Decidual Leukocyte Involvement in Human Spiral Artery Remodeling

By

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Abstract

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The decidualized endometrium harbors abundant leukocyte populations that are proposed to regulate critical processes at the maternal fetal interface including transformation of decidual spiral arteries. The work in this thesis investigated the leukocyte subtypes in the decidua throughout the course of this vascular transformation. A particular focus was the role of the uterine Natural Killer (uNK) cells and macrophages in an in vitro model of vascular remodeling. A significant infiltration of uNK cells and macrophages, matrix metalloproteinase-2/9 activity, and evidence of apoptosis and phagocytosis were observed in remodeling arterioles. From first to second trimester, FACS analysis demonstrated dramatic changes in the decidual leukocyte subpopulations, including the decline of uNK cells and macrophages and substantial increase in T lymphocytes and neutrophils. These data demonstrate an integral role of uNK cells and macrophages in early vascular remodeling and provide evidence of unique and complex immune interactions in the decidual microenvironment during human pregnancy.
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List of Abbreviations

Ang – angiopoetin
ANOVA – analysis of variance
APC – antigen-presenting cell
APC’ – allophycocyanin
CCL – CC chemokine ligand
CD – cluster of differentiation
CLA – common leukocyte antigen
CTL – cytotoxic T lymphocyte
CXCL – CXC chemokine ligand
DAPI – 4’, 6-diamidino-2-phenylindole
DCM – decidual conditioned media
DC-SIGN – dendritic cell-specific ICAM-3 grabbing non-integrin
DMSO – dimethyl sulfoxide
ECM – extracellular matrix
EGF – epidermal growth factor
en/inEVT – endovascular/interstitial extravillous trophoblast
FasL – Fas Ligand
FITC – fluorescein isothiocyanate
FMO – fluorescence-minus-one
FSC – forward scatter
GM-CSF – granulocyte macrophage colony-stimulating factor
GnRH – gonadotropin releasing hormone
hCG – human Chorionic Gonadotropin
HLA – human leukocyte antigen
hPL – human Placental Lactogen
ICAM – intercellular adhesion molecule
IFN-γ – interferon-γ
IHC – immunohistochemistry
IL – interleukin
IUGR – intra-uterine growth restriction
KIR – killer immunoglobulin-like receptors
LIF – leukemia inhibitory factor
LM – lysozyme muramidase
Mac-1 – macrophage receptor 1
M-CSF – macrophage colony stimulating factor
MFI – mean fluorescence intensity
MHC – major histocompatibility complex
MMP – matrix metalloproteinase
MMR – macrophage mannose receptor
PBBx – placental bed biopsies
PBS – phosphate buffered saline
PCM – placental conditioned media
PDC – placental decidual co-culture
PE – phycoerythrin
PerCP – peridinin chlorophyll protein

PIGF – placental growth factor

SEM – standard error of the mean

SMA – smooth muscle actin

SSC – side scatter

STIPS – Second Trimester Interruption of Pregnancy clinic

TCR – T cell receptor

TGF-β – transforming growth factor-β

TIMP – tissue inhibitor of metalloproteinase

TNF-α – tumor necrosis factor-α

TRAIL – TNF-related apoptosis-inducing ligand

Treg – regulatory T lymphocytes

TUNEL - terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling

uNK – uterine Natural Killer

VEGF – vascular endothelial growth factor

VSMC – vascular smooth muscle cells
Chapter 1

Introduction
1.1 The Utero-Placental Interface

Post implantation, the human hemochorial placenta establishes a connection from the fetus to the maternal uterus in order to ensure that adequate maternal blood perfuses the placenta and nourishes the growing fetus. The placenta sequesters $O_2$, nutrients and hormones from maternal blood and excretes $CO_2$ and wastes for excretion by the mature maternal renal system. This interface between the fetal and maternal systems is highly complex with cooperative and protective roles performed by both to ensure a successful pregnancy with a healthy mother and baby. Many changes take place in the maternal system in order to accommodate and support, yet protect against the demands of the developing fetus. For example, the mother increases her blood volume 1.5 times, her cardiac output increases and her systemic vasculature is remodeled. The most dramatic changes occur in the uterine vasculature where the myometrial and decidual spiral arteries are transformed by invading trophoblast to become large sinusoids that supply high flow, low pressure maternal blood to the intervillous space and placenta. Maternal uterine blood flow increases from 40mL/min in the non-pregnant uterus to 450-700mL/min by term (2). Much research in recent years has focused on the cellular mechanisms that mediate these uterine vascular changes because failure of spiral artery transformation is thought to contribute to the pathological pregnancy disorders, specifically preeclampsia and intra-uterine growth restriction (IUGR).

Additionally, how the mother’s immune system is able to tolerate a genetically dissimilar fetus, the “immunological paradox” of pregnancy, remains to be fully understood [reviewed in (3)]. Several maternal adaptations have been suggested to contribute to this tolerance. It is known that 40% of decidual cells are leukocytes which are specialized and supportive of pregnancy. Increasing evidence suggests that these leukocytes play integral roles in both normal and aberrant maternal adaptations to pregnancy. In the following sections, this thesis will provide possible explanations regarding how the various immune cells may influence the utero-placental interface. Particular focus is given to the role of decidual
leukocytes that are abundant during the critical period of spiral artery remodeling in the first and second trimesters of pregnancy.

1.1.1 Decidualization

Beginning as early as day 23 of the menstrual cycle, in the late secretory phase, the endometrium (uterine lining) displays early signs of hormonally-induced changes in the stromal cells, as well as a large infiltration of pregnancy-specific uterine Natural Killer (uNK) cells (4). If fertilization does not occur, progesterone levels fall and inflammatory cells (macrophages, neutrophils and mast cells) infiltrate, facilitating endometrial breakdown (5) and resulting in shedding of the functionalis layer of the endometrium (menses).

If pregnancy does occur, then decidualization is maintained and decidual stromal cells undergo hypertrophy and are characterized by increased cytoplasmic organelles and branched processes (4). Evidence of decidualization is seen first near the spiral arteries and progresses into the surrounding tissue (4). Decidualized stromal cells produce a variety of hormones, angiogenic growth factors, cytokines, extracellular matrix substances and nutrients (6) distinct from those produced by endometrial stromal cells. This process of cellular differentiation is primarily regulated by progesterone (4), as demonstrated by the ability of progestin-implants in mice (7) and progestin-only contraceptives in women (8) to induce and maintain a decidualized endometrial phenotype. In addition to high progesterone levels, human Chorionic Gonadotropin (hCG) and human Placental Lactogen (hPL) produced by the developing placenta also maintain decidualization (9, 10). As well, the increase in uNK cells observed in the secretory phase of the menstrual cycle becomes more pronounced with immune cells comprising 40% of all decidual cells in early pregnancy. This interesting characteristic of the decidua will be elaborated upon in section 1.2.
1.1.2 Implantation and Placental Invasion

The ovum released from the ovary is fertilized in the fallopian tube and enters the uterine cavity where it undergoes a series of cell divisions forming 2-, 4- and 8-cell masses before forming a morula [Figure 1.1 A: modified from (11)]. The inner cells of the morula differentiate to form the inner cell mass of the blastocyst and the outer cells form the trophectoderm, which will later develop into the placenta (Figure 1.1 B). The hormonally primed late secretory endometrium is receptive to implantation of the blastocyst. Complex communications between the maternal cells and the blastocyst regulate successful implantation via secretion of cytokines by the uterine epithelium including epidermal growth factor (EGF) (12) and leukemia inhibitory factor (LIF) (13), and expression of the EGF receptor by the trophectoderm (14). Once the blastocyst forms its initial attachment to the uterine epithelium, the trophectoderm layer differentiates into syncytiurn and cytotrophoblast layers (Figure 1.1 D). The decidual lining at the site of implantation, the decidua basalis, experiences the greatest extent of trophoblast invasion and decidualization (Figure 1.1 E, F). The decidua further from the implantation site, the decidua parietalis, does not experience the same degree of change in response to the developing placenta (Figure 1.1 E, F). The decidua capsularis is the layer that forms around the amniotic cavity and the chorion (Figure 1.1 E, F).
Figure 1.3 Trophoblast differentiation. The early chorionic placental villi grow in size, number, and complexity forming a highly branched tree-like structure by the second trimester. Most of this structure is exposed to the intervillous space and does not contact the decidua (floating villi), while some parts attach the placenta to the decidua (anchoring villi). While the cytotrophoblast and syncytiotrophoblast of the floating villi form a physical barrier between fetal blood vessels within the villi and the maternal blood in the intervillous space. The complex branching of the placenta ensures a huge surface area for efficient gas and nutrient exchange between maternal and fetal blood (Figure 1.2).

As the placenta grows, the villous trophoblast in the anchoring columns proliferate and differentiate into invasive extravillous trophoblast (EVT) that invade into the decidual stroma as interstitial EVT (inEVT) and the maternal vasculature as endovascular EVT (enEVT; Figure 1.3). The invasion of EVT has been implicated in maternal tolerance of the hemi-allogeneic fetus and of particular focus in this thesis, transformation of decidual arteries to ensure increased blood flow to the placenta.

Healthy pregnancy results from a balance between under and over invasion of trophoblast into the decidua. Under invasion of EVT means that the myometrial arteries remain intact and unremodeled,
thus compromising uterine maternal blood flow [as seen in pathologies such as preeclampsia and IUGR (15)]. In contrast increased invasion of trophoblast into the deep myometrium results in placental accreta or even percreta where the trophoblast penetrate the uterine wall (4).

Several factors aid trophoblast in their invasion. Firstly, the invasive EVT express matrix metalloproteinases (MMPs) capable of degrading extracellular matrix (ECM) and facilitate their own movement between cells and into the decidua (16). As well, EVT downregulate expression of their major histocompatibility (MHC) Class I molecules (human leukocyte antigen (HLA)-C, -E, & -G) and do not express either the MHC Class I molecules HLA-A or –B or MHC Class II molecules and are therefore less likely to initiate a maternal immune response (17). This tolerance may also be partially mediated by the interaction of the killer immunoglobulin-like receptors (KIR) and CD94/NKG2 receptors on uNK cells and trophoblastic MHC molecules, preventing NK cell-mediated lysis of the trophoblast (17, 18). Interestingly, trophoblast isolated from first trimester placenta have been shown to recruit uNK cells by production of the CXCL12 chemokine (19, 20), which may help to promote this tolerant interaction.

Similarly, there are several factors that inhibit the invasiveness of EVT through their action on the activity or secretion of MMPs. Tissue inhibitors of metalloproteinases (TIMPs) decrease MMP activity and are expressed by decidual cells as well as by the invasive trophoblast themselves (16). Additionally, uNK cells secrete transforming growth factor (TGF)-β, which decreases MMP secretion and induces production of TIMPs (21). The balance between pro- and anti-invasive factors are thought to play a role in the terminal differentiation of the EVT at the decidual myometrial junction, where they become embedded in a fibrin matrix and take on a non-invasive polygonal phenotype as pregnancy progresses.
1.1.3 Decidual Spiral Artery Remodeling

Coincident with trophoblast invasion, the muscular, tightly coiled decidual spiral arteries undergo extensive changes including loss of their medial vascular smooth muscle cells (VSMC) and most of their intimal endothelial monolayer. The result is dilated and denervated vessels that are no longer under vasomotor control and allow increased uterine blood flow under systemic arterial pressure to perfuse the intervillous space (4). This maternal adaptation ensures adequate fetal nutrition and decreased velocity of blood flow; changes critical to the health of the placental tissues and fetal development.

Throughout the process of decidualization, the spiral arteries grow in size and acquire a more coiled morphology. In the non-pregnant endometrium these vessels do not open to the uterine cavity at the epithelial surface (Figure 1.4). As the placenta establishes itself during pregnancy, the terminal ends of the spiral arteries eventually open up to the intervillous space around day 40 post coitum (p.c.) (4). At the decidual-myometrial junction, the spiral arteries branch from the uterine radial arteries, measuring between 200 and 300 µm in diameter (22) (Figure 1.4). As they become arterioles and capillaries closer to the decidual epithelial surface (Figure 1.4), their diameter is reduced and they possess far
fewer smooth muscle layers. The vessel density in the decidua basalis is reduced to allow increased vascular and luminal surface area as these vessels dilate and become larger throughout remodeling (23). As well, studies from pregnant hysterectomies have shown that at the centre of the placental attachment EVT invasion is deepest (24), extending 2-3 mm into the myometrium (25) and targets the greatest number of spiral arteries (26). These vessels dilate to several times their original diameter (4, 27) and display reduced coiling (28) to accommodate the increased blood volume (Figure 1.4).

As decidualization occurs and invasive interstitial EVT penetrate the decidual stroma, these trophoblast accumulate around the arterioles (26) and are thought to prime the arterioles for remodeling by endovascular EVT as both smooth muscle cell loss and fibrinoid deposition are seen before endovascular EVT presence in the lumen and wall of the vessels (29). Eventually, the endothelium is replaced by endovascular EVT, which acquire an endothelial-like phenotype as they reline the arterioles. There are two theories of how these endovascular EVT populate the vascular lumen. The first theory suggests that invasive interstitial EVT that cluster around the arterioles intravasate from the interstitium to the vessel lumen (30). The second theory proposes that the EVT migrate intravascularly potentially from endovascular trophoblast plugs, exert their effects from the arteriole lumen relining the vessels and extravasating into the vessel wall (reviewed in (31)). Angiopoetin (Ang)-2 is also highly expressed by trophoblast (32, 33), which, depending upon the surrounding cytokine environment, promotes inflammation and/or angiogenesis by destabilizing mature vessels (34). The tyrosine kinase receptor for Ang-2, Tie-2, is expressed on endothelial cells (34), providing a plausible mechanism by which trophoblast may further remodel the spiral arteriole wall, increasing lumen dilation, new vessel growth, localized inflammation and leukocyte recruitment. However, Craven et al (35) have shown that decidual spiral artery remodeling begins in the absence of cellular interaction with EVT, in the endometrium of ectopic pregnancies and decidua parietalis, as a response of the maternal system to pregnancy. This finding suggests that another cell type may play an initiating role in remodeling and mediate the early vascular changes.
Ultimately, spiral arteries are remodeled into dilated reservoirs and lose the majority of their VSMC and endothelial cells. The mechanisms by which these changes may occur have been investigated in animal studies and models using isolated spiral and myometrial arteries. A model of trophoblast invasion in the mammary fat pad of severe combined immunodeficient mice demonstrated that trophoblast interaction with vessel walls resulted in apoptosis of the endothelial and smooth muscle cells (36). In *in vitro* studies, trophoblast invasion of isolated myometrial arteries (37) and trophoblast incorporation into isolated spiral artery walls (38) resulted in VSMC and endothelial cell apoptosis, respectively. Additionally, isolated primary trophoblast cells have been shown to secrete cytokines from the tumor necrosis factor (TNF) family, including Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL), and can induce apoptosis of aortic VSMC over a 24-hour period (39). These authors concede that although this mechanism accounts for trophoblast contribution to VSMC death, other decidual cells are likely to contribute as well to the apoptosis and loss of vascular cells. TRAIL-producing lymphocytes have also been shown to mediate VSMC apoptosis from isolated atherosclerotic plaques (40), and thus, it is possible that leukocytes play a role in inducing apoptosis in the decidual arteries as well.

Apoptotic VSMC and endothelial cells have been reported in remodeling arterioles from *in vivo* decidua basalis specimens and studies of isolated spiral and myometrial arteries cultured with trophoblast (38, 41, 42). Additionally some of these studies have also demonstrated that uNK closely associated with the decidual arteries produce MMP-9 (41, 43). Leukocytes are integral to several tissue remodeling processes including inflammation, wound healing and cancer progression through their secretion of proteases, including MMPs, which induce local destruction of ECM. Trophoblast and decidual leukocyte-derived MMPs may contribute to remodeling of perivascular ECM and cell migration, thus facilitating angiogenesis and the necessary restructuring of ECM surrounding spiral arteries. Dissolution of vascular ECM is thought to alter mechanotransduction signaling of VSMC and endothelial cells, resulting in apoptosis (anoikis) and/or dedifferentiation (44, 45). Therefore, secretion of MMPs by trophoblast and leukocytes may be critical to spiral
artery remodeling analogous to their roles in tumor angiogenesis (46), metastasis (47), and wound repair, by mediating tissue synthesis and destruction (48).

In the decidua, chemokines that attract macrophages and uNK cells are relatively abundant (36, 49) emphasizing the importance of these cells. The VSMC and endothelial cells of spiral arteries express chemokines that attract leukocytes (49, 50) and more recent work indicates that these chemokines produced by the vessels themselves are recruiting uNK cells and macrophages to the vascular wall (51). Another important function of MMPs is their ability to convert chemokines into their active forms (52) suggesting that MMPs may also contribute to leukocyte recruitment to the spiral arteries. As well, the decidual macrophages and uNK cells produce angiogenic factors such as vascular endothelial growth factor (VEGF), placental growth factor (PIGF), and Ang-2 (53, 54) suggesting that these cells in particular may be mediators of spiral artery remodeling. This and other specific functions of leukocyte subtypes within the decidual environment will be investigated in the following sections.

1.2 The Role of Maternal Leukocytes in Pregnancy

One of the most perplexing aspects of pregnancy is how the hemi-allogeneic fetus manages to evade its mother’s immune system. As the fetal trophoblast are the only cells that directly contact maternal cells, many theories have revolved around the expression of self-recognition molecules. The absence of classical HLA-A and HLA-B and MHC Class II molecules on trophoblast as well as their relatively low expression of non-classical HLA-C, -E, and -G may provide a partial explanation, but another important consideration is the adaptation of the maternal leukocytes themselves over the course of gestation. It has been proposed that an inflammatory environment is necessary for the attachment and invasion of the blastocyst (55) and that cells of the innate immune system, macrophages in particular (56), are educated by HLA-G expressed by trophoblast and contribute to development of immune tolerance (57). Despite tolerance of the fetus however, the
maternal immune system remains capable of responding to a host infection and trophoblast exposure to bacterial or viral antigens can actively recruit maternal immune cells utilizing toll-like receptors, all 10 of which are expressed by placenta (55, 58). It is also possible that the unique composition of leukocytes at the maternal-fetal interface plays a role in immunosuppression as the decidua lacks antibody producing B lymphocytes and memory T lymphocytes of the adaptive immune system (59), and under the influence of progesterone maintains a predominantly anti-inflammatory cytokine environment (60, 61). Presumably, the systemic inflammatory environment is also altered in pregnancy, as evidenced by the suppression or exacerbation of autoimmune disorders related to deficiency or excess of Th2 cytokines, respectively (62). Clearly, the distribution and relationship of maternal immune cells with the developing fetus and the role of each cell type in the progression of pregnancy is highly complex and dependent upon their activation state and environment dictated phenotype. The following sections will discuss the leukocyte types found in the decidua, describe their classical roles in both physiological and pathological states, and present and expand upon their proposed roles in pregnancy.

