesis are analogous to my education at UCSF; both have provided a solid foundation for productive future investigations.

Bibliography

Abel, M.H., S.K. Smith, and D.T. Baird. 1980. Suppression of concentration of endometrial prostaglandin in early intra-uterine and ectopic pregnancy in women. *J Endocrinol.* 85:379-86.

Afonso, S., L. Romagnano, and B. Babiarz. 1997. The expression and function of cystatin C and cathepsin B and cathepsin L during mouse embryo implantation and placentation. *Development.* 124:3415-25.

Alexander, C.M., E.J. Hansell, O. Behrendtsen, M.L. Flannery, N.S. Kishnani, S.P. Hawkes, and Z. Werb. 1996. Expression and function of matrix metalloproteinases and their inhibitors at the maternal-embryonic boundary during mouse embryo implantation. *Development.* 122:1723-36.

Allan, D.S., M. Colonna, L.L. Lanier, T.D. Churakova, J.S. Abrams, S.A. Ellis, A.J. McMichael, and V.M. Braud. 1999. Tetrameric complexes of human histocompatibility leukocyte antigen (HLA)-G bind to peripheral blood myelomonocytic cells. *Journal of Experimental Medicine.* 189:1149-56.

Allavena, P., G. Bianchi, D. Zhou, J. van Damme, P. Jílek, S. Sozzani, and A. Mantovani. 1994. Induction of natural killer cell migration by monocyte chemotactic protein-1, -2 and -3. *European Journal of Immunology.* 24:3233-6.

Allavena, P., C. Paganin, I. Martin-Padura, G. Peri, M. Gaboli, E. Dejana, P.C. Marchisio, and A. Mantovani. 1991. Molecules and structures involved in the adhesion of natural killer cells to vascular endothelium. *Journal of Experimental Medicine.* 173:439-48.

Angiolillo, A.L., C. Sgadari, D.D. Taub, F. Liao, J.M. Farber, S. Maheshwari, H.K. Kleinman, G.H. Reaman, and G. Tosato. 1995. Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo. *Journal of Experimental Medicine.* 182:155-62.

Anson-Cartwright, L., K. Dawson, D. Holmyard, S.J. Fisher, R.A. Lazzarini, and J.C. Cross. 2000. The glial cells missing-1 protein is essential for branching

morphogenesis in the chorioallantoic placenta [see comments]. *Nature Genetics.* 25:311-4.

Aplin, J.D., N.A. Hey, and R.A. Graham. 1998. Human endometrial MUC1 carries keratan sulfate: characteristic glycoforms in the luminal epithelium at receptivity. *Glycobiology.* 8:269-76.

Apte, R.S., D. Sinha, E. Mayhew, G.J. Wistow, and J.Y. Niederkorn. 1998. Role of macrophage migration inhibitory factor in inhibiting NK cell activity and preserving immune privilege. *Journal of Immunology.* 160:5693-6.

Arcuri, F., M. Cintorino, R. Vatti, A. Carducci, S. Liberatori, and L. Paulesu. 1999. Expression of macrophage migration inhibitory factor transcript and protein by first-trimester human trophoblasts. *Biology of Reproduction.* 60:1299-303.

Bacon, K.B., B.A. Premack, P. Gardner, and T.J. Schall. 1995. Activation of dual T cell signaling pathways by the chemokine RANTES. *Science.* 269:1727-30.

Baggiolini, M., B. Dewald, and B. Moser. 1994. Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. *Advances in Immunology.* 55:97-179.

Baggiolini, M., B. Dewald, and B. Moser. 1997. Human chemokines: an update. *Annu Rev Immunol.* 15:675-705.

Bainbridge, D.R., S.A. Ellis, and I.L. Sargent. 1999. Little evidence of HLA-G mRNA polymorphism in Caucasian or Afro- Caribbean populations. *J Immunol.* 163:2023-7.

Balasch, J., and J.A. Vanrell. 1987. Corpus luteum insufficiency and fertility: a matter of controversy. *Hum Reprod.* 2:557-67.

Bamberger, A.M., H.M. Schulte, I. Thuneke, I. Erdmann, C.M. Bamberger, and S.L. Asa. 1997. Expression of the apoptosis-inducing Fas ligand (FasL) in human first and third trimester placenta and choriocarcinoma cells. *Journal of Clinical Endocrinology and Metabolism.* 82:3173-5.

Barak, Y., M.C. Nelson, E.S. Ong, Y.Z. Jones, P. Ruiz-Lozano, K.R. Chien, A. Koder, and R.M. Evans. 1999. PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol Cell.* 4:585-95.

Barker, D.J. 1999. Fetal origins of cardiovascular disease. *Annals of Medicine*. 31 Suppl 1:3-6.

Barker, D.J. 2000. In utero programming of cardiovascular disease. *Theriogenology*. 53:555-74.

Barmat, L.I., H.C. Liu, S.D. Spandorfer, A. Kowalik, C. Mele, K. Xu, L. Veeck, M. Damario, and Z. Rosenwaks. 1999. Autologous endometrial co-culture in patients with repeated failures of implantation after in vitro fertilization-embryo transfer. *J Assist Reprod Genet*. 16:121-7.

Bass, K.E., H. Li, S.P. Hawkes, E. Howard, E. Bullen, T.K. Vu, M. McMaster, M. Janatpour, and S.J. Fisher. 1997. Tissue inhibitor of metalloproteinase-3 expression is upregulated during human cytotrophoblast invasion in vitro. *Dev Genet*. 21:61-7.

Bazan, J.F., K.B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D.R. Greaves, A. Zlotnik, and T.J. Schall. 1997. A new class of membrane-bound chemokine with a CX3C motif. *Nature*. 385:640-4.

Behrman, R.E. 1995. Issues in children's health. *Bulletin of the New York Academy of Medicine*. 72:575-85.

Bejarano, M.T., R. de Waal Malefyt, J.S. Abrams, M. Bigler, R. Bacchetta, J.E. de Vries, and M.G. Roncarolo. 1992. Interleukin 10 inhibits allogeneic proliferative and cytotoxic T cell responses generated in primary mixed lymphocyte cultures. *International Immunology*. 4:1389-97.

Ben-Baruch, A., L. Xu, P.R. Young, K. Bengali, J.J. Oppenheim, and J.M. Wang. 1995. Monocyte chemotactic protein-3 (MCP3) interacts with multiple leukocyte receptors. C-C CKR1, a receptor for macrophage inflammatory protein-1 alpha/Rantes, is also a functional receptor for MCP3. *Journal of Biological Chemistry*. 270:22123-8.

Benirschke, K., and P. Kaufmann. 1991. Early development of the human placenta. *In* Pathology of the human placenta. K. Benirschke and P. Kaufmann, editors. Springer-Verlag, New York. 13-21.

Benirschke, K.a.K., P. 2000. Pathology of the Human Placenta. Springer-Verlag, New York.

Bergh, P.A., and D. Navot. 1992. The impact of embryonic development and endometrial maturity on the timing of implantation. *Fertil Steril.* 58:537-42.

Bhatt, H., L.J. Brunet, and C.L. Stewart. 1991. Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proc Natl Acad Sci U S A.* 88:11408-12.

Biassoni, R., C. Bottino, R. Millo, L. Moretta, and A. Moretta. 1999. Natural killer cell-mediated recognition of human trophoblast. *Seminars in Cancer Biology.* 9:13-8.

Bladt, F., D. Riethmacher, S. Isenmann, A. Aguzzi, and C. Birchmeier. 1995. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud [see comments]. *Nature.* 376:768-71.

Bluman, E.M., K.J. Bartynski, B.R. Avalos, and M.A. Caligiuri. 1996. Human natural killer cells produce abundant macrophage inflammatory protein-1 alpha in response to monocyte-derived cytokines. *Journal of Clinical Investigation.* 97:2722-7.

Boklage, C.E. 1990. Survival probability of human conceptions from fertilization to term. *Int J Fertil.* 35:75, 79-80, 81-94.

Boyd, J.D., and W.J. Hamilton. 1967. Development and structure of the human placenta from the end of the 3rd month of gestation. *Journal of Obstetrics and Gynaecology of the British Commonwealth.* 74:161-226.

Boyd, J.G.a.H., W.J. 1970. The Human Placenta. Heffer, Cambridge.

Braga, V.M., and S.J. Gendler. 1993. Modulation of Muc-1 mucin expression in the mouse uterus during the estrus cycle, early pregnancy and placentation. *J Cell Sci.* 105:397-405.

Bremme, K., M. Lagerström, O. Andersson, S. Johansson, and P. Eneroth. 1990. Influences of maternal smoking and fetal sex on maternal serum oestriol, prolactin, hCG, and hPI levels. *Archives of Gynecology and Obstetrics*. 247:95-103.

Brooke, O.G., H.R. Anderson, J.M. Bland, J.L. Peacock, and C.M. Stewart. 1989. Effects on birth weight of smoking, alcohol, caffeine, socioeconomic factors, and psychosocial stress. *Bmj (Clinical Research Ed.)*. 298:795-801.

Brosens, I., and H.G. Dixon. 1966. The anatomy of the maternal side of the placenta. *Journal of Obstetrics and Gynaecology of the British Commonwealth*. 73:357-63.

Brosens, I.A. 1977. Morphological changes in the utero-placental bed in pregnancy hypertension. *Clin Obstet Gynaecol*. 4:573-93.

Buck, G.M., L.E. Sever, R.E. Batt, and P. Mendola. 1997. Life-style factors and female infertility. *Epidemiology*. 8:435-41.

Bulmer, J.N., and P.M. Johnson. 1985. Antigen expression by trophoblast populations in the human placenta and their possible immunobiological relevance. *Placenta*. 6:127-40.

Bulmer, J.N., M. Longfellow, and A. Ritson. 1991a. Leukocytes and resident blood cells in endometrium. *Annals of the New York Academy of Sciences*. 622:57-68.

Bulmer, J.N., L. Morrison, P.M. Johnson, and A. Meager. 1990. Immunohistochemical localization of interferons in human placental tissues in normal, ectopic, and molar pregnancy. *American Journal of Reproductive Immunology*. 22:109-16.

Bulmer, J.N., L. Morrison, M. Longfellow, A. Ritson, and D. Pace. 1991b. Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies. *Hum Reprod*. 6:791-8.

Bulmer, J.N., L. Morrison, and J.C. Smith. 1988. Expression of class II MHC gene products by macrophages in human uteroplacental tissue. *Immunology*. 63:707-14.

Bulmer, J.N.a.J., P.M. 1984. Macrophage populations in the human placenta and amniochorion. *Clinical Experimental Immunology*. 57:393-403.

Burrows, T.D., A. King, and Y.W. Loke. 1993. Expression of adhesion molecules by human decidual large granular lymphocytes. *Cellular Immunology*. 147:81-94.

Burrows, T.D., A. King, and Y.W. Loke. 1995. The role of integrins in adhesion of decidual NK cells to extracellular matrix and decidual stromal cells. *Cellular Immunology*. 166:53-61.

Burton, G.J., M.E. Palmer, and K.J. Dalton. 1989. Morphometric differences between the placental vasculature of non-smokers, smokers and ex-smokers. *British Journal of Obstetrics and Gynaecology*. 96:907-15.

Butcher, E.C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell*. 67:1033-6.

Butcher, E.C., and L.J. Picker. 1996. Lymphocyte homing and homeostasis. *Science*. 272:60-6.

Campbell, J.J., E.F. Foxman, and E.C. Butcher. 1997. Chemoattractant receptor cross talk as a regulatory mechanism in leukocyte adhesion and migration. *European Journal of Immunology*. 27:2571-8.

Campbell, J.J., J. Hedrick, A. Zlotnik, M.A. Siani, D.A. Thompson, and E.C. Butcher. 1998. Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science*. 279:381-4.

Campbell, S., H.R. Swann, M.W. Seif, S.J. Kimber, and J.D. Aplin. 1995. Cell adhesion molecules on the oocyte and preimplantation human embryo. *Hum Reprod*. 10:1571-8.

Castellucci, M., A. Celona, H. Bartels, B. Steininger, V. Benedetto, and P. Kaufmann. 1987. Mitosis of the Hofbauer cell: possible implications for a fetal macrophage. *Placenta*. 8:65-76.

Castellucci, M., Schweikhart, G., Kaufmann, P. and Zaccheo, D. 1984. The stromal architecture of immature intermediate villus of the human placenta: functional and clinical implications. *Gynecol. Obstet. Invest.* 18:95-99.

Castellucci, M., Zaccheo, D. and Pescetto, G. 1980. A three-dimensional study of the normal human placental villous core. I. The Hofbauer cells. *Cell Tissue Research.* 210:235-247.

Castellucci, M.a.K., P. 1982. A three-dimensional study of the normal human placental villous core: II. Stromal architecture. *Placenta.* 3:269-285.

