Investigation of the role of intrauterine interleukin 6 and interleukin 8 in normal early pregnancy and sporadic miscarriage

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Abstract

Successful pregnancy requires tight regulation of two important processes: spiral artery remodelling and invasion of uterine tissue by placental extravillous trophoblast (EVT); disruption of these processes occurs in pregnancy complications such as sporadic miscarriage (SM). Maternal factors, including cytokines and growth factors, may play a role in regulating these fundamental events. The biological function of interleukins (IL)-6 and -8 in other physiological processes suggest that these cytokines maybe important mediators in uteroplacental tissues in early pregnancy.

The aim of this study was to investigate intrauterine IL-6, IL-8 and their receptors at the fetal-maternal interface in early pregnancy; specifically, to investigate their role in regulation of EVT invasion and spiral artery remodelling events and determine whether levels of cytokines are altered in SM.

IL-6, IL-8 and their receptors were localised in placental bed in the first half of pregnancy by immunohistochemistry. $CD56^+$, $CD14^+$, $CD10^+$ and $CD8^+$ cells were isolated from 8-10 and 12-14 weeks gestational age decidua using positive immunomagnetic separation and secretion of IL-6 and IL-8 was measured and compared by ELISA. All decidual cell types investigated secreted both IL-6 and IL-8. Decidual $CD14^+$ cells were the most abundant source of both cytokines. Secretion of IL-6 by $CD10^+$ cells ($P=0.005$) and IL-8 by $CD56^+$ cells ($P=0.02$) was higher at 12-14 weeks compared with 8-10 weeks GA. Compared with healthy pregnant decidua, secretion of IL-6 and IL-8 protein by both $CD14^+$ (IL-6 $P=0.05$; IL-8 $P<0.0001$) and $CD56^+$ cells (IL-6 $P=0.02$; IL-8 $P=0.003$) was reduced in SM ($\leq12^{+6}$ weeks gestation).

The functional role of these cytokines in early pregnancy was assessed with invasion assays and a chorionic plate artery (CpA) model of artery remodelling. IL-6 did not stimulate EVT invasion *in vitro* but did cause phosphorylation of kinase proteins involved in trophoblast cell signalling and reduced EVT secretion of RANTES. IL-6 and IL-8 both contributed to misalignment of VSMC in CpA ($P=0.04$; $P=0.001$ respectively) and IL-6 increased the separation of CpA VSMC layers alone ($P=0.02$) and combined to its receptor sIL-6Rα ($P=0.001$). $CD56^+$ and $CD14^+$ leucocytes were observed in the walls of spiral arteries in the first half of pregnancy with $CD14^+$ cells producing increased Ang-2 ($P=0.002$) and decreased Ang-1 at 12-14 weeks compared with 8-10 weeks GA ($P=0.03$).

Production of IL-6 and IL-8 within the placental bed in early pregnancy and reduced levels in SM, together with their effects on EVT and VSMCs suggests a possible involvement in controlling trophoblast function and contribution in regulating spiral artery remodelling events.

<u>Acknowledgements</u>

Abbreviations

ABC	Avidin-biotin complex
ANOVA	Analysis of variance
APC	Antigen presenting cell
APES	Aminopropyltriethoxysilane
APPAP	Alkaline phosphatase anti-alkaline phosphatase
CD	Cluster of differentiation
CpA	Chorionic plate artery
Cs	Charcoal stripped
CTB	Cytotrophoblast
CXCR	Chemokine receptor
DAB	3'-Diaminobenzidine
ECM	Extracellular matrix
eEVT	Endovascular extravillous trophoblast
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbant sandwich assay
ERK	Extracellular receptor signalling kinase
ERPC	Evacuation of retained products of conception
EVT	Extravillous trophoblast
FCS	Fetal calf serum
FFPE	Formalin fixed paraffin embedded
GA	Gestational age
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H & E	Haematoxylin and Eosin
HLA	Human leucocyte antigen
HUVEC	Human umbilical vein endothelial cells
iEVT	interstitial extravillous trophoblast
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-6Rα	Interleukin-6 receptor alpha
intEVT	intramural extravillous trophoblast
KIR	Killer immunoglobulin receptor
LIF	Leucocyte inhibitory factor
LILR	Leucocyte immunolglobulin like receptor
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibilty complex
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDGF	Platelet derived growth factor
PlGF	Placenta growth factor
POC	Product of conception
qRT-PCR	Quantitative real time polymerase chain reaction
RANTES	Regulated upon activation normal T cell expressed and secreted
RM	Recurrent miscarriage
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SEM	Standard error of the mean
sFlt-1	Soluble fms-like tyrosine kinase 1
SM	Sporadic miscarriage
SpA	Spiral artery
STAT	Signalling transducers and activators of transcription
TBS	Tris buffered saline
TGF-β	Transforming growth factor beta
Th	T helper cell
TIMP	Tissue inhibitor of matrix metalloproteinases
TNFα	Tumour necrosis factor alpha
TOP	Termination of pregnancy
uNK	Uterine natural killer
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle

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Chapter 1

Introduction

1.   Introduction

1.1 Overview of normal placental development

1.1.1 Implantation

Successful implantation depends on an ideal environment in the maternal endometrium reviewed by Loke et al., (1995). The endometrium achieves this by undergoing rapid, spontaneous changes during the luteal phase of the menstrual cycle, resulting in a decidualised endometrial stroma that provides the optimal environment for embryo implantation. This is a dynamic process involving a complex network of coordinating events regulated by autocrine, paracrine and endocrine factors, although the molecular interactions are not fully understood. Existing data derived from knock out animal studies have provided some insight of the physiological functions essential for embryo implantation; however, *in vivo* implantation studies in humans are not possible, resulting in a relative lack of understanding of defective implantation in humans.

Successful oocyte fertilisation ceases the menstrual cycle. The uterus becomes sensitive to implantation by the initial secretion of progesterone from the ovaries; known as the receptive phase, this allows the endometrium to decidualise and is characterised by smooth projections of uterine epithelium named pinopodes. Even if fertilisation is successful, implantation and successful pregnancy is not guaranteed; the maternal tissues and newly formed conceptus require a symbiotic chemical balance of growth factors, hormones and immunomodulators for implantation to occur. During this time the cells of the zygote undergo a series of mitotic cellular divisions resulting in the blastocyst, with successful implantation occurring approximately 6-7 days after fertilisation.

After fertilisation (between 3-6 days post fertilisation) the conceptus begins to undergo a series of cell divisions first reaching the morula (16-cell stage) then dividing into a 32-cell blastocyst consisting of two different cell populations: apical cells and basal cells. Following successful attachment of the blastocyst to the receptive endometrium, placentation is initiated. The surrounding hull of apical cells of the blastocyst contains an inner cell mass of basal cells that rapidly begin to proliferate and differentiate into an embryo and the amnion (Vitiello and Patrizio, 2007) , while the

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outer apical cells of the trophectoderm eventually give rise to the chorion. The blastocyst then attaches to the uterine endometrium by the embryonic pole (Boyd, 1970). Implantation involves attachment of the apical plasma membranes of the trophoblast cells to the apical plasma membranes of uterine epithelia (Aplin, 1991) and the blastocyst invades the uterine epithelium and implants into the endometrial stroma (Figure 1.1). During this time at day 8 post conception (the prelacunar stage; Figures 1.1a and 1.1b) trophoblast cells of the embryonic pole begin to proliferate and differentiate into two distinctive inner and outers layer of trophoblast cell populations encasing the developing mass of embryonic cells and amnion, termed embryoblast (Heuser, 1941). The outer layer of trophoblast cells fuse together to form a multinucleated trophoblast population which is in direct contact with the maternal tissues, known as syncytiotrophoblast cells, and the inner layer which consists of a proliferating mass of trophoblast cells known as cytotrophoblast cells, which are temporarily mononuclear (Figure 1.1) and contribute to other trophoblast populations (which will be discussed later in this chapter). Between 8-12 days after fertilisation small vacuoles known as lacunae begin to form in the syncytium (the lacunar stage; Figure 1.1c); these continue to enlarge to form a network bathed in maternal blood, which will eventually form the intervillous space. At day 12 post conception the blastocyst is fully embedded into the maternal endometrium and the uteroplacental circulation is established (Figures 1.1c and 1.1d).
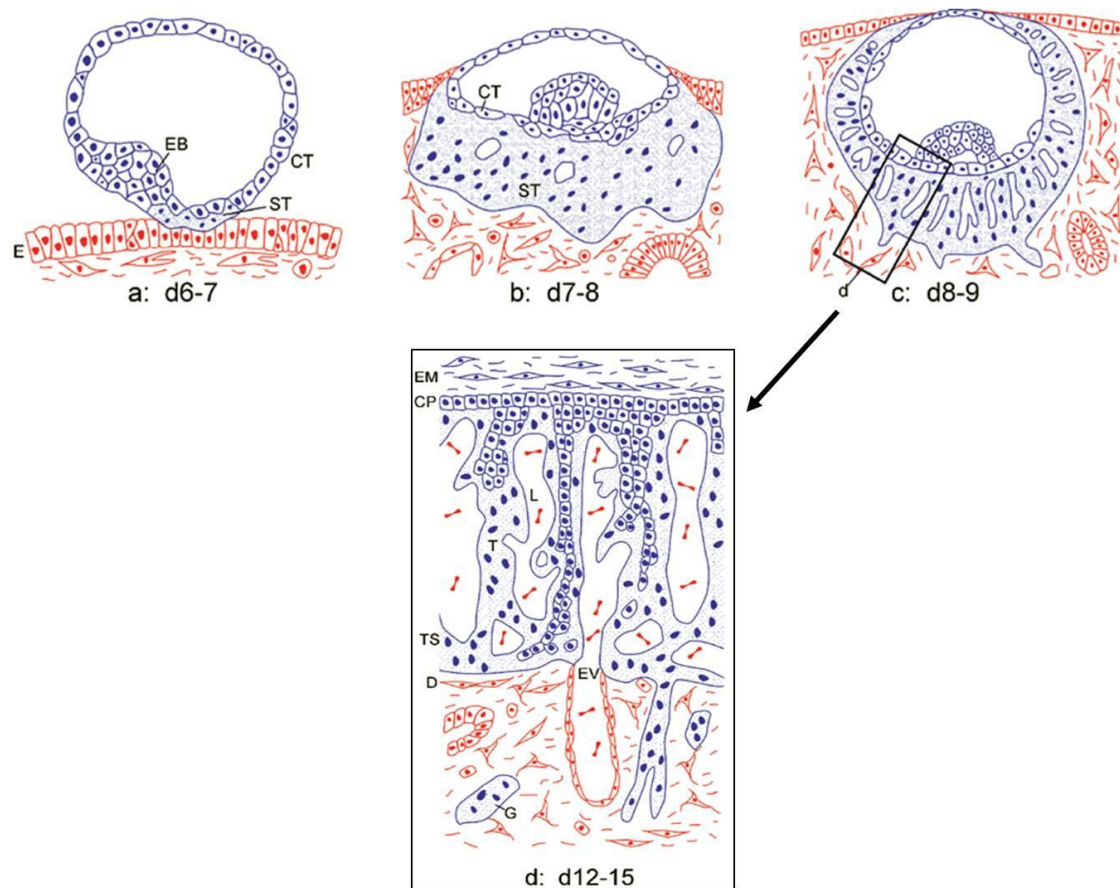
Figure 1.1 Blastocyst implantation and early placental development
Simplified drawings showing blastocyst implantation and early placental development: a and b:
prelacunar stages; c: lacunar stage (formation of lacunae); d: formation of early intervillous
space. Maternal tissues are in shown in red and fetal tissues are in blue. Key: E: endometrial
epithelium; EB: embryoblast; CT: cytotrophoblast; ST: syncytiotrophoblast; EM: embryonic
mesenchyme; CP: chorionic plate; T: primary villi; L: maternal blood lacunae; TS: trophoblast
shell; EV: endometrial vessel; D: decidua; G: trophoblast giant cell. Image source: modified
from (Kaufmann, 1992).

1.1.2 Development of placenta

The placenta is an important organ that maintains and protects the developing fetus. It
plays a number of critical roles, from nutrient exchange and waste secretion to
sequestering the developing fetus from dangerous pathogens and the maternal
immune system. In addition, the placenta serves as an active endocrine hub producing
and secreting growth factors, cytokines and hormones essential for a successful
pregnancy.

1.1.2.1 Chorionic villi

Formation of the chorionic villi and the fetal circulation is evident from 3 weeks after
fertilisation, developing from the trophoblastic shell. The chorionic plate becomes the

definitive placenta (Figure 1.1d). The chorionic villi arise from the chorionic plate and are either free floating in the intervillous space or become anchored to the maternal decidua.