1.2.1 Uterine Natural Killer Cells: A Unique Uterine Leukocyte

The most dominant leukocyte subtype in the decidua in early pregnancy is the unique uNK cell, constituting between 50 and 70% of all decidual immune cells (63, 64). These cells appear to be under hormonal control as they infiltrate during the menstrual cycle coincident with increased progesterone, remain while progesterone levels are high (Figure 1.5: 1) and are not observed either before menarche or post-menopause (59). Uterine NK cell phenotype (CD56$^{\text{bright}}$, CD16$, CD3^{-}$) is distinct from that of their peripheral blood NK cell (PBNK: CD56$^{\text{dim}}$, CD16$^{+}$) counterparts (17). These cells exhibit significantly reduced cytotoxicity compared to PBNK cells (65, 66) and several differences in their gene expression profiles including selective expression of immunomodulatory proteins by uNK cells (67). These characteristics, taken together with their progressive decline after midgestation suggest a supportive and important role for uNK cells in early pregnancy.
Figure 1.5 uNK cell interactions in the decidua. 1: Increased levels of progesterone (P4) in the late secretory phase and maintained in pregnancy promote infiltration and proliferation of uNK cells. 2: Both decidual stromal cells and vascular cells produce chemokines capable of recruiting uNK cells. 3: Uterine NK cells produce angiogenic factors suggesting a role in decidual arteriole remodeling and angiogenesis. 4: Interaction of HLA-C ligands on extravillous trophoblast (EVT) and killer immunoglobulin receptors (KIR) on uNK cells has the ability to both promote and inhibit EVT invasion. 5: Both interaction of EVT HLA ligands with CD94/NKG2 inhibitory receptors on uNK cells and DC, DC-SIGN with ICAM-3 on uNK cells result in production of cytokines that prevent maturation of DC cells. 6: The interaction of DC-SIGN and ICAM-3 may also serve to both occupy DC receptors and spatially distance DC cells from T cells, thus preventing DC-T binding and antigen presentation.

An essential role for uNK cells in the murine implantation site has been identified through several studies of mice deficient in uNK cells, uNK cell-derived interferon (IFN)-γ, or IFN-γ signaling. These mice experience defects in implantation and impaired decidual artery remodeling (68-71).

As uNK cells accumulate post-ovulation and in early pregnancy, they become larger and more granular than either their pre-ovulatory precursors or PBNK cells (59). In fact, it is unclear whether the uNK cells in the pregnant uterus originate primarily by proliferation of local uterine progenitor cells or recruitment from NK cells in the peripheral circulation. It has been demonstrated that stromal TGF-β can differentiate CD56<sup>dim</sup>CD16<sup>+</sup> cells into CD56<sup>bright</sup>CD16<sup>-</sup> (72), which suggests that PBNK cells are capable of differentiating into uNK cells after recruitment to the decidua. Furthermore, vascular and decidual cells
express several chemokines, including CX3CL-1 (fractalkine), CCL-21 (6Ckine), CXCL10 (MDC), CXCL11, CXCL12 (20, 49, 73-75) with activity towards uNK cells (Figure 1.5: 2), however, the receptors for these proteins, CXCR3 and CXCR4, are not expressed on all uNK cells (19), suggesting that two distinct cell populations may exist only some of which are recruited. In contrast, expression of nuclear proliferation antigen by uNK cells supports the theory that in situ proliferation occurs as well (76). Interestingly, CXCR3 and CXCR4 are also expressed by trophoblast suggesting that vascular and decidual cells may also have chemotactic effects on trophoblast invasion.

1.2.1.1 UNK Cells and Trophoblast Invasion
Regardless of their origin, the uNK cells that accumulate in the decidua secrete CCL8, CXCL10 and CCL5, which promote EVT invasion by interaction with their CXCR1 and CXCR3 receptors (53) suggesting that bi-directional communication occurs between uNK and trophoblast at the utero-placental interface. In addition to the prevention of cytolysis of trophoblast, the interactions of uNK KIR and CD94/NKG2 receptors and HLA ligands of the invading interstitial EVT are believed to dictate depth of invasion (Figure 1.5: 4) and trophoblast contribution to spiral artery remodeling (18). As well, expression of both inhibitory and activating KIRs specific for HLA-C on EVT declines over the first trimester and is influenced by the decidual microenvironment indicating that this interaction is critical early in pregnancy (77).

1.2.1.2 UNK cells and Vascular Remodeling
There has been no conclusive evidence that uNK cells are directly involved in human spiral artery remodeling although they are known to be critical in development of the murine spiral arteries (78). Recently, studies have reported an intimate relationship between uNK cells and remodeling arteries in first trimester decidua basalis biopsies. These cells were observed in close proximity to early and mid stage remodeling arterial walls, in the absence of endovascular EVT, proposing a role for uNK cells in mediating the early remodeling events in human decidua (41). This clustering, combined with their production of MMP-9 (41, 43) and angiogenic factors such as VEGF, PIGF and Ang-2 (79-81) provide strong
support for an integral role of uNK cells in angiogenic development of the spiral arteries (Figure 1.5: 3). In fact, a positive correlation was reported between micro vessel density and both the number of uNK cells and VEGF levels in the first trimester decidua (82).

1.2.1.3 UNK Cells and Immune Tolerance
Tolerance of the hemi-allogeneic fetus is critical to progression of pregnancy and while uNK cells are not potent activators of systemic immune responses, they demonstrate reduced ability to lyse cells (15% of PBNKs) and mobilize their perforin-containing granules to cellular synapses (65). Additionally, in vitro experiments exposing uNK cells to HLA-G/E, which are expressed by EVT, resulted in a shift of cytokine production from Th1-type to Th2-type (83) suggesting a potential mechanism by which uNK cells may induce a tolerant decidual environment (Figure 1.5: 5,6).

Therefore, the complex cellular communication between uNK and trophoblast is proposed to dictate depth of invasion, mediate remodeling of the maternal vasculature and contribute to suppression of an immune response against the fetal trophoblast.

1.2.2 Lymphocyte Populations in the Decidua
The immune system consists of two branches, the innate and the adaptive. The innate system consists of antigen-presenting cells (APC) such as macrophages and dendritic cells, NK cells and neutrophils that are programmed to respond to standard pathogen surface molecules and immediately initiate a localized, cytolytic response. The adaptive system educates the peripheral lymph nodes, creating antibodies and memory cells, often in response to signals from the innate reaction. In particular, the APCs of the innate system often process antigens from the foreign cell, and present small peptide sequences using their MHC self-recognition molecules to elicit a response by T lymphocytes. Recent research has identified several different types of T cells, some of which have a role in the non-specific innate system, but most of which make up the complex cascade of events in response to a specific antigen (84).
The two dominant classical CD3⁺ T lymphocytes are the helper T cell (CD4⁺) and the cytotoxic T cell (CD8⁺). As their names imply, the former has a productive and regulatory role, while the latter is primarily involved in killing effector cells. One of the most important roles of the T lymphocytes is producing a variety of cytokines that may either progress or dampen an immune response. Cytokine microenvironments play an integral role in dictating both phenotype and cellular response of local immune cells. While many cell types in each tissue can produce cytokines, the major types of cytokines are named for their potent producer, the helper T cell. CD4 helper T cells interact with MHC Class II molecules and their subsequent cytokine production dictates the type of immune response that will occur (cell-mediated vs humoral, inflammatory vs anti-inflammatory, tolerant vs cytotoxic). These cytokines also direct the interaction of cytotoxic T lymphocytes with MHC Class I complexes and determine whether these cells will be sensitized towards target cells (84).

The two opposing groups of helper T cell cytokines are Th1 and Th2. Th1 cytokines tend to be pro-inflammatory often initiating cytotoxic T cells and include interleukin (IL)-2, IFN-γ, lymphotoxin and TNF-α. Th2 cytokines tend to be anti-inflammatory or tolerant and include IL-4, -5, -6, -10, and -13. Th2 cytokines initiate antibody production by B lymphocytes in typical immune environments.

Studies of the decidua in early pregnancy have determined that CD3⁺ T cells account for approximately 10% of decidual leukocytes, but can range from 5 to 25% in individual patients, while the antibody-producing B lymphocytes are virtually absent (59). Normal pregnancy involves a shift in the Th1/Th2 cytokine ratio from a Th1 dominant environment during implantation, to a Th2 dominant cytokine environment as pregnancy progresses [reviewed in (85)], peaking in the second trimester, and a Th1 cytokine environment again at term (55). There are some inflammatory cytokines produced during normal pregnancy (13, 86) suggesting that they are necessary for implantation, however cytokines such as IFN-γ are only detectable in early pregnancy in mice, while Th2 cytokines are present at the
maternal fetal interface throughout gestation (87). This indicates a more critical role for these Th2 cytokines, analogous perhaps to IL-4 and IL-10 promotion of allograft tolerance in organ transplantation when, in contrast, the Th1 cytokines IL-2 and IFN-γ promote allograft rejection (88). In line with this reasoning, Th1 cytokines have been suggested to inhibit trophoblast invasion while Th2 cytokines may help to promote trophoblast invasion and can support differentiation of endometrial NK cells (85). Isolated primary trophoblast cells have been shown to induce migration of T cells by production of CXCL16 (89) and are able to directly attach to decidual T cells (90). As well, T cells express the only receptor for CXCL16, CXCR6, and thus support a direct, local communication between T cells and EVT (Figure 1.6: 6). Additionally, evidence exists to suggest that progesterone both inhibits production of Th1 cytokines and stimulates the production of Th2 cytokines including LIF, IL-4, IL-10 and macrophage colony stimulating factor (M-CSF) by decidual T cells (Figure 1.6: 1) and these cytokines contribute to the maintenance of healthy pregnancy (91). Regulation of T lymphocytes and their local interactions with other cells are summarized in Figure 1.6.

Interestingly, macrophages and dendritic cells are found in close association with T cells in the decidua (91), which in a typical inflammatory environment would stimulate a classical immune response. However, several factors prevent this from occurring in healthy pregnancy. First, the anti-inflammatory cytokine environment seems to act primarily on the innate immune cells, such as the antigen-presenting cells mentioned, to facilitate cooperation and potentially control the maternal immune reaction to the hemi-allogeneic fetus (92). Second, there is evidence of apoptosis of lymphocytes (93) which may be mediated by FasL expressed on trophoblast (94) thus providing a mechanism of trophoblast-regulated inhibition of T cell activation (Figure 1.6: 5). Finally, compared to peripheral blood, the decidua harbors fewer T cells, which have relatively low T cell receptor expression and therefore, may be less likely to interact with MHC complexes presenting fetal antigens (95-97).
Figure 1.6 T lymphocyte interactions in the decidua. 1: Progesterone shifts T helper cell cytokine production, stimulating Th2 cytokine production and decreasing Th1 cytokines. 2: Immature DC (not expressing CD83) can present antigen to T cells, but instead of eliciting an activated T cell response induce Ag-specific tolerance. 3: The increased Th2 cytokine production (specifically by macrophage) and decreased Th1 cytokines (specifically by mature DC) inhibit classical activation of T cells and promote the differentiation of naïve T cells to a regulatory/suppressive phenotype. 4: EVT inhibit NKT cell cytotoxicity by interaction of their HLA-E ligands with the NKG2 receptors on the NKT cells. 5: Reciprocal apoptosis has been demonstrated through a Fas-FasL mediated interaction between EVT and T lymphocytes. 6: EVT produce chemokine CXCL16 that induces T cell migration towards EVT by specific interaction with its receptor, CCR6.

Even when antigen presentation to T cells is successful, there are adaptations observed that may help to prevent successful activation of an immune response during pregnancy, by production of atypical, inert antibodies. In situations where Th2 responses typically occur, such as infection by microorganisms, pregnancy, and allergies, there is increased production of asymmetric antibodies, which are unable to effectively initiate a typical immune response and result in reduced clearance of antigens and phagocytosis (98). An increase in proportion of asymmetric antibodies in peripheral circulation has been reported in women during the first trimester of pregnancy and circulating levels returned to normal by 20-30 days post partum. In addition, asymmetric antibodies accounted for up to 70% of all the antibodies observed in the placental bed at term with anti-paternal antibodies occurring in a 5:1 asymmetric to symmetric ratio (98). These observations suggest a
complicated, selective method of immunosuppression and may help to explain both why establishment of a Th2 anti-inflammatory cytokine environment occurs and how it contributes to induced tolerance of fetal antigens.

Another unique aspect of the decidua is its enrichment of regulatory and atypical lymphocytes compared to peripheral blood and the pre-ovulatory endometrium. These T cell subsets include CD4⁺CD25⁺ regulatory T cells (99), γδ T cells (100), and Natural Killer T lymphocytes (NKT cells) (101). For the most part, the cells present are those usually abundant at mucosal sites, likely contributing to defense against pathogens and tolerance of the foreign fetal trophoblast.

The regulatory T (Treg) cells are a subset of CD4⁺ cells that are mainly involved in tolerance, suppression and other regulatory actions rather than in immunity (102) and express the IL-2 receptor (CD25) (103). These cells are highly specific to the tissue they are in (104) and act to prevent an immune response either at its outset or while it is taking place (105). Tregs can be activated by immature dendritic cells (104) and are involved in self-tolerance, preventing autoimmunity (105). Studies have reported an increase in Tregs in peripheral blood in the first and second trimester of pregnancy (106). An even greater increase is observed in the decidua accounting for about 14% of CD4⁺ T cells in the human and up to 30% of all T cells in mice (99, 106, 107). Peripheral CD4⁺CD25⁺ regulatory T cells have been shown to inhibit production of IL-2 by T cells thus preventing their activation (108). It has also been proposed that the tolerogenic CD4⁺CD25bright Tregs in the decidua downregulate a local immune response (109). Depletion of Tregs in mice results in failure of pregnancy (107), demonstrating that these cells are critical and may act to inhibit an immune response against the semi-allogeneic conceptus.

The γδ T cells are an example of cells that are typically found at mucosal sites rather than in peripheral blood and are found in greater proportions in the uterine decidua (110). These cells can recognize antigen without MHC complexes or CD4/8 receptors [reviewed in (105)]. They do express NKG2 receptors, and while a few act through the activating
receptors, the decidual $\gamma\delta$ T cells primarily act through the inhibitory receptors and have a suppressive role. In the first trimester, human decidual $\gamma\delta$ T cells demonstrate high mRNA expression of the Th2 cytokines IL-10, TGF-$\beta$1, IL-6 and IL-1$\beta$ (111), suggesting a suppressive role in maternal tolerance of the fetus. In fact, these cytokines have been implicated in differentiation of $\alpha\beta$ T cells towards a more regulatory/suppressive phenotype. Dysregulation of cytokine production by lymphocytes, including decreased IL-10 and increased IL-2, are associated with pregnancy pathologies including preeclampsia (112).

CD56$^+$CD$8^{\text{dim}}$ NKT cells have similar characteristics to both NK cells and T lymphocytes (89). They are found in small numbers in the decidua (101) and can express either $\alpha\beta$ or $\gamma\delta$ T cell receptors (113). NKT cells have been identified in peripheral blood, bone marrow and liver and are immunomodulatory in infection, cancer and transplantation through their production of cytokines (114). Two major types of NKT cells are described in the decidua, invariant and non-invariant. Invariant NKT cells express both activating and inhibitory NKG2 receptors, which have been implicated in tumor pathogenesis and systemic inflammation. These NK cell receptors interact with HLA-E expressed by EVT to inhibit NKT cell cytotoxic capacity (115) (Figure 1.6: 4). Similar to Tregs, invariant NKT cells have been shown to interact with dendritic cells to influence the Th1/Th2 balance and dampen the immune response (116). The non-invariant NKT cells of the decidua have been shown to produce factors that promote a Th2 cytokine environment and support trophoblast growth and invasion (117). Therefore, this unique cell type may serve as yet another potential mediator of maternal tolerance and trophoblast invasion.

1.2.3 Neutrophils and Human Reproductive Tissues

Neutrophils are short-lived cells of the innate immune system that have potent antibacterial capability. They do not reside in tissues, but infiltrate during times of wound healing and infection, recruited by tissue macrophages upon encountering a pathogen (118). Recently, neutrophils with more tolerogenic and angiogenic properties have been reported to participate in tumor progression (119). These functions are similar to described roles for
neutrophils in the uterus. While not detected in first trimester decidua or myometrium (64), neutrophils are observed throughout the menstrual cycle (5). During menstruation there is a large infiltration of innate immune cells including macrophages, activated mast cells, and neutrophils, which are credited with influencing endometrial breakdown, likely through production of MMPs (5). As well, neutrophils have been implicated in the process of pharmacologic and spontaneous abortion perhaps through a mechanism similar to shedding of the endometrium (120). In contrast, neutrophils later in the cycle are thought to promote vascular repair in the endometrium and vasculogenesis. During this period they are found within vessel lumens adherent to the endothelium and in close contact with proliferating endothelial cells (121, 122). These cells are thought to contribute to endometrial repair because of their potent production of angiogenic factors such as VEGF (123, 124). Since neutrophils are so important in endometrial building and breakdown, it is possible they have a role in later gestation or in pathologies, despite not being reported in first trimester uterine tissues.

1.2.4 Decidual Macrophages have Supportive Roles in Pregnancy

Macrophages are another dominant subtype of immune cells in the decidua and account for at least 15% of all decidual leukocytes in the first trimester (63). The primary role of macrophages is to phagocytose foreign pathogens, infected and malignant cells (125). Additionally, they present antigen to T cells, participate in angiogenesis and tissue remodeling and often exhibit varied phenotypes and tissue-specific functions (126). The heterogeneity and activation state of macrophage populations are determined by their local cytokine environment (127). Thus, the Th1/Th2 balance so critical at the maternal-fetal interface likely dictates the macrophage phenotype and its contribution to healthy pregnancy.

In a typical inflammatory environment, such as a response to microbial molecules or cancer cells, macrophages are stimulated by IFN-γ to produce pro-inflammatory cytokines (IL-1, IFN-γ, TNF-α) further contributing to the Th1 environment. They exert anti-proliferative and cytotoxic effects, process and present antigens, and ultimately induce a
Th1-polarized T-cell response (128, 129). Macrophages activated through this classical pathway are termed M1 macrophages and participate primarily in host response to infection/pathogen. Macrophages can also be non-classically/alternatively activated (M2) by Th2-type cytokines (IL-4, IL-10, IL-13) (129) in tolerant or chronic situations such as the low-grade inflammation associated with obesity (130). M2 macrophages highly express the surface molecules IL-1Rα, IL-10, and macrophage-mannose receptor (MMR: CD206) as compared to M1 macrophages, which have low expression of these molecules (128). These and other phenotypes of M2 macrophages have led to the proposal that these cells participate in tissue repair and host defense in a productive way, induced by a more tolerogenic environment (131). As well, a specific M2 tumor-associated macrophage population has been identified and is thought to be a precipitating factor in tumor-mediated angiogenesis and metastasis because of their pro-tumor activities such as secretion of growth factors, matrix remodeling and suppression of adaptive immunity (132, 133). It is therefore plausible that similar to their roles in tissue remodeling and tumor progression, decidual macrophages possess an M2 phenotype and contribute to decidual angiogenesis and spiral artery remodeling.