Chakraborty, I., S.K. Das, J. Wang, and S.K. Dey. 1996. Developmental expression of the cyclo-oxygenase-1 and cyclo-oxygenase-2 genes in the peri-implantation mouse uterus and their differential regulation by the blastocyst and ovarian steroids. *J Mol Endocrinol.* 16:107-22.

Chantakru, S., W.A. Kuziel, N. Maeda, and B.A. Croy. 2001. A study on the density and distribution of uterine Natural Killer cells at mid pregnancy in mice genetically-ablated for CCR2, CCR5 and the CCR5 receptor ligand, MIP-1 alpha. *J. Reprod. Immunol.* 49:33-47.

Chaouat, G., E. Menu, G. Delage, J.F. Moreau, L. Khrishnan, L. Hui, A.A. Meliani, J. Martal, R. Raghupathy, C. Lelaidier, and et al. 1995. Immuno-endocrine interactions in early pregnancy. *Hum Reprod.* 10 Suppl 2:55-9.

Charo, I.F., S.J. Myers, A. Herman, C. Franci, A.J. Connolly, and S.R. Coughlin. 1994. Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proceedings of the National Academy of Sciences of the United States of America.* 91:2752-6.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry.* 162:156-9.

Christmas, S.E., J.N. Bulmer, A. Meager, and P.M. Johnson. 1990. Phenotypic and functional analysis of human CD3- decidual leucocyte clones. *Immunology.* 71:182-9.

Chumbley, G., A. King, N. Holmes, and Y.W. Loke. 1993. In situ hybridization and northern blot demonstration of HLA-G mRNA in human trophoblast populations by locus-specific oligonucleotide. *Human Immunology*. 37:17-22.

Clark, D.E., S.K. Smith, A.M. Sharkey, H.M. Sowter, and D.S. Charnock-Jones. 1996. Hepatocyte growth factor/scatter factor and its receptor c-met: localisation and expression in the human placenta throughout pregnancy. *J Endocrinol.* 151:459-67.

Cohen, J. 1991. The efficiency and efficacy of IVF and GIFT. *Hum Reprod.* 6:613-8.

Cohen, P.E., L. Zhu, and J.W. Pollard. 1997. Absence of colony stimulating factor-1 in osteopetrotic (csfmop/csfmop) mice disrupts estrous cycles and ovulation. *Biol Reprod.* 56:110-8.

Colonna, M., J. Samaridis, M. Cella, L. Angman, R.L. Allen, C.A. O'Callaghan, R. Dunbar, G.S. Ogg, V. Cerundolo, and A. Rolink. 1998. Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. *Journal of Immunology*. 160:3096-100.

Combadiere, C., S.K. Ahuja, and P.M. Murphy. 1995. Cloning and functional expression of a human eosinophil CC chemokine receptor [published erratum appears in J Biol Chem 1995 Dec 15;270(50):30235]. *Journal of Biological Chemistry*. 270:16491-4.

Combadiere, C., S.K. Ahuja, H.L. Tiffany, and P.M. Murphy. 1996. Cloning and functional expression of CC CKR5, a human monocyte CC chemokine receptor selective for MIP-1(alpha), MIP-1(beta), and RANTES. *Journal of Leukocyte Biology*. 60:147-52.

Combadiere, C., K. Salzwedel, E.D. Smith, H.L. Tiffany, E.A. Berger, and P.M. Murphy. 1998. Identification of CX3CR1. A chemotactic receptor for the human CX3C chemokine fractalkine and a fusion coreceptor for HIV-1. *Journal of Biological Chemistry*. 273:23799-804.

Copeman, J., R.N. Han, I. Caniggia, M. McMaster, S.J. Fisher, and J.C. Cross. 2000. Posttranscriptional regulation of human leukocyte antigen G during human extravillous cytotrophoblast differentiation. *Biology of Reproduction*. 62:1543-50.

Crisa, L., M.T. McMaster, J.K. Ishii, S.J. Fisher, and D.R. Salomon. 1997. Identification of a thymic epithelial cell subset sharing expression of the class Ib HLA-G molecule with fetal trophoblasts. *Journal of Experimental Medicine*. 186:289-98.

Critchley, H.O., R.L. Jones, R.G. Lea, T.A. Drudy, R.W. Kelly, A.R. Williams, and D.T. Baird. 1999. Role of inflammatory mediators in human endometrium during progesterone withdrawal and early pregnancy. *Journal of Clinical Endocrinology and Metabolism*. 84:240-8.

Critchley, H.O., R.W. Kelly, R.G. Lea, T.A. Drudy, R.L. Jones, and D.T. Baird. 1996. Sex steroid regulation of leukocyte traffic in human decidua. *Human Reproduction*. 11:2257-62.

Cross, J.C. 1998. Formation of the placenta and extraembryonic membranes. *Ann N Y Acad Sci*. 857:23-32.

Cross, J.C., Z. Werb, and S.J. Fisher. 1994. Implantation and the placenta: key pieces of the development puzzle. *Science*. 266:1508-18.

Croy, B.A., A.A. Ashkar, K. Minhas, and J.D. Greenwood. 2000. Can murine uterine natural killer cells give insights into the pathogenesis of preeclampsia? *Journal of the Society for Gynecologic Investigation*. 7:12-20.

Croy, B.A., and C. Chapeau. 1990. Evaluation of the pregnancy immunotrophism hypothesis by assessment of the reproductive performance of young adult mice of genotype scid/scid.bg/bg. *J Reprod Fertil*. 88:231-9.

Croy, B.A., and Y. Kiso. 1993. Granulated metrial gland cells: a natural killer cell subset of the pregnant murine uterus. *Microscopy Research and Technique*. 25:189-200.

Croy, B.A., J.A. Luross, M.J. Guimond, and J.S. Hunt. 1996. Uterine natural killer cells: insights into lineage relationships and functions from studies of pregnancies in mutant and transgenic mice. *Natural Immunity*. 15:22-33.

Csapo, A.I., and M. Pulkkinen. 1978. Indispensability of the human corpus luteum in the maintenance of early pregnancy. Luteectomy evidence. *Obstet Gynecol Surv*. 33:69-81.

Csapo, A.I., M.O. Pulkkinen, B. Ruttner, J.P. Sauvage, and W.G. Wiest. 1972. The significance of the human corpus luteum in pregnancy maintenance. I. Preliminary studies. *Am J Obstet Gynecol.* 112:1061-7.

Cullinan, E.B., S.J. Abbondanzo, P.S. Anderson, J.W. Pollard, B.A. Lessey, and C.L. Stewart. 1996. Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. *Proc Natl Acad Sci U S A.* 93:3115-20.

Cyster, J.G. 1999. Chemokines and cell migration in secondary lymphoid organs. *Science.* 286:2098-102.

Dallenbach-Hellweg, G. 1981. The normal histology of the endometrium. Springer-Verlag, Berlin.

Damsky, C., A. Sutherland, and S. Fisher. 1993a. Extracellular matrix 5: adhesive interactions in early mammalian embryogenesis, implantation, and placentation. *Faseb J.* 7:1320-9.

Damsky, C., A. Sutherland, and S. Fisher. 1993b. Extracellular matrix 5: adhesive interactions in early mammalian embryogenesis, implantation, and placentation. *Faseb Journal.* 7:1320-9.

Damsky, C.H., and S.J. Fisher. 1998. Trophoblast pseudo-vasculogenesis: faking it with endothelial adhesion receptors. *Curr Opin Cell Biol.* 10:660-6.

Damsky, C.H., M.L. Fitzgerald, and S.J. Fisher. 1992. Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. *J Clin Invest.* 89:210-22.

Damsky, C.H., C. Librach, K.H. Lim, M.L. Fitzgerald, M.T. McMaster, M. Janatpour, Y. Zhou, S.K. Logan, and S.J. Fisher. 1994. Integrin switching regulates normal trophoblast invasion. *Development.* 120:3657-66.

Das, S.K., N. Das, J. Wang, H. Lim, B. Schryver, G.D. Plowman, and S.K. Dey. 1997a. Expression of betacellulin and epiregulin genes in the mouse uterus temporally by

the blastocyst solely at the site of its apposition is coincident with the "window" of implantation. *Dev Biol.* 190:178-90.

Das, S.K., X.N. Wang, B.C. Paria, D. Damm, J.A. Abraham, M. Klagsbrun, G.K. Andrews, and S.K. Dey. 1994. Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF- receptor in implantation. *Development.* 120:1071-83.

Das, S.K., S. Yano, J. Wang, D.R. Edwards, H. Nagase, and S.K. Dey. 1997b. Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the mouse uterus during the peri-implantation period. *Dev Genet.* 21:44-54.

Daugherty, B.L., S.J. Siciliano, J.A. DeMartino, L. Malkowitz, A. Sirotina, and M.S. Springer. 1996. Cloning, expression, and characterization of the human eosinophil eotaxin receptor. *Journal of Experimental Medicine.* 183:2349-54.

Dawood, M.Y. 1994. Corpus luteal insufficiency. *Curr Opin Obstet Gynecol.* 6:121-7.

De, S.K., M.T. McMaster, and G.K. Andrews. 1990. Endotoxin induction of murine metallothionein gene expression. *Journal of Biological Chemistry.* 265:15267-74.

Deniz, G., S.E. Christmas, R. Brew, and P.M. Johnson. 1994. Phenotypic and functional cellular differences between human CD3- decidual and peripheral blood leukocytes. *J Immunol.* 152:4255-61.

Depraetere, V., and P. Golstein. 1997. Fas and other cell death signaling pathways. *Seminars in Immunology.* 9:93-107.

DiFranza, J.R., and R.A. Lew. 1995. Effect of maternal cigarette smoking on pregnancy complications and sudden infant death syndrome. *J Fam Pract.* 40:385-94.

Ding, Y.Q., L.J. Zhu, M.K. Bagchi, and I.C. Bagchi. 1994. Progesterone stimulates calcitonin gene expression in the uterus during implantation. *Endocrinology.* 135:2265-74.

Dolan-Mullen, P., G. Ramirez, and J.Y. Groff. 1994. A meta-analysis of randomized trials of prenatal smoking cessation interventions. *Am J Obstet Gynecol.* 171:1328-34.

Duc-Goiran, P., T.M. Mignot, C. Bourgeois, and F. Ferre. 1999. Embryo-maternal interactions at the implantation site: a delicate equilibrium. *Eur J Obstet Gynecol Reprod Biol.* 83:85-100.

Dudley, D.J., S. Spencer, S. Edwin, and M.D. Mitchell. 1995. Regulation of human decidual cell macrophage inflammatory protein-1 alpha (MIP-1 alpha) production by inflammatory cytokines. *American Journal of Reproductive Immunology.* 34:231-5.

Dungy, L.J., T.A. Siddiqi, and S. Khan. 1991. Transforming growth factor-beta 1 expression during placental development. *American Journal of Obstetrics and Gynecology.* 165:853-7.

Edmonds, D.K., K.S. Lindsay, J.F. Miller, E. Williamson, and P.J. Wood. 1982. Early embryonic mortality in women. *Fertil Steril.* 38:447-53.

Ellard, G.A., F.D. Johnstone, R.J. Prescott, W. Ji-Xian, and M. Jian-Hua. 1996. Smoking during pregnancy: the dose dependence of birthweight deficits. *Br J Obstet Gynaecol.* 103:806-13.

Ellis, S.A., M.S. Palmer, and A.J. McMichael. 1990. Human trophoblast and the choriocarcinoma cell line BeWo express a truncated HLA Class I molecule. *J Immunol.* 144:731-5.

Enders, A.C., and B.F. King. 1991. Early stages of trophoblastic invasion of the maternal vascular system during implantation in the macaque and baboon. *American Journal of Anatomy.* 192:329-46.

Enders, A.C., and A. Lopata. 1999. Implantation in the marmoset monkey: expansion of the early implantation site. *Anat Rec.* 256:279-99.

Enders, A.C., and S. Schlafke. 1969. Cytological aspects of trophoblast-uterine interaction in early implantation. *Am J Anat.* 125:1-29.

Enders, A.C.a.K., B.F. 1970. The cytology of Hofbauer cells. *Anatomical Records.* 167:231-252.

Engelhardt, H., B.A. Croy, and G.J. King. 1997. Role of uterine immune cells in early pregnancy in pigs. *Journal of Reproduction and Fertility. Supplement.* 52:115-31.

Fazleabas, A.T., K.M. Donnelly, S. Srinivasan, J.D. Fortman, and J.B. Miller. 1999. Modulation of the baboon (Papio anubis) uterine endometrium by chorionic gonadotrophin during the period of uterine receptivity. *Proc Natl Acad Sci U S A.* 96:2543-8.

Ferry, B.L., I.L. Sargent, P.M. Starkey, and C.W. Redman. 1991. Cytotoxic activity against trophoblast and choriocarcinoma cells of large granular lymphocytes from human early pregnancy decidua. *Cellular Immunology.* 132:140-9.