After 4 weeks the basic structure of the placenta is formed. The human placenta is a disc like structure comprising many tree like projections resulting in a branching system of chorionic villi, which increases from 20 weeks of gestation (Boyd, 1970) Chorionic villi are in contact with maternal blood and develop into a mass of tissue that makes up the mature placenta, with the fetal circulation extending within the villous stromal mesenchyme. New villous branches bud from existing chorionic villi, ensuring a maximum surface area in contact with maternal blood for absorption of nutrients, exchange of gases and waste secretion. Thus the chorionic villi are the main functional units of the placenta. Mature chorionic villi consist of a mesenchymal core embedded with fetal capillaries and the two layers of trophoblast cells; the mononucleated cytotrophoblast cells which are encased by mutlinucleated syncytiotrophoblast cells (Figure 1.2).

Placental macrophages known as Hofbauer cells were first identified more than 150 years ago (Kastschenko, 1885) as large ovoid cells with abundant cytoplasm that are present in the chorionic villous core of the placenta from at least 8 weeks gestation until term (Boyd, 1970). They appear in the placental villi prior to the establishment of fetal circulation, suggesting that they are derived from mesenchymal stem cells in early pregnancy; evidence indicates that they have an M2 type phenotype (Joerink et al., 2011). Despite numerous investigations, the function of Hofbauer cells remains largely unknown but a role protecting the fetus against immune attack has been proposed (Uren and Boyle, 1990, Castellucci et al., 2000). Immunohistochemical studies of first trimester placentas demonstrating close contact with endothelial progenitor cells and placental vessels have led to suggestions of a role in early placental vasculogenesis rather than an immunological role (Seval et al., 2007). This also relates to previous studies revealing that placental macrophages express high levels of VEGF-A (Sharkey et al., 1993).

1.1.2.2 Villous trophoblast cells

Following successful implantation the cytotrophoblast cells differentiate into villous cytotrophoblast cells (vCTB). The vCTB cells fuse to form the multinuclear syncytiotrophoblast layer which covers the floating chorionic villi in the intervillous space where the villi are bathed in maternal blood allowing sufficient nutrient and gas exchange for the developing fetus (Ferretti et al., 2007). The syncytiotrophoblast cell layer is also involved in the maintenance of pregnancy by releasing factors that increase progesterone levels such as human chorionic gonadotrophin (hCG) and human placental lactogen (hPL) (Vitiello and Patrizio, 2007). Cytotrophoblast cells form columns covered by a syncytiotrophoblast cell layer and they begin to proliferate. As the cells proliferate they break through the syncytiotrophoblast layer and the cytotrophoblast columns extend from the tips of floating chorionic villi and eventually attach to the maternal decidua, anchoring the chorionic villi to the uterine tissues.

1.1.2.3 Non-villous trophoblast cells

During the third month of pregnancy in parts of the established placenta the chorionic villi on the anembryonic pole of the conceptus regress thus allowing the definitive placenta to develop which is bathed in maternal blood. The non-villous areas of the outer placenta are comprised of non-villous cytotrophoblast cells which are a heterogeneous population (Bulmer and Johnson, 1985) and exist as a multilayered epithelium in direct contact with maternal stroma -that do not invade the adjacent decidua. This is termed the chorion laeve contrasting with the chorion frondosum of the villous placenta. The role of the non-villous cytotrophoblast cells within the chorion laeve remains unclear.
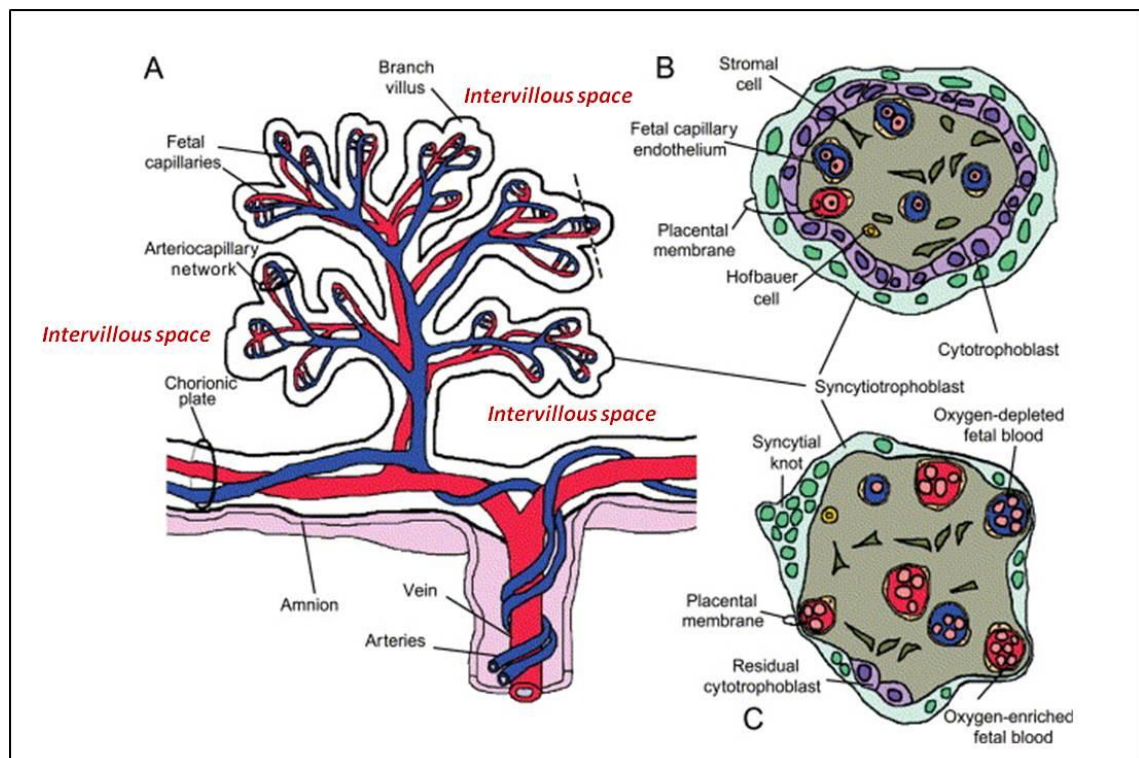
Figure 1.2 Fetal placental circulation and a representative drawing of a chorionic villous
A: Representative drawing of a mature chorionic villus showing the fetal placental circulation;
B: The dotted line shows the position of a representative drawing of a cross section through a
chorionic villus at approximately 10 weeks gestational age; C: Cross section through a chorionic
villus at full term (Modified from (Gude et al., 2004)).

1.1.3 Extravillous trophoblast cells

Upon attachment and anchorage of chorionic villi to the decidua, the anchored villous

cytotrophoblast cells form columns and begin to differentiate from a proliferative

phenotype into a distinct invasive phenotype known as extravillous trophoblast (EVT)

cells (Fitzgerald et al., 2008). The two trophoblast subtypes can be distinguished by

phenotypic differences in their expression of various cell adhesion molecules (CAM),

MHC antigens and secretion of growth factors (Norwitz et al., 2001). The

cytotrophoblast cell columns that protrude from the villi spread laterally to form the

cytotrophoblast shell from which invading EVT arise (Kurman et al., 1984, Loke et al.,

1995) .

1.1.3.1 EVT invasion

Classical histological methods used on placental bed biopsies between 8-18 weeks

gestational age taken from pregnant hysterectomy samples have been the most

influential early studies on trophobast invasion (Pijnenborg et al., 1980, Pijnenborg et

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al., 1982, Pijnenborg et al., 1983). EVT from the cytotrophoblast shell invade the uterine decidua and myometrium and fuse to form multinucleated giant cells. These studies provide evidence that the process of EVT invasion occurs from week 8 of gestation right through to 18 weeks gestation, and this has been confirmed by spiral artery Doppler studies (Matijevic et al., 1995).

The invading EVT infiltrate the maternal uterine tissues by two separate pathways; the interstitial route associated with active degradation of the extracellular matrix by secretion of matrix metalloproteinases (MMPs), thereby allowing interstitial EVT (iEVT) cells to migrate through the decidua to the inner myometrium (Lash et al., 2005); and the route taken by a subtype of EVT known as endovascular EVT (eEVT) which accumulate to form plugs within the lumen of the spiral artery and which, in early pregnancy, plug the outlet of the spiral arteries (SpA) into the intervillous space (Figure 1.3).

EVT which have been observed aligning adjacent to SpAs have been termed perivascular EVT and EVT cells present embedded in the walls of spiral arteries have been termed intramural EVT. These cells can be distinguished as they differ in morphology to iEVT, adopting a more dendritic spindle shape compared with the rounded interstitial EVT cell (Kurman et al., 1984).

The origin of eEVT has been debated with disagreement regarding whether this EVT subpopulation has a route of invasion which originates from interstitial invasion, or whether they invade by a separate pathway altogether and represent a distinct EVT population. It has been suggested that eEVT originate from invading iEVT which penetrate the SpA vessel wall from the outside, rather than by retrograde migration intraluminally (Kaufmann et al., 2003). However, in a recent review (Pijnenborg et al., 2011) emphasised that intraluminal trophoblast cells that have been identified in SpA cross sections are cytotrophoblastic in origin (Boyd, 1970) indicating an endovascular pathway, although Pijnenborg et al. (2011) suggested that there is a possibility that eEVT are derived from both intraluminal trophoblast and iEVT; perivascular EVT clusters have been observed close to decidual SpA indicating the possibility of transmural trophoblast migration, whereas the sparse dispersion of EVT and reduced concentration of EVT near SpAs within myometrium suggests that interstitial EVT may

be responsible for eEVT at deeper levels (Pijnenborg et al., 2006a). Phenotypic differences between eEVT and iEVT, such as the expression of NCAM (CD56) by eEVT (Burrows et al., 1994) also provide evidence for the argument that eEVT are a distinct trophoblast population differing from iEVT. In support of an interstitial route for endovascular trophoblast invasion is that fusion of iEVT into multinucleate trophoblast giant cells in the decidua may result in iEVT losing their invasive phenotype and therefore are unable to penetrate the walls of SpAs; this has been considered as a mechanism for inhibiting eEVT invasion and the presence of large clusters of trophoblast giant cells which have been observed in pre-eclampsia, may equate to the shallow invasion observed in these pregnancies (Stanek and Biesiada, 2012).

Recently a potential new subtype of EVT cell has been hypothesised as an alternative route to the wider known routes of trophoblast invasion; named as endoglandular EVT invasion (Moser et al., 2010). In this study EVT was detected in uterine glands using confrontation co-culture assays, suggesting that the glandular lumina are able to open up into the intervillous space to provide key growth factors for the developing embryo prior to the establishment of maternal blood flow into the intervillous space.
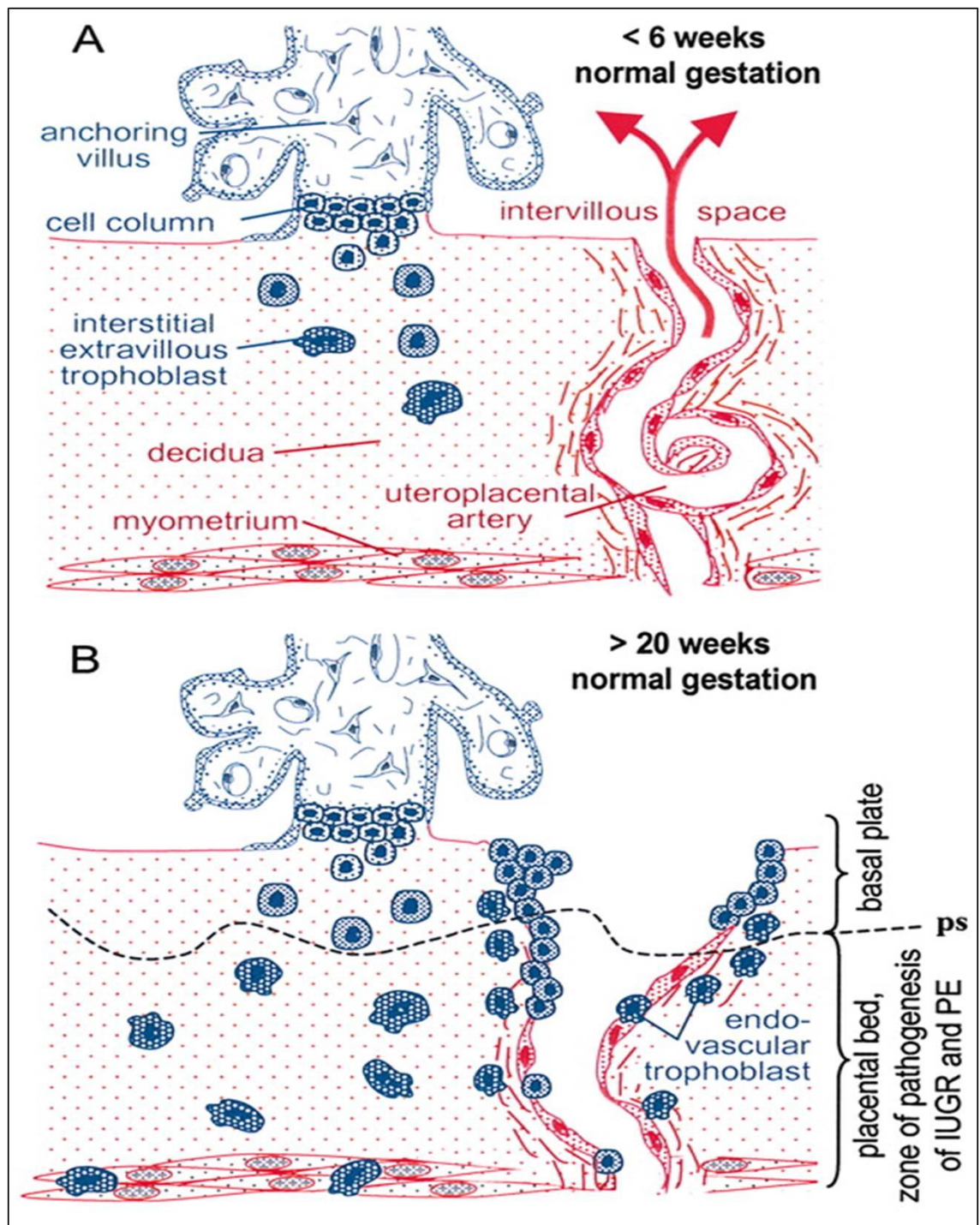
Figure 1.3 Schematic representation of trophoblast invasion
Schematic representation of interstitial and endovascular trophoblast invasion in human pregnancy before week 6 of gestation (A) and after week 20 of normal gestation (B). Blue: fetal tissues. Red: maternal tissues. ps: zone of placental separation, where the basal plate (decidua basalis above, attached to the placenta) separates from the placental bed (remaining in the uterus after delivery) (Image taken from (Kaufmann et al., 2003)).