While decidual macrophages have been shown, in vitro, to have both antigen-presenting and immunosuppressive capacities (134), they possess several characteristics that support the latter as their dominant role in pregnancy. The high levels of progesterone and cooperation of IL-10, IL-13, and IL-4 inhibit classical macrophage activation and secretion of TNF-α (85, 88) (Figure 1.7: 1). In turn, they also secrete much higher levels of anti-inflammatory IL-10 than either blood monocytes or non-monocytic decidual cells, demonstrating a more suppressive function (135). In addition, decidual macrophages have been shown to inhibit activation of classical T cells, suppress Th1 cells and induce differentiation of regulatory T cells (136, 137) (Figure 1.7: 4). Macrophages in the murine uterus have also been identified as immunosuppressive (138). Therefore, their sensitivity to cytokine signals may make decidual macrophages a primary target and producer of the Th2 cytokines at the maternal-fetal interface (139).
Another indicator of a tolerant phenotype of decidual macrophages is their unique surface marker expression. Macrophages have very high expression of dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN: CD209), MMR (CD206), and endoglin (CD105) and it has been suggested that these cells are involved in tissue homeostasis during pregnancy (140-142) (Figure 1.7: 3). Both DC-SIGN and MMR are implicated in immunomodulation and indicate a potential role for macrophages in the clearing of extracellular matrix breakdown products (142) (Figure 1.7: 3). Th2 cytokine IL-13 can induce DC-SIGN expression by macrophages further providing a potential influence of the decidual cytokine environment on macrophage phenotype (143). Endoglin, a component of the TGF-β receptor (144), may contribute to TGF-β mediated tissue remodeling and suppression of an immune response (145, 146).

**Figure 1.7 Macrophage interactions in the decidua.** 1: Progesterone and increased Th2 cytokine production inhibits classical macrophage activation, decreasing macrophage production of TNF-α and increasing IL-10. 2: HLA-DR on macrophages can interact with HLA-G expressed by EVT and inhibit EVT invasion. 3: ICAM-3, MMR (CD206), and endoglin (CD105) expression by macrophages are thought to aid in matrix remodeling and promoting a phagocytic rather than antigen presenting phenotype, resulting in clearance of ECM breakdown products during remodeling. 4: Interaction of macrophage ICAM-3 with DC-SIGN, as well as the cytokine production by macrophages results in inhibition of classical T cell activation and promotion of naïve T cells to differentiate into a more suppressive/regulatory phenotype.
While decidual macrophages have tolerogenic properties, they also strongly express the HLA Class II molecule HLA-DR, involved in antigen presentation, particularly in the first half of pregnancy (142). *In vitro* studies have shown that trophoblast-derived cytokines induce monocyte migration and macrophages are important for trophoblast survival (55). However, activated macrophages were also shown to inhibit trophoblast migration (147). Additionally, while they possess both capabilities, macrophages have been described as more phagocytic than antigen-presenting during early pregnancy (55). The complex communication between decidual macrophages and trophoblast outlined above combined with their proximity to one another at the maternal fetal interface indicates that decidual macrophages have an important role in regulation of trophoblast invasion (Figure 1.7: 2).

These data when taken together suggest potent roles for decidual macrophages in remodeling of the decidual matrix and arteries, regulation of trophoblast invasion and immunosuppression.

### 1.2.5 Dendritic Cells, Antigen Presentation and Tolerance

Another type of antigen-presenting cell found in the decidua is the dendritic cell. Typical dendritic cells are found in mucosal and epithelial surfaces of the body to act as primary mediators of a response to foreign pathogens. They become activated during inflammation by Th1 pro-inflammatory cytokines, such as IL-1 and TNF- α (125), taking up antigens using DEC-205 and MMR C-type lectin endocytosis receptors (148) (Figure 1.8: 3). As they mature, their MHC Class II surface molecules translocate from intracellular to extracellular (149) and present processed epitopes to naïve T cell populations (125). Mature dendritic cells express the co-stimulatory molecules CD80, CD86 and CD83 during antigen presentation while producing cytokines such as IL-12 to activate T cells (125) (Figure 1.9). Mature CD83⁺ dendritic cells are extremely potent APCs (150) and are the only “professional” APC that are capable of activating T cells from a naïve state, rather than needing memory or already activated T cells to elicit an immune response, as in the case of macrophages. While dendritic cells are sometimes difficult to distinguish from macrophages, they are far less phagocytic and more densely express MHC Class II loci.
Additionally, while dendritic cells can capture antigen at any maturity level, induction of an activated T cell response requires a mature CD83\(^+\) phenotype (Figure 1.8: 4). Presentation of antigen by immature DC-SIGN\(^+\) dendritic cells induces antigen-specific tolerance by the T cells (152) (Figure 1.8: 2). This dendritic cell-mediated tolerance can be promoted by exposure to a Th2 environment abundant in IL-10 (153).

It is likely that DC-SIGN\(^+\) cells have an important role in the decidua of early pregnancy, as they are not detected in non-pregnant human endometrium. As well, approximately 10% of this abundant myeloid population expresses the proliferation marker Ki67, indicating that they are recruited from peripheral circulation and proliferate in situ in early pregnancy (140). Studies in mice have corroborated this, demonstrating a significant increase in dendritic cells in the early decidua compared to both the non-pregnant endometrium and later pregnancy (154).

In first trimester decidua, many of the APCs are monocyte-derived macrophage/dendritic cell-like with varying expression of DC-SIGN, CD14, DEC-205, HLA-DR, CD68, and even low CD4 expression (140) (Figure 1.9). Most express DC-SIGN, but some are DC-SIGN\(^-\) CD14\(^-\) DEC-205\(^-\). Many of these immature cells appear able to differentiate into either...
macrophages or dendritic cells (155), or may alternate between cell types depending upon the cytokine environment, as has been demonstrated using in vitro experiments (143) (Figure 1.9). Thus, regulation of dendritic cell maturity may reside in the cytokine environment of the decidua. Most of the cytokines produced by uNK and decidual macrophages are Th2-promoting, such as IL-10 and TGF-β [reviewed in (85)], and are immunosuppressive.

Interaction of trophoblast HLA-E with uNK inhibitory CD94/NKG2A receptors induces uNK secretion of IL-10 and GM-CSF, which has been shown to prevent maturation of immature dendritic cells (156) (Figure 1.5: 5). Dendritic cells and uNK cells are observed in close contact in the decidua and so it is possible that another mechanism of inhibition occurs through binding of DC-SIGN with its ligand, intercellular adhesion molecule (ICAM)-3, which is highly expressed by uNK cells (157) (Figure 1.8: 1). This interaction may prevent

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**Figure 1.9 Monocyte differentiation and surface marker expression.** Peripheral blood mononuclear cells (PBMC) can give rise to two types of antigen presenting cells (APC): macrophages and dendritic cells (DC). Surface marker expression on progenitors and APCs are outlined as reported by studies in vivo, in vitro, in tumors or in decidua. M1 macrophages are classically activated by Th1 cytokines. M2 macrophages are alternatively activated by Th2 cytokines. DC maturation relies upon presence of pathogens and/or Th1 cytokines.
DC-SIGN from binding target cells such as trophoblast, prevent interaction of dendritics with T cells, or may simply help uNK to exert their cytokine effect on these cells. Regardless, if this communication helps to maintain an immature dendritic cell phenotype, as previously mentioned, trophoblastic peptides could in fact be presented to T cells in order to induce tolerance. Some studies have demonstrated the ability of HLA-G, expressed by invasive trophoblast, to inhibit dendritic cell function in the mouse (158). The suppression of dendritic cell potency during pregnancy extends to their mature phenotype as well. Despite less than 1% of decidual leukocytes being CD83+ mature dendritics, these cells retain their immunostimulatory capacity and can effectively present antigen similar to their peripheral monocyte-derived counterparts (159, 160). However, decidual CD83+ dendritics have decreased secretion of the T cell activating cytokine IL-12. This unique characteristic may help prime naïve CD4+ T cells to acquire a Th2 phenotype and help to further direct the decidual Th1/Th2 balance (160).

Furthermore, an essential role for dendritic cells in implantation and early pregnancy has been defined using dendritic cell-depleted mice that retain their macrophage, NK and T cell populations. Following dendritic cell depletion at gestational d4, these mice exhibit impaired implantation and fetal resorption as well as decreased angiogenesis, blood volume and vascular permeability at the implantation site (161). In contrast, an increase in dendritic cells (both DC-SIGN+ and CD83+) was observed in placental bed biopsies of preeclamptic compared to normal pregnancies (162). As well, significantly greater numbers of CD83+ dendritic cells are harbored in decidua from recurrent miscarriages compared to age-matched controls (163). This increase in mature APCs suggests a more inflammatory environment in these pregnancies and indicates potential for a more acute/hostile recognition of trophoblast and effective presentation of fetal antigens to activated T cells, resulting in a detrimental maternal immune response. Therefore, it is clear that maintenance of dendritic cells in their immature state, as a mechanism of induced tolerance of fetal trophoblast antigen, is a critical component of healthy pregnancy.
1.3 Rationale and Hypothesis

1.3.1 Rationale

The large enrichment of leukocytes in the secretory endometrium and decidua suggests important roles for these unique cells during pregnancy. In the first trimester these immune cells have been identified as primarily uNK cells and macrophages, with smaller subpopulations of CD4 T cells, CD8 T cells, Tregs, γδ T cells, NKT cells, and dendritic cells (54, 63, 64, 164, 165) [reviewed in (90)]. Several groups have proposed a role for these atypical cells in various processes at the maternal fetal interface including decidualization, spiral artery remodeling, trophoblast invasion and maternal tolerance of the hemi-allogeneic fetus (43, 53, 166). Mechanisms of leukocyte regulation of remodeling may involve secretion of MMPs and angiogenic factors which have been shown to be involved in restructuring of the decidual stroma in other processes such as trophoblast invasion and menses. As well, a few groups have previously described the early stages of vascular remodeling in the absence of luminal trophoblast (35, 41), however there has been no definitive delineation of the temporal sequence of events in this process.

The experiments in this thesis employed a placental-decidual co-culture model, adapted from previous work by Vicovac et al. (167), developed to allow for the study of the complex process of spiral artery remodeling in intact decidual explants. EVT invasion into arterioles, endothelial cell and VSMC loss, and relining of the vessels by endovascular EVT has been previously reported in this model (168). This model was used to study the temporal interaction of leukocytes with remodeling arterioles, elucidating the mechanistic role of different cells and the influence of various factors on remodeling.

Furthermore, while certain populations of decidual leukocytes have been investigated early in pregnancy, decidual vascular remodeling is not complete until 20 weeks of gestation. Thus, another objective was to perform a detailed characterization of decidual lymphocyte, granulocyte and APC phenotypes in the first 20 weeks of gestation during this dynamic process. As well, immune cells have been postulated to influence many aspects of the
decidual environment beyond arteriole remodeling. Therefore determining the normal distribution and phenotype of the immune cells present in the decidua will serve as a basis for understanding normal development of the maternal fetal interface and comparison in future analysis to pathologic pregnancies.

1.3.2 Hypothesis

The overall hypothesis of this thesis is that decidual leukocytes are key mediators of several critical processes at the maternal fetal interface during pregnancy. The hypothesis of the in vitro PDC model study was that uNK cells and macrophages infiltrate the decidual arteries and participate in early vascular remodeling through specific mechanisms inducing vascular cell priming, destruction, and clearance. The hypothesis of the flow cytometry immunophenotyping experiments was that decidual leukocyte subtypes have specific distributions that change with advancing gestational age and phenotypes that reflect their involvement in spiral artery and tissue remodeling, trophoblast invasion, and maternal immune tolerance of the semi-allogeneic conceptus.

The specific aims of this thesis are:

1) To quantify a time course of vascular remodeling in the PDC model.
2) To describe and quantify the relationship of uNK cells and macrophages with arterioles before and during remodeling.
3) To investigate mechanisms of uNK cell- and macrophage-mediated transformation of the decidual spiral arterioles.
4) To determine phenotypes of decidual leukocytes and the characteristic changes in their distribution over the first 20 weeks of gestation.
Chapter 2

Leukocyte-Mediated Vascular Remodeling in vitro

The results presented in this chapter were submitted as a manuscript to the American Journal of Pathology in November 2009.

**Title:** Vascular-leukocyte interactions: mechanisms of human decidual spiral artery remodeling in vitro.

**Authors:** Aleah D Hazan*, Samantha D Smith*, Rebecca L Jones, Wendy Whittle, Stephen J Lye, and Caroline E Dunk.

Dr. Samantha Smith from the University of Manchester conducted the experiments summarized in Figures 2.7 and 2.9 A-D.
2 Leukocyte-Mediated Vascular Remodeling in vitro

2.1 Introduction

Following human blastocyst implantation, EVT arise from placental villi and invade the decidualizing maternal endometrium (decidua), where they participate in remodeling of the spiral arteries. During remodeling, the spiral arteries undergo extensive changes including loss of their vasoactive medial VSMCs and most of their intimal endothelial monolayer. This transforms the muscular, tightly coiled decidual spiral arteries into dilated sinusoids, enabling increased uterine blood volume to perfuse the placenta, a process essential for successful establishment of utero-placental circulation and a healthy pregnancy. These changes are thought to be induced by the EVT, which invade the spiral arteries, eventually reline the vessels and acquire an endothelial-like phenotype (169). Failure of spiral arteries in the third of the myometrium closest to the decidua to undergo appropriate remodeling has been described in patients with preeclampsia and IUGR (15).

Prior to embryo implantation the secretory endometrium initiates decidualization, a process accompanied by high progesterone levels and a large infiltration of innate immune cells. By early pregnancy leukocytes comprise 40% of all decidual cells. Specialized uNK cells and macrophages constitute 70% and 20% of decidual leukocytes respectively (63, 64). Both decidual macrophages and uNK cells produce vasoactive factors, including VEGF, PlGF and Ang-2, which are proposed to contribute to decidual vascular remodeling (53, 54). Similarly, a specific M2 tumor associated macrophage population is thought to be the precipitating factor in tumor-mediated angiogenesis and metastasis as they possess many protumor activities including secretion of growth factors, matrix remodeling and suppression of adaptive immunity (132, 133). We suggest that the decidual macrophage may play a similar role in decidual angiogenesis and spiral artery remodeling. Multiple studies have identified an essential role for uNK cells in the murine implantation site; mice deficient in either uNK or IFN-γ signaling exhibit implantation abnormalities and defects of maternal artery remodeling (68-71). In humans, communication between uNK cell
receptors and interstitial EVT is believed to dictate depth of trophoblast invasion (18). However, no conclusive evidence exists to implicate uNK cells directly in human vascular transformation.

We recently reported an intimate relationship between uNK cells, macrophages, and remodeling arteries in biopsies of first trimester decidua basalis (41). Leukocytes were observed in close proximity to early and mid stage remodeling arterial walls, in the absence of either interstitial and endovascular EVT, providing the first evidence for a putative role for leukocytes in mediating human vascular remodeling (41). Moreover we demonstrated that uNK cells and macrophages within the vascular wall expressed MMP-7 and -9. MMPs are key proteases in the reproductive system and are known to be important for processes such as trophoblast invasion (21, 170, 171) and focal degradation of the endometrial ECM during menstruation (172). We suggested that leukocyte-derived MMPs contribute to vascular remodeling, consistent with their reported roles in tumor angiogenesis (46) and metastasis (47). However, in these in vivo specimens we were unable to conclusively describe the direct temporal relationship of the leukocytes with progression of vascular remodeling or directly test mechanistic functionality. We have developed a placental-decidual co-culture (PDC) model, adapted from previous work by Vicovac et al. (1995) (167), that allows us to study the dynamic processes of vascular remodeling in intact decidual explants. Using this model, we have previously reported EVT invasion into arterioles, endothelial cell and VSMC loss, and relining of the vessels by endovascular EVT (168).

We hypothesized that uNK cells and macrophages participate in artery remodeling through specific mechanisms inducing vascular cell priming, destruction and clearance. We employed our PDC model to define the temporal, spatial, and mechanistic relationships of uNK cells and macrophages with spiral arteries during remodeling.
2.2 Methods

2.2.1 Tissue Collection

Placentae and decidua parietalis (without prior invasion) were obtained, following written informed consent, from patients undergoing first trimester elective terminations at the Morgantaler Clinic and Mount Sinai Hospital. The Mount Sinai Hospital Research Ethics Board (Toronto) approved collections of human tissues. Tissue was collected in cold phosphate buffered saline (PBS) and dissected at 6-9 weeks \((n=16)\) according to the criteria of the Carnegie classification evaluating characteristics of embryonic/fetal parts.

2.2.2 Placental Decidual Co-Culture

PDC were established in triplicate by placement of placental villi on the apical epithelial surface of patient-matched decidual explants. Briefly small fragments of placental villi (15-20 mg wet weight) were dissected from the placenta, teased apart, and selected for the presence of EVT cell columns. Thickness and integrity of decidua parietalis was assessed and the most intact pieces (not shredded during vacuum suction) were dissected into 2-3 mm² cubes for culture with or without a placental explant. Decidual explants were placed with the apical epithelial surface uppermost in Millicell-CM culture dish inserts pore size 0.4 \(\mu\)m, (Fisher, Ottawa, Canada) pre-coated with 0.2mL undiluted phenol red-free Matrigel substrate (Becton Dickinson, Mississauga, Canada). The matrigel was allowed to solidify prior to the placement of the corresponding placental villous explant in contact with the decidual epithelial surface. Explants were cultured in serum-free Dulbecco’s Modified Eagle Medium (DMEM)-Ham’s F-12 media (Invitrogen, Burlington, Canada) supplemented with 20 ng/mL progesterone, 300 pg/mL 17\(\beta\)-estradiol, (Sigma, Canada) and 100 \(\mu\)g/mL normocin (Cedarlane laboratories, Burlington, Canada) at 3% \(O_2/5% CO_2\). Culture media was changed every 48 hours. PDC (from a single patient) were established in triplicate for each treatment point. Adjacent explants of decidua parietalis were cultured in the absence of placenta in order to confirm that there was no trophoblast invasion prior to the establishment of the culture or degradation of blood vessels due to the culture conditions.
Decidua-only controls from adjacent tissues and placental-decidual explants were maintained in culture for 3 or 6 days.

2.2.3 MMP-2/9 Inhibition

Given the MMP-9 expression in VSMC, endothelial cells, uNK cells, and macrophages, as well as the active MMP-2/9 detected in VSMC and leukocytes in both in vivo decidua basalis and PDC model specimens, I decided to use the PDC model to investigate function of MMP-2/9. I carried this out by manipulating culture conditions with the addition of a specific MMP-2/9 inhibitor in order to determine the role of these proteinases on vascular remodeling processes.

In order to minimize damage to the decidual epithelial surface decidual explants were immobilized in a Petri dish and injected at 2 basal stromal sites with 50nM MMP-2/9 Inhibitor II (Calbiochem, Darmstadt, Germany) or vehicle (0.1% dimethyl sulfoxide (DMSO); Sigma, Oakville, Ontario, Canada) (50µL/site) using a 700 series Microlitre Hamilton syringe with cemented needle gauge 22s (VWR, Mississauga, Ontario, Canada) followed by a 30 minute incubation at 37°C, prior to establishment of PDC as described above (n=3).

2.2.4 Fixation and Processing

PDC replicates and matching decidua-only controls were fixed in 4% paraformaldehyde for 1 hour at room temperature and rinsed 3x in cold Ca^{2+} and Mg^{2+} free PBS on a shaker for 20 minutes and stored in PBS at 4°C until processing. Explants were dehydrated by a gradient of ethanol in PBS solutions from 70% to 100%. Experiments were cleared in xylene for 1 hour, excess xylene was removed by paraffin infiltration for 3 hours and embedded in paraffin wax using an embedding machine.