Ferry, B.L., P.M. Starkey, I.L. Sargent, G.M. Watt, M. Jackson, and C.W. Redman. 1990. Cell populations in the human early pregnancy decidua: natural killer activity and response to interleukin-2 of CD56-positive large granular lymphocytes. *Immunology.* 70:446-52.

Firulli, A.B., D.G. McFadden, Q. Lin, D. Srivastava, and E.N. Olson. 1998. Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. *Nat Genet.* 18:266-70.

Fishel, S.B., R.G. Edwards, J.M. Purdy, P.C. Steptoe, J. Webster, E. Walters, J. Cohen, C. Fehilly, J. Hewitt, and G. Rowland. 1985. Implantation, abortion, and birth after in vitro fertilization using the natural menstrual cycle or follicular stimulation with clomiphene citrate and human menopausal gonadotropin. *J In Vitro Fert Embryo Transf.* 2:123-31.

Fisher, S.J., T.Y. Cui, L. Zhang, L. Hartman, K. Grahl, G.Y. Zhang, J. Tarpey, and C.H. Damsky. 1989. Adhesive and degradative properties of human placental cytotrophoblast cells in vitro. *Journal of Cell Biology.* 109:891-902.

Flynn, A., J.H. Finke, and M.A. Loftus. 1985. Comparison of interleukin 1 production by adherent cells and tissue pieces from human placenta. *Immunopharmacology.* 9:19-26.

Foxman, E.F., J.J. Campbell, and E.C. Butcher. 1997. Multistep navigation and the combinatorial control of leukocyte chemotaxis. *Journal of Cell Biology.* 139:1349-60.

Foxman, E.F., E.J. Kunkel, and E.C. Butcher. 1999. Integrating conflicting chemotactic signals. The role of memory in leukocyte navigation. *Journal of Cell Biology.* 147:577-88.

Franci, C., L.M. Wong, J. Van Damme, P. Proost, and I.F. Charo. 1995. Monocyte chemoattractant protein-3, but not monocyte chemoattractant protein-2, is a functional ligand of the human monocyte chemoattractant protein-1 receptor. *Journal of Immunology.* 154:6511-7.

Frauli, M., and H. Ludwig. 1987. Immunocytochemical identification of mitotic Hofbauer cells in cultures of first trimester human placental villi. *Archives of Gynecology and Obstetrics.* 241:47-51.

Friedman, S.A., R.N. Taylor, and J.M. Roberts. 1991. Pathophysiology of preeclampsia. *Clin Perinatol.* 18:661-82.

Gao, J.L., D.B. Kuhns, H.L. Tiffany, D. McDermott, X. Li, U. Francke, and P.M. Murphy. 1993. Structure and functional expression of the human macrophage inflammatory protein 1 alpha/RANTES receptor. *Journal of Experimental Medicine.* 177:1421-7.

García-Velasco, J.A., and A. Arici. 1999. Chemokines and human reproduction. *Fertility and Sterility.* 71:983-93.

Gardner, D.K., W.B. Schoolcraft, L. Wagley, T. Schlenker, J. Stevens, and J. Hesla. 1998. A prospective randomized trial of blastocyst culture and transfer in in- vitro fertilization [see comments]. *Hum Reprod.* 13:3434-40.

Geiselhart, A., J. Dietl, K. Marzusch, P. Ruck, M. Ruck, H.P. Horny, E. Kaiserling, and R. Handgretinger. 1995. Comparative analysis of the immunophenotypes of decidual and peripheral blood large granular lymphocytes and T cells during early human pregnancy. *American Journal of Reproductive Immunology.* 33:315-22.

Genbacev, O., K.E. Bass, R.J. Joslin, and S.J. Fisher. 1995. Maternal smoking inhibits early human cytotrophoblast differentiation. *Reprod Toxicol.* 9:245-55.

Genbacev, O., R. Joslin, C.H. Damsky, B.M. Polliotti, and S.J. Fisher. 1996. Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. *Journal of Clinical Investigation*. 97:540-50.

Genbacev, O., E. DiFederico, M. McMaster, and S.J. Fisher. 1999a. Invasive cytotrophoblast apoptosis in pre-eclampsia. *Hum Reprod*. 14 Suppl 2:59-66.

Genbacev, O., and R.K. Miller. 1993. Three-dimensional culture of placental villous tissue explants -- an in vitro model for peri-implantation. *Methods in Toxicology*. 3:205-244.

Genbacev, O., S.A. Schubach, and R.K. Miller. 1992. Villous culture of first trimester human placenta--model to study extravillous trophoblast (EVT) differentiation. *Placenta*. 13:439-61.

Genbacev, O., Y. Zhou, J.W. Ludlow, and S.J. Fisher. 1997. Regulation of human placental development by oxygen tension. *Science*. 277:1669-72.

Genbacev, O., Y. Zhou, M.T. McMaster, J.W. Ludlow, C.H. Damsky, and S.J. Fisher. 1999b. Oxygen regulates human cytotrophoblast proliferation, differentiation, and invasion: implications for endovascular invasion in normal pregnancy and preeclampsia. *In* Embryo implantation: molecular, cellular and clinical aspects. D.D. Carson, editor. Springer, New York. 39-53.

Genbacev, O., M.T. McMaster, and S.J. Fisher. 2000. A repertoire of cell cycle regulators whose expression is coordinated with human cytotrophoblast differentiation. *American Journal of Pathology*. 157:1337-51

Gerhardt, A., R.E. Scharf, M.W. Beckmann, S. Struve, H.G. Bender, M. Pillny, W. Sandmann, and R.B. Zotz. 2000. Prothrombin and factor V mutations in women with a history of thrombosis during pregnancy and the puerperium. *N Engl J Med*. 342:374-80.

Giancarlo, B., S. Silvano, Z. Albert, A. Mantovani, and P. Allavena. 1996. Migratory response of human natural killer cells to lymphotactin. *European Journal of Immunology*. 26:3238-41.

Giudice, L.C., and J.C. Irwin. 1999. Roles of the insulin-like growth factor family in nonpregnant human endometrium and at the decidual: trophoblast interface. *Semin Reprod Endocrinol.* 17:13-21.

Giudice, L.C., S.I. Tazuke, and L. Swiersz. 1998. Status of current research on endometriosis. *J Reprod Med.* 43:252-62.

Glover, D.M., D. Brownstein, S. Burchett, A. Larsen, and C.B. Wilson. 1987. Expression of HLA class II antigens and secretion of interleukin-1 by monocytes and macrophages from adults and neonates. *Immunology.* 61:195-201.

Godiska, R., D. Chantry, C.J. Raport, S. Sozzani, P. Allavena, D. Leviten, A. Mantovani, and P.W. Gray. 1997. Human macrophage-derived chemokine (MDC), a novel chemoattractant for monocytes, monocyte-derived dendritic cells, and natural killer cells. *Journal of Experimental Medicine.* 185:1595-604.

Godkin, J.D., and J.J. Dore. 1998. Transforming growth factor beta and the endometrium. *Rev Reprod.* 3:1-6.

Gogolin-Ewens, K.J., C.S. Lee, W.R. Mercer, and M.R. Brandon. 1989. Site-directed differences in the immune response to the fetus. *Immunology.* 66:312-7.

Goldstein, D.P., and R.S. Berkowitz. 1994. Current management of complete and partial molar pregnancy. *J Reprod Med.* 39:139-46.

Goldstein, J., M. Braverman, C. Salafia, and P. Buckley. 1988. The phenotype of human placental macrophages and its variation with gestational age. *American Journal of Pathology.* 133:648-59.

Goodnow, C.C., and J.G. Cyster. 1997. Lymphocyte homing: the scent of a follicle. *Current Biology.* 7:R219-22.

Gosling, J., F.S. Monteclaro, R.E. Atchison, H. Arai, C.L. Tsou, M.A. Goldsmith, and I.F. Charo. 1997. Molecular uncoupling of C-C chemokine receptor 5-induced chemotaxis and signal transduction from HIV-1 coreceptor activity. *Proceedings of the National Academy of Sciences of the United States of America.* 94:5061-6.

Graham, C.H., and P.K. Lala. 1992. Mechanisms of placental invasion of the uterus and their control. *Biochem Cell Biol.* 70:867-74.

Griffiths, G.M., and C. Mueller. 1991. Expression of perforin and granzymes in vivo: potential diagnostic markers for activated cytotoxic cells. *Immunology Today.* 12:415-9.

Guimond, M.J., J.A. Luross, B. Wang, C. Terhorst, S. Danial, and B.A. Croy. 1997. Absence of natural killer cells during murine pregnancy is associated with reproductive compromise in TgE26 mice. *Biology of Reproduction.* 56:169-79.

Guimond, M.J., B. Wang, and B.A. Croy. 1998. Engraftment of bone marrow from severe combined immunodeficient (SCID) mice reverses the reproductive deficits in natural killer cell-deficient tg epsilon 26 mice. *Journal of Experimental Medicine.* 187:217-23.

Gulan, G., E.R. Podack, D. Rukavina, L. Gudelj, G. Rubesa, O. Petrovic, P.M. Johnson, and S.E. Christmas. 1997. Perforin-expressing lymphocytes in peripheral blood and decidua of human first-trimester pathological pregnancies. *American Journal of Reproductive Immunology.* 38:9-18.

Guleria, I., and J.W. Pollard. 2000. The trophoblast is a component of the innate immune system during pregnancy. *Nature Medicine.* 6:589-93.

Guller, S., and L. LaChapelle. 1999. The role of placental Fas ligand in maintaining immune privilege at maternal-fetal interfaces. *Seminars in Reproductive Endocrinology.* 17:39-44.

Gunn, M.D., S. Kyuwa, C. Tam, T. Kakiuchi, A. Matsuzawa, L.T. Williams, and H. Nakano. 1999. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization [see comments]. *Journal of Experimental Medicine.* 189:451-60.

Gunn, M.D., V.N. Ngo, K.M. Ansel, E.H. Ekland, J.G. Cyster, and L.T. Williams. 1998. A B-cell homing chemokine made in lymphoid follicles activates the BLR1 receptor. *Nature.* in press.

Haller, H., F. Tedesco, D. Rukavina, O. Radillo, L. Gudelj, and A.E. Beer. 1995. Decidual-trophoblast interactions: decidual lymphoid cell populations in basal and parietal decidua. *Journal of Reproductive Immunology*. 28:165-71.

Hamilton, W.J., Boyd, J.D., and Mossman, H.W. 1972. Human Embryology. Prenatal development of form and function. W. Heffer & Sons Ltd., Cambridge.

Handyside, A.H., and C.M. Ogilvie. 1999. Screening oocytes and preimplantation embryos for aneuploidy. *Curr Opin Obstet Gynecol*. 11:301-5.

Handyside, A.H., P.N. Scriven, and C.M. Ogilvie. 1998. The future of preimplantation genetic diagnosis. *Hum Reprod*. 13 Suppl 4:249-55.

Harvey, M.B., K.J. Leco, M.Y. Arcellana-Panlilio, X. Zhang, D.R. Edwards, and G.A. Schultz. 1995. Proteinase expression in early mouse embryos is regulated by leukaemia inhibitory factor and epidermal growth factor. *Development*. 121:1005-14.

Haynes, M.K., R.L. Wapner, L.G. Jackson, and J.B. Smith. 1997. Phenotypic analysis of adhesion molecules in first-trimester decidual tissue from chorion villus samples. *American Journal of Reproductive Immunology*. 38:423-30.

Head, J.R. 1996. Uterine natural killer cells during pregnancy in rodents. *Natural Immunity*. 15:7-21.

Hedrick, J.A., V. Saylor, D. Figueroa, L. Mizoue, Y. Xu, S. Menon, J. Abrams, T. Handel, and A. Zlotnik. 1997. Lymphotactin is produced by NK cells and attracts both NK cells and T cells in vivo. *Journal of Immunology*. 158:1533-40.

Hertig, A.J., J. Rock, E.C. Adams, and M.C. Menkin. 1959. Thirty-four fertilized human ova, good, bad and indifferent, recovered from 210 women of known fertility: a study of biologic wastage in early human pregnancy. *Pediatrics*. 23:202-11.

Hertig, A.T., and J. Rock. 1973. Searching for early fertilized human ova. *Gynecol Invest*. 4:121-39.

Hey, N.A., R.A. Graham, M.W. Seif, and J.D. Aplin. 1994. The polymorphic epithelial mucin MUC1 in human endometrium is regulated with maximal expression in the implantation phase. *J Clin Endocrinol Metab.* 78:337-42.

Hiby, S.E., A. King, A.M. Sharkey, and Y.W. Loke. 1997. Human uterine NK cells have a similar repertoire of killer inhibitory and activatory receptors to those found in blood, as demonstrated by RT-PCR and sequencing. *Molecular Immunology.* 34:419-30.

Hill, J.A. 1994. Sporadic and recurrent spontaneous abortion. *Curr Probl Obstet Gynecol.* 17:113-64.