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1.1.3.2 Waves of invasion

Waves of invasion refer predominantly to endovascular trophoblast invasion. There is debate as to whether endovascular EVT invasion through the decidua into the myometrium is an ongoing process or as to whether it occurs in two separate waves; with wave one occurring in the first trimester up to 8-10 weeks gestation and wave two occurring in the first half of the second trimester from 14-18 weeks gestation with a break in between wave one and wave two during which there is no significant additional trophoblast invasion.

Evidence supporting the two wave concept using complete hysterectomy samples of known gestational age showed that the earliest gestation that decidual spiral arteries were completely filled with eEVT was at 10 weeks gestation whereas the earliest observation of eEVT infiltrating inner myometrial SpAs was observed after 14 weeks gestation(Pijnenborg et al., 1983) suggesting a temporary break between the two waves of EVT invasion – the mechanisms that would underlie such a two wave process of invasion are not known.

1.1.3.4 Trophoblast differentiation

As mentioned previously, during placental development, the cytotrophoblast stem cells in the chorionic villi differentiate into two distinct trophoblast populations; syncytiotrophoblast and extravillous trophoblast. The syncytiotrophoblast remain epithelial whilst the extravillous trophoblast acquires a distinct phenotype, losing epithelial markers such as α-integrin 6 but retaining cytokeratins (reviewed by (Vicovac and Aplin, 1996)) and up regulating α5β1 and α1β1 integrins (Damsky et al., 1994).

Human trophoblast differentiation has been difficult to explore *in vivo* undoubtedly because of the difficulty and ethical issues around obtaining normal pregnancy tissue. Furthermore, the human placenta has a highly specific structure rendering laboratory animal studies of limited value. Therefore, *in vitro* studies have been necessary to investigate trophoblast differentiation. Differentiation from proliferative CTB into invasive iEVT is thought to be influenced by a variety of factors including extra cellular matrix contact and maternal endometrial components. In recent years it has come to light that oxygen tension has an important effect on the placental and embryonic

development. *In vitro* studies have shown that physiological factors such as hypoxic conditions can trigger cytotrophoblast cells of the placenta to proliferate and form cytotrophoblast cell columns; as the extravillous trophoblast cells encounter increasing oxygen levels in the maternal uterine decidua they are stimulated to differentiate into an invasive phenotype (Genbacev et al., 1997).

During the first 10-12 weeks of gestation the endovascular EVT plug the outflow of the uterine spiral arteries, preventing maternal blood flow entering the intervillous space and therefore creating a physiological hypoxic environment (Hustin and Schaaps, 1987). The low partial oxygen pressure is thought to be the driving force for substantial development of the early placenta and is thus crucial for fetal development. Rodesch et al. (1992) observed a significantly lower level of oxygen tension between the placenta (17.9 mmHg) and the endometrium (39.6mmHg) at 8-10 weeks gestation which balanced out at 12-13 weeks gestation due to loosening of the luminal trophoblast plug, thus allowing flow of maternal blood into the intervillous space. Overall it is believed that plugging of the mouths of spiral arteries by endovascular trophoblast cells is responsible for creating this hypoxic state. However, the phenotypic shift from proliferative trophoblast to invasive trophoblast requires a high oxygen state raising the question of how the endovascular trophoblast cells invade the arteries primarily? Pijnenborg et al. (2006a) have suggested that blastocyst implantation is associated with temporary high oxygen concentrations allowing invasion of EVT which is then followed by lower concentrations due to the sealing of the outflow of arteries protecting the feto-placental unit from oxidative stress. Although oxygen gradients are believed to play a role in controlling the invasive potential of EVT, it is not clear why the EVT target the uterine spiral arteries rather than the uterine veins (Pijnenborg et al., 1980). Reports have suggested that venous endothelial cells express an ephrin protein named EPHB4 which may act to repel invading trophoblast (Red-Horse et al., 2005). In addition it has also been suggested that chemoattractants produced by the vascular smooth muscle cells of the spiral arteries may play a role in recruiting the endovascular trophoblast to their lumen (Whitley and Cartwright, 2009). At the end of the first trimester, from 10-12 weeks gestational age onwards, the maternal-placental circulation is established and the

invasive endovascular EVT proceed to migrate in a retrograde direction down the spiral arteries into the myometrial artery segments (James et al., 2006).

1.2 <u>Maternal-fetal interface</u>

1.2.1 The placental bed

Maternal fetal contact is initiated at the time of implantation of the blastocyst into the uterine epithelium and the placental bed is formed (Figure 1.3B). Underlying the attached placenta is the uterine wall which includes the cytotrophoblast shell, the decidualised endometrium and inner third of the myometrium. The placental bed is characterised microscopically by the presence of EVT in both decidual and myometrial stroma and SpAs.

Spiral arteries run perpendicular to the basal plate of the maternal fetal interface. The placental bed contains approximately 150 spiral arteries providing channels for sufficient blood flow to the intervillous space.

1.2.1.1 Decidua and Decidualisation

Successful pregnancy requires a highly receptive endometrium during the implantation window which involves a symbiotic signalling process between the blastocyst and the mother (Aplin, 2000). Decidualisation of the endometrium regulates placentation and provides maternal immune tolerance against fetal antigens, thus preventing rejection of the developing fetus (Pijnenborg, 2002, Salker et al., 2010). Throughout each menstrual cycle the endometrium prepares itself for the chance of an implanting blastocyst before fertilisation of the oocyte has occurred. The endometrium undergoes rapid changes of remodelling during the mid-secretory phase of the menstrual cycle in a process known as decidualisation, providing an optimal environment for embryo implantation. If fertilisation does not occur the endometrium will shed during a process known as menstruation. However, if fertilisation of the oocyte does occur, rising levels of progesterone trigger the endometrium to undergo further decidualisation (Schindler, 1997) and the levels of progesterone remain high during pregnancy to maintain the decidua. Decidualisation (governed by oestrogen and progesterone), involves the extensive proliferation and differentiation of fibroblast

cells into enlarged glycogen filled-endometrial stromal cells characteristic of the decidua (Kurita et al., 2001).

The decidua consists of three anatomical regions in relation to their positions to the site of implantation. At approximately 8-10 weeks gestation the decidua that encapsulates the developing fetus is termed *decidua capsularis.* The *decidua parietalis* lines the entire body of the pregnant uterus and the *decidua basalis* is the maternal aspect underlying the placenta that is located between the chorionic sac and myometrium (Figure 1.4A). After week 12 of gestation the *decidua capsularis* comes into contact with the *decidua parietalis* and these start to fuse together until the uterine cavity is gradually obliterated (Figure 1.4B).

Functional roles for the decidua have been suggested; these include providing a source of growth factors and cytokines to regulate trophoblast invasion (Brosens et al., 2002); reviewed in section 1.1.5), to facilitate the exchange of nutrients to the developing fetus as well as protecting the fetus from the maternal immune system (Croy et al., 2002).
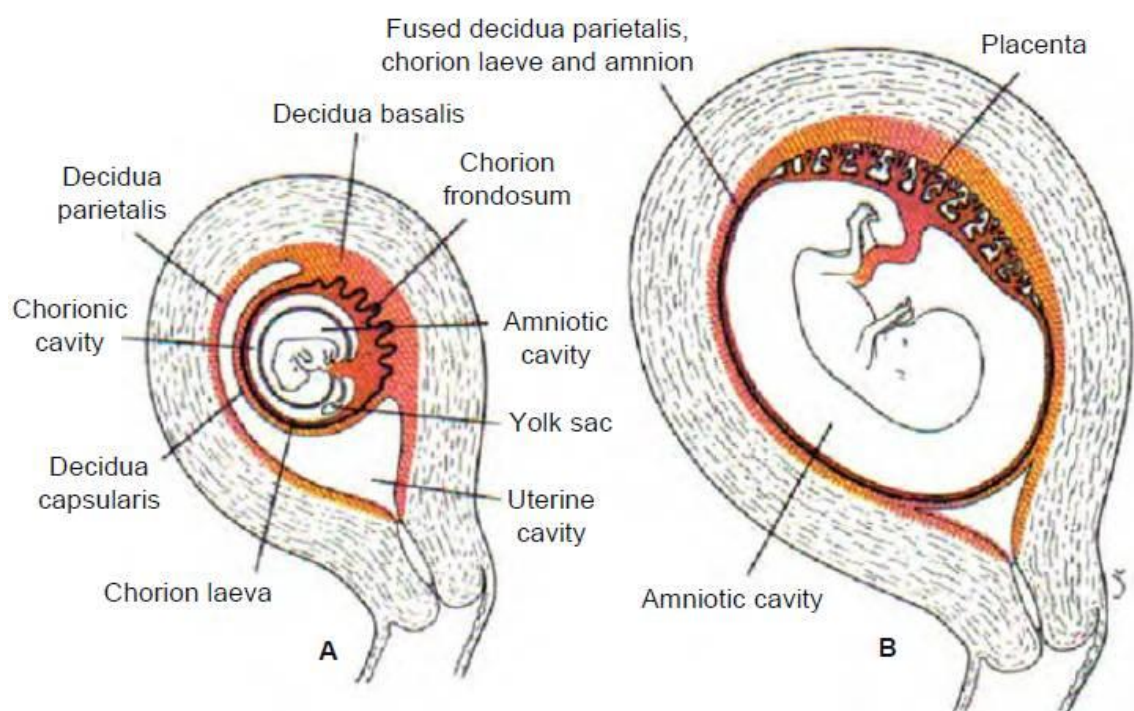
Figure 1.4 Areas of decidua in the developing stages of first half of pregnancy
Areas of the decidua in relationship to the developing fetus and progressive obliteration of the uterine cavity. A: 6-8weeks gestation; B: 16 weeks gestation. Image taken from Langman's Medical Embryology (2004).

1.2.2 Establishment of uteroplacental circulation

Invasion of EVT through the decidua, myometrium and uterine spiral arteries is essential for successful human pregnancy. Invasion of EVT into the spiral arteries is required to establish a low-pressure, continuous flow of blood supply to the intervillous space, providing nutrients for the developing fetus.

1.2.2.1 Spiral artery remodelling

Spiral arteries begin to develop from endometrial radial arteries in the secretory phase of the menstrual cycle (Figure 1.5), when progesterone levels are increased (Ferenczy et al., 1979). In the non-pregnant uterus the spiral arteries consist of thick muscular walls with a well defined elastic lamina, which is lost as the artery penetrates the endometrium (Robertson and Manning, 1974). During the menstrual cycle, if implantation of the blastocyst into the endometrium does not occur, the spiral arteries regress and are lost during menstruation. However, if implantation does occur then spiral arterioles and arteries develop further and supply the placenta and fetus with the required nutrients vital for development.

The spiral arteries and arterioles achieve this by undergoing extensive remodelling into highly dilated structures that lack muscle and elastic (Pijnenborg et al., 2006b). During early pregnancy the spiral arteries responses include vessel dilation, loss of vascular smooth muscle cell (VSMC) arrangement and vacuolation of the media (Craven et al., 1998). The arteries are termed 'spiral' because they are highly coiled, narrow, muscular structures (Figure 1.5). The coiling increases their surface area to achieve maximum blood flow (a 3-4 fold increase) for the demands of exchanging respiratory gases and nutrients from mother to fetus. Thus, the purpose of the remodelling of the coiled vessels is to ensure that an adequate volume of blood at a low pressure enters the intervillous space (Harris and Aplin, 2007). The process of spiral artery remodelling is a reversible one where the musculo-elastic structure of the vessels, consisting of VSMCs, elastic and extracellular matrix are displaced with fibrinoid material containing intramural trophoblast cells, resulting in large, dilated low resistance vessels (Pijnenborg et al., 1980). Figure 1.6 illustrates the various stages of spiral artery remodelling. Another characteristic of spiral arteries undergoing transformation is endothelial cell swelling and the displacement of SpA endothelium by eEVT cells.