NB: Only explants that had attached to the decidual epithelial surface by day 1 and remained attached at the time of collection and through fixation were processed.
Occasionally the multiple solution changes during processing and paraffin-embedding resulted in the loss of the placental explant, as observed in some photomicrographs.

2.2.5 Immunohistochemistry

Paraffin-embedded explants were sectioned to 5 µm using a microtome, adhered to Superfrost++ slides using a warm water bath and dried in an oven at 60°C overnight. Slides were de-paraffinized in xylene (3 x 5min) and re-hydrated in a series of ethanol in PBS solutions with decreasing concentrations of ethanol from 100% to 50% and rinsed in PBS (2 x 5min). Hematoxylin & eosin (H&E) stain was performed on every 10th section to assess morphology of experiments. From PBS slides were quickly stained with Harris Hematoxylin Solution (<2min), dipped in 1% glacial acetic acid in 70% ethanol and washed in tap water for 2min. Slides were stained with eosin for 1 min and rinsed in PBS before being dehydrated through the ethanol gradient series and cleared in xylene (2 x 2min) before being coverslipped using Permount. Slides for immunohistochemical (IHC) stain were incubated in 3% Hydrogen Peroxide in methanol solution for 40 minutes to quench endogenous peroxidase activity. Antigen retrieval was accomplished by microwave pretreatment with either 10mM sodium citrate (pH6.0) or 1mM ethylenediamine tetraacetic acid (EDTA: pH9.0), or incubation with 0.02% Triton in PBS for 10 min at room temperature. All slides were incubated with Dako protein blocking solution for 1 hour at room temperature to block non-specific binding.

All primary and secondary antibody usage conditions are summarized in Table 2.1. Slides were washed in PBS (2 x 5min) after application of both primary and secondary antibodies. Slides were developed using labeled streptavidin biotin (LSAB)-horseradish peroxidase (HRP) (1hr) (Dako, Mississauga, Ontario, Canada) and 3,3-diaminobenzidine+ (DAB) in diluting solution (Dako, Canada). Slides were washed in ddH₂O, counterstained lightly with Harris Hematoxylin Solution (Sigma, Canada) and dehydrated in an ascending ethanol series, cleared in xylene and mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA).
Table 2.1 Details of antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Source</th>
<th>Dilution</th>
<th>Ag Retrieval</th>
<th>Specificity</th>
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<tr>
<td>Cytokeratin</td>
<td>Mouse</td>
<td>DAKO</td>
<td>0.17 µg/ml</td>
<td>Microwave: 10mM Sodium (Na) Citrate pH6</td>
<td>Epithelial cells incl. trophoblast</td>
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<tr>
<td>CD31 (PECAM-1)</td>
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<td>DAKO</td>
<td>1.3 µg/ml</td>
<td>Microwave: NaCitrate</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Mouse</td>
<td>DAKO</td>
<td>0.035 µg/ml</td>
<td>Microwave: NaCitrate</td>
<td>Smooth muscle cells</td>
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<tr>
<td>CD45</td>
<td>Mouse</td>
<td>DAKO</td>
<td>0.35 µg/ml</td>
<td>Microwave: NaCitrate</td>
<td>All leukocytes</td>
</tr>
<tr>
<td>CD56</td>
<td>Mouse</td>
<td>DAKO</td>
<td>0.3 µg/ml</td>
<td>Microwave: 1mM EDTA</td>
<td>uNK cells</td>
</tr>
<tr>
<td>CD68</td>
<td>Mouse</td>
<td>Novocastra Laboratories</td>
<td>0.5 µg/ml</td>
<td>Microwave: NaCitrate</td>
<td>Macrophages</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Mouse</td>
<td>Calbiochem</td>
<td>4 µg/ml</td>
<td>Microwave: NaCitrate</td>
<td>MMP-9</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Rabbit</td>
<td>Biomedia</td>
<td>1 µg/ml</td>
<td>Microwave: NaCitrate</td>
<td>Phagocytic cells</td>
</tr>
<tr>
<td>anti-mouse IgG- biotin</td>
<td>Goat</td>
<td>Dako</td>
<td>0.024 µg/ml</td>
<td>Matched to primary antibody</td>
<td>Mouse IgG</td>
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<td>Mouse</td>
<td>Dako</td>
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<td>Matched to primary antibody</td>
<td>All IgG</td>
</tr>
<tr>
<td>Isotype</td>
<td></td>
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Photomicrographs of immunohistochemical staining were captured using an Olympus BX61 Upright Microscope and an Olympus DP70 12.5 mega pixel camera with accompanying Olympus software (Olympus America Inc., Center Valley, Pennsylvania, USA).

2.2.6 Image Analysis and Quantification

Serial sections of PDCs and corresponding decidua-only controls from day 3 and 6 of culture immunostained for α-smooth muscle actin (SMA), CD45, CD56 and CD68 were scanned at 16X and 100X magnification (Visiopharm Integrator System Version 3.0.8.0, Visiopharm, Horsholm, Denmark). Adjacent digital photomicrographs were stitched together automatically by the Visiopharm software to create composite images of the entire PDC tissue section. Image analysis was conducted using Visiopharm’s Visiomorph analysis software which established pixel classification parameters based on pigment for
stroma, positive DAB stain and vessel lumen. These parameters were then applied to each image and PDC decidua were divided into 3 depths (0-500, 500-1000, and 1000-2000 µm) from the epithelial surface (Figure 2.1). Veins and glands were excluded from the analysis by morphology as examined using the vascular markers CD31 and α-SMA. To identify changes in the decidual arterioles, areas of VSMC and vessel lumens were calculated by Visiomorph at each depth and expressed as a ratio (α-SMA/lumen). For each patient sample, triplicate α-SMA/lumen measurements from replicate co-cultures were calculated and averaged.

In addition we determined leukocyte association with arteries in decidua-only controls, PDCs prior to remodeling, and during active remodeling. Decidual arterioles were designated by morphology as: unremodeled (multiple, well-organized layers of smooth muscle, intact endothelium and narrow lumen), actively remodeling (disrupted VSMC and/or disorganized medial VSMC and endothelial desquamation and/or loss), or advanced remodeling (complete loss of VSMC, few/no endothelial cells and dilated lumen) arteries. On average, the medial wall thickness of unremodeled arterioles from both co-cultures and decidua-only controls was ~15 µm. A line was drawn to denote the lumen of each vessel and concentric rings were automatically executed at 15 µm distances from this line by the Visiomorph software (Figure 2.1). Immunohistochemical staining was classified by pixel pigment (as described above) to calculate area of leukocyte staining (CD45, CD56, & CD68), which was then expressed as a proportion of each concentric zone area (i.e. area of CD45+/0-15 µm concentric zone area and area of CD45+/15-30 µm concentric zone area), excluding the arteriole lumen area.
2.2.7 Statistical Analysis

We checked our data for distribution using SigmaStat (Systat Software Inc., San Jose, CA) and found that it was not normally distributed. Therefore, statistical analyses for non-parametric data were performed using PRISM (GraphPad Software Inc., La Jolla, CA) including Kruskal-Wallis Analysis of Variance (ANOVA) with Dunn’s post hoc test for multiple comparisons and occasionally Mann-Whitney t-tests as indicated. Data are presented as medians and inter-quartile ranges for each time point or vascular remodeling stage in box and whisker plots. Whiskers represent 10 and 90 % inclusive.
2.3 Results

2.3.1 Temporal Vascular Remodeling in Human Placental-Decidual Co-Culture

The time course of decidual vascular changes induced by the presence of placental explants was examined (Figure 2.2 A). At the site of placental-decidual contact, cytokeratin$^+$ trophoblast was consistently observed in anchoring columns arising from the tips of placental villi and attaching to the decidual epithelial surface (Figure 2.2 B). Serial sections of a representative PDC at day 6 demonstrated progressive remodeling along a vessel from the point of placental contact (Figure 2.2 D: CD31, E: $\alpha$–SMA and F: cytokeratin). Superficial portions of arterioles (closest to the decidual epithelial surface) showed features of more advanced remodeling (Figure 2.2 right of $Ep$) than distal portions of the same vessels, evidenced by little or no remaining endothelium, disorganized, sporadic VSMC and EVT in their lumen. Mid-portions of the vessels showed evidence of active remodeling in the absence of intraluminal EVT (Fig.2.2 -Ml). Active remodeling is characterized by endothelial desquamation and shedding into the vessel lumen (Fig.2.2 D$^*$), as well as disruption and loss of VSMC (Fig.2.2 E). Deep arteries (Fig.2.2 -De) in PDCs remained largely untransformed, with intact VSMC and endothelial layers, and no EVT invasion, similar to decidua-only control arterioles (Fig.2.2 G,H). Veins were excluded based on morphological features such as thin layers of VSMC, intact flattened endothelium and dilated lumens (Fig.2.2 I,J). All arterioles in decidua-only control cultures were intact and unremodeled. The size of the arterioles observed depended upon their stage of remodeling and position within the decidua. Most of the un-remodeled arterioles had a diameter of between 25 and 50 $\mu$m regardless of whether they were found in the superficial (in decidua-only controls) or deep (in PDC) decidua. Remodeling arterioles typically had a greater lumen diameter in the range of 50 to 100 $\mu$m at their narrowest cross-section. Lengthwise sections through vessels can make them appear even larger.
Figure 2.2 Decidual spiral artery remodeling in the placental-decidual co-culture model. A: Representative photomicrograph of a PDC. B: An EVT column (cytokeratin) attaching to the decidual epithelial surface (bracket). C: Isotype control. D-F: Representative serial sections from a day 6 PDC. Progressive remodeling occurs along an artery in the direction of trophoblast (F: cytokeratin) invasion (left to right). The segment of the artery at the superficial epithelial decidual surface (Ep) contains EVT in the lumen (arrowhead: F) and is more fully remodeled. Early vascular remodeling is observed in the mid-segment of the artery (Mi) by endothelial cell (D: CD31,*) swelling and disruption, and VSMC loss (E: α-SMA, open arrowheads) in the absence of trophoblast. Deep vessels (De) demonstrate no evidence of remodeling. G-H: Intact arterioles from the superficial portion of decidua-only cultures have no trophoblast present (G: cytokeratin) and possess multiple layers of vascular smooth muscle (H: α-SMA). I-J: Veins were excluded from analysis by morphology. Serial sections demonstrate a thin endothelial layer (I: CD31) and sporadic VSMC (J: α-SMA, arrows). Scale bars: 100 µm.
Quantification of vascular remodeling in PDCs at day 3 and 6 along with corresponding decidua-only controls is presented as median and inter-quartile ranges in Figure 2.3. Decreased α-SMA/lumen ratios resulted from the loss of VSMC and lumen dilation of the remodeling arterioles. No statistical differences in α-SMA/lumen ratios for decidua-only controls were observed between 3 and 6 days, so the data were pooled for graphical representation. Within 500µm of the decidual epithelial surface, a ten-fold decrease in the α-SMA/lumen ratio of PDCs at both day 3 (p<0.05) and 6 (p<0.05) was observed compared to decidua-only controls. No significant difference existed between day 3 and 6 PDCs at this first depth, indicating that maximal VSMC loss occurs within the first 3 days in culture. The α-SMA/lumen ratio between 500-1000µm was also significantly decreased at both day 3 (p<0.05) and 6 (p<0.01) compared to decidua-only controls, with no significant difference between time points. Beyond 1000 µm there was a trend towards a decrease in α-SMA/lumen ratio with time in culture, using pooled decidua-only controls. Interestingly, when α-SMA/lumen ratios from day 6 PDCs were compared to their internal experiment-matched day 6 controls at 1000-2000 µm depth, a significant decrease in the PDC α-SMA/lumen ratio was found (p<0.05). Similar comparison of day 3 PDCs to their internal experimental controls demonstrated no difference, indicating a progressive, ongoing process of remodeling over time in these cultures.
2.3.2 Leukocyte Association with Arterioles Differs with Progression of Remodeling

Decidual spiral arterioles from PDCs (day 3 and 6) were designated as unremodeled, early, active or advanced remodeling based on the characteristic changes in vascular cells (Figure 2.4). Unremodeled arterioles possessed an intact CD31+ endothelial lining (Figure 2.4 A), 3-4 layers of α-SMA+ VSMC (Figure 2.4 B) and no evidence of cytokeratin+ trophoblast (Figure 2.4 C). Early to active remodeling arterioles were characterized by endothelial cell swelling, desquamation and sloughing off into the lumen (Figure 2.4 E,I). These arterioles exhibited varying degrees of VSMC hypertrophy, disorganization and loss (Figure 2.4 F,J) with no evidence of EVT (Figure 2.4 G,K). This was not observed in veins.

Figure 2.4 An active role for leukocytes in vascular remodeling. A-D: Serial sections of unremodeled decidual arterioles (Un-Rem) far from the point of placental contact in the PDC basal decidua. Leukocytes (D) are present in stroma and excluded from vessels with intact smooth muscle layers. E-L: Serial sections of early (Early Rem: E-H) and actively (Active Rem: I-L) remodeling arterioles. Veins (V) possess more flattened endothelium (E) compared to early (E: A) or actively (I) remodeling arterioles. Infiltration of leukocytes are observed around and within the remodeling vessel walls (H,L). M-P: Serial sections of an advanced remodeled (Adv Rem) arteriole relined with trophoblast (M). Fewer leukocytes are present in the vessel wall (P). Scale bars: A-D, I-P: 100µm E-H: 200µm.
which possessed an intact flattened endothelium and a single smooth muscle layer (Figure 2.4 E, I). Advanced remodeling arterioles were observed within the first 500 µm depth of the decidua at day 6 and were relined with EVT (Fig 2.4 O). Minimal endothelium and VSMC remained within the vascular wall of these arterioles (Figure 2.4 M, N). Throughout remodeling distinct changes in leukocyte distribution were observed. While leukocytes were abundant in the stroma, these cells were excluded from the walls of unremodeled arterioles (Figure 2.4 D). In the early stages of remodeling, leukocytes cluster around the arterioles compared to their stromal distribution (Figure 2.4 H). Within the wall of actively remodeling arterioles, a large infiltration of CD45+ leukocytes was observed (Figure 2.4 L). Fewer leukocytes were detected in proximity to advanced remodeling arterioles (Figure 2.4 P).

2.3.2.1 Macrophage and uNK Infiltrate Actively Remodeling Spiral Arterioles
Leukocytes (CD45+) sub classified as CD56+ uNK cells and CD68+ macrophages were observed infiltrating the vascular walls of actively remodeling arterioles by immunohistochemistry (Figure 2.5). Remodeling arterioles with some remaining VSMC layers (Figure 2.5 A) contained CD45+ leukocytes (Figure 2.5 B), uNK cells (Fig 2.5 C) and macrophages (Figure 2.5 D) around and within their vascular walls. Increased disruption of VSMC (Figure 2.5 E) was associated with denser infiltration of leukocytes (Figure 2.5 F), uNK cells (Figure 2.5 G) and macrophages (Figure 2.5 H), many of which crossed the endothelium and accumulated in the lumen of actively remodeling arterioles. Leukocytes did not infiltrate decidua-only control vessels with intact VSMC (Figure 2.5 I-L).

2.3.2.2 Quantification of Leukocyte Association with Decidual Arterioles
By day 3, the superficial portions of the arterioles (<1000µm from the decidual epithelial surface) exhibited characteristics of active remodeling. Similar observations were made in larger, deeper spiral arteries (>1000µm) at day 6. These arterioles displaying evidence of remodeling were compared to deep arterioles from PDCs and superficial arterioles from decidua-only controls. The concentric zones 30-60µm from vessel lumens were examined
during optimization and no significant differences in area of CD45$^+$ leukocytes was found between the 15-30µm zone and those further from vessels. Measurements taken >30µm from the vessel lumen also frequently included leukocytes clustered around neighbouring vessels. Therefore only the two 15µm concentric distances closest to the arterioles were used in this comparison ensuring that enough area was examined to account for the intact muscular arterial wall and to ensure exclusion of leukocytes not directly associated with the vessel under investigation. Upon quantification, no significant differences in the area of CD45$^+$ staining surrounding decidua-only control arterioles compared to untransformed PDC arterioles are found (Figure 2.6). There were, however, significantly increased proportions of CD45$^+$ staining within the 0-15µm zone of actively remodeling arteriole lumens at both day 3 (Fig 2.6 A: n=31 arteries) and 6 (Figure 2.6 B: n=27) compared to arterioles from decidua-only cultures (d3 n=20; d6 n=25) or unremodeled arterioles from the same experiment (d3 n=28; d6 n=22) (p<0.001 for all). In more advanced remodeling arterioles containing trophoblast (d6 n=8), the proportion of CD45$^+$ staining in the 0-15µm

**Figure 2.5 Leukocyte subtypes involved in active vascular remodeling.** A-D: Serial sections of an example vessel in an early stage of remodeling (Early Rem). Vessel possesses a single remaining ring of smooth muscle (A). The majority of leukocytes (B) in close association with the vessel are CD56$^+$ uNK (C) with a few CD68$^+$ macrophage (D). E-H: Serial sections of an artery in a more progressed stage of remodeling (Active Rem). (E) Smooth muscle layers are absent. VSMC are disrupted, sporadic and shed into vessel lumen. (F) Leukocytes are densely clustered within the arterial wall and lumen. Many uNK cells (G) and macrophages (H) are present in artery wall and lumen. I-L: Serial sections of a decidua-only control vessel (Dec Con). (I) Intact vessel possesses multiple layers of smooth muscle. Both uNK and macrophage (K & L) are present in the decidual stroma but do not demonstrate a close association with the vessel. Scale bars: 100 µm.
Figure 2.6 Quantification of leukocytes associated with remodeling arterioles and unremodeled arterioles from PDCs and decidua-only controls. Black bars: Decidua-only control culture arterioles (day 3: n=20, day 6: n=25). Striped bars: Unremodeled arterioles from PDC (day 3: n=28, day 6: n=22). White bars: Actively remodeling arterioles from PDC (day 3: n=31, day 6: n=27). Leukocyte infiltration was assessed by area of CD45 staining and expressed as a ratio of the concentric ring areas 0-15µm and 15-30µm from arteriole lumens. A significant increase in CD45+ leukocyte/ concentric ring area was observed at the 0-15µm distance on both days 3 (A) and 6 (B). A significant increase in the proportion of uNK cells in the 0-15µm zone surrounding actively remodeling vessels was observed at day 3 (C) and 6 (D). A significant increase in the proportion of macrophages within the 0-15µm ring surrounding actively remodeling vessels was observed at both day 3 (E) and 6 (F). In contrast to day 3, a significant increase in the proportion of macrophages within the 15-30µm zone around actively remodeling arteries was observed at d6 (F). *: p<0.05, **: p<0.01, ***: p<0.001.
zone was decreased as compared to actively remodeling vessels within the same depth and from the same experiments (n=27, p<0.05). There were no significant differences in area of CD45$^+$ staining between the 15-30µm zones around decidua-only controls, unremodeled, early remodeling, or advanced remodeling arterioles at day 3 or 6.