Hill, J.A., H.M. Faris, I. Schiff, and D.J. Anderson. 1988. Characterization of leukocyte subpopulations in the peritoneal fluid of women with endometriosis. *Fertil Steril.* 50:216-22.

Hill, J.A., K. Polgar, and D.J. Anderson. 1995. T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion. *Jama.* 273:1933-6.

Himmelberger, D.U., B.W. Brown, Jr., and E.N. cohen. 1978. Cigarette smoking during pregnancy and the occurrence of spontaneous abortion and congenital abnormality. *American Journal of Epidemiology.* 108:470-9.

Honore, L.H. 1997. Pathology of female infertility. *Curr Opin Obstet Gynecol.* 9:37-43.

Hornung, D., I.P. Ryan, V.A. Chao, J.L. Vigne, E.D. Schriock, and R.N. Taylor. 1997. Immunolocalization and regulation of the chemokine RANTES in human endometrial and endometriosis tissues and cells. *Journal of Clinical Endocrinology and Metabolism.* 82:1621-8.

Howard, M., A. O'Garra, H. Ishida, R. de Waal Malefyt, and J. de Vries. 1992. Biological properties of interleukin 10. *Journal of Clinical Immunology.* 12:239-47.

Howatson, A.G., M. Farquharson, A. Meager, A.M. McNicol, and A.K. Foulis. 1988. Localization of alpha-interferon in the human feto-placental unit. *Journal of Endocrinology.* 119:531-4.

Huang, J.C., D.Y. Liu, S. Yadollahi, K.K. Wu, and M.Y. Dawood. 1998. Interleukin-1 beta induces cyclooxygenase-2 gene expression in cultured endometrial stromal cells. *J Clin Endocrinol Metab.* 83:538-41.

Hunt, J.S., D. Vassmer, T.A. Ferguson, and L. Miller. 1997. Fas ligand is positioned in mouse uterus and placenta to prevent trafficking of activated leukocytes between the mother and the conceptus. *Journal of Immunology.* 158:4122-8.

Huppertz, B., S. Kertschanska, A.Y. Demir, H.G. Frank, and P. Kaufmann. 1998. Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta. *Cell Tissue Res.* 291:133-48.

Hutter, H., A. Hammer, G. Dohr, and J.S. Hunt. 1998. HLA expression at the maternal-fetal interface. *Dev Immunol.* 6:197-204.

Huxley, A. 1971. The fifth philosopher's song. *In* The collected poetry of Aldous Huxley. D. Watt, editor. Harper & Row, New York. 106.

Idris, N., and K.L. Carraway. 1999. Sialomucin complex (Muc4) expression in the rat female reproductive tract. *Biol Reprod.* 61:1431-8.

Imai, T., K. Hieshima, C. Haskell, M. Baba, M. Nagira, M. Nishimura, M. Kakizaki, S. Takagi, H. Nomiyama, T.J. Schall, and O. Yoshie. 1997. Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell.* 91:521-30.

Imakawa, K., R.V. Anthony, M. Kazemi, K.R. Marotti, H.G. Polites, and R.M. Roberts. 1987. Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. *Nature.* 330:377-9.

Janatpour, M.J., M.T. McMaster, O. Genbacev, Y. Zhou, J. Dong, J.C. Cross, M.A. Israel, and S.J. Fisher. 2000. Id-2 regulates critical aspects of human cytotrophoblast differentiation, invasion and migration [In Process Citation]. *Development.* 127:549-58.

Janatpour, M.J., M.F. Utset, J.C. Cross, J. Rossant, J. Dong, M.A. Israel, and S.J. Fisher. 1999a. A repertoire of differentially expressed transcription factors that offers

insight into mechanisms of human cytotrophoblast differentiation. *Dev Genet.* 25:146-57.

Janatpour, M.J., M.F. Utset, J.C. Cross, J. Rossant, J. Dong, M.A. Israel, and S.J. Fisher. 1999b. A repertoire of differentially expressed transcription factors that offers insight into mechanisms of human cytotrophoblast differentiation. *Developmental Genetics.* 25:146-57.

Jaschevatzky, O.E., A. Shalit, S. Grunstein, J. Kaplanski, and A. Danon. 1983. Increased decidual prostaglandin E concentration in human abortion. *Br J Obstet Gynaecol.* 90:958-60.

Jen, Y., K. Manova, and R. Benezra. 1997. Each member of the Id gene family exhibits a unique expression pattern in mouse gastrulation and neurogenesis. *Dev Dyn.* 208:92-106.

Jeng, G.T., J.R. Scott, and L.F. Burmeister. 1995. A comparison of meta-analytic results using literature vs individual patient data. Paternal cell immunization for recurrent miscarriage. *Jama.* 274:830-6.

Jin, K., H.N. Ho, T.P. Speed, and T.J. Gill, 3rd. 1995. Reproductive failure and the major histocompatibility complex [see comments]. *Am J Hum Genet.* 56:1456-67.

Johnson, J.D., D.P. Houchens, W.M. Kluwe, D.K. Craig, and G.L. Fisher. 1990. Effects of mainstream and environmental tobacco smoke on the immune system in animals and humans: a review. *Critical Reviews in Toxicology.* 20:369-95.

Johnston, C. 1981. Cigarette smoking and the outcome of human pregnancies: a status report on the consequences. *Clinical Toxicology.* 18:189-209.

Jones, R.L., R.W. Kelly, and H.O. Critchley. 1997. Chemokine and cyclooxygenase-2 expression in human endometrium coincides with leukocyte accumulation. *Human Reproduction.* 12:1300-6.

Ju, S.T., K. Matsui, and M. Ozdemirli. 1999. Molecular and cellular mechanisms regulating T and B cell apoptosis through Fas/FasL interaction. *International Reviews of Immunology.* 18:485-513.

Kamat, B.R., and P.G. Isaacson. 1987. The immunocytochemical distribution of leukocytic subpopulations in human endometrium. *American Journal of Pathology*. 127:66-73.

Kamimura, S., K. Eguchi, M. Yonezawa, and K. Sekiba. 1991. Localization and developmental change of indoleamine 2,3-dioxygenase activity in the human placenta. *Acta Med Okayama*. 45:135-9.

Kauma, S., D. Matt, S. Strom, D. Eierman, and T. Turner. 1990. Interleukin-1 beta, human leukocyte antigen HLA-DR alpha, and transforming growth factor-beta expression in endometrium, placenta, and placental membranes. *American Journal of Obstetrics and Gynecology*. 163:1430-7.

Keirse, M.J.N.C. 1979. Endogenous prostaglandins in human parturition. *In* Human parturition. M.J.N.C. Keirse, A.B.M. Anderson, and J. Bennebroek-Gravenhorst, editors. Leiden University Press, Leiden, The Netherlands. 101-42.

Kelly, R.W., G.G. Carr, and S.C. Riley. 1997. The inhibition of synthesis of a beta-chemokine, monocyte chemotactic protein-1 (MCP-1) by progesterone. *Biochemical and Biophysical Research Communications*. 239:557-61.

Kelly, R.W., P. Illingworth, G. Baldie, R. Leask, S. Brouwer, and A.A. Calder. 1994. Progesterone control of interleukin-8 production in endometrium and chorio-decidual cells underlines the role of the neutrophil in menstruation and parturition. *Human Reproduction*. 9:253-8.

Khan, S., H. Katabuchi, M. Araki, R. Nishimura, and H. Okamura. 2000. Human villous macrophage-conditioned media enhance human trophoblast growth and differentiation in vitro. *Biology of Reproduction*. 62:1075-83.

Kilby, M.D., S. Afford, X.F. Li, A.J. Strain, A. Ahmed, and M.J. Whittle. 1996. Localisation of hepatocyte growth factor and its receptor (c-met) protein and mRNA in human term placenta. *Growth Factors*. 13:133-9.

Kim, C.H., L.M. Pelus, E. Appelbaum, K. Johanson, N. Anzai, and H.E. Broxmeyer. 1999. CCR7 ligands, SLC/6Ckine/Exodus2/TCA4 and CKbeta-11/MIP-3beta/ELC, are chemoattractants for CD56(+)CD16(-) NK cells and late stage lymphoid progenitors. *Cellular Immunology*. 193:226-35.

King, A., D.S. Allan, M. Bowen, S.J. Powis, S. Joseph, S. Verma, S.E. Hiby, A.J. McMichael, Y.W. Loke, and V.M. Braud. 2000a. HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. *European Journal of Immunology*. 30:1623-31.

King, A., N. Balendran, P. Wooding, N.P. Carter, and Y.W. Loke. 1991. CD3- leukocytes present in the human uterus during early placentation: phenotypic and morphologic characterization of the CD56++ population. *Dev Immunol.* 1:169-90.

King, A., C. Birkby, and Y.W. Loke. 1989a. Early human decidual cells exhibit NK activity against the K562 cell line but not against first trimester trophoblast. *Cellular Immunology*. 118:337-44.

King, A., C. Boocock, A.M. Sharkey, L. Gardner, A. Beretta, A.G. Siccardi, and Y.W. Loke. 1996a. Evidence for the expression of HLAA-C class I mRNA and protein by human first trimester trophoblast. *Journal of Immunology*. 156:2068-76.

King, A., T. Burrows, S. Verma, S. Hiby, and Y.W. Loke. 1998a. Human uterine lymphocytes. *Hum Reprod Update*. 4:480-5.

King, A., T.D. Burrows, S.E. Hiby, J.M. Bowen, S. Joseph, S. Verma, P.B. Lim, L. Gardner, P. Le Bouteiller, A. Ziegler, B. Uchanska-Ziegler, and Y.W. Loke. 2000b. Surface expression of HLA-C antigen by human extravillous trophoblast. *Placenta*. 21:376-87.

King, A., L. Gardner, and Y.W. Loke. 1996b. Evaluation of oestrogen and progesterone receptor expression in uterine mucosal lymphocytes. *Human Reproduction.* 11:1079-82.

King, A., L. Gardner, A. Sharkey, and Y.W. Loke. 1998b. Expression of CD3 epsilon, CD3 zeta, and RAG-1/RAG-2 in decidual CD56+ NK cells. *Cell Immunol.* 183:99-105.

King, A., and Y.W. Loke. 1990. Human trophoblast and JEG choriocarcinoma cells are sensitive to lysis by IL-2-stimulated decidual NK cells. *Cellular Immunology.* 129:435-48.

King, A., and Y.W. Loke. 1991. On the nature and function of human uterine granular lymphocytes [see comments]. *Immunology Today*. 12:432-5.

King, A., V. Wellings, L. Gardner, and Y.W. Loke. 1989b. Immunocytochemical characterization of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle. *Human Immunology*. 24:195-205.

King, A., P. Wooding, L. Gardner, and Y.W. Loke. 1993. Expression of perforin, granzyme A and TIA-1 by human uterine CD56+ NK cells implies they are activated and capable of effector functions. *Human Reproduction*. 8:2061-7.

Kitaura, M., T. Nakajima, T. Imai, S. Harada, C. Combadiere, H.L. Tiffany, P.M. Murphy, and O. Yoshie. 1996. Molecular cloning of human eotaxin, an eosinophil-selective CC chemokine, and identification of a specific eosinophil eotaxin receptor, CC chemokine receptor 3. *Journal of Biological Chemistry*. 271:7725-30.

Kleinman, J.C., and A. Kopstein. 1987. Smoking during pregnancy, 1967-80. *AJPH*. 77:823-825.

Kliman, H.J., J.E. Nestler, E. Sermasi, J.M. Sanger, and J.F.d. Strauss. 1986. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology*. 118:1567-82.

Kline, J., Z.A. Stein, M. Susser, and D. Warburton. 1977. Smoking: a risk factor for spontaneous abortion. *New England Journal of Medicine*. 297:793-6.

Koch, A.E., P.J. Polverini, S.L. Kunkel, L.A. Harlow, L.A. DiPietro, V.M. Elner, S.G. Elner, and R.M. Strieter. 1992. Interleukin-8 as a macrophage-derived mediator of angiogenesis [see comments]. *Science*. 258:1798-801.

Kovats, S., E.K. Main, C. Librach, M. Stubblebine, S.J. Fisher, and R. DeMars. 1990. A class I antigen, HLA-G, expressed in human trophoblasts. *Science*. 248:220-3.

Kramer, M.S. 1987. Determinants of low birth weight: Methodological assessment and meta-analysis. *Bull WHO*. 65:663-737.

Krebs, C., L.M. Macara, R. Leiser, A.W. Bowman, I.A. Greer, and J.C. Kingdom. 1996. Intrauterine growth restriction with absent end-diastolic flow velocity in the

umbilical artery is associated with maldevelopment of the placental terminal villous tree. *Am J Obstet Gynecol.* 175:1534-42.

Kruse, A., R. Hallmann, and E.C. Butcher. 1999a. Specialized patterns of vascular differentiation antigens in the pregnant mouse uterus and the placenta. *Biol Reprod.* 61:1393-401.