Complete re-endothelialisation of the spiral arteries has been reported at term (Khong et al., 1992), although it is disputed to what extent SpAs lose their endothelial cell lining at earlier stages of pregnancy and it is likely that endothelial cells and eEVT cells coexist in a spatial and temporal manner.

Early spiral artery remodelling changes are associated with interstitial EVT in decidua where they begin to disrupt the media; VSMCs start to separate and become hypertrophic, suggesting that interstitial EVT are 'priming' the SpA for endovascular trophoblast invasion. In addition there is now recent evidence to support trophoblast-independent spiral artery remodelling by decidual cells before trophoblast invasion into the maternal tissues occurs (Smith et al., 2009). By 10 weeks of gestation eEVT has caused severe disruption to the artery media (Pijnenborg et al., 1983); the vascular walls of decidual spiral arteries have completely diminished; the arteries have lost the internal elastic lamina, and their walls are then replaced by deposition of fibrinoid material which maintains the integrity of the vessel. The intramural trophoblast cells in the walls of the spiral arteries are embedded in amorphous fibrinoid material and ultrastructural observations suggest that this fibrinoid is derived from degenerative trophoblast cells, maternal serum leakage and elastic lamina residue (De Wolf et al., 1973).

1.2.2.2 Origin of intramural EVT

The initial view of the origin of intramural EVT was that this trophoblast population derived from the lumen of SpA and therefore, are endovascular in origin, as they become embedded in the vessel walls (Brosens et al., 1967). This was supported by observations of trophoblast cells located in the intima rather than the media of myometrial spiral arteries at 16-22 weeks of gestation (De Wolf et al., 1980). However, conflicting evidence from immunohistochemical studies has demonstrated that intramural EVT lack expression of human chorionic gonadotrophin (hCG) in common with the interstitial EVT population, but contrasting the endovascular EVT population (Kurman et al., 1984).

INTERVILLOUS SPACE OF THE PLACENTA

BASAL PLATE

DECIDUA

Basal Artery

Spiral Arteries

Basal

MYOMETRIUM

Radial

Arcuate

PERITONEUM
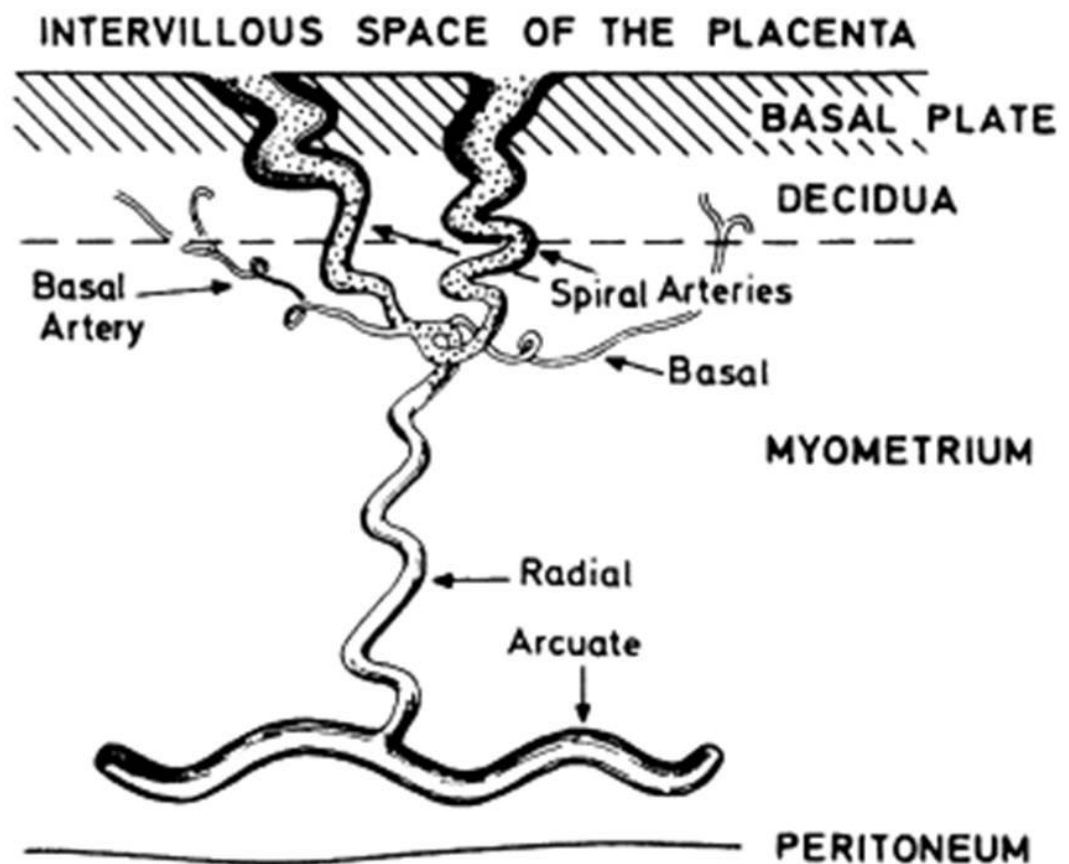
Figure 1.5 Vascular anatomy of a pregnant uterus
Image taken from Brosens et al. (1967).

1.2.3 Control of trophoblast invasion

The invasive nature of interstitial EVT occurs through proteolytic action. This is achieved by their ability to secrete a class of zinc-dependent endopeptidases known as matrix metalloproteinases (MMPs), cathepsins and urokinase-type plasminogen activators (uPA) (Bischof et al., 2000). Degradation of the extracellular matrix allows movement of the EVT between the decidual and myometrial compartments. EVT cells share some characteristics with malignant cells; their ability to migrate and invade tissue and their capacity to sequester themselves from the host's immune system (Ferretti et al., 2007). However, in contrast to the uncontrolled invasion of malignant cells, the invasion of EVT cells through uterine tissues is a tightly controlled process limited both to spatially (localised to the decidua and inner third of the myometrium) and developmentally (invasion ceases during the second trimester of pregnancy) (Meisser et al., 1999). Disruptions in this tightly controlled environment can lead to

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placental deficiencies which affect the maternal vascular homeostasis resulting in pregnancy complications such as early miscarriage, pre-eclampsia, intrauterine growth restriction and placenta accreta. Despite the importance of trophoblast invasion in pregnancy very little is understood about the factors that control this process, although decidual factors are likely to play an important role. Several growth factors and cytokines, some secreted by trophoblast themselves, are believed to be responsible for regulating this process by modulating protease activity, disrupting cell adhesion and inducing cell apoptosis (Lash et al., 2005), thereby affecting trophoblast migration, proliferation and differentiation into an non-invasive phenotype (Fitzgerald et al., 2008) so that trophoblast invasion ceases in the inner third of the myometrium.

1.2.4 Control of spiral artery remodelling

Remodelling of the decidual segments of spiral arteries is generally complete by 10-12 weeks' gestation and continues in the myometrial segments up until around week 20-22 of gestation (Pijnenborg et al., 2006b). Spiral artery remodelling is crucial for a successful pregnancy: failure of spiral artery remodelling has been implicated in the pathogenesis of pre-eclampsia (Pijnenborg et al., 1991), intrauterine growth restriction (Khong et al., 1986), second trimester miscarriage (Khong et al., 1987, Ball et al., 2006a) and preterm delivery (Kim et al., 2003). Despite its importance in early pregnancy, the molecular mechanisms that are involved in spiral artery remodelling remain unclear. Lack of appropriate physiological and pathological models hinders studies. A number of possible mechanisms such as cellular migration, dedifferentiation, macrophage phagocytosis and apoptosis may be responsible for vascular smooth muscle displacement. It is likely that several of these mechanisms work together in a co-ordinated fashion to regulate the process of spiral artery remodelling.
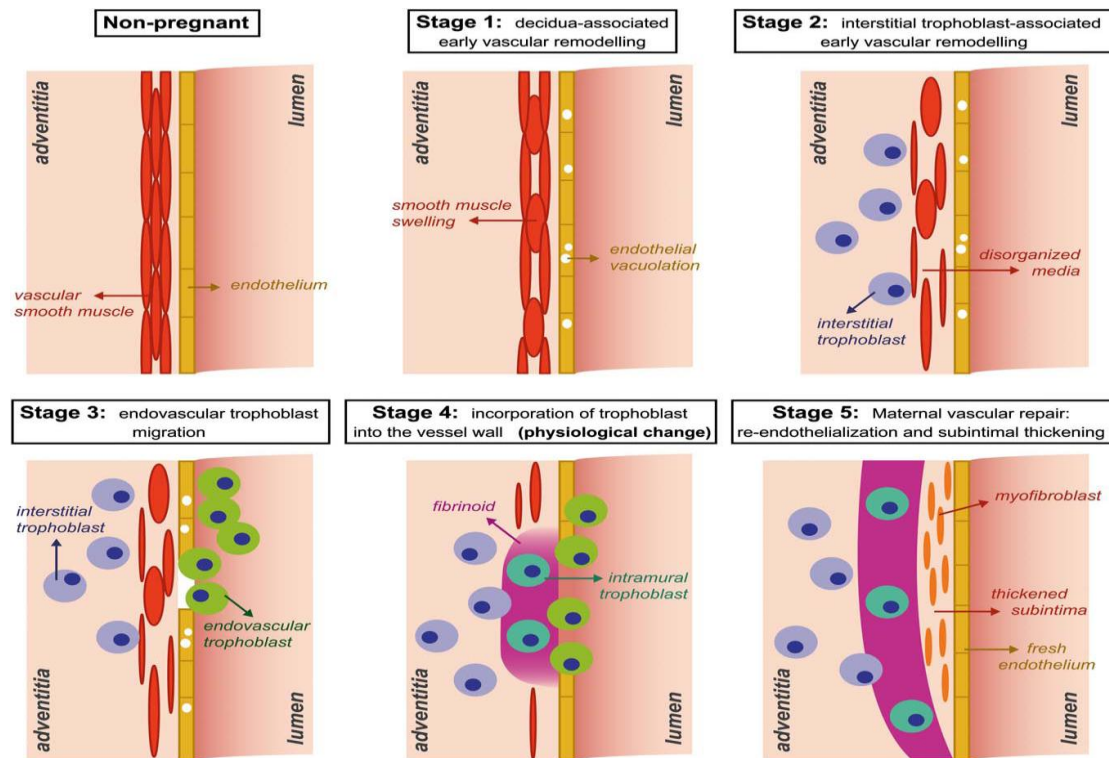
Figure 1.6 The different stages of uterine spiral artery remodelling
The arteries begin to develop in the secretory phase of the menstrual cycle in non-pregnant endometrium. The earliest stage in vascular remodelling during pregnancy (stage 1) shows endothelial vacuolation and slight swelling of the VSMCs. Interstitial trophoblast then begins to invade the surrounding stromal tissue which is associated with further disorganisation of the VSMC layer (stage 2). Only in stage 3 does the endovascular trophoblast appear leading to intramural trophoblast becoming embedded within a fibrinoid layer, replacing the VSMCs (stage 4). In the final stage of spiral artery remodelling the endothelium has re-generated (Pijnenborg et al., 2006b).

1.2.4.1 Fate of SpA VSMCs

There are numerous ways in which both endovascular and interstitial trophoblast may influence spiral artery remodelling. Interstitial trophoblast is likely to influence the behaviour of the VSMCs; it has been observed that invasion of interstitial trophoblast through the extracellular matrix of the decidua liberates growth factors that could influence vessel wall structure as well as facilitating transit of the endovascular trophoblast along the arteries (Harris and Aplin, 2007). Endothelial changes are likely to be influenced by the endovascular trophoblast which transiently replaces the endothelial cells lining the spiral arteries. The loss of endothelial cell adherence may lead to anoikis which in turn affects the composition of the ECM which could affect the differentiation state of the VSMC (Whitley and Cartwright, 2009). The close relationship between the endothelial cells and the VSMCs is essential for maintaining

the integrity of the vasculature and disruptions in this relationship are thought to be the likely cause of remodelling in the spiral arteries (Whitley and Cartwright, 2009). There have also been observations of vascular smooth muscle cell reorganisation occurring in the presence of intramural trophoblast where fibrinoid displaces the muscle of the vessel wall (Kam et al., 1999). Recent *in vitro* data suggest that the trophoblast is responsible for SpA VSMC apoptosis (Cartwright et al., 2007, Smith et al., 2009) although VSMC apoptosis is rarely detected *in vivo* in the placental bed (Bulmer et al., 2012). However, the limitation in assessment of apoptosis is that it is a transient process with cell loss and evidence may therefore be difficult to detect. The contribution of apoptosis to loss of spiral artery smooth muscle remains a cause for debate. Migration and de-differentiation of VSMCs have both been suggested as well as apoptosis (Bulmer et al., 2012).