Image analysis was used to quantify proportion of positive CD56 and CD68 staining surrounding vessels similar to that performed for the common leukocyte antigen (CD45). A substantial and significant increase in both uNK cells and macrophages within the 0-15µm zone of actively remodeling arterioles compared to either decidua-only control arterioles (Figure 2.6 C,D: CD56 d3&6 p<0.001; E,F: CD68 d3 p<0.01, d6 p<0.001) or unremodeled PDC arterioles (CD56 d3&6 p<0.001; CD68 d3&6 p<0.001) was observed. At day 3, no differences existed in uNK cell and macrophage proportions in the outer (15-30µm) zone between the different remodeling stages. By day 6, more macrophages were observed in the outer zones of actively remodeling vessels compared to either decidua-only controls (p<0.05) or unremodeled PDC arteries (p<0.01).

2.3.3 MMP-9 Expression During Vascular Remodeling

MMP-9 expression by uNK cells, macrophages, vascular cells and EVT was examined by dual immunofluorescence (Figure 2.7). VSMC of early remodeling arterioles (Figure 2.7 B,C) were MMP-9$^+$, as were CD56$^+$ uNK cells infiltrating actively remodeling arterioles (Figure 2.7 E,F). Furthermore, the enEVT present in advanced remodeling arterioles (Figure 2.7 H,I) were also MMP-9$^+$. Decidual stromal (st), glandular epithelial and endothelial cells were MMP-9$^+$ (data not shown). No staining for MMP-9 was detected in the negative control (Figure 2.7 J). In situ zymography revealed MMP-2/9 (gelatinase) activity in infiltrating leukocytes (Figure 2.7 K, enlarged in L) associated with remodeling arterioles. MMP-2/9 activity was also observed within the VSMC of the disrupted vascular wall (Figure 2.7 N, enlarged in O).
Figure 2.7 Matrix-metalloproteinase expression and activity in remodeling arteries. Dual immunofluorescence (arrowheads) of cell markers (red) and MMP-9 (A,D,G; green). VSMC of early remodeling arteries (B), CD56 in the vascular wall of actively remodeling arteries (E) and enEVT of advanced remodeling arteries (H) all demonstrate co-localization with cytoplasmic MMP-9 (C,F,I respectively; yellow). MMP-9 was also expressed by decidual stromal cells (st). J: Negative control for dual immunofluorescence (anti-mouse FITC and DAPI). K,N: In situ zymography revealed gelatinase reactivity (GEL: green; nuclei: red) in both leukocytes (K) and VSMC (N) (arrows) that were identified in serial sections (data not shown) of actively remodeling vessels, enlarged in L and O. M: Negative control for in situ zymography. Scale bars: 50µm.
2.3.4 MMP-2/9 Inhibition Impairs Decidual Vascular Remodeling in Co-Culture

In order to investigate the role of MMP-2 and MMP-9 in vascular remodeling, we micro-injected decidual explants with MMP-2/9 Inhibitor II or vehicle (0.1% DMSO) and cultured them with placenta for four days. Remodeling of the superficial arterioles at the epithelial surface was observed in the vehicle-injected control PDCs (Figure 2.8 B) similar to that seen in PDCs with no treatment. In PDCs injected with MMP-2/9 Inhibitor II however, the arterioles remained unremodeled with multiple intact VSMC layers although the placental explant remained in contact (Figure 2.8 C). Decidual leukocytes (CD45+) did not infiltrate arterioles of the MMP-2/9 Inhibitor II injected PDCs (Figure 2.8 D). Image analysis demonstrated significantly greater α-SMA/lumen ratios within both 0<500µm (p<0.01) and 500<1000µm (p<0.05) depths of MMP-2/9 Inhibitor II-injected PDCs compared to vehicle-injected PDC controls (Figure 2.8 A; n=3).
Figure 2.8 Inhibition of MMP-2/9 prevents decidual spiral artery remodeling. A: Quantification of α-SMA/lumen ratios demonstrated a failure of vascular transformation in MMP-2/9 inhibited PDC (striped bars: n=3) in comparison to vehicle-injected PDC controls (white bars: n=3). Representative CK and α-SMA stained images of PDCs injected with vehicle (B, D) or 50nM MMP-2/9 Inhibitor II (C, E). F: CD45 stained vehicle-injected PDC showing the difference in leukocyte clustering between arterioles close to placental contact and deep in the decidua. G: CD45 stained serial section of MMP-2/9 Inhibitor II injected PDC shows that leukocytes are not recruited to arterioles and do not invade the arterial wall. Scale bars: B,D: 200µm C,E-G: 100µm.
2.3.5 Evidence for Leukocyte-Mediated Mechanisms of Vascular Remodeling

Potential mechanisms contributing to the rapid, progressive loss of both endothelium and VSMC were investigated. Apoptotic cells were identified by positive terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL+) in the wall of actively remodeling arterioles as both endothelial cells (CD31; Figure 2.9 A,B) and VSMC (α-SMA; Figure 2.9 C,D) by dual immunofluorescence. Toluidine blue staining of semi-thin sections showed apoptotic vascular cells displaying condensed pyknotic nuclei. Large granular leukocytes (likely uNK cells) were observed within the arteriole lumen and macrophages were embedded in the vascular wall (Fig 2.9 E).

In early remodeling arterioles/arteries (>500 μm), we observed features of VSMC dedifferentiation, characterized by a gradual decline in α-SMA immunostaining of cells at increasing distances from the remaining intact rings of VSMC (Fig 2.9 F). Additionally, these arterioles were characterized by macrophage accumulation and clusters of lysozyme muramidase+ cells, indicating phagocytosis of peripheral vascular cells (Fig 2.9 G, H).
2.4 Discussion

In this chapter novel data of vascular-leukocyte communication and potential mechanisms mediating early human decidual spiral artery remodeling is presented. In the PDC, remodeling was initiated in arterioles close to the site of placental contact (epithelial decidual surface) and progressed along the length of the vessels. By day 3 of culture, EVT were present in the superficial portions of the arterioles, however vascular remodeling and significant leukocyte infiltration and accumulation was observed in deeper portions of the same arterioles. Additionally, α-SMA/lumen ratios were similar at all depths in decidua-only controls confirming that the decreased α-SMA/lumen ratios in superficial PDC decidua were induced by the presence of the placental explant. In more advanced remodeling arterioles demonstrating significant loss of VSMC and endothelial cells and containing enEVT, there were significantly fewer leukocytes within the perivascular area suggesting a transient involvement with remodeling arterioles during the period of vascular cell loss. Similarly, in our previous work on in vivo decidua basalis arteries, we observed significant accumulation of uNK cells and macrophages in the early to mid stages of remodeling in the absence of enEVT; but not in vessel with enEVT present (41). Thus, our PDC model demonstrates that early decidual vascular remodeling occurs prior to enEVT presence and is associated with a large infiltration of leukocytes. The progression of vascular remodeling and leukocyte infiltration is summarized in Figure 2.10.
This conclusion serves to elaborate on the current dogma that credits invasion of EVT to be primarily responsible for “physiological transformation” of the spiral arteries. Much of this data comes from placental bed biopsies, including the decidual-myometrial junction, and decidual basalis specimens where VSMC disruption is observed in the presence of both inEVT and enEVT (26, 31). In our PDC model, we only observed invasion of enEVT into arterioles, not inEVT into stroma, which is a limitation of this model. This is likely due to the inability of EVT to penetrate the intact decidual epithelial surface and when placental explants are placed against the basal surface of the decidua, Moser et al have shown that robust interstitial invasion occurs (173). Importantly, the decidua parietalis does not harbor many inEVT in early pregnancy. Therefore, these results suggest that enEVT are able to contribute to vascular remodeling independent of inEVT.
Other detailed studies of early implantation sites have provided convincing evidence of subtle remodeling and VSMC disruption within decidual arteries prior to direct cellular contact of vascular cells with invading EVT (26, 35). These authors also described a leukocytic infiltration of decidual vessels, which was recently described as a ‘decidualization-associated’ vascular change (31). The current study extends this work by clearly demonstrating that although vascular changes are observed prior to visible EVT within the arteriole lumen, placentally-derived mechanisms are required to initiate vascular remodeling. It has also recently been shown that primary EVT stimulated with gonadotropin releasing hormone produce CXCL8, which promotes uNK cell trans-well migration (174). The PDC model also verifies that it is the resident decidual leukocyte population that participates in remodeling as blood flow is absent and de novo leukocyte recruitment cannot occur in our model. Moreover, we suggest that enEVT can communicate with stromal leukocytes without direct EVT-leukocyte cellular contact, potentially via changes in VSMC chemokine profiles. This hypothesis is supported by a number of studies demonstrating that VSMC and endothelium of spiral arteries express several chemokines including CCL14, 16, 21, 22 and CX3CR1 (49, 50). Furthermore, we have reported that the uNK cell and macrophage chemoattractants CXCL12 and CCL14 are localized to the VSMC of actively remodeling decidual arteries in vivo (51). These recent findings, when taken together with the data presented in this manuscript, provide evidence for complex chemokine communication between EVT, VSMC and decidual leukocytes at the cellular level during vascular remodeling and demonstrate the ability of primed vessels to recruit leukocytes from the stroma.

Interestingly, many leukocytes are observed within the lumen of the arterioles, in addition to those that penetrate the vascular wall. Extending the debate of how the uNK cells populate the decidua, it was previously proposed that while uNK cells are clustered around arteries, the uNK cells within the vascular wall, in contact with endothelial cells, and found as single cells within the lumen are in the process of diapedesis (175). While this recruitment of uNK cells into the decidua from peripheral blood may occur in vivo, this data provides strong evidence that the resident decidual leukocytes may intravasate into the
vasculature as remodeling occurs. This is supported by the detection of granulated metrial gland cells in the lungs of mice during pregnancy (176) and the chemoattraction of uNK cells by EVT mentioned above.

Several mechanisms have been suggested to mediate VSMC and endothelial cell loss during vascular remodeling including migration, dedifferentiation and apoptosis. In PDCs the vascular layers of early remodeling arteries exhibit loosening in their outermost portions and ‘loosened’ VSMC stain weaker for α-SMA with increasing distance from the vascular wall. Similar observations have been made in placental bed biopsies from early pregnancy where isolated VSMC were found at a distance from vessels, particularly in vessels colonized by enEVT (177). Furthermore, we have shown that active MMP-9 is highly expressed by infiltrating uNK cells, macrophages, and in actively remodeling arterioles reinforcing our previous observations, and those of others, that uNK cells associated with decidual arteries produce MMP-9 (41, 43, 178). Production of MMP-9 by leukocytes within the disorganized VSMC layers of remodeling arteries may initiate vascular matrix and basement membrane destruction, allowing leukocytes to infiltrate, and facilitating VSMC dispersal and dedifferentiation as is observed during vascular remodeling (42, 177). Interestingly, when decidual explants from PDC were injected with an inhibitor of MMP-2/9 activity prior to co-culture with a placental explant, the decidual arterioles retained intact VSMC layers and little or no evidence of remodeling was observed. Loss of cellular anchoring to ECM also triggers a form of apoptotic cell death known as anoikis (45); this may represent an alternative mechanism for VSMC and endothelial apoptosis during active decidual artery remodeling. MMPs fulfill important roles during inflammation, unrelated to matrix destruction, modulating chemokine activity via proteolytic cleavage. Of particular relevance, MMP-9 can cleave and inactivate chemokine CXCL5, whilst increasing CXCL8 potency in neutrophil chemoattraction (52). Interestingly in the presence of MMP-2/9 inhibitor leukocytes did not accumulate or infiltrate the vasculature suggesting that they were not able to respond to the placental stimulus and lending further support to the functional role for active MMPs in leukocyte recruitment, trophoblast invasion, and vascular remodeling. Localized MMP-9 secretion may also contribute to the tight regulation of
leukocyte infiltration and cessation of local inflammation in the advanced stages of decidual remodeling.

Studies using isolated myometrial spiral arteries have demonstrated that trophoblast cells secrete cytokines from the TNF family, including Fas-ligand and TRAIL, that can induce apoptosis of aortic VSMC over a 24-hour period (37, 39). In contrast, some studies of placental bed biopsies (PBBx) have not identified apoptosis in perivascular cells (179). Importantly, in this manuscript we demonstrate co-localization of TUNEL reactivity with both VSMC and endothelial cells, coincident with early medial smooth muscle disruption and leukocyte accumulation in the PDCs. This corroborates our recent in vivo evidence of apoptotic VSMC and endothelial cells in remodeling decidual arteries prior to EVT presence (41). However, the number of TUNEL+ cells is unlikely to account for the full magnitude of VSMC loss. Alternatively, plasticity or dedifferentiation of VSMC from a functional (contractile) to a synthetic (proliferative) phenotype (180) may account for the extensive vascular disorganization observed in our model. VSMC susceptibility to apoptotic stimuli, and thus rapidity of vascular transformation, may also be influenced by differences between the contractile and proliferative phenotypes of VSMC (181). Another mechanism that may contribute to the paucity of TUNEL positivity is the rapid phagocytic clearance of apoptotic cells by macrophages in order to minimize inflammation. Our finding of lysozyme muramidase+ cells surrounding vessels with early signs of VSMC disruption supports an active role for macrophages in decidual vascular remodeling. Interestingly, uNK cells have also been shown to secrete IFN-γ (86), which can activate macrophages to be phagocytic (182) and may also function to recruit macrophages to the remodeling arteries. These mechanisms suggest participation of uNK cells and macrophages in VSMC disruption, death and clearance in remodeling.

These results extend our understanding of the temporal sequence of events and mechanisms of remodeling, strongly supporting an integral role for uNK cells and macrophages in decidual vascular transformation. As demonstrated by the failure of vascular remodeling induced by inhibition of MMP-2/9 activity, we further support the body
of literature that implicates these important proteases in maintenance and progression of healthy pregnancy. Furthermore, these experiments demonstrate how the PDC model can be manipulated to investigate the role of regulatory factors, such as cytokines, proteases or hormones, in mediating the physiological process of remodeling. Study of the normal physiological process of decidual spiral artery transformation is critical in order to identify causative factors of impaired vascular remodeling and reduced utero-placental perfusion associated with preeclampsia and IUGR.
Chapter 3

Immunophenotyping of Decidual Leukocyte Populations in the First and Second Trimesters

The flow cytometry data presented in this chapter was acquired in the Laboratory Medicine and Pathology Flow Cytometry Centre by Ms. Annie Bang.
3 Immunophenotyping of Decidual Leukocyte Populations in the First and Second Trimesters

3.1 Introduction

Increasing evidence suggests that the abundant maternal decidual leukocyte populations are highly specialized to the time of pregnancy, play key roles in the establishment of the utero-placental circulation, and contribute to tolerance of the genetically dissimilar fetus (3). However, these complex changes remain to be fully understood and most studies are focused on characterization of a particular cell type within a defined period of gestation (usually 1st or 3rd trimester). In other tissue remodeling processes including inflammation, wound healing, and cancer progression, alternatively activated leukocytes secrete angiogenic factors and MMPs facilitating angiogenesis and remodeling of blood vessels (46-48, 122, 128, 133, 183). In the decidua high levels of chemokines are secreted, especially by the spiral artery VSMC and endothelial cells suggesting that recruitment of leukocytes to remodeling spiral arteries is critical (36, 49, 50). We have shown both in vivo and in vitro that uNK cells and macrophages infiltrate remodeling spiral arterioles and contribute to the loss of smooth muscle cells and increase in lumen size [(41) & Chapter 2].

In the first trimester of pregnancy, uterine NK cells are the major cell population representing between 50 – 70% of all decidual leukocytes. The second largest populations are the decidual macrophages and T lymphocytes, which constitute between 15-20% and 10% of decidual leukocytes, respectively. The remaining cells include variable percentages of dendritic cells and subpopulations of T lymphocytes including regulatory T cells, γδ T cells and NKT cells (54, 99-101). These smaller subpopulations of leukocytes may mediate the other dominant roles such as regulation of trophoblast invasion and maternal tolerance as they have potent cytotoxic, antigen-presenting and immune-stimulatory capacities (53, 166). Importantly, the high progesterone levels of healthy pregnancy direct many of these leukocyte types to maintain a tolerant or suppressive phenotype (61, 62, 85, 120). Recent
technical advances in the field of multi-colour flow cytometry now provide an opportunity to perform a detailed characterization of all the decidual leukocyte subpopulation phenotypes at one time. This is necessary to 1) define the changes that occur between first and second trimester and 2) establish a control population in order to determine what aberrant changes may occur prior to and/or in response to pregnancy complications.

The uterine NK cells observed in pregnancy exhibit a distinct phenotype (CD56^{bright}, CD16^-, CD3^-) and significantly reduced cytotoxicity compared to their peripheral blood counterparts PBNK cells (CD56^{dim}, CD16^+) (17, 65, 66). These unique cells appear in the late secretory endometrium, when progesterone levels begin to rise, and increase throughout the first trimester coincident with sustained high progesterone (59). The communication of uNK cells with trophoblast has been thoroughly investigated demonstrating uNK prevention of trophoblast cytolysis, reciprocal chemokine-mediated recruitment, and interaction of uNK KIR and CD94/NKG2 receptors with EVT HLA ligands dictating depth of trophoblast invasion and contribution to spiral artery remodeling (18, 53). The essential role for uNK cells was first demonstrated in uNK-deficient mice, which display implantation defects and impaired spiral artery remodeling (69, 78). Subsequent studies have shown that uNK cells also mediate angiogenesis by secreting VEGF, VEGF-C and Ang-2 (81, 184). Similarly we have recently reported a close association of uNK cells with remodeling arteries in the first trimester human decidua prior to presence of trophoblast and coincident with evidence of apoptosis and uNK-derived MMP production, implicating these cells in vascular cell apoptosis and extra-cellular matrix destruction ([41] & Chapter 2]. UNK cells are known to decrease in number in the second trimester in both humans and mice (63, 78), however, enticing preliminary observations suggest that the surviving uNK cells may also differentiate and/or express different factors [personal communication, Dr. Anne Croy]. One study has reported higher levels of IL-6 and lower levels of IL-4 secretion by uNK cells in the early second trimester compared to the first trimester (185).
Studies of the decidua in early pregnancy have determined that CD3+ T lymphocytes account for approximately 10% of decidual leukocytes, but can range from 5 to 25% in individual patients, while the antibody-producing B lymphocytes are virtually absent (59). The two major subpopulations of T lymphocytes are the helper T cell (CD4+) and the cytotoxic T cell (CTL: CD8+). As their names imply, the former has a productive and regulatory role, while the latter is primarily involved in killing effector cells. One of the most important roles of the T helper cells is production of Th1 and Th2 cytokines that may either progress or dampen an immune response. Th1 cytokines tend to be pro-inflammatory often initiating CTLs and include IL-2, IFN-γ, lymphotoxin and TNF-α. Th2 cytokines tend to be anti-inflammatory and include IL-4, -5, -6, -10, and -13. Normal pregnancy is characterized by an inflammatory Th1 phenotype in the early first trimester that changes to a more tolerant Th2 cytokine environment promoted by high levels of progesterone in the second trimester (85, 91).

The much smaller population of Tregs (CD4+CD25+) are mainly involved in self-tolerance, preventing autoimmunity activated by immature dendritic cells (102, 104, 105). The increase of these cells in peripheral blood and decidua in early mouse and human pregnancy, combined with the failed pregnancies observed in Treg-depleted mice suggest a critical role for these cells in healthy pregnancy likely serving to inhibit maternal immune recognition of the hemi-allogeneic fetus (99, 106, 107).