Kruse, A., R. Hallmann, and E.C. Butcher. 1999b. Specialized patterns of vascular differentiation antigens in the pregnant mouse uterus and the placenta. *Biology of Reproduction.* 61:1393-401.

Kruse, A., M.J. Merchant, R. Hallmann, and E.C. Butcher. 1999c. Evidence of specialized leukocyte-vascular homing interactions at the maternal/fetal interface. *European Journal of Immunology.* 29:1116-26.

Kumar, S., L.J. Zhu, M. Polihronis, S.T. Cameron, D.T. Baird, F. Schatz, A. Dua, Y.K. Ying, M.K. Bagchi, and I.C. Bagchi. 1998. Progesterone induces calcitonin gene expression in human endometrium within the putative window of implantation. *J Clin Endocrinol Metab.* 83:4443-50.

Lagow, E., M.M. DeSouza, and D.D. Carson. 1999. Mammalian reproductive tract mucins. *Hum Reprod Update.* 5:280-92.

Lairmore, M.D., P.S. Cuthbert, L.L. Utley, C.J. Morgan, C.S. Dezzutti, C.L. Anderson, and D.D. Sedmak. 1993. Cellular localization of CD4 in the human placenta. Implications for maternal-to-fetal transmission of HIV. *Journal of Immunology.* 151:1673-81.

Lala, P.K., and C.H. Graham. 1990. Mechanisms of trophoblast invasiveness and their control: the role of proteases and protease inhibitors. *Cancer Metastasis Rev.* 9:369-79.

Lambers, D.S., and K.E. Clark. 1996. The maternal and fetal physiologic effects of nicotine. *Semin Perinatol.* 20:115-26.

Lanier, L.L. 1998. NK cell receptors. *Annual Review of Immunology.* 16:359-93.

Lanier, L.L. 1999. Natural killer cells fertile with receptors for HLA-G? *Proc Natl Acad Sci U S A*. 96:5343-5.

Lanier, L.L. 2000. The origin and functions of natural killer cells. *Clinical Immunology*. 95:S14-8.

Lanier, L.L., B. Corliss, and J.H. Phillips. 1997. Arousal and inhibition of human NK cells. *Immunological Reviews*. 155:145-54.

Lanier, L.L., A.M. Le, C.I. Civin, M.R. Loken, and J.H. Phillips. 1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *Journal of Immunology*. 136:4480-6.

Lawrence, M.B., E.L. Berg, E.C. Butcher, and T.A. Springer. 1995. Rolling of lymphocytes and neutrophils on peripheral node addressin and subsequent arrest on ICAM-1 in shear flow. *Eur J Immunol*. 25:1025-31.

Leach, R.E., R. Khalifa, N.D. Ramirez, S.K. Das, J. Wang, S.K. Dey, R. Romero, and D.R. Armant. 1999. Multiple roles for heparin-binding epidermal growth factor-like growth factor are suggested by its cell-specific expression during the human endometrial cycle and early placentation. *J Clin Endocrinol Metab*. 84:3355-63.

Lee, C.S., K. Gogolin-Ewens, and M.R. Brandon. 1988. Identification of a unique lymphocyte subpopulation in the sheep uterus. *Immunology*. 63:157-64.

Lehrach, H., D. Diamond, J.M. Wozney, and H. Boedtker. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry*. 16:4743-51.

Lessey, B.A. 1998. Endometrial integrins and the establishment of uterine receptivity. *Hum Reprod*. 13 Suppl 3:247-58; discussion 259-61.

Lessey, B.A., and J.T. Arnold. 1998. Paracrine signaling in the endometrium: integrins and the establishment of uterine receptivity. *J Reprod Immunol*. 39:105-16.

Letterio, J.J., and A.B. Roberts. 1998. Regulation of immune responses by TGF-beta. *Annual Review of Immunology*. 16:137-61.

Li, C.Q., R.A. Windsor, L. Perkins, R.L. Goldenberg, and J.B. Lowe. 1993. The impact on infant birth weight and gestational age of cotinine-validated smoking reduction during pregnancy [see comments]. *Jama.* 269:1519-24.

Librach, C.L., S.L. Feigenbaum, K.E. Bass, T.Y. Cui, N. Verastas, Y. Sadovsky, J.P. Quigley, D.L. French, and S.J. Fisher. 1994. Interleukin-1 beta regulates human cytotrophoblast metalloproteinase activity and invasion in vitro. *J Biol Chem.* 269:17125-31.

Librach, C.L., Z. Werb, M.L. Fitzgerald, K. Chiu, N.M. Corwin, R.A. Esteves, D. Grobelny, R. Galardy, C.H. Damsky, and S.J. Fisher. 1991. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. *J Cell Biol.* 113:437-49.

Lim, H., R.A. Gupta, W.G. Ma, B.C. Paria, D.E. Moller, J.D. Morrow, R.N. DuBois, J.M. Trzaskos, and S.K. Dey. 1999a. Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARdelta. *Genes Dev.* 13:1561-74.

Lim, H., L. Ma, W.G. Ma, R.L. Maas, and S.K. Dey. 1999b. Hoxa-10 regulates uterine stromal cell responsiveness to progesterone during implantation and decidualization in the mouse. *Mol Endocrinol.* 13:1005-17.

Lim, H., B.C. Paria, S.K. Das, J.E. Dinchuk, R. Langenbach, J.M. Trzaskos, and S.K. Dey. 1997. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell.* 91:197-208.

Lin, P.Y., S.V. Joag, J.D. Young, Y.S. Chang, Y.K. Soong, and T.T. Kuo. 1991. Expression of perforin by natural killer cells within first trimester endometrium in humans. *Biology of Reproduction.* 45:698-703.

Liu, H.C., J. Cohen, M. Alikani, N. Noyes, and Z. Rosenwaks. 1993. Assisted hatching facilitates earlier implantation. *Fertil Steril.* 60:871-5.

Liu, L., and R.M. Roberts. 1996. Silencing of the gene for the beta subunit of human chorionic gonadotropin by the embryonic transcription factor Oct-3/4. *J Biol Chem.* 271:16683-9.

Loetscher, P., M. Seitz, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1996. Activation of NK cells by CC chemokines. Chemotaxis, Ca2+ mobilization, and enzyme release. *Journal of Immunology*. 156:322-7.

Loke, Y.W.a.K., A. 1995. Uterine mucosal leukocyte. *In* Human Implantation. Cell Biology and Immunology. Y.W.a.K. Loke, A., editor. Cambridge University Press, Cambridge.

Long, E.O. 1999. Regulation of immune responses through inhibitory receptors. *Annual Review of Immunology*. 17:875-904.

López-Botet, M., T. Bellón, M. Llano, F. Navarro, P. García, and M. de Miguel. 2000. Paired inhibitory and triggering NK cell receptors for HLA class I molecules. *Human Immunology*. 61:7-17.

Luo, J., R. Sladek, J.A. Bader, A. Matthyssen, J. Rossant, and V. Giguere. 1997. Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-beta. *Nature*. 388:778-82.

Luster, A.D., S.M. Greenberg, and P. Leder. 1995. The IP-10 chemokine binds to a specific cell surface heparan sulfate site shared with platelet factor 4 and inhibits endothelial cell proliferation. *Journal of Experimental Medicine*. 182:219-31.

Luster, A.D., J.C. Unkeless, and J.V. Ravetch. 1985. Gamma-interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. *Nature*. 315:672-6.

Ma, L., G.V. Benson, H. Lim, S.K. Dey, and R.L. Maas. 1998a. Abdominal B (AbdB) Hoxa genes: regulation in adult uterus by estrogen and progesterone and repression in mullerian duct by the synthetic estrogen diethylstilbestrol (DES). *Dev Biol*. 197:141-54.

Ma, Q., D. Jones, P.R. Borghesani, R.A. Segal, T. Nagasawa, T. Kishimoto, R.T. Bronson, and T.A. Springer. 1998b. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*. 95:9448-53.

Maathuis, J.B., and R.W. Kelly. 1978. Concentrations of prostaglandins F2alpha and E2 in the endometrium throughout the human menstrual cycle, after the administration of clomiphene or an oestrogen-progestogen pill and in early pregnancy. *J Endocrinol.* 77:361-71.

Maghazachi, A.A. 1997. Role of the heterotrimeric G proteins in stromal-derived factor-1alpha-induced natural killer cell chemotaxis and calcium mobilization [published erratum appears in Biochem Biophys Res Commun 1997 Aug 28;237(3):759]. *Biochemical and Biophysical Research Communications.* 236:270-4.

Maghazachi, A.A., A. al-Aoukaty, and T.J. Schall. 1994. C-C chemokines induce the chemotaxis of NK and IL-2-activated NK cells. Role for G proteins. *Journal of Immunology.* 153:4969-77.

Maghazachi, A.A., A. Al-Aoukaty, and T.J. Schall. 1996. CC chemokines induce the generation of killer cells from CD56+ cells. *Eur J Immunol.* 26:315-9.

Maghazachi, A.A., B.S. Skalhegg, B. Rolstad, and A. Al-Aoukaty. 1997. Interferon-inducible protein-10 and lymphotactin induce the chemotaxis and mobilization of intracellular calcium in natural killer cells through pertussis toxin-sensitive and -insensitive heterotrimeric G-proteins. *Faseb Journal.* 11:765-74.

Manaseki, S., and R.F. Searle. 1989. Natural killer (NK) cell activity of first trimester human decidua. *Cellular Immunology.* 121:166-73.

Manjunath, R., K. Ozato, and A.B. Mukherjee. 1985. Stimulation of alloreactive cytotoxic T cells by paternal major histocompatibility antigens during murine pregnancy. *Biol Reprod.* 33:668-78.

Mann, G.B., K.J. Fowler, A. Gabriel, E.C. Nice, R.L. Williams, and A.R. Dunn. 1993. Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell.* 73:249-61.

Marions, L., and K.G. Danielsson. 1999. Expression of cyclo-oxygenase in human endometrium during the implantation period. *Mol Hum Reprod.* 5:961-5.

Martin, K.L., D.H. Barlow, and I.L. Sargent. 1998. Heparin-binding epidermal growth factor significantly improves human blastocyst development and hatching in serum-free medium. *Hum Reprod.* 13:1645-52.

Marx, L., P. Arck, M. Kapp, C. Kieslich, and J. Dietl. 1999. Leukocyte populations, hormone receptors and apoptosis in eutopic and ectopic first trimester human pregnancies. *Human Reproduction.* 14:1111-7.

Marzusch, K., P. Ruck, A. Geiselhart, R. Handgretinger, J.A. Dietl, E. Kaiserling, H.P. Horny, G. Vince, and C.W. Redman. 1993. Distribution of cell adhesion molecules on CD56++, CD3-, CD16- large granular lymphocytes and endothelial cells in first-trimester human decidua. *Human Reproduction.* 8:1203-8.

Mastroianni, L., Jr. 1999. The fallopian tube and reproductive health. *J Pediatr Adolesc Gynecol.* 12:121-6.

Matsushima, K., C.G. Larsen, G.C. DuBois, and J.J. Oppenheim. 1989. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. *Journal of Experimental Medicine.* 169:1485-90.

McGrath, K.E., A.D. Koniski, K.M. Maltby, J.K. McGann, and J. Palis. 1999. Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. *Developmental Biology.* 213:442-56.

McMaster, M., Y. Zhou, S. Shorter, K. Kapasi, D. Geraghty, K.H. Lim, and S. Fisher. 1998. HLA-G isoforms produced by placental cytotrophoblasts and found in amniotic fluid are due to unusual glycosylation. *J Immunol.* 160:5922-8.

McMaster, M.T., K.E. Bass, and S.J. Fisher. 1994. Human trophoblast invasion. Autocrine control and paracrine modulation. *Ann N Y Acad Sci.* 734:122-31.

McMaster, M.T., C.L. Librach, Y. Zhou, K.H. Lim, M.J. Janatpour, R. DeMars, S. Kovats, C. Damsky, and S.J. Fisher. 1995. Human placental HLA-G expression is restricted to differentiated cytotrophoblasts. *J Immunol.* 154:3771-8.

Medawar, P.B. 1953. Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. *Symp Soc Exp Biol.* 7:320-38.

Meekins, J.W., R. Pijnenborg, M. Hanssens, I.R. McFadyen, and A. van Asshe. 1994. A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies. *Br J Obstet Gynaecol.* 101:669-74.

Meeusen, E., A. Fox, M. Brandon, and C.S. Lee. 1993. Activation of uterine intraepithelial gamma delta T cell receptor-positive lymphocytes during pregnancy. *European Journal of Immunology.* 23:1112-7.

Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Research.* 12:7035-56.

Mi, S., X. Lee, X. Li, G.M. Veldman, H. Finnerty, L. Racie, E. LaVallie, X.Y. Tang, P. Edouard, S. Howes, J.C. Keith, Jr., and J.M. McCoy. 2000. Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. [Comment In: Nature. 2000 Feb 17;403(6771):715, 717]. *Nature.* 403:785-9.