1.2.4.2 Factors responsible for SpA remodelling

Although it has been suggested that interstitial and endovascular EVT are responsible for complete spiral artery remodelling, others have suggested that the arterial modifications are initiated in a maternal response to pregnancy prior to trophoblast invasion along the lumen of the spiral artery vessels (Craven et al., 1998). This maternal response of arterial modification has been termed trophoblast-independent remodelling and appears to be a decidual component that is independent of trophoblast. This tophoblast-independent phase occurs in the early stages of pregnancy, with trophoblast-dependent spiral artery remodelling starting at approximately 6-8 weeks of gestation (Pijnenborg et al., 2006b) (Figure 1.7).

Histological evidence shows that in the absence of EVT the vessels are surrounded by a cell type within decidua termed uterine natural killer (uNK) cells (Bulmer et al., 1991) which are described in further detail in section 1.4.2. There is speculation that the secretion of growth factors such as vascular endothelial growth factor (VEGF)-C and transforming growth factor-beta1 (TGF-β1) by uNK cells initiate the decidual-associated remodelling in preparation for trophoblast infiltration to allow the final stages of transformation to take place (Lash et al., 2006c).

Craven et al. (1998) have demonstrated changes in spiral artery vessel structure in the absence of trophoblast. The initial changes included swelling and vacuolation of the

medial smooth muscle cells, increased vascular permeability and increased endothelial cell activation leading to vessel dilation (Figure 1.6, Stage 1). It also has been documented that progesterone may have an indirect effect on vascular remodelling by its effects on recruitment of cells such as lymphocytes, macrophages and uNK cells to the endometrium associated with the ability to up-regulate stromal cell chemokine expression (Sentman et al., 2004). In agreement with data that uNK cells play an important role in early events of spiral artery remodelling, evidence from transgenic mice deficient in uNK cells has shown failure of decidual artery remodelling and implantation site abnormalities (Ashkar et al., 2000). It has also been reported that in human placental bed biopsies uNK cells are localised in association with vessels undergoing remodelling (Bulmer and Lash, 2005). These data and the observation that uNK cells diminish in number after 20 weeks' gestation when SpA changes are almost complete provides support for the suggestion that these cells play a role in the spiral artery remodelling process can be made. However, the confinement of uNK cells to the endometrium needs to be considered since trophoblast invasion and spiral artery remodelling extend through to the inner third of the myometrium where uNK cells are not a prominent feature; this may reflect a role for these cells in early SpA vascular changes and priming of SpA VSMCs for the later trophoblast-dependent alterations which mediate transformation of myometrial segments of spiral arteries.
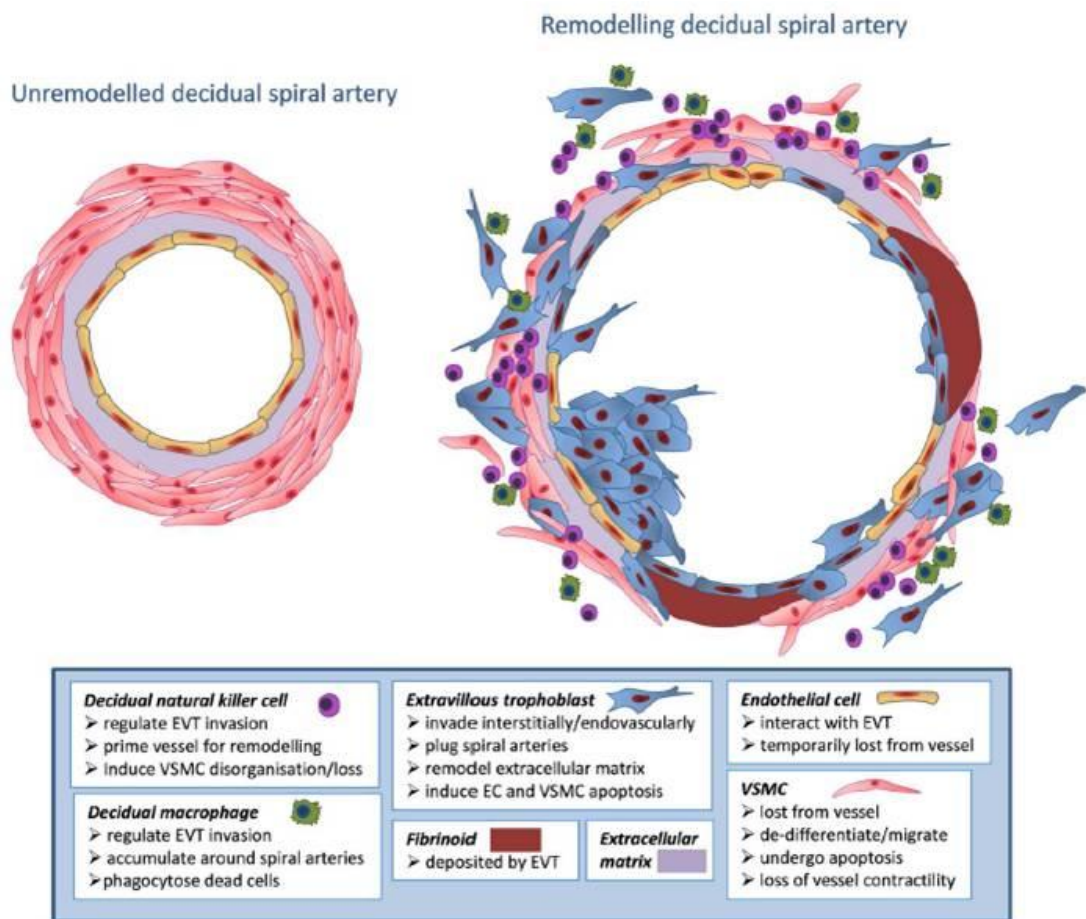
Figure 1.7 Process of decidual spiral artery remodelling
Representation of process of decidual artery remodelling. An un-remodelled artery has intact
endothelium and a VSMC cell layer making it a low flow, high resistant vessel. During
pregnancy the spiral arteries undergo remodelling initially brought about by maternal immune
cells and completed by invading EVT. VSMC layers and endothelium are lost and are replaced
with embedded intramural trophoblast and fibrinoid material, changing the integrity of the
vessel and thereby creating a high flow, low resistant vessel. Endothelial displacement is
transient and is replaced by the third trimester. Image taken from Cartwright et al. (2010).

1.2.5 Aberrant trophoblast invasion and deficient spiral artery remodelling

Spiral artery remodelling plays an important role in the establishment and

maintenance of successful pregnancy and understanding the mechanisms which

control this process may improve understanding of important pregnancy complications

which are associated with aberrant trophoblast invasion.

These processes involve a complex network of molecular and cellular interactions

which at each stage could be compromised leading to pregnancy complications and

disorders. Several pregnancy disorders including recurrent and spontaneous

miscarriage, pre-eclampsia, premterm labour and intrauterine growth restriction have

been associated with aberrant trophoblast invasion and/or deficient spiral artery remodelling.

Recurrent miscarriage (RM) is defined as three or more consecutive pregnancy losses before 20 weeks gestation (Wilcox et al., 1988) and affects approximately 1 in 300 women. Known causes of 20-50% of RM cases are associated with infection, endocrine disturbances, endometrial defects and abnormal embryo karyotype (Li et al., 2002). Although the causes of the remaining 50% of RM cases are unknown, there is a majority of evidence suggesting an immune involvement (reviewed by Laird et al. (2003) and Bansal (2010)). Women suffering RM have displayed higher uterine blood flow resistance compared with healthy successful pregnancies indicating insufficient establishment of the uteroplacental circulation, which may be the result of aberrant trophoblast invasion due to defective immune tolerance mechanisms (Habara et al., 2002). Decreased levels of EVT HLA-G expression has been identified in RM (Emmer et al., 2002) suggesting a down regulation of uNK cell 'self' recognition causing a detrimental anti-fetal immune response by production of pro-inflammatory Th1 type cytokines. It has been proposed that an imbalance in Th1/Th2 reactivity in RM influences immunisation of paternal antigens (Szpakowski et al., 2000) and recently increased numbers of Th17 cells in both decidual tissues and maternal blood with increased levels of IL-17 in peripheral blood have been implicated in women suffering RM suggesting that Th17 cells destroy the conceptus by mediating maternal immune tolerance (Liu et al., 2011). A reduction in placental VEGF has also been observed in women suffering RM which could prevent the maturation of blood vessels, therefore affecting uteroplacental circulation and preventing a viable pregnancy (Vuorela et al., 2000). Recent reports identify specific paternal genetic patterns in HLA-C expression and maternal NK recognition which has been associated with increased risk of RM, indicating that successful placentation relies on a coordinated balance of NK inhibition and activation of trophoblast through HLA-C recognition (Hiby et al., 2008).

Pre-eclampsia is a disease which is characterised by gestational hypertension and proteinuria, which has serious health consequences for both mother and baby; it is a major cause of maternal mortality and morbidity (Barton et al., 2001) and can cause fetal mortality (Sibai et al., 1995). Although pre-eclampsia is often not evident until the

third trimester, the disease is established during the first half of pregnancy during stages of trophoblast invasion and spiral artery remodelling. In the initial stages of the disease defective spiral artery remodelling is caused by shallow eEVT invasion in decidual spiral artery segments and a lack of invasion into the myometrium in early second trimester, meaning that myometrial spiral arteries do not undergo physiological change or remodelling, suggesting that the 'second wave' of trophoblast invasion does not occur (Brosens et al., 1972, Pijnenborg et al., 1991). Observations of term placental bed biopsies revealed a higher proportion of un-remodelled SpAs in pre eclamptic patients compared with normal pregnancies (Kim et al., 2003), providing evidence that impaired SpA remodelling is either a causative factor or an effect of the disease. A lack of spiral artery remodelling may be due to delayed endovascular trophoblast invasion; eEVT was detected in the lumen of third trimester decidual spiral arteries in patients with pre-eclampsia, while an absence of intraluminal eEVT is apparent in normal pregnancies beyond the second trimester (Khong et al., 1986).

Progression of the disease is characterised by endothelial cell dysfunction with deficient production of nitric oxide (Roberts and Lain, 2002) causing vasoconstriction and reducing the flow of blood into the intervillous space, increasing oxidative stress to the placenta, resulting in uteroplacental ischemia. Placental ischemia has been suggested to evoke an inflammatory response by the release of many cytokines and growth factors from the placenta and further affect endothelial fuction. Secretion of placental TNF-α and circulating levels of VEGF agonist (sFlt1) have been associated with endothelial dysfunction in pre-eclampsia (Maynard et al., 2003, Hung et al., 2004).

Fetal intrauterine growth restriction (IUGR) is a major cause of fetal morbidity and mortality; 50% of stillbirths at term are associated with IUGR (Froen et al., 2004). IUGR is defined as a failure of the fetus to reach its genetically predetermined growth potential and is characterised by abnormal fetal weight, size, and symmetry. As well as genetic predisposition, optimal fetal growth is also dependent on a regulated combination of maternal, fetal, placental and external factors (such as maternal smoking, consumption of alcohol and use of recreational drugs during pregnancy). Irregularities of such factors contribute to uteroplacental dysfunction which is the most common cause of IUGR (Baschat et al., 2000). Placental dysfunction is likely to

occur in the first trimester of pregnancy which suggests defective trophoblast adherence and invasion, therefore affecting SpA changes; such changes have been histologically observed in the placental bed in IUGR (Hustin et al., 1983). Such insufficiencies of uterplacental perfusion impinge on nutrient transfer to the fetus (Baschat and Hecher, 2004) and the condition may manifest early in pregnancy.

Pregnancy disorders such as RM, pre-eclampsia and IUGR are multifactorial but some studies indicate an association with restricted eEVT invasion and failure of SpA remodelling and that pregnancy outcome is directly linked with the timing and extent of these processes. Therefore, investigation into the regulation of these processes at the maternal fetal-interface in early pregnancy will likely provide insight into a better understanding in the mechanisms involved in pregnancy pathology.

1.3 Immunology of normal pregnancy

1.3.1 Immunological paradox of pregnancy

The fetus can be considered to be semi-allogenic to the mother due to contribution of human leucocyte antigen (HLA) genes from each parent. Fetal tissue expressing paternal alloantigens would be expected to stimulate a maternal T-cell immune response; however, a detrimental T-cell response is not initiated and the fetus escapes rejection. This unique process which allows a genetically and immunologically foreign entity to survive and evade the maternal immune system is described as the immunological paradox of pregnancy (Billingham et al., 1953, Damber et al., 1977).