Another subset of lymphocytes found in small numbers in the decidua are the NKT cells, which possess some characteristics of both NK cells and T lymphocytes (89, 101). However, in pregnancy interaction of NKT cell NKG2 receptors with EVT HLA-E inhibits their own cytotoxic capacity (115). As well, decidual NKT cells (CD56+CD8dim) have been shown to produce factors that promote a Th2 cytokine environment and support trophoblast growth and invasion (116, 117). Thus, the unique populations of lymphocytes at the maternal fetal interface may work in concert to mediate critical processes, in particular maternal immune tolerance.
Macrophages are the second most abundant population of decidual leukocytes accounting for 15-20% in the first trimester (63). Classically activated macrophages (M1) produce pro-inflammatory cytokines, phagocytose foreign pathogens and infected/malignant cells, and present antigen to T cells, ultimately inducing a host T-cell response to an infection/pathogen (125, 128, 129). These M1 macrophages express markers of classical activation such as CD163. Alternative activation of macrophages (M2) occurs in response to Th2 cytokines and these cells participate in angiogenesis, tissue remodeling, and other tissue-specific functions (126, 129). The M2 macrophages highly express surface molecules IL-1Rα, IL-10, and MMR (CD206) (128). Interestingly, the heterogeneity and phenotype of macrophages is sensitive to local cytokines (127). Therefore, the dominant Th2 cytokine environment at the maternal fetal interface may promote decidual macrophages to acquire an M2 phenotype contributing to suppression of a local immune response (139), decidual angiogenesis and spiral artery remodeling. Studies of decidual macrophages have described increased IL-10 and reduced TNF-α production, immunomodulatory capacity, an ability to induce differentiation of naïve T cells to become Tregs, antigen presentation, inhibition of trophoblast invasion, and clearance of ECM breakdown products (142, 147). Many of these reported functions provide evidence for a supportive role for macrophages in pregnancy contributing to maintenance of the decidual tissue environment.

Another important decidual leukocyte population are the dendritic cells. Reports have varied on the percentage of dendritic cells in the decidua, ranging from 1-2% up to 20%. This discrepancy may be due to the overlapping expression of identifying surface molecules by both dendritic cells and macrophages. As these two cell types have common precursors, it is in their mature states and different primary functions that they diverge. Dendritic cells are less phagocytic and far more potent antigen presenters than macrophages (151). In fact, mature CD83+ dendritic cells are the only APC capable of activating naïve T cells (150). Less than 1% of decidual leukocytes are CD83+; the majority are immature DC-SIGN+ (CD209) dendritic cells. This phenotype is likely maintained by the high levels of Th2 cytokines, which have been shown to prevent maturation of dendritics
(156). While dendritic cells of any maturity can capture antigen using DEC-205 (CD205) and MMR (CD206) antigen-uptake receptors (148), presentation by immature DC-SIGN expressing dendritic cells to T cells induces antigen-specific tolerance (152). Thus, production of Th2 cytokines such as IL-10, TGF-β, and GM-CSF by uNK cells and decidual macrophages serves as a mechanism of protection from the maternal immune response (85, 153). Furthermore, an essential role for dendritic cells was identified following observation of both implantation and early pregnancy failures in dendritic cell-depleted mice that retained their macrophage, NK, and T cell populations (161). Therefore these cells are yet another example of how the Th2 cytokine environment influences maternal tolerance.

While not detected in first trimester decidua or myometrium (64), cyclic fluctuations in endometrial neutrophils are observed throughout the menstrual cycle (5). These cells do not usually reside in tissues, but infiltrate during times of wound healing and infection, recruited by tissue macrophages upon encountering a pathogen (118). During menstruation, there is a large infiltration of innate immune cells including macrophage, activated mast cells, and neutrophils, which are credited with influencing endometrial breakdown, likely through production of MMPs (5). Increased numbers of neutrophils are also observed in the decidua and myometrium at the onset of labour in mice [Shynlova, unpublished data] further supporting an important role for these cells in tissue remodeling of the uterus.

We hypothesize that the decidual leukocytes possess characteristic phenotypes that both support and maintain pregnancy and that both the distribution of cell types and phenotype will change from early to mid pregnancy with the evolving requirements of pregnancy. This following study will provide a detailed immunophenotyping and distribution profile of decidual leukocytes from the first and second trimesters of pregnancy.
3.2 Methods

3.2.1 Tissue Collection
Following informed consent, first (n=8) and second (n=9) trimester placental and decidual tissues were collected from patients of the Morgentaler clinic and the Mount Sinai Hospital Second Trimester Interruption of Pregnancy (STIPS) clinic. Second trimester patients from the STIPS clinic were classified by obstetrical history and uterine artery pulsatile index determined by Doppler ultrasound. The research nurses collect the portions of decidua that remain intact post-procedure, and therefore, samples contain varying amounts of decidua basalis versus parietalis. Typically, earlier samples contain more decidua parietalis as this portion begins to regress and is much thinner by mid-pregnancy. The entire sample that is collected is used for leukocyte isolation.

3.2.2 Decidual Leukocyte Isolation
Decidual tissues were placed into a 50mL tube and rinsed repeatedly with aliquots of cold, sterile PBS with Ca\(^{2+}\) and Mg\(^{2+}\) until the wash solution was blood-free. The rinsed tissue was removed into a tissue culture dish, weighed and minced finely using no.21 scalpels; minced tissue was flushed repeatedly with cold, sterile PBS using wide-tipped 25mL pipettes, collected into the pipette and placed in a 100\(\mu\)m sieve. This process was repeated 3-5 times to ensure that tissue was minced as finely as possible and leukocytes were released from the decidual stroma. The single cell suspension was centrifuged for 10 minutes at 2000 rotations per minute (rpm) at 4°C to remove excess blood and debris. The cell pellet was re-suspended in RPMI-1640 media supplemented with 10% fetal bovine serum and antibiotics (100 mg/mL amphotericin B, 100 mg/mL streptomycin & 100 U/mL penicillin; all from Sigma, Oakville, ON, Canada), passed through a 70\(\mu\)m filter and incubated in a 6-well tissue treated plate at 37°C for 20 minutes to reduce fibroblast contamination of the final cell suspension. This incubation time was selected as it minimized loss of leukocytes such as macrophages from the cell suspension. At incubation times greater than 1 hour, macrophages adhered to the plates. Cells not attached to the plate were collected and passed through a 40\(\mu\)m filter to further remove
debris and cell aggregates. After a PBS wash cells were centrifuged and resuspended in erythrocyte lysis buffer solution (Qiagen) for 2 x 15 min on ice to lyse red blood cells. Cells were washed in PBS and centrifuged to remove excess erythrocyte lysis buffer from the cell suspension. The cell pellet was resuspended in Dako protein block solution and kept on ice for 1 hour, during which time antibody combinations were prepared in 1.5mL eppendorf tubes.

3.2.3 Flow Cytometry Cell Preparation

3.2.3.1 Monoclonal Antibodies

The fluorochrome-conjugated mouse anti-human monoclonal antibodies (BD Biosciences) used to immunophenotype decidual leukocytes by flow cytometry are listed in Table 3.1 with their conjugated fluorochrome, immunoglobulin sub-class and specificity. Antibodies were combined to create panels as outlined in Table 3.2 in order to identify phenotypes of the different cell types investigated. Optimal antibody concentrations were determined by titration of volumes similar to those recommended by the manufacturer.

3.2.3.2 Controls

Unstained, isolated decidual leukocytes were used to determine any auto-fluorescence of the tissue-derived cells. Fluorescence-minus-one (FMO) controls were established for each antibody panel by incubating the cell suspension with the panel of interest but omitting one antibody at a time in each tube. The FMO control shows the relative background and overlap of the remaining fluorophores into the fluorescence detector of the omitted antibody. These controls assist in discriminating the positive-fluorescence signal from the negative, and are critical when evaluating dim cell populations. Isotype controls were established (one for each panel) using isotype matched antibodies without specificity in the same concentration as the antigen-specific antibodies. The isotype controls (i.e. APC’-conjugated IgG1κ) are matched to the antibody of interest by immunoglobulin sub-type and fluorochrome (Table 3.1) and demonstrate that positive fluorescence is not due to
non-specific binding of antibodies to the cells of interest and false positive detection of fluorescent signal.

Table 3.1 Flow cytometry antibody (Ab) information.

<table>
<thead>
<tr>
<th>Ab Specificity</th>
<th>Fluorochrome</th>
<th>Immunoglobulin</th>
<th>Leukocyte Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>APC'-Cy7</td>
<td>Mouse IgG1κ</td>
<td>Common Leukocyte Antigen, CLA</td>
</tr>
<tr>
<td>CD3</td>
<td>Alexa Fluor 488</td>
<td>MsIgG1</td>
<td>T Lymphocytes</td>
</tr>
<tr>
<td>CD4</td>
<td>PE'</td>
<td>MsIgG1κ</td>
<td>Helper T Lymphocytes</td>
</tr>
<tr>
<td>CD8</td>
<td>PE</td>
<td>MsIgG1κ</td>
<td>Cytotoxic T Lymphocytes</td>
</tr>
<tr>
<td>CD11b</td>
<td>PE-Cy5</td>
<td>MsIgG1κ</td>
<td>αMβ2 Integrin (Mac-1)</td>
</tr>
<tr>
<td>CD14</td>
<td>PE-Cy7</td>
<td>MsIgG2κ</td>
<td>Monocytes/Macrophages (Ø)</td>
</tr>
<tr>
<td>CD15</td>
<td>FITC</td>
<td>MsIgM</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>CD25</td>
<td>PE-Cy5</td>
<td>MsIgG1κ</td>
<td>Regulatory T Lymphocytes</td>
</tr>
<tr>
<td>CD56</td>
<td>PE-Cy7</td>
<td>MsIgG1κ</td>
<td>Uterine Natural Killer Cells</td>
</tr>
<tr>
<td>CD83</td>
<td>APC'</td>
<td>MsIgG1κ</td>
<td>Mature Antigen Presenting DC</td>
</tr>
<tr>
<td>CD163</td>
<td>PE</td>
<td>MsIgG1κ</td>
<td>Intermediate / Antigen Uptake Receptor DC</td>
</tr>
<tr>
<td>CD205</td>
<td>PE</td>
<td>MsIgG2κ</td>
<td>M2 Alternative Ø Activation/Antigen Uptake Receptor</td>
</tr>
<tr>
<td>CD206</td>
<td>APC'</td>
<td>MsIgG1κ</td>
<td>Immature Dendritic Cells (DC)</td>
</tr>
<tr>
<td>CD209</td>
<td>PerCP-Cy5.5</td>
<td>MsIgG2κ</td>
<td>MHC Class II Molecules</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>PE-Cy5</td>
<td>MsIgG2κ</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 Antibody (Ab) panel to identify leukocyte subpopulations.

<table>
<thead>
<tr>
<th>Tube</th>
<th>N</th>
<th>Ab 1</th>
<th>Ab 2</th>
<th>Ab 3</th>
<th>Ab 4</th>
<th>Ab 5</th>
<th>Ab 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>11</td>
<td>CD45</td>
<td>CD3</td>
<td>CD4</td>
<td>CD8</td>
<td>CD11b</td>
<td>CD56</td>
</tr>
<tr>
<td>Lymphocytes (Treg)</td>
<td>6</td>
<td>CD45</td>
<td>CD3</td>
<td>CD4</td>
<td>CD8</td>
<td>CD25</td>
<td>CD56</td>
</tr>
<tr>
<td>Monocytes/Macrophages &amp; Neutrophils</td>
<td>17</td>
<td>CD45</td>
<td>CD14</td>
<td>CD15</td>
<td>HLA-DR</td>
<td>CD163</td>
<td>CD206</td>
</tr>
<tr>
<td>Macrophages/Neutrophils (Activation)</td>
<td>6</td>
<td>CD45</td>
<td>CD14</td>
<td>CD15</td>
<td>CD11b</td>
<td>CD163</td>
<td>CD206</td>
</tr>
<tr>
<td>Dendritic Cells</td>
<td>17</td>
<td>CD45</td>
<td>CD83</td>
<td>CD205</td>
<td>CD209</td>
<td>CD14</td>
<td></td>
</tr>
<tr>
<td>DCs (and Monocytes)</td>
<td>6</td>
<td>CD45</td>
<td>CD83</td>
<td>CD205</td>
<td>CD209</td>
<td>CD14</td>
<td></td>
</tr>
</tbody>
</table>

3.2.3.3 Immunophenotyping

Cells were counted to verify that at least 1 million cells per mL and between 300,000 and 1 million cells per tube were present. The cell suspension in protein block was evenly divided into the unstained, experimental, FMO, and isotype tubes with antibody combinations prepared as described. Cells were incubated with antibodies in the dark at 4°C for 30
minutes, washed with PBS (5 minutes at 2000rpm, 4°C) and re-suspended in a stabilizing-fixative buffer (containing 1% formaldehyde, BD Biosciences) and left in the dark at 4°C overnight. The stabilizing-fixative was used to prevent the dissociation of the component dyes in the tandem fluorochromes (ie. APC'-Cy7).

3.2.4 Flow Cytometry Instrument Setup and Sample Data Collection

Prepared cells were analyzed by flow cytometry within 48 hours (typically 12-24 hrs) of labeling using a FACSARia flow cytometer equipped with two excitation lasers, a 15mW 488 nm (blue) solid state laser and a 15mW 633 nm (red) helium-neon laser, coupled to its corresponding detection optics assembly (Table 3.3), a 70 µm nozzle under 70 psi sheath pressure and FACSDiva software, version 6.1 (BD Biosciences). Baseline PMT voltages were established at the start of the project using BD™ Cytometer Setup and Tracking Beads (CS&T beads) and FACSDiva software; and verified with unstained cells and cells stained with the full antibody panel. To allow for doublet discrimination, area, height and width measurements of light scatter signals, forward scatter (FSC) and 90 degree side scatter (SSC), were collected; only area measurements were collected for all other fluorescence signals. Threshold gates were set on FSC and CD45 fluorescence, when present, to exclude debris and non-leukocyte events.

Table 3.3 Excitation lasers and detection filters for each fluorochrome.

<table>
<thead>
<tr>
<th>Antibody Specificity</th>
<th>Fluorochrome</th>
<th>Excitation Laser</th>
<th>Emission detector filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>AlexaFluor 488</td>
<td>488 nm</td>
<td>530/30</td>
</tr>
<tr>
<td>CD4</td>
<td>APC’</td>
<td>635 nm</td>
<td>660/20</td>
</tr>
<tr>
<td>CD8</td>
<td>PE</td>
<td>488 nm</td>
<td>576/26</td>
</tr>
<tr>
<td>CD11b</td>
<td>PE-Cy5</td>
<td>488 nm</td>
<td>675/20</td>
</tr>
<tr>
<td>CD14</td>
<td>PE-Cy7</td>
<td>488 nm</td>
<td>780/60</td>
</tr>
<tr>
<td>CD15</td>
<td>FITC</td>
<td>488 nm</td>
<td>530/30</td>
</tr>
<tr>
<td>CD25</td>
<td>PE-Cy5</td>
<td>488 nm</td>
<td>675/20</td>
</tr>
<tr>
<td>CD45</td>
<td>APC’-Cy7</td>
<td>635 nm</td>
<td>780/60</td>
</tr>
<tr>
<td>CD56</td>
<td>PE-Cy7</td>
<td>488 nm</td>
<td>780/60</td>
</tr>
<tr>
<td>CD83</td>
<td>APC’</td>
<td>635 nm</td>
<td>660/20</td>
</tr>
<tr>
<td>CD163</td>
<td>PE</td>
<td>488 nm</td>
<td>576/26</td>
</tr>
<tr>
<td>CD205</td>
<td>PE</td>
<td>488 nm</td>
<td>576/26</td>
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<tr>
<td>CD206</td>
<td>APC’</td>
<td>635 nm</td>
<td>660/20</td>
</tr>
<tr>
<td>CD209</td>
<td>PerCP-Cy5.5</td>
<td>488 nm</td>
<td>695/40</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>PE-Cy5</td>
<td>488 nm</td>
<td>675/20</td>
</tr>
</tbody>
</table>
3.2.4.1 Data Analysis

Flow cytometry data were collected as .FCS 3.0 data files and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Cellular data from each tube was analyzed consistently within patient samples by first identifying single leukocytes based on their light scatter properties and positive CD45 signal (common leukocyte antigen: CLA) and then identifying the leukocyte subpopulations based on the antibody panel used.

In order to analyze only single cell events from the collected data in each tube, a contiguous gating tree strategy was applied. Gate 1 was based on forward scatter-area (FSC-A) and side scatter-area (SSC-A) to eliminate debris, dead cells and very large cellular aggregates (Figure 3.1a). Gates 2 and 3 were drawn on height versus width of SSC (Figure 3.1b) and FSC (Figure 3.1c), respectively, to include the full range of height signal but a narrow range of width signal in order to eliminate any aggregates of cells. As a CD45 threshold trigger was applied during data acquisition, CD45 versus SSC-A plot of cells remaining in Gate 3 was drawn to verify that the CD45 mean fluorescent intensity (MFI) was $>10^2$ (Figure 3.1d).

Once gating was performed FMO, isotype, and unstained controls were used to ensure correct identification of a positive fluorescent signal for each cell population (Figure 3.2). Unstained cells were used to identify negative and auto-fluorescence of decidual leukocytes alone (Figure 3.2 A). Isotype controls (Figure 3.2 B) and FMOs (Figure 3.2 C: CD3, D: CD8) were used to determine where gates should be placed to separate positive
cell populations from non-specific binding, background and antibody interaction. The use of these different controls results in the proper identification of positive (ie. CD3⁺) and double-positive (ie. CD3⁺CD8⁺) cell populations in the experimental tubes (Figure 3.2 E) where decidual leukocytes are incubated with all the antibodies of interest for each cell type (lymphocytes, monocytes & granulocytes and dendritic cells).

Additionally, I used CD19 as a marker of B cells and since they should be absent in the decidua this provides a good indicator of maternal blood contamination. The percentage of B cells was always very low (<2%) and should be less than 5% (59).

3.2.5 Statistical Analysis

In order to determine whether changes in leukocyte phenotypes occurred with advancing gestational age Linear Regression analyses were performed using each replicate and individual data point for the cell populations of interest. Data are presented as raw proportions of the total decidual leukocyte (CD45⁺) population using the line of best fit to indicate trends/correlations and correlation coefficients to indicate tightness of data. Comparisons of first and second trimester mean leukocyte proportions were analyzed by unpaired t-test and data are presented as mean ± standard error of the mean (SEM).
3.3 Results

Immunophenotyping by flow cytometry was conducted on leukocytes isolated from decidual tissue samples at 5 to 20 weeks of gestation (n=17). Specific subpopulations of CD45⁺ leukocytes were evaluated and then analyzed by both linear regression (with advancing gestation age: summarized in figures) and unpaired t-tests comparing first and second trimester (data presented as mean±SEM in text). To help visualize the difference in cell populations, pseudocolour dot plots are used to illustrate the fluorescent intensity and density of specific cell populations.