Miki, A., Y. Ishikawa, T. Yabe, H. Furukawa, T. Fujii, K. Tokunaga, T. Akaza, K. Tadokoro, Y. Taketani, and T. Juji. 1998. Human lymphocytes obtained from decidual tissue express killer activatory receptors as well as killer inhibitory receptors: analysis using a single strand conformation polymorphism method. *American Journal of Reproductive Immunology.* 39:271-8.

Miller, J.F., E. Williamson, J. Glue, Y.B. Gordon, J.G. Grudzinskas, and A. Sykes. 1980. Fetal loss after implantation. A prospective study. *Lancet.* 2:554-6.

Miller, W.L. 1998. Steroid hormone biosynthesis and actions in the materno-feto-placental unit. *Clin Perinatol.* 25:799-817, v.

Milne, S.A., H.O. Critchley, T.A. Drudy, R.W. Kelly, and D.T. Baird. 1999. Perivascular interleukin-8 messenger ribonucleic acid expression in human endometrium varies across the menstrual cycle and in early pregnancy decidua. *Journal of Clinical Endocrinology and Metabolism.* 84:2563-7.

Mincheva-Nilsson, L., V. Baranov, M.M. Yeung, S. Hammarstrom, and M.L. Hammarstrom. 1994. Immunomorphologic studies of human decidua-associated lymphoid cells in normal early pregnancy. *J Immunol.* 152:2020-32.

Mincheva-Nilsson, L., S. Hammarstrom, and M.L. Hammarstrom. 1992. Human decidual leukocytes from early pregnancy contain high numbers of gamma delta+ cells and show selective down-regulation of alloreactivity. *J Immunol.* 149:2203-11.

Mitchell, S.N., and S.K. Smith. 1992. The effect of progesterone and human interferon alpha-2 on the release of PGF2 alpha and PGE from epithelial cells of human proliferative endometrium. *Prostaglandins.* 44:457-70.

Morales, J., B. Homey, A.P. Vicari, S. Hudak, E. Oldham, J. Hedrick, R. Orozco, N.G. Copeland, N.A. Jenkins, L.M. McEvoy, and A. Zlotnik. 1999. CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proceedings of the National Academy of Sciences of the United States of America.* 96:14470-5.

Mosmann, T.R., and R.L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol.* 7:145-73.

Mowbray, J.F., and J.L. Underwood. 1985. Immunology of abortion. *Clin Exp Immunol.* 60:1-7.

Muller, A., B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, and S.N. Wagner. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature.* 410:24-5.

Munn, D.H., M. Zhou, J.T. Attwood, I. Bondarev, S.J. Conway, B. Marshall, C. Brown, and A.L. Mellor. 1998. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science.* 281:1191-3.

Myers, S.J., L.M. Wong, and I.F. Charo. 1995. Signal transduction and ligand specificity of the human monocyte chemoattractant protein-1 receptor in transfected embryonic kidney cells. *Journal of Biological Chemistry.* 270:5786-92.

Nagasawa, T., S. Hirota, K. Tachibana, N. Takakura, S. Nishikawa, Y. Kitamura, N. Yoshida, H. Kikutani, and T. Kishimoto. 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature.* 382:635-8.

Nagasawa, T., H. Kikutani, and T. Kishimoto. 1994. Molecular cloning and structure of a pre-B-cell growth-stimulating factor. *Proceedings of the National Academy of Sciences of the United States of America.* 91:2305-9.

Nagler, A., L.L. Lanier, S. Cwirla, and J.H. Phillips. 1989. Comparative studies of human FcRIII-positive and negative natural killer cells. *Journal of Immunology.* 143:3183-91.

Nagler, A., L.L. Lanier, and J.H. Phillips. 1990. Constitutive expression of high affinity interleukin 2 receptors on human CD16-natural killer cells in vivo. *Journal of Experimental Medicine.* 171:1527-33.

Nakamura, Y., and Y. Ohta. 1990. Immunohistochemical study of human placental stromal cells. *Human Pathology.* 21:936-40.

Nakayama, H., I.C. Scott, and J.C. Cross. 1998. The transition to endoreduplication in trophoblast giant cells is regulated by the mSNA zinc finger transcription factor. *Dev Biol.* 199:150-63.

Nasu, K., T. Sugano, N. Matsui, H. Narahara, Y. Kawano, and I. Miyakawa. 1999. Expression of hepatocyte growth factor in cultured human endometrial stromal cells is induced through a protein kinase C-dependent pathway. *Biol Reprod.* 60:1183-7.

Nemlander, A., E. Saksela, and P. Häyry. 1983. Are "natural killer" cells involved in allograft rejection? *European Journal of Immunology.* 13:348-50.

Neote, K., D. DiGregorio, J.Y. Mak, R. Horuk, and T.J. Schall. 1993. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. *Cell.* 72:415-25.

Nieto, M., J.M. Frade, D. Sancho, M. Mellado, A.C. Martinez, and F. Sánchez-Madrid. 1997. Polarization of chemokine receptors to the leading edge during lymphocyte chemotaxis. *Journal of Experimental Medicine.* 186:153-8.

Nieto, M., F. Navarro, J.J. Perez-Villar, M.A. del Pozo, R. González-Amaro, M. Mellado, J.M. Frade, A.C. Martínez, M. López-Botet, and F. Sánchez-Madrid. 1998. Roles

of chemokines and receptor polarization in NK-target cell interactions. *Journal of Immunology*. 161:3330-9.

Nikas, G., and A. Psychoyos. 1997. Uterine pinopodes in peri-implantation human endometrium. Clinical relevance. *Ann N Y Acad Sci*. 816:129-42.

Nishikawa, K., S. Saito, T. Morii, K. Hamada, H. Ako, N. Narita, M. Ichijo, M. Kurahayashi, and K. Sugamura. 1991. Accumulation of CD16-CD56+ natural killer cells with high affinity interleukin 2 receptors in human early pregnancy decidua. *Int Immunol.* 3:743-50.

Norwitz, E.R. 1998. Multiple pregnancy: trends past, present, and future. *Infertil Reprod Med Clinics North America.* 9:351-69.

Norwitz, E.R., J.N. Robinson, and J.T. Repke. 1999. Prevention of preeclampsia: is it possible? *Clin Obstet Gynecol.* 42:436-54.

Norwitz, E.R., and T. Wilson. 2000. Secretory component: a potential regulator of endometrium/decidual prostaglandin production in early human pregnancy. *Am J Obstet Gynecol.* in press.

O'Brien, J.M., J.R. Barton, and E.S. Donaldson. 1996. The management of placenta percreta: conservative and operative strategies. *Am J Obstet Gynecol.* 175:1632-8.

O'Campo, P., M.V. Davis, and A.C. Gielen. 1995. Smoking cessation interventions for pregnant women: review and future directions. *Semin Perinatol.* 19:279-85.

Oliveira, L.H., S.V. Leandro, M.E. Fonseca, and L.M. Dias. 1986. A new technique for the isolation of placental phagocyte cells and a description of their macrophage properties after in vitro culture. *Brazilian Journal of Medical and Biological Research.* 19:249-55.

Oshiro, B.T., R.M. Silver, J.R. Scott, H. Yu, and D.W. Branch. 1996. Antiphospholipid antibodies and fetal death. *Obstet Gynecol.* 87:489-93.

Overpeck, M., and A. Moss. 1991. Children's exposure to environmental cigarette smoke before and after birth: health of our nation's children, United States 1988. *ask Evangeline.*

Pace, D., L. Morrison, and J.N. Bulmer. 1989. Proliferative activity in endometrial stromal granulocytes throughout menstrual cycle and early pregnancy. *Journal of Clinical Pathology.* 42:35-9.

Palmieri, S.L., W. Peter, H. Hess, and H.R. Scholer. 1994. Oct-4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. *Dev Biol.* 166:259-67.

Paradowska, E., Z. Blach-Olszewska, and E. Gejdel. 1997. Constitutive and induced cytokine production by human placenta and amniotic membrane at term. *Placenta.* 18:441-6.

Paria, B.C., S.K. Das, and S.K. Dey. 1998. Embryo implantation requires estrogen-directed uterine preparation and catecholestrogen-mediated embryonic activation. *Adv Pharmacol.* 42:840-3.

Paria, B.C., Y.M. Huet-Hudson, and S.K. Dey. 1993. Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. *Proc Natl Acad Sci U S A.* 90:10159-62.

Paul, P., N. Rouas-Freiss, I. Khalil-Daher, P. Moreau, B. Riteau, F.A. Le Gal, M.F. Avril, J. Dausset, J.G. Guillet, and E.D. Carosella. 1998. HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. *Proceedings of the National Academy of Sciences of the United States of America.* 95:4510-5.

Pavia, C., P.K. Siiteri, J.D. Perlman, and D.P. Stites. 1979a. Suppression of murine allogeneic cell interactions by sex hormones. *J Reprod Immunol.* 1:33-8.

Pavia, C., P.K. Siiteri, J.D. Perlman, and D.P. Stites. 1979b. Suppression of murine allogeneic cell interactions by sex hormones. *Journal of Reproductive Immunology.* 1:33-8.

Peyron, R., E. Aubeny, V. Targosz, L. Silvestre, M. Renault, F. Elkik, P. Leclerc, A. Ulmann, and E.E. Baulieu. 1993. Early termination of pregnancy with mifepristone (RU 486) and the orally active prostaglandin misoprostol. *N Engl J Med.* 328:1509-13.

Pfarrer, C., L. Macara, R. Leiser, and J. Kingdom. 1999. Adaptive angiogenesis in placentas of heavy smokers [letter] [see comments]. *Lancet.* 354:303.

Phillips, G.D., R.A. Whitehead, A.M. Stone, M.W. Ruebel, M.L. Goodkin, and D.R. Knighton. 1993. Transforming growth factor beta (TGF-B) stimulation of angiogenesis: an electron microscopic study. *J Submicrosc Cytol Pathol.* 25:149-55.

Pijnenborg, R., W.B. Robertson, I. Brosens, and G. Dixon. 1981. Review article: trophoblast invasion and the establishment of haemochorial placentation in man and laboratory animals. *Placenta.* 2:71-91.

Pike, I.L., A.J. Ammit, and C. O'Neill. 1992. Actions of platelet activating factor (PAF) on gametes and embryos: clinical aspects. *Reprod Fertil Dev.* 4:399-410.

Ponath, P.D., S. Qin, T.W. Post, J. Wang, L. Wu, N.P. Gerard, W. Newman, C. Gerard, and C.R. Mackay. 1996. Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils [see comments]. *Journal of Experimental Medicine.* 183:2437-48.

Poropatich, C., M. Rojas, and S.G. Silverberg. 1987. Polymorphonuclear leukocytes in the endometrium during the normal menstrual cycle. *International Journal of Gynecological Pathology.* 6:230-4.

Premack, B.A., and T.J. Schall. 1996. Chemokine receptors: gateways to inflammation and infection. *Nature Medicine.* 2:1174-8.

Pröll, J., A. Blaschitz, H. Hutter, and G. Dohr. 1999. First trimester human endovascular trophoblast cells express both HLA-C and HLA-G. *American Journal of Reproductive Immunology.* 42:30-6.

Psychoyos, A. 1986. Uterine receptivity for nidation. *Ann N Y Acad Sci.* 476:36-42.

Queenan, J.T., Jr., L.C. Kao, C.E. Arboleda, A. Ulloa-Aguirre, T.G. Golos, D.B. Cines, and J.F.d. Strauss. 1987. Regulation of urokinase-type plasminogen activator production by cultured human cytotrophoblasts. *J Biol Chem.* 262:10903-6.

Raab, G., K. Kover, B.C. Paria, S.K. Dey, R.M. Ezzell, and M. Klagsbrun. 1996. Mouse preimplantation blastocysts adhere to cells expressing the transmembrane form of heparin-binding EGF-like growth factor. *Development.* 122:637-45.

Rajagopalan, S., and E.O. Long. 1999. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *Journal of Experimental Medicine*. 189:1093-100.

Raport, C.J., J. Gosling, V.L. Schweickart, P.W. Gray, and I.F. Charo. 1996. Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1beta, and MIP-1alpha. *Journal of Biological Chemistry*. 271:17161-6.

Rappolee, D.A., and Z. Werb. 1988. Secretory products of phagocytes. *Current Opinion in Immunology*. 1:47-55.

Rappolee, D.A., and Z. Werb. 1992. Macrophage-derived growth factors. *Current Topics in Microbiology and Immunology*. 181:87-140.

Redman, C.W. 1983. HLA-DR antigen on human trophoblast: a review. *Am J Reprod Immunol*. 3:175-7.

Redman, C.W., G.P. Sacks, and I.L. Sargent. 1999. Preeclampsia: an excessive maternal inflammatory response to pregnancy. *Am J Obstet Gynecol*. 180:499-506.