In 1953 comparisons were proposed between allografts in organ transplantation and the fetus; however, it was highlighted that pregnancy contradicted the immunology concept of allogeneic rejection (Billingham et al., 1953). The fetus, like the allograft is non-self; it is semiallogeneic but despite this the fetus is able to survive, whereas an allogeneic allograft is rejected. Explanations proposed in early studies were that the maternal immune response was suppressed during pregnancy, that the fetus did not express mature antigens and that the placenta served as an immune barrier between mother and fetus by lacking major histocompatibility complex (MHC) gene products/antigens. However, later studies revealed that the fetus is in fact immunogenic and does evoke a maternal immune response; this was demonstrated in

studies in mouse (Hoskin and Murgita, 1989), proving that the fetus expresses mature antigens and that the mother is immunologically aware of the pregnancy. Beer and Billingham (1971) proposed five concepts to explain this immunological paradox:

1) Fetal and maternal blood circulations are separate.
2) The uterus is an immunological privileged site.
3) An immunological barrier exists at the maternal-fetal interface – paternal alloantigens are not expressed or are sequestered.
4) Decidual inhibition of alloreactivity.
5) Production of immunosuppressive factors by the fetus and mother.

Recent studies have proved or refuted some of these hypotheses; evidence of an anti-inflammatory and immunosuppressive response at the maternal-fetal interface has been reported. However, contrary to this is evidence that direct contact between fetal cells and maternal cells is associated with immune recognition, providing evidence that the developing fetus and the mother have an interactive relationship.

1.3.2 Trophoblast major histocompatibility complex (MHC) expression

During normal pregnancy chorionic villi become bathed in maternal blood and cells from the placenta invade the maternal uterine tissues. To avoid a hostile immune response from the mother, the placenta ensures a safe immunological barrier from maternal immune attack. Trophoblast cells are the first lineage derived from the developing embryo. They are extraembryonic in nature containing unmethylated DNA (Ohgane et al., 2002), express retroviral proteins (Mi et al., 2000) and have a genetic contribution from the father and thus are semi-allogenic to the mother. However, human villous trophoblast cells do not express either usual class I or class II MHC antigens (Faulk and Temple, 1976, Sunderland et al., 1981) suggesting that these cells are sequestered when encountered by maternal blood permitting survival of the developing fetus. Extravillous trophoblast cells are not confined to the chorionic villi, obviously since they are extravillous and are able to invade the maternal tissues without evoking an immune attack. Villous stromal cells do express MHC antigens so the lack of exposure to blood in the intervillous space relies on the intact syncytiotrophoblast cell layer, once syncytiotrophoblast layer is broken, the villous stroma is exposed to maternal immune cells in the intervillous space.

EVT cells do not express the highly polymorphic HLA class I molecules HLA-A and HLA-B but they do express the less polymorphic HLA-C (Moffett-King, 2002) and unusual non-classical truncated class I MHC molecules, HLA-E and HLA-G (Hunt et al., 1988, King et al., 2000a), which are invariant and show limited polymorphism (Carosella, 2000). HLA-G expression is almost entirely specific to EVT cells and due to its limited polymorphism; it is unlikely to be recognised by the adaptive maternal immune system. However, HLA-G recognition is specific to maternal NK leucocytes through killer inhibitory receptor (KIR) expression and this recognition prevents EVT cytotoxity from NK cells. Complete lack of MHC class I molecules would expose the EVT to cell lysis (Hunt et al., 2000, Fournel et al., 2000) and also further down regulates a maternal T cell cytotoxic response to the placental cells (Bainbridge et al., 2000).

HLA-G expression is essential for successful pregnancy outcome (Fuzzi et al., 2002) decreased EVT HLA-G expression has been implicated in miscarriage (Emmer et al., 2002) and the presence of a number of rare HLA-G isoforms have been detected in women suffering from recurrent miscarriage (van der Ven et al., 2000, Aldrich et al., 2000, Pfeiffer et al., 2001). The functions of HLA-G remain unclear but aside from protection against maternal leucocytes, other roles of HLA-G have been proposed such as promoting uNK cell proliferation and interferon (IFN)-γ production (van der Meer et al., 2004) suggesting a regulatory role in EVT invasion (Lash et al., 2006b) and modification of the maternal immune response by suppressing allogeneic lymphocyte proliferation (Apps et al., 2007).

1.3.3 Th1/Th2 paradigm

CD4$^+$ Helper T lymphocytes (Th) contribute towards immunity as well as producing cytokines. They are classified into different functional subsets; Th1 and Th2. In the normal immune system there is a balance between in Th1/Th2 responses. A Th1 response produces cytokines such as IFN-γ, TNF-α, and interleukin (IL)-2 which promote cell mediated immunity and this response will predominate over Th2, whereas a Th2 response provides humoral immunity by the production of IL-4, IL-5, IL-9, IL10 and IL-13. Another CD4$^+$ T lymphocyte subpopulation has also been identified that do not secrete either Th1 or Th2 type cytokines but produce TGF-β and IL-10

which down regulates the effects of Th1 cells; this population of cells has been named Th3 (Borish and Steinke, 2003).

Wegmann et al. (1993) proposed that during pregnancy a Th2 response prevails, whereas a Th1 response is detrimental to pregnancy and many studies, particularly in mouse pregnancy, have provided evidence of this. TNF-α serum levels are elevated in pre-term labour and pre-eclampsia (Casey et al., 1989). TNF-α is considered to be harmful for pregnancy; increased levels are associated with embryonic demise in mouse studies (Clark et al., 2004) and TNF-α affects feto-placental development by inhibiting trophoblast invasion (Bauer et al., 2004, Huber et al., 2006, Otun et al., 2011). Lash et al. (2006b) have also demonstrated the inhibitory effects of IFN-γ on EVT invasion by apoptosis and decreased protease activity. The Th1-type immunity was considered to be incompatible for successful human pregnancy (Raghupathy, 1997), favouring Wegmann's hypothesis. Th1 cytokine dominance has been reported in RM compared with normal pregnancies (Raghupathy et al., 2000), although this has been disputed by (Bates et al., 2002) who reported a Th2 shift in RM. Challenging the concept of a Th2 bias in normal pregnancy are data proving that IFN-γ is essential for successful pregnancy outcome as it is pivotal in SpA remodelling (Ashkar et al., 2000, Croy et al., 2003, Murphy et al., 2009, Robson et al., 2012). Furthermore a study using a Th2 knockout (IL-4, IL-5, IL-9 and IL-13 ko) mouse showed normal reproductive outcome (Fallon et al., 2002). Additional cytokines such as IL-15 and IL-18 have been observed at the maternal-fetal interface (Chaouat et al., 2002, Murphy et al., 2009) that are not classed Th1 nor Th2 type cytokines, again challenging the paradigm and adding to its complexity.

These data, combined with many studies investigating the Th1/Th2 paradigm, suggest that the immune responses at the maternal-fetal interface is a complex one, which may be time specific, dynamic and continuous rather than focusing on maternal tolerance alone.

1.3.4 Cytokines in normal pregnancy

Human endometrium produces a wide variety of pro-inflammatory cytokines throughout the proliferative and secretory phases of the menstrual cycle (Tabibzadeh, 1991). These cytokines are believed to play a significant role in the modulation of the

uterine environment preparing the uterus for implantation of the developing conceptus and formation of a functional placenta for the establishment of pregnancy.

Prevention of maternal rejection of the fetus requires a regulated environment which occurs primarily at the maternal-fetal interface and within the uterine tissues. In normal systems naive CD4^{+} T-cells are the major producers of cytokines and are divided into three subsets Th1, Th2 and Th3 (see section 1.3.3). However, a plethora of Th1 and Th2 cytokines are produced by trophoblast cells, maternal T lymphocytes, stromal cells, epithelial cells, uNK cells, macrophages and other maternal leucocytes, localised at the maternal-fetal interface (Vince and Johnson, 2000) and maintenance and development of the fetal-placental unit may be dependent on these cytokines. The presence of these cytokines at the maternal-fetal interface influence the local environment by regulating processes such as implantation, placental development, cytotrophoblast proliferation, angiogenesis, EVT invasion, SpA remodelling, cellular growth and apoptosis (Piccinni et al., 2000, Drake et al., 2001, Dimitriadis et al., 2005, Lash et al., 2006c, Murphy et al., 2009). However, this is a complex area of research due to the pleiotrophy and redundancy of the cytokine network and a wide range of results have been reported; some known functions of specific cytokines are summarised below in sections 1.3.4.1 and 1.3.4.2.

1.3.4.1 Cytokines and implantation

It is likely that a complex mix of cytokines in the endometrium is involved in blastocyst implantation. Studies have focused on IL-1 and receptors for IL-1 have been reported to be distributed throughout the human endometrium and are present at the maternal-fetal interface, suggesting a role in the implantation of the blastocyst (Simon et al., 1998). Cytokines may increase the receptivity of the endometrium allowing communication with the developing embryo. Gp130 cytokines, including IL-6 and leukaemia inhibitory factor (LIF), play important roles in implantation; LIF knockout mice failed to initiate decidualisation and therefore failed to support implantation; LIF^{-/-} mice injected with exogenous LIF rescued implantation (Stewart et al., 1992). Laird et al. (1997) have linked successful implantation with LIF in humans. Women with unexplained infertility produced lower levels of endometrial LIF in the luteal phase of the menstrual cycle compared with controls and this has subsequently been confirmed

(Ledee-Bataille et al., 2002). IL-6 deficient mice have shown reduced fertility and reduced numbers of viable implantation sites (Robertson, 2000).Together with LIF, IL-6 and IL-1, IL-11 has also been suggested to be important in pregnancy; *in vitro* studies have shown that IL-11 stimulates decidualisation of human endometrial stromal cells (Dimitriadis et al., 2002) and primary decidual cells *in vitro* secrete IL-11 and regulate trophoblast invasion (von Rango et al., 2004). Impaired synthesis of endometrial epithelial IL-11 has also been implicated in women suffering recurrent miscarriage (Linjawi et al., 2004) providing further evidence for the importance of this cytokine in early pregnancy.

There are many other factors alongside gp130 cytokines that are involved in implantation such as progesterone, a variety of chemokines and other cytokines such as TNF-α, TGF-β1 and IL-15 (Dimitriadis et al., 2005), indicating the complexity of this event with multiple contributors to the processes involved.

1.3.4.2 Cytokines and trophoblast invasion

The mechanisms that control EVT invasion in early pregnancy remains widely misunderstood although it is likely that several cytokines and growth factors derived from the decidua and trophoblasts themselves are involved (Meisser et al., 1999, Bischof et al., 2000, Lala and Chakraborty, 2003, Lash et al., 2006c, Naruse et al., 2010). Moreover various decidual cells, such as uNK cells, decidualised endometrial stromal cells and macrophages secrete both inhibitory and stimulating factors that may control EVT invasion (outlined in Figure 1.8; Knofler and Pollheimer (2012)). TNF-α and IFN-γ are cytokines which are associated with the inhibition of trophoblast invasion by triggering cell death and also inhibiting protease enzymes (Ho et al., 1999, Knofler et al., 2000, Lash et al., 2006b, Otun et al., 2011). TGF-β1, 2 and 3 have been reported to inhibit trophoblast invasiveness by reducing trophoblast protease activity (Lash et al., 2005). There is also evidence indicating that a Th2 type response prevents trophoblast apoptosis (Aschkenazi et al., 2002) suggesting that Th2 cytokines may stimulate invasion; this coincides with studies of IL-6 (Jovanovic and Vicovac, 2009, Dubinsky et al., 2010), IL-8 (Hanna et al., 2006, De Oliveira et al., 2010), IL-11 (Paiva et al., 2007) and IL-1β (Gonzalez et al., 2011, Prutsch et al., 2012), all of which have been reported to stimulate trophoblast invasion.