3.3.1 Uterine Natural Killer cells decline with advancing gestational age

In the first trimester, uNK cells comprise approximately 50% of all leukocytes in the decidua. This proportion decreases by nearly half between first (5-12 weeks, n=8, 47.7±2.6) and second (13-20 weeks, n=9, 27.0±2.9) trimester (p<0.0001). When proportions were analyzed by linear regression for gestational age, there was a significant negative correlation with advancing gestational age (Figure 3.3 A: r= -0.749, p= 0.0005).

![Figure 3.3 Uterine Natural Killer cell populations decrease with advancing gestation. A: There was a significant decrease in uNK cell proportion of total decidual leukocytes from early to mid pregnancy (p=0.0005; r= -0.749). This decrease is illustrated by pseudocolour dot plots of CD45 (CLA; x-axis) vs. CD56 (uNK; y-axis) fluorescence intensity at 7 (B) and 19 (C) weeks of gestation.](image)
This decline is also illustrated by the decreased density and tightness of the CD56\(^+\) cell population shown in the CD45 (CLA; \text{x-axis}) vs. CD56 (uNK; \text{y-axis}) dot plot at 19 weeks (Figure 3.3 C) compared to 7 weeks (Figure 3.3 B).

3.3.2 Decidual lymphocyte populations increase from early to mid pregnancy

The T lymphocytes account for approximately 10% of the total decidual leukocytes in the first trimester (10.0±1.1) and increase two-fold (p= 0.0005) by the second trimester (20.2±1.9). Lymphocytes demonstrate a positive correlation, represented by an increasing slope across gestation (Figure 3.4 A: p= 0.0007) and a correlation coefficient of 0.739. This increase in T cells can be clearly identified by the difference in density of cells between CD3 (T cells; \text{y-axis}) vs. CD45 (\text{x-axis}) dot plots at 7 (Figure 3.4 B: 11.4\%) and 19 weeks (Figure 3.4 C: 29\%). When we investigated the proportion of helper (CD4) vs. cytotoxic (CD8) T cells a significant increase in CD4\(^+\) cell proportion over the first 20 weeks of gestation was found (Figure 3.4 D: r= 0.723, p= 0.0015). There was no corresponding increase in CD8\(^+\) cells across the same gestation time frame (Figure 3.4 E: r= 0.0965, p= 0.722). The CD3\(^+\) T lymphocyte population in the first trimester is almost entirely accounted for by the CD4\(^+\) T helper cell (Th: 4.2±0.5) and CD8\(^+\) T cytotoxic cell (Tc: 5.0±0.7) populations. While there is a significant, two-fold increase in CD4\(^+\) cells in the second trimester (p= 0.0026; 8.6±1.1) to account for this, the lack of a significant increase in CD8\(^+\) cells (7.5±2.3; p= 0.316) leaves a portion of the second trimester CD3\(^+\) population unaccounted for by Th and Tc cells. When the CD3\(^+\) population is shown on a dot plot of CD4\(^+\) (\text{y-axis}) vs. CD8\(^+\) (\text{x-axis}) markers, it is clear that these cell populations are distinct from one another (Figure 3.4 F: 7 weeks) and present in nearly equal proportions.

Therefore, we next investigated the CD4:CD8 or Th:Tc cell ratio as a characteristic measure of the adaptive immune environment. Although there is a trend to increase, this ratio does not significantly change across gestation (Figure 3.4 G; p= 0.1353; r= 0.39). The mean ratios are consistently >1 in both first (1.2±0.2) and second trimester (1.5±0.3) and are not significantly different between trimesters (p= 0.4336).
Upon recognizing the unaccounted for CD3+ T cell population, I included CD25 in the antibody panel to identify CD4+CD25+ Tregs as a potential contributor to the increased lymphocyte population. We found a small population that represents approximately 3% of decidual leukocytes (6-14 weeks, n=6, 2.9±0.4) and does not significantly change across the gestational ages investigated so far (Figure 3.4 H). These cells more dimly express...
CD4 than CD3⁺CD4⁺CD25⁻CD8⁻ cells and represent less than 50% of the CD3⁺CD4⁺ cells (Figure 3.4 I; 10 weeks).

Another subset of lymphocytes is the NK T population. The mean proportion of these cells does not significantly change from first (2.2±0.7) to second (3.5±1.0) trimester (p= 0.29) and does not demonstrate a significant correlation with gestational age (Figure 3.5 A: r= 0.137, p= 0.6136). This small portion of CD56⁺ NK cells express low levels of the CD8 cell surface receptor and can be clearly seen as a separate population in the CD56 (y-axis) vs. CD8 (x-axis) dot plot shown in Figure 3.5 B (15 weeks).

![Figure 3.5 Decidual NKT cell populations. A: A subset of CD56⁺ NK cells dimly express the CD8 cell surface receptor and are present in low numbers in the first 20 weeks of pregnancy (r= 0.137, p= 0.6136). This unique population is clearly distinct from the typical uNK (CD56⁺) and cytotoxic T cell (CD8⁺) populations, seen in B (15 weeks).]

3.3.3 A novel second trimester decidual neutrophil population

Perhaps the most striking difference in cell types across gestation is the large and statistically significant increase in CD15⁺ neutrophils (Figure 3.6 A: r= 0.696, p=0.0027). In the first trimester of pregnancy, these cells represent 7.9±1.5 percent of total decidual leukocytes; a proportion that significantly increases to 27.9±4.8 in the second trimester (p=0.0014). This large infiltration of neutrophils is clearly illustrated by the CD15⁺ cells seen in the CD15 (y-axis) vs. CD45 (x-axis) dot plots at 7 (Figure 3.6 B; 2.73%) and 19 weeks (Figure 3.6 C; 24.9%).
3.3.4 Decidual macrophages decline with advancing gestation

Decidual macrophages comprise the second largest subpopulation of leukocytes during the first trimester and decrease with advancing gestation. This is demonstrated by a significant decrease in both CD14+ (Figure 3.7 A: r= -0.537, p=0.0321) and HLA-DR+ (Figure 3.7 B: r= -0.5, p=0.046) cells over the first half of gestation. This population significantly decreases from approximately 30-35% of decidual leukocytes in the first trimester to 15-20% in the second (CD14: 30.3±5.4 to 15.4±2.7, p=0.0265; HLA-DR: 35.0±3.6 to 19.6±3.2, p=0.0064). These proportions are consistent as the majority of decidual macrophages are double positive for CD14 and HLA-DR, as seen in the CD14 (y-axis) vs. HLA-DR (x-axis) dot plot (Figure 3.7 C: 9 weeks). The proportion of cells positive for the classical (CD163) and alternative (CD206) macrophage activation markers do not significantly change over the first 20 weeks of gestation (Figure 3.7 D, E) and make up...
4.3±0.5 and 4.1±0.4% of decidual leukocytes, respectively. Interestingly, expression of these surface molecules remains constant despite the decrease in CD14⁺ and HLA-DR⁺ cells. Therefore the proportion of CD14⁺HLA-DR⁺ cells that are also positive for CD163 and CD206 demonstrates a positive correlation across the first half of gestation (Figure 3.7 F: r=0.592, p=0.02) from 6.7±0.9% in the first trimester to 17.6±3.3% in the second trimester (p=0.011). This data suggests that there is a phenotypic shift in the decidual macrophages between the first and second trimesters. Alternatively one phenotype may persist while the other declines.

![Figure 3.7 Macrophage populations decrease with advancing gestation](image)

In order to elucidate potential gestational differences and investigate macrophage recruitment from the periphery, dot plots of CD14 vs. HLA-DR expression are presented at increasing gestational ages from 6 to 19 weeks in Figure 3.8. A very early sample has many CD14⁺HLA-DR⁻ cells (Figure 3.8 A: 6 weeks) that appear to differentiate and become activated as nearly all CD14⁺ cells are CD14⁺HLA-DR⁺ after 8 weeks (Figure 3.8 B, C, D,
E). This needs to be corroborated by increasing the number of samples investigated at this early gestational age. There is a greater proportion of CD14^-HLA-DR^+ cells at 8 and 9 weeks (Figure 3.8 B: 19.1% and C: 9.3%) than at later gestational ages. Interestingly, a small but distinct CD14^-HLA-DR^- cell population presents again by 16 weeks and is detected in 18 and 19 week samples as well (Figure 3.8 F, G, H). This population may represent the small amount of blood contamination that is inevitable in the second trimester samples. More samples are needed to delineate the true pattern of CD14 and HLA-DR expression in these cells.

3.3.5 Decidual dendritic cells maintain an immature phenotype

Dendritic cells present in the decidua were immunophenotyped using the markers CD209 (DC-SIGN), CD205 (DEC-205), and CD83 to identify maturation by potential for antigen uptake and presentation. In the first 20 weeks of gestation there was no significant change in the proportions of either the large CD209^+ immature dendritic cell population (Figure 3.9 A: 24.6±2.7%) or the small CD83^+ mature dendritic cell population (Figure 3.9 C: 1.6±0.2%). There was however a significant negative correlation of CD205^+ intermediate...
dendritic cell proportion with advancing gestation age \( (r = -0.603, p=0.0104) \). This represented a significant reduction by nearly half from first (14.3±2.5%) to second (7.8±1.1%) trimester (\( p=0.024 \)).

3.3.6 Activation State of Decidual Leukocytes

Activation status of the decidual leukocytes was determined by expression of \( \alpha_\text{M}\beta_2 \) integrin (Mac-1: CD11b). This integrin was detected on several of the decidual leukocyte subpopulations and varied between patient samples. Despite a trend towards an increase, expression of CD11b did not significantly change over the first 20 weeks of gestation (Figure 3.10 A: \( r=0.35, p=0.17 \)). The CD11b+ cell population accounts for 39.3±3.4% of total decidual leukocytes. Differential expression by the major leukocyte subtypes is shown at the gestational age when each is most abundant (Figure 3.10). In late gestation the large CD15+ neutrophil population strongly express CD11b (Figure 3.10 B: 14 weeks). As these cells infiltrate the decidua around 13 weeks, they may contribute to the increasing trend. CD14+ macrophages also express CD11b (Figure 3.10 C: 10 weeks) and may account for the majority of CD11b cells in the first trimester. Interestingly, the CD56+ uNK cells (Figure
3.10 D: 8 weeks) dominant in the first trimester decidua and CD3⁺ lymphocytes (Figure 3.10 E: 16 weeks) which increase in the second trimester do not express CD11b.

Figure 3.10 Decidual leukocyte activation marker (mac-1 integrin) expression. Expression of CD11b by decidual leukocytes does not significantly change in the first 20 weeks of gestation (A: p=0.17; r=0.35). CD15⁺ neutrophils (B: 14 weeks) highly express CD11b and CD14⁺ macrophages (C: 10 weeks) dimly express CD11b while most CD56⁺ uNK cells (D: 8 weeks) and CD3⁺ T lymphocytes (E: 16 weeks) are CD11b⁻.
3.4 Discussion

In this chapter, multi-colour flow cytometry immunophenotyping was employed to provide quantitative information about the leukocyte composition of the decidua. The leukocyte phenotypes and distributions display characteristic differences from early to mid pregnancy coincident with changes in the specific requirements and adaptations of the maternal fetal interface. Some of the most significant changes we observed between first and second trimester were the increased proportion of CD3+ T lymphocytes, in particular CD4+ T helper cells, differentiated M2 macrophages, and the dramatic increase in neutrophils. We also observed significant decreases in the proportions of uNK cells and several markers for immature macrophages and dendritic cells. Both macrophages and neutrophils strongly expressed the Mac-1 integrin, which is a marker for activation of peripheral leukocyte populations as they extravasate from the vasculature.

One of the most substantial changes from first to second trimester is the gradual decrease in proportion of uNK cells. Many studies have similarly reported that the uNK cell population in the decidua is greatly decreased by term [reviewed in (186)] however no studies have identified when this decline occurs. In mice, uNK cells are known to decline after mid-gestation (78). The flow cytometry results presented in this chapter support the mouse data, by demonstrating that in humans, a reduction in uNK cell proportion by nearly half from ~50% of decidual leukocytes in the first trimester to only ~25% in the second trimester occurs. While the cyclic changes in uNK cells in the endometrium are under the influence of progesterone (59, 61), the progressive decline must occur by another mechanism as progesterone levels are maintained and increase with advancing gestation. Since uNK cells have proposed roles in both immune tolerance, communicating with EVT and dendritic cells to suppress a maternal response, and spiral artery remodeling, their abundance in the first trimester indicates that this is the critical period of their contribution to these processes. Since spiral artery remodeling is complete by approximately 20 weeks of gestation, the uNK cell population probably continues to steadily decline into the later second and early third trimester. Additionally, the homogeneity of the uNK cell population observed in first trimester is reduced in the second trimester when the cells less brightly
express CD56. A functional shift or dedifferentiation of uNK cells in the second trimester is supported by studies that suggest the receptors critical to regulation of trophoblast invasion and immunotolerance are downregulated after the first trimester (77) and reports of differential cytokine production between the first and second trimesters (185).

Interestingly, the decrease in uNK cell proportion is coincident with a large infiltration of neutrophils. No other research groups have described decidual neutrophils in mid or late human pregnancy. In early pregnancy, neutrophils are known to comprise a small population of decidual leukocytes, which our first trimester findings (7%) support. The novel increase to >25% of decidual leukocytes suggests an important role at mid pregnancy. Neutrophils have been previously described in the cycling endometrium. In the late stages of the cycle they are potent producers of angiogenic factors and are thought to promote vascular repair and vasculogenesis (123, 124). The neutrophils in the second trimester may perform a similar role aiding in the repair of late stage remodeling spiral arteries. It is known that following loss of the VSMC and replacement of the endothelium by trophoblast a re-endothelialization occurs (22). In addition, research in tumor progression has identified two populations of neutrophils: a protumor type with both tolerogenic and angiogenic properties, and in contrast a more classical inflammatory neutrophil subtype with antitumor properties (119). As the cytokine environment is predominantly anti-inflammatory and tolerance-promoting at the maternal fetal interface a unique supportive phenotype of neutrophils involved in vascular repair would make sense. The cells appear to be recruited from the circulation as they only infiltrate after the maternal circulation opens to the intervillous space. These cells may be recruited due to low grade inflammation associated with the remodeling arteries and CD11b expression provides a mechanism of extravasation and may differentiate in situ post-recruitment. Subsequent studies in the laboratory have confirmed that decidual neutrophils are indeed resident in second trimester decidua and express a different chemokine receptor profile to that found in peripheral blood in that they down-regulate both CXCR1 and CXCR2 (IL-8 receptors) and up-regulate CXCR3 and CXCR4 (CXCL10/IP-10 and CXCL11/SDF-1 receptors) [Amsalem, unpublished data].
Another major change in the decidual leukocyte populations between first and second trimester is the 2-fold increase in T lymphocytes from 10 to 20%. Human studies have consistently described a “static” 10% of T cells during the menstrual cycle and in the early implantation site [reviewed in (186)]. However, studies in mice have also identified an increase in decidual T cells in the second half of pregnancy (187). Our findings support these mouse studies and contribute further knowledge about decidual T lymphocyte populations during human pregnancy. In the first trimester, the total percentage of CD3+ T cells is almost entirely accounted for by the roughly equal CD4 helper T cell and CD8 cytotoxic T cell populations. Interestingly, the proportion of cytotoxic T cells does not significantly change with advancing gestation, while the proportion of helper T cells is doubled. This is of interest because of the instrumental role that the helper T cells play in dictating the high Th2:Th1 cytokine ratio in the decidua: a property that is considered to contribute to the maintenance of successful pregnancy (60, 85). Another parameter we investigated was the CD4:CD8 ratio. This is a common measure of immune system health and a marker of infection. In peripheral blood of healthy individuals this ratio is rarely observed below 1. The most concerning change in this ratio is a decrease, which indicates either an increase in cytotoxic T cells, a decrease in helper T cells, or both all of which are common responses to EBV, pneumonia, influenza, HSV, and HIV infections, or cancer chemotherapy (84). In our decidual samples the mean CD4:CD8 ratios did not significantly change with advancing gestation and were consistently around or above a ratio of 1. This consistent result is interesting and may serve as a baseline for complicated pregnancies or changes at term in future studies.

Additionally, two smaller subpopulations of lymphocytes were investigated to determine the phenotype of the cells that comprise the remainder of the increase in CD3+ T cells. The addition of the CD25 marker for identification of Tregs was used in the most recent experiments, but only detected on a small proportion of cells and does not demonstrate any relationship with gestational age. The CD4+CD25+ cells are an interesting population that is being thoroughly investigated by many other groups for their roles in immune suppression and maternal tolerance. The other uncommon lymphocyte subset we
investigated was the NKT cell population. While the proportion of these CD56^+CD8^{dim} cells was quite variable, accounting for between 1 and 8% of decidual leukocytes, there was no increasing trend and there seems to be a great deal of patient variability in both percentage as well as identification/separation of the population. While this population may help to contribute to the increased T cell population observed, in the future I will investigate other potential contributing lymphocyte subsets such as γδ T cells and Th17 cells. Th17 cells are capable of inducing tumor regression (potentially analogous to inhibiting EVT invasion) and secreting IL-17, which is able to recruit, activate, and induce migration of neutrophils (188). This population has been described in peripheral blood during pregnancy (CD4^+CD25^+:Th17 ratio increases, but not in preeclampsia) but has not been investigated in the decidua. It will be interesting to see if these cells perhaps have a role in early pregnancy.

The final two types of leukocytes immunophenotyped fall into the category of APCs: macrophages and dendritic cells. These cells differentiate from a common monocyte precursor that expresses CD14, DC-SIGN (CD209) and HLA-DR. Macrophages typically lose DC-SIGN expression as they acquire a predominantly phagocytic role and dendritic cells typically lose their CD14 expression as they mature, take up antigen and eventually become mature potent antigen presenters (126, 149, 151, 155). Thus, the traditional markers of differentiation for both cell types were investigated in this study and a decrease in the cells co-expressing CD14, HLA-DR, and DEC-205 (CD205) was observed between first and second trimester decidua. There was a trend towards a decrease in the expression of DC-SIGN (CD209) as well. The fluorochromes for the HLA-DR and CD209 antibodies overlap and so could not be combined in the same panels but most CD14 cells were positive for both HLA-DR and CD209, supporting recent reports of APCs with a monocyte-derived macrophage/dendritic cell-like phenotype (140). This and other studies have described overlap in expression of activation, antigen uptake and presentation markers between decidual macrophages and dendritic cells (125, 189, 190). These unclassifiable cells were also described to sometimes possess DEC-205, an antigen uptake receptor characteristic of dendritic cells of intermediate maturity (155).
receptor is part of the mannose receptor family, as is MMR (CD206), which was also found on a small proportion of decidual leukocytes, and is typically an antigen uptake receptor expressed by alternatively activated M2 macrophages (128, 129). However, both of these antigen uptake receptors have at different times been described on macrophages as well as dendritic cells. Thus far, the major APCs described in first trimester decidua have been macrophages (59, 63). It is possible that the immature cells identified in this and other studies remain able to differentiate into either macrophages or dendritic cells depending upon their cytokine environment (155). Since Th2 cytokines are the dominant influence, it is possible that these cells are maintained in a previously un-described phenotype as they are in a distinct environment from the Th1 cytokine environment of a typical inflammatory response. In fact, it appears that many of the tolerant and immature receptors are expressed on both types of cells and that the only definitive markers of each are CD163 on classically activated macrophages and CD83 on mature dendritic. However both of these are expressed by relatively few cells in the decidua at early and mid pregnancy. The major conclusion that can be made regarding this ambiguous immature cell type is that there is a decline in their proportion (HLA-DR, CD14) and antigen uptake capacity (DEC-205). The combination of the Th2 cytokine environment maintaining their immature state, the absence of classical self-recognition HLA molecules (HLA-A, HLA-B) on EVT and the ability of immature cells to induce antigen-specific tolerance by T cells suggests that a critical period of maternal recognition of trophoblast and suppression of an immune response occurs in the first trimester, when the proportion of these cells is highest. Their decline in the second trimester may help to prevent interaction with the growing population of T cells, preventing presentation to T cells and stimulation of a systemic immune response.