Riley, P., L. Anson-Cartwright, and J.C. Cross. 1998. The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. *Nat Genet*. 18:271-5.

Riley, S.C., R. Leask, T. Chard, N.C. Wathen, A.A. Calder, and D.C. Howe. 1999. Secretion of matrix metalloproteinase-2, matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases into the intrauterine compartments during early pregnancy. *Mol Hum Reprod*. 5:376-81.

Riteau, B., C. Menier, I. Khalil-Daher, C. Sedlik, J. Dausset, N. Rouas-Freiss, and E.D. Carosella. 1999. HLA-G inhibits the allogeneic proliferative response. *J Reprod Immunol*. 43:203-11.

Ritson, A., and J.N. Bulmer. 1987. Endometrial granulocytes in human decidua react with a natural-killer (NK) cell marker, NKH1. *Immunology*. 62:329-31.

Robb, L., R. Li, L. Hartley, H.H. Nandurkar, F. Koentgen, and C.G. Begley. 1998. Infertility in female mice lacking the receptor for interleukin 11 is due to a defective uterine response to implantation. *Nat Med.* 4:303-8.

Robert, C.J., and C.R. Lowe. 1975. Letter: Where have all the conceptions gone? *Lancet.* 1:636-7.

Roberts, J.M., and C.W. Redman. 1993. Pre-eclampsia: more than pregnancy-induced hypertension [published erratum appears in Lancet 1993 Aug 21;342(8869):504] [see comments]. *Lancet.* 341:1447-51.

Roberts, R.M., A.D. Ealy, A.P. Alexenko, C.S. Han, and T. Ezashi. 1999. Trophoblast interferons. *Placenta.* 20:259-64.

Roberts, R.M., D.W. Leaman, and J.C. Cross. 1992. Role of interferons in maternal recognition of pregnancy in ruminants. *Proc Soc Exp Biol Med.* 200:7-18.

Rocca, B., A.L. Loeb, J.F. Strauss, 3rd, R. Vezza, A. Habib, H. Li, and G.A. FitzGerald. 2000. Directed vascular expression of the thromboxane A2 receptor results in intrauterine growth retardation. *Nat Med.* 6:219-21.

Romero, R., R. Gomez, M. Mazor, S. Ghezzi, and B.H. Yoon. 1997. The preterm labor syndrome. *In* Preterm labor. M. Elder, R.S. Lamont, and R. Romero, editors. Churchill-Livingstone, New York. 29-49.

Roth, I., D.B. Corry, R.M. Locksley, J.S. Abrams, M.J. Litton, and S.J. Fisher. 1996. Human placental cytotrophoblasts produce the immunosuppressive cytokine interleukin 10. *J Exp Med.* 184:539-48.

Ruck, P., K. Marzusch, E. Kaiserling, H.P. Horny, J. Dietl, A. Geiselhart, R. Handgretinger, and C.W. Redman. 1994. Distribution of cell adhesion molecules in decidua of early human pregnancy. An immunohistochemical study. *Laboratory Investigation.* 71:94-101.

Rukavina, D., G. Rube*sa, L. Gudelj, H. Haller, and E.R. Podack. 1995. Characteristics of perforin expressing lymphocytes within the first trimester decidua of human pregnancy. *American Journal of Reproductive Immunology.* 33:394-404.

Runic, R., C.J. Lockwood, Y. Ma, B. Dipasquale, and S. Guller. 1996. Expression of Fas ligand by human cytotrophoblasts: implications in placentation and fetal survival. *Journal of Clinical Endocrinology and Metabolism.* 81:3119-22.

Rush, D., and E.H. Kass. 1972. Maternal smoking: a reassessment of the association with perinatal mortality. *American Journal of Epidemiology.* 96:183-96.

Ryan, I.P., and R.N. Taylor. 1997. Endometriosis and infertility: new concepts. *Obstet Gynecol Surv.* 52:365-71.

Saito, S., T. Kasahara, S. Sakakura, H. Umekage, N. Harada, and M. Ichijo. 1994. Detection and localization of interleukin-8 mRNA and protein in human placenta and decidual tissues. *Journal of Reproductive Immunology.* 27:161-72.

Saito, S., T. Morii, M. Enomoto, S. Sakakura, K. Nishikawa, N. Narita, and M. Ichijo. 1993. The effect of interleukin 2 and transforming growth factor-beta 2 (TGF-beta 2) on the proliferation and natural killer activity of decidual CD16- CD56bright natural killer cells. *Cellular Immunology.* 152:605-13.

Saito, S., S. Sakakura, M. Enomoto, M. Ichijo, K. Matsumoto, and T. Nakamura. 1995. Hepatocyte growth factor promotes the growth of cytotrophoblasts by the paracrine mechanism. *J Biochem (Tokyo).* 117:671-6.

Saito, S., H. Umekage, K. Nishikawa, T. Morii, N. Narita, M. Enomoto, S. Sakakura, N. Harada, M. Ichijo, and H. Morikawa. 1996. Interleukin 4 (IL-4) blocks the IL-2-induced increased in natural killer activity and DNA synthesis of decidual CD16-CD56bright NK cells by inhibiting expression of the IL-2 receptor alpha, beta, and gamma. *Cellular Immunology.* 170:71-7.

Salazar-Mather, T.P., T.A. Hamilton, and C.A. Biron. 2000. A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense. *Journal of Clinical Investigation.* 105:985-93.

Sargent, I.L., J. Arenas, and C.W. Redman. 1987. Maternal cell-mediated sensitisation to paternal HLA may occur, but is not a regular event in normal human pregnancy. *J Reprod Immunol.* 10:111-20.

Sauer, M.V., R.J. Paulson, and R.A. Lobo. 1992. Reversing the natural decline in human fertility. An extended clinical trial of oocyte donation to women of advanced reproductive age. *JAMA*. 268:1275-9.

Schall, T.J., K. Bacon, R.D. Camp, J.W. Kaspari, and D.V. Goeddel. 1993. Human macrophage inflammatory protein alpha (MIP-1 alpha) and MIP-1 beta chemokines attract distinct populations of lymphocytes. *Journal of Experimental Medicine*. 177:1821-6.

Schall, T.J., and K.B. Bacon. 1994. Chemokines, leukocyte trafficking, and inflammation. *Current Opinion in Immunology*. 6:865-73.

Schatz, F., G. Krikun, R. Runic, E.Y. Wang, V. Hausknecht, and C.J. Lockwood. 1999. Implications of decidualization-associated protease expression in implantation and menstruation. *Semin Reprod Endocrinol*. 17:3-12.

Schlafke, S., and A.C. Enders. 1975. Cellular basis of interaction between trophoblast and uterus at implantation. *Biol Reprod*. 12:41-65.

Schmidt, C., F. Bladt, S. Goedecke, V. Brinkmann, W. Zschiesche, M. Sharpe, E. Gherardi, and C. Birchmeier. 1995. Scatter factor/hepatocyte growth factor is essential for liver development. *Nature*. 373:699-702.

Schrocksnadel, H., G. Baier-Bitterlich, O. Dapunt, H. Wachter, and D. Fuchs. 1996. Decreased plasma tryptophan in pregnancy. *Obstet Gynecol*. 88:47-50.

Schust, D.J., D.J. Anderson, and J.A. Hill. 1996. Progesterone-induced immunosuppression is not mediated through the progesterone receptor. *Hum Reprod*. 11:980-5.

Scott, I.C., L. Anson-Cartwright, P. Riley, D. Reda, and J.C. Cross. 2000. The HAND1 basic helix-loop-helix transcription factor regulates trophoblast differentiation via multiple mechanisms. *Mol Cell Biol*. 20:530-41.

Seppala, M. 1999. Glycodelins: biological activity in reproduction and differentiation. *Curr Opin Obstet Gynecol*. 11:261-3.

Sernee, M.F., H.L. Ploegh, and D.J. Schust. 1998. Why certain antibodies cross-react with HLA-A and HLA-G: epitope mapping of two common MHC class I reagents. *Mol Immunol.* 35:177-88.

Sheth, K.V., G.L. Roca, S.T. al-Sedairy, R.S. Parhar, C.J. Hamilton, and F. al-Abdul Jabbar. 1991. Prediction of successful embryo implantation by measuring interleukin-1- alpha and immunosuppressive factor(s) in preimplantation embryo culture fluid. *Fertil Steril.* 55:952-7.

Shiverick, K.T., and C. Salafia. 1999. Cigarette smoking and pregnancy I: ovarian, uterine and placental effects. *Placenta.* 20:265-72.

Simon, C., A. Mercader, A. Frances, M.J. Gimeno, M.L. Polan, J. Remohi, and A. Pellicer. 1996. Hormonal regulation of serum and endometrial IL-1 alpha, IL-1 beta and IL-1ra: IL-1 endometrial microenvironment of the human embryo at the apposition phase under physiological and supraphysiological steroid level conditions. *J Reprod Immunol.* 31:165-84.

Simpson, J.L. 1980. Genes, chromosomes, and reproductive failure. *Fertil Steril.* 33:107-16.

Slowey, M.J., H.G. Verhage, and A.T. Fazleabas. 1994. Epidermal growth factor, transforming growth factor-alpha, and epidermal growth factor receptor localization in the baboon (Papio anubis) uterus during the menstrual cycle and early pregnancy. *J Soc Gynecol Investig.* 1:277-84.

Smith, J.A., R.C. Burton, M. Barg, and G.F. Mitchell. 1978. Maternal alloimmunisation in pregnancy. In vitro studies of T cell- dependent immunity to paternal alloantigens. *Transplantation.* 25:216-20.

Sol-Church, K., J. Frenck, D. Troeber, and R.W. Mason. 1999. Cathepsin P, a novel protease in mouse placenta. *Biochem J.* 343:307-309.

Somersalo, K., O. Carpén, and E. Saksela. 1994. Stimulated natural killer cells secrete factors with chemotactic activity, including NAP-1/IL-8, which supports VLA-4- and VLA-5-mediated migration of T lymphocytes. *European Journal of Immunology.* 24:2957-65.

Sopori, M.L., C.C. Gairola, A.J. DeLucia, L.R. Bryant, and S. Cherian. 1985. Immune responsiveness of monkeys exposed chronically to cigarette smoke. *Clinical Immunology and Immunopathology*. 36:338-44.

Spandorfer, S., and Z. Rosenwaks. 1999. The impact of maternal age and ovarian age on implantation efficacy. *In* Embryo implantation: molecular, cellular and clinical aspects. D.D. Carson, editor. Springer, New York. 12-9.

Speroff, L., R.H. Glass, and N.G. Kase. 1994. Clinical Gynecologic Endocrinology and Infertility. Williams & Wilkins, Baltimore. 231-50.

Springer, T.A. 1995. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annual Review of Physiology*. 57:827-72.

Staples, L.D., Heap, R.B., Wooding, F.B.P. et al. 1983. Migration of leucocytes into the uterus after removal of ovarian progesterone during early pregnancy in the sheep. *Placenta*. 4:339-350.

Starkey, P.M. 1993. The decidua and factors controlling placentation. Blackwell Scientific Publications, Oxford.

Starkey, P.M., I.L. Sargent, and C.W. Redman. 1988. Cell populations in human early pregnancy decidua: characterization and isolation of large granular lymphocytes by flow cytometry. *Immunology*. 65:129-34.

Stewart, C.L., and E.B. Cullinan. 1997. Preimplantation development of the mammalian embryo and its regulation by growth factors. *Dev Genet*. 21:91-101.

Stewart, C.L., P. Kaspar, L.J. Brunet, H. Bhatt, I. Gadi, F. Kontgen, and S.J. Abbondanzo. 1992. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature*. 359:76-9.

Stewart-Akers, A.M., J.S. Krasnow, and J.A. DeLoia. 1997. Decidual leukocyte populations in ectopic pregnancies. *Fertility and Sterility*. 68:1103-7.

Strieter, R.M., S.L. Kunkel, D.A. Arenberg, M.D. Burdick, and P.J. Polverini. 1995a. Interferon gamma-inducible protein 10 (IP-10), a member of the C-X-C

chemokine family, is an inhibitor of angiogenesis. *Biochemical and Biophysical Research Communications*. 210:51-7.

Strieter, R.M., P.J. Polverini, S.L. Kunkel, D.A. Arenberg, M.D. Burdick, J. Kasper, J. Dzuiba, J. Van Damme, A. Walz, D. Marriott, and et al. 1995b. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *Journal of Biological Chemistry*. 270:27348-57.

Surveyor, G.A., S.J. Gendler, L. Pemberton, S.K. Das, I. Chakraborty, J. Julian, R.A. Pimental, C.C. Wegner, S.K. Dey, and D.D. Carson. 1995. Expression and steroid hormonal control of Muc-1 in the mouse uterus. *Endocrinology*. 136:3639-47.

Sutton, L., M. Gadd, D.Y. Mason, and C.W. Redman. 1986. Cells bearing class II MHC antigens in the human placenta and amniochorion. *Immunology*. 58:23-9.