This area has remained inconclusive and many reports are contradictory. This may be an effect of studying cytokines in isolation. Furthermore the source of trophoblast cells and the cell lines used in these studies have varied, thus adding to the difficulty of elucidating the mechanisms that are involved in the control of trophoblast invasion.
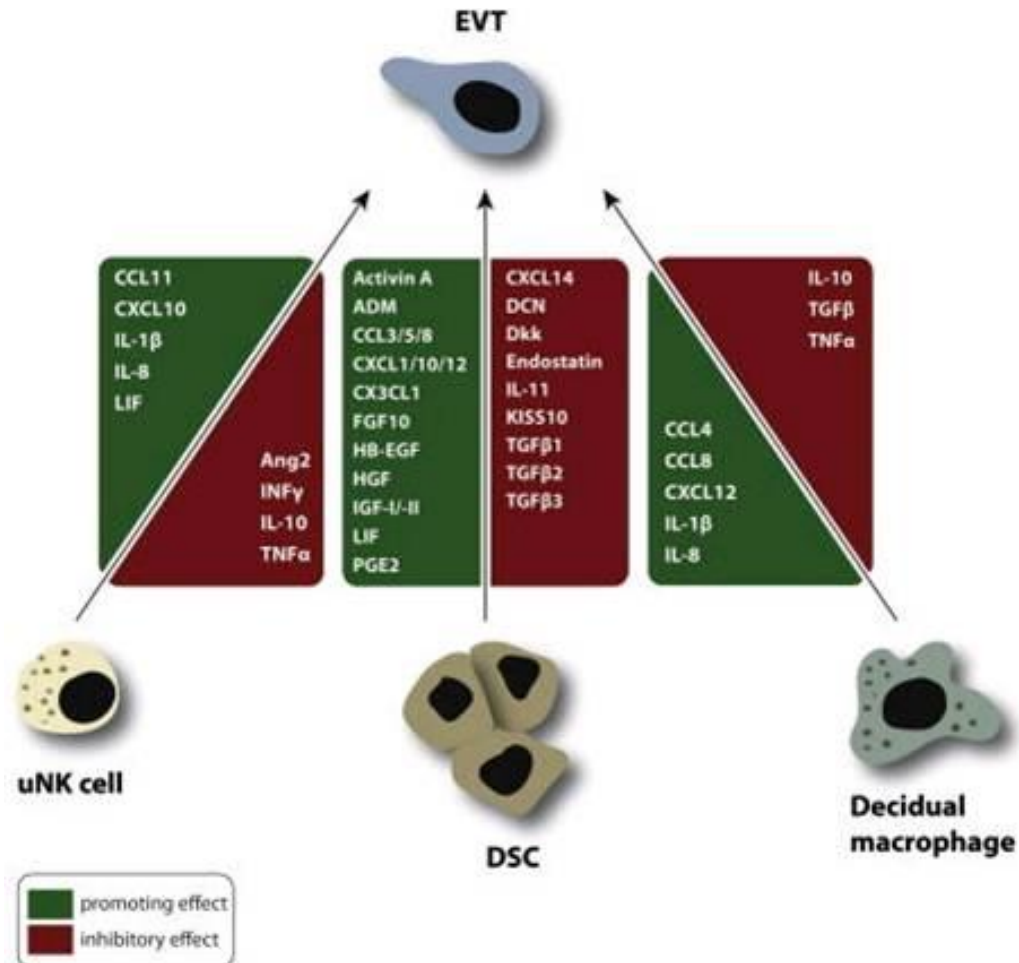
Figure 1.8 Inhibitory and stimulating factors influencing trophoblast invasion
Summary of inhibitory and stimulating factors secreted by predominant cells of the decidua; uNK cells, decidual stromal cells and macrophages which influence trophoblast invasive behaviour. Image from Knofler and Pollheimer (2012).

1.4 Decidual cell populations

1.4.1 Leucocyte populations and distribution

The human endometrium is a complex mucosal tissue and has a unique immune cell component (Eriksson et al., 2004). This component prepares the maternal environment to adapt to the implanting fetus by providing immune surveillance against invading pathogens and regulating immune responses to the semi-allogeneic

fetus. This balance of immune regulation is necessary for successful pregnancy and is controlled by an intrauterine leucocyte population which differs from peripheral blood leucocyte populations adding to the immune paradigm.

Maternal leucocytes are an important component of the endometrium. They are present during the proliferative phase and early secretory phase of the menstrual cycle but increase in number substantially at the time of expected implantation and continue to rise in early pregnancy (Bulmer et al., 1991), accounting for approximately 30-40% of the stromal cells within the decidualised endometrium lining the uterus during pregnancy. Three major decidual leucocyte populations have been identified in pregnancy; uterine natural killer cells, macrophages and T lymphocytes (Bulmer et al., 1991); dendritic cells are less abundant (Gardner and Moffett, 2003) with NKT cells (Tsuda et al., 2001) and T regulatory cells (Heikkinen et al., 2004) accounting for a small subset of the decidual leucocyte population but with equal functional importance. All of these endometrial leucocytes are present at the implantation site where they encounter the invading trophoblast. In contrast B lymphocytes are rare in the decidua throughout pregnancy.

The explanation for the increasing numbers of leucocytes around the time of expected implantation (mid secretory phase) in a fertilised cycle still remains unknown. Suggestions for this increase include hormonal fluctuations, differentiation of precursor cells and proliferation of existing leucocytes or leucocyte recruitment from other sites. There has been extensive investigation of the role of endometrial leucocytes in pregnancy ranging from immune tolerance to regulation of trophoblast invasion and spiral artery remodelling. Uterine natural killer cells and macrophages have been implicated in pregnancy disorders such as pre-eclampsia, fetal growth restriction and recurrent abortion suggesting that these leucocytes have an important role in normal pregnancy.

1.4.2 Uterine natural killer cells

Human uterine natural killer (uNK) cells are the most prominent leucocyte population within early pregnant decidua and they rapidly increase in number during the first trimester of pregnancy, accounting for over 70% of the decidual stromal leucocyte

population during the first half of pregnancy with uNK cell numbers decreasing after week 20 of gestation (Bulmer et al., 1991).

1.4.2.1 Phenotypic features of uterine natural killer cells

Human natural killer (NK) cells originate from stem cell haematopoietic precursors (Colucci et al., 2003). Distinct lineages of NK cells arise depending on their location; in tissues, lymphoid and non-lymphoid organs and peripheral blood where they may differ in phenotype and function.

Uterine natural killer cells have been classified as a 'natural killer' cell subtype due to their cytoplasmic granules similar to that of the large granular lymphocyte appearance of peripheral blood (PB) NK cells. They were first recognised in the 1920s (Bulmer and Lash, 2005) and further characterised in non-pregnant and pregnant endometrium using phloxine tartrazine staining of cytoplasmic granules (Hamperl and Hellweg, 1958). The majority of NK cells are characterised by their expression of CD56 cell membrane protein and the lack of CD3 expression and uterine natural killer cells are now characterised by their CD56 expression. Bulmer et al. (1991) quantified the numbers of phloxine tartrazine granulated cells and CD56$^+$ cells in non-pregnant and early pregnant endometrium and the results were comparable. This major subset of NK cells in the uterus predominantly have the phenotype CD56^{bright} CD16^{negative}, similar to an NK cell phenotype observed in the gastrointestinal tract and secondary lymph nodes (Drayton et al., 2006, Sentman et al., 2007), whereas over 90% of the peripheral blood NK cell population are CD56^{dim} CD16^{positive}, the remaining 10% being CD56^{bright} CD16^{dim} (Cooper et al., 2001). In contrast with uNK cells, which have cytoplasmic granules, peripheral blood CD56^{bright} CD16^{dim} NK cells are usually agranular. The CD56 antigen expressed on human NK cells is an isoform of the human neural cell adhesion molecule with an unknown function, but the CD56^{dim} NK cell population is highly cytotoxic while the CD56^{positive} population are strong producers of cytokines (Cooper et al., 2001, Szekeres-Bartho, 2008). Uterine NK cells express a variety of other cell surface markers that reflect their unique phenotypic and functional properties (Cooper et al., 2001); these are summarised in Table 1.1. The CD16 cell surface molecule is a low affinity FcγR, which binds to antibody coated targets resulting in a signal for cellular cytotoxicity (Cooper et al., 2001). CD16 along with other cell surface molecules

gives rise to the antibody dependent cytotoxic potential of NK cells, whereas the release of perforin (Rukavina et al., 1995) and granulysin (Nakashima et al., 2008) by uNK cells is associated with cell mediated killing, although this NK cell mediated apoptosis has never been observed in normal human decidua unless activated *in vitro* with IL-2 or IL-15 (Verma et al., 2000, Kopcow et al., 2005). Unlike peripheral blood NK cells, uNK cells lack expression of CD16, the consequence of this difference affects the functional antibody dependent cytotoxity of these cells and uNK cells. Furthermore, uNK cells have lower cytotoxic activity than PBNK cells despite their content of perforin (Kopcow et al., 2005).

CD9 and KIRs (killer immunoglobulin-like receptors) are other cell surface markers used to distinguish between CD56^{bright} PBNK cells and uNK cells as these markers are more widely expressed on the latter, along with CD9 expression (Eriksson et al., 2004). CD9 is believed to be involved in various cellular and physiological functions such as cellular migration and invasion (Boucheix and Rubinstein, 2001). The expression of cell surface receptors by uNK cells may also modulate the maternal immune response at the time of implantation and placentation; they are able to recognise the unusual trophoblast MHC class I ligands, such as HLA-C, HLA-G and HLA-E and reproductive success may primarily depend on this allorecognition (King et al., 1996a, Koopman et al., 2003). KIR expression by uNK cells is highly variable and allows for recognition with polymorphic HLA-C ligands expressed by EVT (Sharkey et al., 2008). The EVT HLA-E ligand is recognised by uNK cells through the lectin-like inhibitory receptor CD94/NKG2A (King et al., 2000b). There is evidence that HLA-G binds to uNK cells by KIR2DL4 receptor, but expression of this receptor by uNK cells is debated (Apps et al., 2008) and it is likely that trophoblast HLA-G binds to LILR (leucocyte immunoglobulin-like receptors) which is expressed by decidual macrophages (discussed further in section 1.4.3) and is only present on a minority of uNK cells (Apps et al., 2007). Uterine natural killer cells are also able to recognise HLA-C expressed by extravillous trophoblast. Each individual pregnancy is characterised by specific combinations of uNK cell KIRs and EVT HLA-C variants; although complex, particular combinations such as maternal KIR AA and fetal HLA-C2 are over represented in pre-eclampsia, intrauterine growth restriction and recurrent miscarriage (Hiby et al., 2010), highlighting that the interaction between EVT

HLA-C and uNK KIRS governs the maternal-fetal immune response and therefore appears to be crucial for reproductive success.

Expressing this mix of highly variable KIRs and the activating receptors NKp30, NKp44, NKG2D, uNK cells remain tolerant towards trophoblast cells and have been suggested to be cytokine producers at the fetal-maternal interface (Kopcow et al., 2005).

Table 1.1 Phenotypic differences and similarities of NK cell surface markers
Phenotypic differences and similarities of expression of cell surface markers by PBNK (peripheral blood NK) and uNK (uterine NK) cells. Table modified from Kalkunte et al. (2008); (+ presence; - absence; +/- variable expression).

Antigen/NK receptors	PBNK	uNK
CD56	dim	bright
CD45	+	+
CD16	+	-
CD69	-	+
CD9	-	+
KIR	+	+
NKp30	+	+
NKp44	-	+
NKp46	+/-	+
NKG2D	+	+
CD94/NKG2A	+/-	+

1.4.2.2 uNK cell localisation and distribution

Uterine NK cells are found in abundance in both decidua basalis and decidua parietalis; they are not a prominent feature in the human myometrium and therefore should perhaps be termed 'endometrial' rather than 'uterine' (Bulmer and Lash, 2005) . During the first trimester of pregnancy the uNK cells are in close association with fetal trophoblast cells (King et al., 1996a), but the presence of uNK cells in endometrium does not depend on the presence of trophoblast and they are also present throughout the menstrual cycle in non-pregnant endometrium (Bulmer and Lash, 2005), albeit in small numbers in proliferative and early secretory phases (Loke et al., 1995). Uterine natural killer cells are also prominent in decidualised endometrium in ectopic

pregnancy where trophoblast is absent (Laskarin et al., 2010) as well as pseudo decidualised endometrium under the influence of progesterone (Le Bouteiller and Piccinni, 2008). Oestrogen and progesterone are thought to play a role in regulating the proliferation and recruitment of uNK cells (Bulmer and Lash, 2005) and uNK cells are particularly prominent in progesterone treated endometrium. However, uNK cells themselves lack expression of the progesterone receptor suggesting that their function is not directly altered by the hormone (King et al., 1996b) but uNK cells do express ER-β1 and Gh receptor proteins indicating that oestrogen and glutocorticoids may up-regulate gene transcription in uNK cells (Henderson et al., 2003). Progesterone plays a role in up-regulating the release of various factors from surrounding stromal cells, such as endometrial derived IL-15 and prolactin (Kao et al., 2002, Mirkin et al., 2005), all of which may influence uNK cell differentiation, function and proliferation (Bulmer and Lash, 2005). The co-localisation of decidualised endometrial stromal cells with uNK cells is striking and uNK cells are also seen in areas of ectopic decidua; this relationship may suggest a fundamental role for decidual stromal cells in the differentiation of uNK cells (Kitaya et al., 2000, Dunn et al., 2002). If pregnancy does not occur, uNK cell numbers begin to diminish during menstruation providing further evidence of direct or indirect hormonal influence on the evolution of these cells. During early pregnancy uNK cells accumulate around invading EVT and remodelling SpAs (Bulmer et al., 1991, Smith et al., 2009) and contribute to the majority of decidual leucocytes into the early second trimester (Williams et al., 2009b). Their numbers progressively dwindle in the second trimester and lower numbers of CD56$^+$ cells are observed in term deciduas (Williams et al., 2009b). This spatial and temporal distribution has led to the suggestion that uNK cells play a role in trophoblast invasion and SpA remodelling.