While there was a decrease in these general markers, more specific markers of macrophage activation, CD206, CD163 did not demonstrate any change with gestational age. Thus, as the number of immature cells decline, the proportion of CD206+CD163+ macrophages within the CD14+HLA-DR+ population demonstrates a positive correlation with gestational age, increasing by nearly 3-fold. Therefore the decidua may retain more mature/differentiated macrophages even though the total proportion is declining. This is
also supported by the phenotypic analysis of “macrophages” where a less heterogeneous CD14\(^+\)-HLA-DR\(^+\) population is observed in the second trimester. As well, the mature dendritic cells detected represent 2-3% of decidual leukocytes and this proportion does not change between early and mid pregnancy. It is possible that these mature/differentiated macrophage and dendritic cell populations are present in the event of an antigenic/pathogenic challenge such as infection to protect both mother and fetus from the invading pathogen. This would support the idea that while progesterone maintains a tolerant environment there are latent cells capable of responding to infection in a classical manner. Finally, several studies have investigated potential differences in immune cell proportions between decidua basalis and decidua parietalis in the first trimester to determine the impact of obtaining decidua basalis vs parietalis on results. Leukocytes were uniformly distributed, except for macrophages, which were sparse in the decidua parietalis (186). This may explain the difference of one sample that had significantly less CD14\(^+\) and HLA-DR\(^+\) cells in early pregnancy when these cells are abundant (7 weeks).

In conclusion these decidual APC populations are very heterogeneous and atypical due to the unique cytokine influences. Therefore, it is hard to distinguish whether the cells expressing different antigen-uptake and activation markers are indeed macrophages or dendritic cells. Our data supports evidence of an intermediate cell type that may possess the ability to differentiate into either macrophages or mature dendritic cells dependent upon the decidual microenvironment. I hypothesize that in pathological pregnancy complications such as preeclampsia and IUGR a more mature phenotype of these cells will predominate, such as CD163\(^+\)CD206\(^-\) macrophages and CD83\(^+\) dendritic cells. Recent studies have reported an increase in both immature and mature dendritic cells in preeclamptic PBBx’s (162).

Lastly, we investigated expression of the activation marker Mac-1 (CD11b) that is a \(\beta\)2 integrin, a constitutive complement receptor (CR3) and a receptor for the vascular ligands ICAM-1 and ICAM-2. Both of these adhesion molecules are expressed by endothelium and ICAM-1 expression by endothelial cells increases in inflammation (191). We found that
Mac-1 integrin expression was quite variable on decidual leukocytes, but there was a trend towards an increase in expression between early and mid pregnancy, likely because of the increased proportions of cells that co-expressed it. In particular we observed uniform bright expression by neutrophils and dim expression by the CD14+ macrophage/dendritic cell-like leukocytes. This integrin is implicated in the extravasation of cells from vessels by adhesion to ICAM-1 & -2 on endothelial cells. Therefore our observations provide an interesting way of delineating which cells may be recruited from peripheral maternal circulation and those that are resident to the decidua or proliferate in situ. Thus, these results help to support the theory of resident populations of uNK and T cells as these specific populations are mostly negative for Mac-1. Interesting studies in mice have shown that at mid gestation maternal vessels have very high expression of ICAM-2 but low expression of inflammatory ICAM-1. This may suggest promotion of peripheral leukocyte adherence and recruitment into the implantation site. By term (d20), ICAM-2 expression is weak as well and the number of Mac-1+ monocytes is also greatly reduced. This suggests that recruitment of peripheral leukocytes is maximal at mid gestation and supports our findings of increased cell types that are likely recruited from maternal circulation. As well, similar to our uNK cell findings mouse uNK cells (GMG) were Mac-1 negative a d9 (175).

As well, CD11b is expressed by all non-lymphocytes as well as T cells once they are activated, so an upregulation of CD11b expression by T cells may indicate a more activated state in the decidua. This will be of particular interest as we investigate the decidual leukocyte populations at term and in pathologies. One potential confounding factor of CD11b detection is that mechanical stress (mincing, centrifuging, or vortexing, prior to fixation) during cell isolation and processing may result in increased upregulated expression. This would result in an increased reporting of Mac-1 integrin however, the highly cell-specific expression by neutrophils and macrophage/dendritic – like cells may indicate that this is not a concern in our experiments. Macrophages typically are involved in recruitment of neutrophils and this would provide a novel function for this abundant population of tissue macrophages during the first trimester and would explain their subsequent decline after the neutrophils being to infiltrate. Neutrophils can positively feedback and are potent promoters of their own recruitment and proliferation (192).
One important note is the critical change in environment between 11-13 weeks when the intervillous space opens to maternal circulation. Not only is this accompanied by an increase in oxygen tension, but it also provides ample opportunity for many peripheral blood leukocytes to be recruited into the decidua. This may explain why there are dramatic changes observed, particularly in uNK cells and neutrophils, at this time. The actual duration of trophoblast plug dissolution and gestational age when this occurs may exhibit patient variability and help to explain the overlapping trends during this time frame. Another factor that may influence our results is the collection of decidua basalis versus parietalis, as studies have shown that a few (but not most) immune cell subpopulations are scarce in the areas of decidua further from the placenta (parietalis).

While these experiments provide information about the leukocyte proportions in the decidua, they do not measure the whole number of leukocytes in the decidua. This may influence the results as a dramatic increase in one population can cause a perceived decline in another population, however, the consistent results and large changes observed in this chapter are very well supported by the literature and their intensity of surface marker expression. Smaller changes in proportions are the most likely to be artifact. However, most clinical measures are now reported in proportions. For example, white blood cell counts used in the detection of infection and leukemia are measured as percentages. Thus, the data presented here likely represent real effects, as even proportional changes will still dictate what the dominant cells and cytokines will be in a given environment. One new measure that may provide some interesting insight would be to measure the weight of the tissue collected prior to leukocyte isolation and determine the whole number of CD45+ cells in that specimen. This would provide a “density” of immune cells per gram of decidua, indicating how much of the decidua is comprised of leukocytes across gestation. While there are clearly many ways in which to evaluate the leukocyte composition of the decidua, the important information lies in how populations change across gestation as this is most likely related to their functional role in the various processes critical within each of these time frames during pregnancy.
This chapter has ultimately uncovered some novel and very interesting changes in the decidual leukocyte populations between early and mid gestation. Further studies of decidual leukocytes from term samples and pathologies will help to complete the story of how these cells cycle, are trafficked and participate in the gestational age-specific processes at the maternal fetal interface. Occasionally, dramatic increases or decreases in certain leukocyte subtypes are observed that may indicate either infection or a pathologic pregnancy that went undiagnosed due to elective termination. These samples support our future hypothesis that deviations from healthy pregnancy immune cell phenotypes may be detected in early pregnancy, meaning that intervention would be possible to address aberrant changes in these critical players in pregnancy.
Chapter 4

Summary and Future Directions
4 Summary and Future Directions

4.1 Summary and Conclusions

In summary this thesis has shown that:

1) In the PDC model, vascular remodeling in decidual explants occurs only when co-cultured with a placental explant and remodeling of arterioles progresses from the point of placental contact towards the basal decidua.

2) Decidual macrophages and uNK cells demonstrate a significant dense infiltration of the vascular wall of remodeling arterioles prior to the invasion of enEVT compared to unremodeled deep vessels, decidual explant controls, and advanced stage remodeling arterioles.

3) Several leukocyte functions may contribute to vascular remodeling as determined using the PDC model, including MMP-mediated ECM breakdown and resultant disruption, apoptosis and phagocytosis of vascular cells.

4) Leukocyte subtypes exhibit characteristic proportions that correlate with advancing gestation and a dramatic shift in leukocyte composition of the decidua occurs around 11 to 13 weeks of pregnancy.

The experiments outlined above have demonstrated and further support a necessary and interesting contribution of the maternal immune system to healthy development of the decidua during pregnancy. In Chapter 2, I demonstrated a quantifiable method for investigating arteriole remodeling in the decidua using the PDC model. These experiments showed that remodeling begins in superficial portions of arterioles at the epithelial surface of the decidua where placental contact is established and progresses towards the basal decidua with time in culture. These changes were not observed in decidual explants that were not co-cultured with placenta. Additionally, this model demonstrates changes
induced by endovascular EVT invasion only, as no interstitial EVT invasion is observed. In the actively remodeling arteries, I observed a statistically significant dense infiltration of both uNK cells and macrophages within the vascular wall coincident with disorganization and loss of VSMC and endothelium. These leukocytes were observed in portions of arterioles upstream of the invading placental trophoblast. In arterioles that were lined with EVT in place of endothelial cells, leukocytes were significantly reduced within the vascular wall compared with actively remodeling arterioles. Furthermore, evidence of VSMC and endothelial cell apoptosis in remodeling arterioles was detected. As well, dense clusters of phagocytic cells were observed in the peripheral areas around early remodeling arterioles that demonstrated VSMC loosening and individual VSMC in the stroma at increasing distances radially from the vascular wall. Work by a collaborating PhD student demonstrated that similar to in vivo observations, the vascular wall and nearby leukocytes in the PDC produce high levels of MMP-2 and MMP-2/9 enzymatic activity. When the functional role of this protease was investigated by inhibiting MMP-2/9 in the PDC model, I found that vascular remodeling and leukocyte infiltration did not occur, and the decidua resembled the decidua-only explants. Taken together, these results support a novel role for leukocytes in vascular priming and the early stage of remodeling prior to invasion and relining of the vessels by trophoblast. Communication between uNK and trophoblast by secretion of chemokines and cytokines may stimulate reciprocal recruitment as no changes are observed in the absence of a placental explant. UNK cells and macrophages also secrete angiogenic factors and proteases that can initiate changes including breakdown of ECM, which will induce VSMC migration away from the vessels and begin apoptosis. These dying vascular cells may signal phagocytosis by macrophages. Thus, I have demonstrated evidence of a novel and integral role for uNK cells and macrophages in the remodeling of spiral arterioles in early pregnancy and provided a model that can be used to investigate the cellular interactions that occur during this process.

In Chapter 3 immunophenotyping of leukocytes isolated from decidual samples between 5 and 20 weeks of pregnancy was performed. These experiments provided a comprehensive characterization of surface markers expressed by decidual leukocytes
across the first and second trimesters. This chapter also summarized the proportions that each cell type comprises of the total CD45+ decidual leukocyte population. In particular, the second trimester profile of decidual leukocytes was the first to directly quantify the total population of the decidua at this time. Most previous investigations of immune cells were from PBBx of early or term pregnancy and have focused on one or two cell types. This comprehensive study has focused on the identification of the various cell types and their proportions of the total leukocyte population. Should future studies yield new evidence of interesting phenotypes or double/triple positive subpopulations then the abundant data collected from the multiple staining within each panel can be reanalyzed to further classify these unique cells. One of the most significant and consistent findings was the pronounced decrease in uNK cells after the first trimester. A loss in CD56 MFI was also observed, supporting the hypothesis of a uNK cell phenotype change in the second trimester. The proportion of the macrophage/dendritic cell-like population that expressed both CD14 and HLA-DR demonstrated a statistically significant negative correlation with increasing gestational age. Interestingly, the critical role for both these cell types in early spiral artery remodeling, proposed in Chapter 2, is further supported by their decline at mid pregnancy at which point the remodeling process is complete. In the same transition from first to second trimester an impressive significant increase in neutrophils occurs. This infiltration occurs around the same time as the trophoblastic plugs disperse permitting maternal blood to perfuse the decidual arterioles and the intervillous space. It is possible that the neutrophils are being recruited from maternal blood at this stage supported by their expression of CD11b. Additionally, the proportion of T lymphocytes doubles between early and mid pregnancy accounted for in part by a large increase in helper T cells. There may be another subset of lymphocytes that helps to account for this increase in CD3+ cells and further investigations will attempt to uncover its identity. In this chapter I have demonstrated that healthy patients have characteristic distributions of leukocytes that change with advancing gestation. In addition, the dominant cell types observed in each trimester are likely related to the local changes and adaptations of the maternal fetal interface during these periods.
4.2 Future Directions

Ch. 2: Leukocyte-mediated vascular remodeling in vitro.

The data presented in Chapter 2 has been submitted as a manuscript to the American Journal of Pathology. We have received positive reviewers comments, which have been incorporated in this thesis. However, future studies that would further test the mechanism of VSMC loss during transformation would be to perform dual immunofluorescence staining of $\alpha$-SMA with desmin or vimentin to determine whether the VSMC observed at a distance from vessels are in fact dedifferentiating to a stromal cell phenotype via myofibroblasts. Furthermore, isolated decidual macrophages will be activated with placental conditioned media (PCM) and placed with VSMC and endothelial cells to determine whether trophoblast-derived factors are capable of stimulating macrophage-mediated phagocytosis of these vascular cells. To confirm the inhibition of MMP-2/9, we will establish a series of co-cultures including PCM in culture with decidua and MMP-2/9 Inhibitor in media rather than injected directly into decidua to see if the inhibitor can still exert its effects. As well, in situ zymography will be performed on the PDC MMP-2/9 Inhibitor experiments and in conditioned medium collected from the cultures to determine if there is truly reduced MMP activity.

Ch. 3: Immunophenotyping of decidual leukocyte populations in the 1st and 2nd trimesters.

While the leukocyte profiles established in Chapter 3 provide comprehensive information regarding the first half of pregnancy, further studies are needed to understand how these populations are distributed in the third trimester. More detailed immunophenotyping will be combined with isolation of specific leukocyte subtypes, analysis of chemokine production and chemokine receptor profiles in an aim to identify 1) how the leukocytes are being recruited, 2) how decidual leukocytes differentiate across gestation, and 3) the cellular functions/interactions of decidual leukocytes in the processes of vascular remodeling. As well, future studies will delineate changes in these populations in pregnancy pathologies including preeclampsia and IUGR compared to age-matched controls.
Firstly, immunohistochemistry will be performed on decidual samples collected at the time of immunophenotyping to determine the localization of the various leukocyte subpopulations. The decidual specimens from first and second trimester will also be compared to PBBx and decidual samples collected from term elective caesarean sections and term and pre-term preeclamptic and IUGR pregnancies. The collection of decidual specimens from these cohorts is underway and I have now performed flow cytometry immunophenotyping of decidual leukocytes from two healthy controls and two IUGR pregnancies. These experiments will comprise a second manuscript submission in combination with the first and second trimester data from Chapter 3 to provide a full gestational age leukocyte phenotype profile and a comparison to the changes observed in pathologies. Preliminary data from term samples also suggest that there may be a differential regulation of leukocyte profile/recruitment depending upon whether the patient is labouring when the sample is collected.

Further phenotyping at more advanced gestational ages will indicate whether the cell populations shift again with the adaptations in the third trimester at the maternal fetal interface. As well, addition of T lymphocyte subpopulation surface markers may help to identify other cells that play an important role in the second trimester and help to account for the increase in CD3\(^+\) cells observed. Another important observation will be how the CD14\(^+\)HLA-DR\(^+\) population changes at term as there is a decrease in the heterogeneity of these cells from the first to second trimester. An increase in macrophages has been reported at term and it will be helpful to provide a phenotype of these cells that may be related to their differing function later in pregnancy. The continued collection of decidual samples at the time of delivery from healthy term, preterm, and preeclamptic or IUGR pregnancies is an important step in identifying aberrant changes that may occur compared to established distributions of leukocytes in healthy patients. This may help to identify predictive markers for future development of uterine vascular pathologies as many of the leukocyte populations have putative angiogenic roles/properties in normal pregnancy. Ultimately, abnormal changes in cell types present or their activation states will provide potential biomarkers that can be used in the early detection of these diseases, prior to
clinical presentation. Additionally, if hostile or activated immune cells are determined to be present and are a causative factor in the progression of these disorders, then treatment could also be developed targeting these cells, their secreted cytokines, or their surface markers responsible for initiating an immune response against the trophoblast.

Additionally, migration experiments will be performed with isolated leukocyte subpopulations to determine migratory stimuli, and triggers able to induce leukocyte-mediated phagocytosis and apoptosis of vascular cells. We will investigate functional differences between leukocyte subtypes from pathologic decidua using in vitro experiments to look at their ability to induce trophoblast apoptosis in addition to the experiments described above. If functional and phenotypic differences of leukocytes present earlier in gestation, these could be used as diagnostic markers of preeclampsia/IUGR development or as targets for therapeutics. A recent study of uNK-induced endothelial cell apoptosis found that uNK cells isolated from decidua in pregnancies at higher risk of developing preeclampsia were less able to induce apoptosis than those isolated from healthy pregnancies (193). This may explain one relationship contributing to the impaired spiral artery remodeling described in uterine-vascular pathologies. Our proposed studies will attempt to uncover other critical cell types and cellular interactions in the development of preeclampsia and IUGR.

Furthermore, our laboratory will continue to perform mechanistic and phenotyping studies of the neutrophil population reported in Chapter 3. These studies will compare the phenotype of second trimester decidual neutrophils to peripheral blood neutrophils collected from women at 16-18 weeks of pregnancy and non-pregnant healthy female volunteers. This detailed immunophenotyping study will examine markers of neutrophils (CD15, CD66b) and chemokine receptors previously reported on neutrophils including IL-8 receptors CD181 (CXCR1) and CD182 (CXCR2), CD183 (CXCR3), CD184 (CXCR4), CD191 (CCR1) and CD195 (CCR5). Preliminary experiments have already demonstrated distinct differences in surface marker expression between decidual and peripheral neutrophil populations and shown that 2nd trimester decidual conditioned media (DCM) can
stimulate peripheral blood neutrophil migration and invasion while 1st trimester DCM has little effect. This project aims to identify the origin of these cells, their chemoattractant or the mechanism of their infiltration into the decidua and their role at the maternal fetal interface including a potential role in angiogenesis or later stages of spiral artery remodeling. These studies are of particular interest because studies have reported increased activation of peripheral neutrophils as well as increased neutrophil infiltration of systemic vasculature in preeclamptic women (194) indicating a role for these cells in pregnancy pathologies as well.

These proposed experiments will delineate the critical leukocytes in development of pathologies and characteristic leukocyte profiles across gestation. These studies are critical and their results will contribute immense knowledge to the field of reproductive immunology. As well, the leukocyte subtypes uncovered as important regulators of maternal fetal interface development at different stages, such as neutrophils, will help to direct the focus of future studies by our and other research groups.
Chapter 5

References