Suwa, S., and H. Friesen. 1969. Biosynthesis of human placental proteins and human placental lactogen (HPL) in vitro. II. Dynamic studies of normal term placentas. *Endocrinology*. 85:1037-45.

Suzuki, N., J. Nakayama, I.M. Shih, D. Aoki, S. Nozawa, and M.N. Fukuda. 1999. Expression of trophinin, tastin, and bystin by trophoblast and endometrial cells in human placenta. *Biol Reprod*. 60:621-7.

Tabibzadeh, S., Q.F. Kong, and A. Babaknia. 1994. Expression of adhesion molecules in human endometrial vasculature throughout the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism*. 79:1024-32.

Tabor, S., Struhl, K., Scharf, S.J., Gelfand, D.H. 1993. Enzymatic manipulation of DNA and RNA. *In* Current Protocols in Molecular Biology. Vol. 1. J.W.a. Sons, editor. K. Jannssen, New York. 3.5.9-3.5.10.

Tafuri, A., J. Alferink, P. Moller, G.J. Hammerling, and B. Arnold. 1995. T cell awareness of paternal alloantigens during pregnancy. *Science*. 270:630-3.

Taga, M., M. Saji, K. Suyama, and H. Minaguchi. 1996. Transforming growth factor-alpha, like epidermal growth factor, stimulates cell proliferation and inhibits prolactin secretion in the human decidual cells in vitro. *J Endocrinol Invest*. 19:659-62.

Tanaka, S., T. Kunath, A.K. Hadjantonakis, A. Nagy, and J. Rossant. 1998. Promotion of trophoblast stem cell proliferation by FGF4. *Science*. 282:2072-5.

Taub, D.D., K. Conlon, A.R. Lloyd, J.J. Oppenheim, and D.J. Kelvin. 1993. Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1 alpha and MIP-1 beta. *Science*. 260:355-8.

Taub, D.D., T.J. Sayers, C.R. Carter, and J.R. Ortaldo. 1995. Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytolysis. *J Immunol.* 155:3877-88.

Tawia, S.A., L.A. Beaton, and P.A. Rogers. 1993. Immunolocalization of the cellular adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule (PECAM), in human endometrium throughout the menstrual cycle. *Human Reproduction*. 8:175-81.

Taylor, H.S., C. Bagot, A. Kardana, D. Olive, and A. Arici. 1999. HOX gene expression is altered in the endometrium of women with endometriosis. *Hum Reprod.* 14:1328-31.

Taylor, H.S., G.B. Vanden Heuvel, and P. Igarashi. 1997. A conserved Hox axis in the mouse and human female reproductive system: late establishment and persistent adult expression of the Hoxa cluster genes. *Biol Reprod.* 57:1338-45.

Tedla, N., H.W. Wang, H.P. McNeil, N. Di Girolamo, T. Hampartzoumian, D. Wakefield, and A. Lloyd. 1998. Regulation of T lymphocyte trafficking into lymph nodes during an immune response by the chemokines macrophage inflammatory protein (MIP)-1 alpha and MIP-1 beta. *Journal of Immunology*. 161:5663-72.

Thellin, O., B. Coumans, W. Zorzi, A. Igout, and E. Heinen. 2000. Tolerance to the foeto-placental 'graft': ten ways to support a child for nine months. *Current Opinion in Immunology*. 12:731-7.

Thorburn, G.D., and J.R. Challis. 1979. Endocrine control of parturition. *Physiol Rev.* 59:863-918.

Tibbetts, T.A., F. DeMayo, S. Rich, O.M. Conneely, and B.W. O'Malley. 1999. Progesterone receptors in the thymus are required for thymic involution during pregnancy and for normal fertility. *Proceedings of the National Academy of Sciences of the United States of America.* 96:12021-6.

Tisljar, K., J. Deussing, and C. Peters. 1999. Cathepsin J, a novel murine cysteine protease of the papain family with a placenta-restricted expression. *FEBS Lett.* 459:299-304.

Toth, F.D., N. Nørskov-Lauritsen, C. Juhl, and P. Ebbesen. 1991. Human trophoblast interferon: pattern of response to priming and superinduction of purified term trophoblast and choriocarcinoma cells. *Journal of Reproductive Immunology.* 19:55-67.

Tsou, C.L., R.P. Gladue, L.A. Carroll, T. Paradis, J.G. Boyd, R.T. Nelson, K. Neote, and I.F. Charo. 1998. Identification of C-C chemokine receptor 1 (CCR1) as the monocyte hemofiltrate C-C chemokine (HCC)-1 receptor. *Journal of Experimental Medicine.* 188:603-8.

Uehara, Y., O. Minowa, C. Mori, K. Shiota, J. Kuno, T. Noda, and N. Kitamura. 1995. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature.* 373:702-5.

Uren, S., and W. Boyle. 1985. Isolation of macrophages from human placenta. *Journal of Immunological Methods.* 78:25-34.

Vallet, J.L., T.S. Gross, M.F. Fliss, and F.W. Bazer. 1989. Effects of pregnancy, oxytocin, ovine trophoblast protein-1 and their interactions on endometrial production of prostaglandin F2 alpha in vitro in perifusion chambers. *Prostaglandins.* 38:113-24.

van der Veen, F., and H. Fox. 1982. The effects of cigarette smoking on the human placenta: a light and electron microscopic study. *Placenta.* 3:243-56.

van der Velde, W.J., J.H. Copius Peereboom-Stegeman, P.E. Treffers, and J. James. 1983. Structural changes in the placenta of smoking mothers: a quantitative study. *Placenta.* 4:231-40.

van der Velde, W.J., J.H. Peereboom-Stegeman, P.E. Treffers, and J. James. 1985. Basal lamina thickening in the placentae of smoking mothers. *Placenta.* 6:329-40.

Vassiliadou, N., and J.N. Bulmer. 1998. Characterization of tubal and decidual leukocyte populations in ectopic pregnancy: evidence that endometrial granulated lymphocytes are absent from the tubal implantation site. *Fertility and Sterility.* 69:760-7.

Vega, W.A., B. Kolody, J. Hwang, and A. Noble. 1993. Prevalence and magnitude of perinatal substance exposures in California [see comments]. *N Engl J Med.* 329:850-4.

Verma, S., A. King, and Y.W. Loke. 1997. Expression of killer cell inhibitory receptors on human uterine natural killer cells. *European Journal of Immunology.* 27:979-83.

Vogiagis, D., and L.A. Salamonsen. 1999. Review: The role of leukaemia inhibitory factor in the establishment of pregnancy. *J Endocrinol.* 160:181-90.

Walsh, R.A. 1994. Effects of maternal smoking on adverse pregnancy outcomes: examination of the criteria of causation. *Hum Biol.* 66:1059-92.

Wang, J., L. Mayernik, J.F. Schultz, and D.R. Armant. 2000. Acceleration of trophoblast differentiation by heparin-binding EGF-like growth factor is dependent on the stage-specific activation of calcium influx by ErbB receptors in developing mouse blastocysts. *Development.* 127:33-44.

Wang, J.M., B. Sherry, M.J. Fivash, D.J. Kelvin, and J.J. Oppenheim. 1993. Human recombinant macrophage inflammatory protein-1 alpha and -beta and monocyte chemotactic and activating factor utilize common and unique receptors on human monocytes. *Journal of Immunology.* 150:3022-9.

Wegmann, T.G., and G.A. Carlson. 1977. Allogeneic pregnancy as immunoabsorbent. *J Immunol.* 119:1659-63.

Wegmann, T.G., H. Lin, L. Guilbert, and T.R. Mosmann. 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon ? *Immunol Today.* 14:353-6.

Werler, M.M., B.R. Pober, and L.B. Holmes. 1985. Smoking and pregnancy. *Teratology.* 32:473-81.

Westergaard, L.G., N. Wiberg, C.Y. Andersen, S.B. Laursen, A. Kliem, J.G. Westergaard, and B. Teisner. 1998. Circulating concentrations of placenta protein 14 during the natural menstrual cycle in women significantly reflect endometrial receptivity to implantation and pregnancy during successive assisted reproduction cycles. *Hum Reprod.* 13:2612-9.

Wheeler, J.M., B.M. Johnston, and L.R. Malinak. 1983. The relationship of endometriosis to spontaneous abortion. *Fertil Steril.* 39:656-60.

Wilcox, A.J., D.D. Baird, and C.R. Weinberg. 1999. Time of implantation of the conceptus and loss of pregnancy. *N Engl J Med.* 340:1796-9.

Wilcox, A.J., C.R. Weinberg, J.F. O'Connor, D.D. Baird, J.P. Schlatterer, R.E. Canfield, E.G. Armstrong, and B.C. Nisula. 1988. Incidence of early loss of pregnancy. *N Engl J Med.* 319:189-94.

Wilson, T., G.C. Liggins, G.P. Aimer, and E.J. Watkins. 1986. The effect of progesterone on the release of arachidonic acid from human endometrial cells stimulated by histamine. *Prostaglandins.* 31:343-60.

Windsor, R.A., J.B. Lowe, L.L. Perkins, D. Smith-Yoder, L. Artz, M. Crawford, K. Amburgy, and N.R. Boyd, Jr. 1993. Health education for pregnant smokers: its behavioral impact and cost benefit. *Am J Public Health.* 83:201-6.

Wistow, G.J., M.P. Shaughnessy, D.C. Lee, J. Hodin, and P.S. Zelenka. 1993. A macrophage migration inhibitory factor is expressed in the differentiating cells of the eye lens. *Proceedings of the National Academy of Sciences of the United States of America.* 90:1272-5.

Witkin, S.S., and A. Chaudhry. 1989. Association between recurrent spontaneous abortions and circulating IgG antibodies to sperm tails in women. *J Reprod Immunol.* 15:151-8.

Xu, C., D. Mao, V.M. Holers, B. Palanca, A.M. Cheng, and H. Molina. 2000. A critical role for murine complement regulator crry in fetomaternal tolerance. *Science.* 287:498-501.

Yasuda, M., K. Takakuwa, A. Tokunaga, and K. Tanaka. 1995. Prospective studies of the association between anticardiolipin antibody and outcome of pregnancy. *Obstet Gynecol.* 86:555-9.

Yelavarthi, K.K., J.L. Fishback, and J.S. Hunt. 1991. Analysis of HLA-G mRNA in human placental and extraplacental membrane cells by in situ hybridization. *Journal of Immunology.* 146:2847-54.

Yoo, H.J., D.H. Barlow, and H.J. Mardon. 1997. Temporal and spatial regulation of expression of heparin-binding epidermal growth factor-like growth factor in the human endometrium: a possible role in blastocyst implantation. *Dev Genet.* 21:102-8.

Yoshie, O., T. Imai, and H. Nomiyama. 1997. Novel lymphocyte-specific CC chemokines and their receptors. *J Leukoc Biol.* 62:634-44.

Yoshimura, T., K. Matsushima, S. Tanaka, E.A. Robinson, E. Appella, J.J. Oppenheim, and E.J. Leonard. 1987. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proceedings of the National Academy of Sciences of the United States of America.* 84:9233-7.

Yu, Z., B.A. Croy, C. Chapeau, and G.J. King. 1993. Elevated endometrial natural killer cell activity during early porcine pregnancy is conceptus-mediated. *Journal of Reproductive Immunology.* 24:153-64.

Yu, Z., B.A. Croy, and G.J. King. 1994. Lysis of porcine trophoblast cells by endometrial natural killer-like effector cells in vitro does not require interleukin-2. *Biology of Reproduction.* 51:1279-84.

Zaccheo, D., V. Pistoia, M. Castellucci, and C. Martinoli. 1989. Isolation and characterization of Hofbauer cells from human placental villi. *Archives of Gynecology and Obstetrics.* 246:189-200.

Zhou, M., and A.L. Mellor. 1998. Expanded cohorts of maternal CD8+ T-cells specific for paternal MHC class I accumulate during pregnancy. *J Reprod Immunol.* 40:47-62.

Zhou, Y., C.H. Damsky, K. Chiu, J.M. Roberts, and S.J. Fisher. 1993. Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts. *J Clin Invest.* 91:950-60.

Zhou, Y., C.H. Damsky, and S.J. Fisher. 1997. Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome ? *J Clin Invest.* 99:2152-64.

Zhu, L.J., K. Cullinan-Bove, M. Polihronis, M.K. Bagchi, and I.C. Bagchi. 1998. Calcitonin is a progesterone-regulated marker that forecasts the receptive state of endometrium during implantation. *Endocrinology.* 139:3923-34.

Zinaman, M.J., E.D. Clegg, C.C. Brown, J. O'Connor, and S.G. Selevan. 1996. Estimates of human fertility and pregnancy loss. *Fertil Steril.* 65:503-9.

Zlotnik, A., J. Morales, and J.A. Hedrick. 1999. Recent advances in chemokines and chemokine receptors. *Critical Reviews in Immunology.* 19:1-47.