1.4.2.3 uNK cell function during pregnancy

The role of uNK cells during early pregnancy remains unclear but their importance in placentation has been demonstrated in both mouse and humans (Manaster and Mandelboim, 2010). This has led to investigation and focus on uNK secreted cytokines and growth factors that may influence EVT invasion. More recently it has been proposed uNK cells may be involved in the early stages of spiral artery remodelling due

to the fact that uNK cells are present in maximum numbers at the time of spiral artery remodelling and reduce in number in the second half of pregnancy (Smith et al., 2009).

Uterine NK cells produce a significant number of cytokines, chemokines and angiogenic growth factors including IL-10, IL-1β, TGF-β1, INF-γ, GM-CSF, LIF and TNF-α (Rieger et al., 2001, von Rango et al., 2003) with production of some cytokines increasing with gestational age (Lash et al., 2010b).

The cytokines produced by uNK cells have been proposed to regulate EVT invasion in early pregnancy. There is evidence that the uNK cell derived cytokines including TNF-α, TGF-β1 and IFN-γ all inhibit trophoblast invasion *in vitro* (Lash et al., 2005, Lash et al., 2006b, Otun et al., 2011). However, other studies have reported stimulation of trophoblast invasion with uNK cell supernatants (Hanna et al., 2006, Lash et al., 2010a), although gestational age differences have been reported (Lash et al., 2010b). A recent study by (De Oliveira et al., 2010) demonstrated that IL-8 secreted by uNK cells increased the invasiveness of EVT using placental explants. These are but a few of the many studies that have investigated the effects of various cytokines and chemokines that are known to be produced by uNK cells on trophoblast invasion, with contradictory reports. This is likely to be explained by the complexity of the cytokine milieu *in vivo*, the different tissues used and their gestational age, as it has been demonstrated that uNK cell cytokine secretion differs with gestational age (Naruse et al., 2010, Lash et al., 2010c). Recent co-culture studies with isolated uNK cells and EVT from the same patient also had an effect on the cytokine secretion profile of uNK cells (Lash et al., 2011); secretion of several cytokines and growth factors was reduced when uNK cells and EVT were in direct contact, suggesting a regulatory mechanism for cytokine secretion in the placental bed.

The role of uNK cells in SpA remodelling has been suggested by numerous immunohistochemical studies; Lash et al. (2006c) have demonstrated that human uNK cells isolated from first trimester decidua are a prominent source of a range of angiogenic growth factors which reduce with increasing gestational age. Reduced numbers of uNK cells in third trimester pregnant decidua have been associated with pre-eclampsia and intrauterine growth restriction (Williams et al., 2009a), suggesting a role for these cells in SpA remodelling. Craven et al. (1998) proposed that early initial

changes in SpA remodelling occur prior to invading trophoblast and it has recently been reported that uNK cells have a possible involvement in mediating the effects of early 'trophoblast-independent' SpA remodelling (Pijnenborg et al., 2006b, Smith et al., 2009, Fraser et al., 2012, Robson et al., 2012, Wallace et al., 2012). Robson et al. (2012) reported evidence of VSMC disruption when chorionic plate arteries from term placentas and myometrial arteries from non-pregnant hysterectomy specimens were treated with uNK cell supernatants from pregnancies at 8-10 weeks gestation. Disruptions in VSMC integrity was observed when both CpA and non-pregnant myometrial SpA vessels were treated with Ang-1, Ang-2, IFN-γ and VEGF-C – all of which are growth factors produced by uNK cells (Lash et al., 2006c), and the addition of Ang-2 inhibitor also abrogated the effects of uNK cell supernatants on VSMC disorganisation in CpA. Altered levels of extra cellular matrix components of the vessel walls were also observed when treated with uNK cell supernatants in both vessel models suggesting that these cells may initiate the early stages of SpA trophoblast independent remodelling by the action of MMPs and via the secretion of Ang-2. Other studies suggest that the early vascular changes in SpAs are initiated by uNK cells through apoptotic mechanisms (Harris et al., 2007, Keogh et al., 2007, Smith et al., 2009, Fraser et al., 2012).

Studies investigating murine pregnancies have also implicated uNK cells in SpA remodelling. This was first demonstrated in the Tgε26 strain of mouse that lacks NK cells; they display abnormal vasculature and irregular decidual organisation along with a small placenta (Guimond et al., 1997). Uterine NK cell derived cytokines, such as interferon-γ (IFN-γ), have been suggested to be important in SpA remodelling. IFN-γ in mouse models modulates vascular modification and decidual integrity (Ashkar et al., 2000), but in humans it has been observed that uNK-cell derived IFN-γ is secreted at low levels (Lash et al., 2006b, Marzusch et al., 1997) and it has little effect on VSMC organisation of non-pregnant myometrial arteries *in vitro* but does alter VSMC organisation of chorionic plate arteries (Robson et al., 2012); it is likely that angiogenic growth factors play a more substantial role. Together with murine studies and investigation with human pregnancies, there is accumulating evidence that uNK cells are a major source of decidual cytokines, which could be involved in the processes of early pregnancy and now numerous studies are focusing on this aspect of their biology.

1.4.2 Decidual T lymphocytes

Approximately 10% of the endometrial stromal leucocyte population comprises CD3$^+$ T lymphocytes in early pregnancy (Bulmer et al., 1991). CD3$^+$ T lymphocytes have been subjected to relatively little research compared with uNK cells and decidual/endometrial macrophages. Endometrial CD3$^+$ T lymphocytes are predominantly CD8$^+$ unlike the peripheral blood population where CD3$^+$ CD4$^+$ T cells are more abundant (3:1 ratio) (Bulmer et al., 1991).

Endometrial T lymphocytes numbers do not alter greatly during the menstrual cycle and pregnancy, leading to the suggestion that these cells do not have a key role in the establishment and maintenance of pregnancy but rather play an important role in providing immune protection against external pathogens (Vassiliadou and Bulmer, 1996). Murine studies have shown that decidual CD8$^+$ T lymphocytes exert immunosuppressive activity by a reduction in cytotoxic activity (Nagarkatti and Clark, 1983, Thomas and Erickson, 1986). However, Scaife et al. (2006) demonstrated that decidual CD8$^+$ T lymphocytes isolated from early pregnancy are capable of cytolysis and evoke a Th1 cytokine response, releasing high levels of IFN-γ.

Decidual T lymphocytes have been reported to express markers such as HLA-DR, CD38 and CD49a unlike peripheral blood T lymphocytes (Geiselhart et al., 1995) and decidual CD8$^+$ T lymphocytes have been reported to stimulate trophoblast invasion *in vitro* (Scaife et al., 2006), suggesting that these cells may have an important role in trophoblast function and normal placental development.

CD4$^+$ T cells are a minor component of the decidual leucocoyte population and immunohistochemical studies have been difficult to identify these cells in the endometrium due to macrophage expression of CD4. To date there have been no specific functional studies of CD4$^+$ T cells in the endometrium or decidua.

1.4.2.1 Natural killer T cells

Human natural killer T (NKT) cells have been identified in early pregnant decidua (Tsuda et al., 2001). NKT cells are characterised by the expression of either a non-variant or invariant T cell receptor, CD161 (C-type lectin) and CD56. NKT cells are present at the maternal-fetal interface during pregnancy and the proportion of non-

variant NKT cells are higher in pregnant decidua compared with NKT cells in peripheral blood of the same patients (Tsuda et al., 2001, Boyson et al., 2002, Uemura et al., 2008).

Peripheral blood NKT cells are potent anti-tumour effector cells (Ohkawa et al., 2001). Unlike uNK cells NKT cells lack expression of KIR CD94/NKG2A and their suggested role in pregnancy is to produce cytokines. NKT cells have been reported to produce large amounts of IL-4 and IFN-γ (Bendelac, 1997, Tsuda et al., 2001); cytokines that have been implicated in pregnancy. Although NKT cells do not express KIRs that enable them to recognise MHC class I molecules, recognition of trophoblast may be achieved through presentation of glycolipids on CD1d receptor expressed by trophoblast cells (Bendelac et al., 2007, Uemura et al., 2008). Numbers of $CD3^+CD56^+$ cells dwindle in decidua at term compared with numbers in first trimester decidua (Williams et al., 2009b) and this observation together with reports of reduced $CD3^+CD56^+$ cell numbers in recurrent miscarriage (Yamamoto et al., 1999), suggests a role for these cells in early pregnancy processes.

1.4.2.2 Regulatory T lymphocytes

Regulatory T lymphocytes (T reg cells) are a highly heterogeneous T cell population and play an important role in suppressing the peripheral T cell response. $CD4^+CD25^+$ T reg cells are the most widely studied T reg population. T reg cells are present at the maternal-fetal interface and 10-15% of decidual $CD4^+$ T lymphocytes are $CD4^+CD25^+$ T reg cells (Heikkinen et al., 2004). Yang et al. (2009) reported an increase of CD25 bright cells in the decidual $CD4^+$ T cell population compared with the peripheral $CD4^+$ T cell population, suggesting a role for these cells in maternal tolerance to fetal alloantigens. Nevertheless, the activation of regulatory T cells and induction of tolerance in pregnancy remains unclear, although reduced numbers of $CD4^+CD25^+$ T reg cells have been associated with sporadic miscarriage (Sasaki et al., 2004), recurrent miscarriage (Yang et al., 2009) and pre-eclampsia (Sasaki et al., 2007).

1.4.3 Decidual macrophages

Macrophages have the ability to alter their antigen presenting status in response to the microenvironment, thereby altering their function allowing them to participate in a

wide range of biological processes including metabolism, vascular remodelling and tissue homeostasis (Gordon, 2007).

Decidual macrophages are the second most abundant leucocyte population and account for approximately 20-25% of all decidual leucocytes in early pregnancy (Bulmer et al., 1988) and are present at the implantation site (Loke and King, 2000). Macrophages are potent antigen presenting cells (APCs) and are more abundant APCs than dendritic cells. They have been characterised in the endometrium and decidua by immunodetection of CD14 cell surface protein and CD68 lysosomal protein (CD14$^+$/CD68$^+$). Despite some similarities with peripheral blood monocytes, evidence regarding the origin of decidual macrophages is lacking. However, increasing evidence suggests that decidual macrophages originate from a myeloid lineage and have a plasticity which allows them to alter their phenotype by functional differentiation to adapt to the local microenvironment of the human decidua.

Histological studies have provided circumstantial evidence that uNK cells along with other leucocytes wirthin the decidua, including decidual macrophages, initiate spiral artery remodelling prior to the invasion of extravillous trophoblast cells (Smith et al., 2009). Decidual leucocytes including decidual macrophages are producers of varied amounts of growth factors, cytokines and proteases which may contribute to vessel remodelling (Plaisier et al., 2009).

Decidual macrophages highly express the MHC class II human leucocyte antigen HLA-DR, allowing antigen presentation to decidual T cells, contributing to maternal-fetal tolerance (Figure 1.9). This allows the invading trophoblast to interact with macrophages in the decidua through a variety of receptor- ligand pairs, thereby activating macrophages in the decidua (Vince and Johnson, 2000, Renaud and Graham, 2008). Leucocyte immunoglobulin-like receptor (LILR) expression has been detected on decidual macrophages (Apps et al., 2007). LILR is a ligand for non-classical HLA-G expressed on EVT; therefore, invasive trophoblast may modulate the function of decidual macrophages or vice versa (Figure 1.9). Decidual macrophages are thought to be incapable of differentiating into dendritic cells, but during early pregnancy a subpopulation of CD14$^+$/HLA-DR$^+$ cells express the immature dendritic cell marker

CD209 (DC-SIGN) (Kammerer et al., 2003) suggesting that these cells have the ability to differentiate into mature (CD83$^+$) dendritic cells.

Decidual macrophages also have an important immunoregulatory role preventing aggressive immune inflammatory responses, thus resulting in a tolerant immune environment that supports placentation, implantation, efficient trophoblast invasion and vascular remodelling (Seavey and Mosmann, 2008). Classic macrophages secrete high amounts of Th1 pro-inflammatory cytokines; TNF-α and IL-12 have been classified as M1 pro-inflammatory macrophages, whereas macrophages which respond to Th2 cytokines such as IL-4 and IL-13 possess an immunosuppressive phenotype and are therefore termed activated macrophages or M2 macrophages. Decidual macrophages have been suggested to have an immunosuppressive role by the secretion of prostaglandins and cytokines such as IL-10 and IL-1 (Goerdt and Orfanos, 1999). Lidstrom et al. (2003), using an enzyme-linked immunosorbent spot-forming cell assay (ELISPOT), demonstrated that decidual macrophages are potent producers of IL-10 which is an immunosuppressive cytokine indicating that macrophages could potentially regulate functions such as trophoblast invasion in early pregnancy (Renaud and Graham, 2008). In keeping with this, gene expression profiles from microarrays have demonstrated that decidual macrophages contain up-regulated transcripts encoding genes for immunosuppressive and tissue remodelling proteins compared with peripheral blood macrophages (Gustafsson et al., 2008). In addition to production of immunosuppressive cytokines, cell lines expressing the trophoblast HLA-G homodimer induce decidual macrophages to secrete large amounts of pro-inflammatory cytokines IL-6 and IL-8 (Li et al., 2009). It has been suggested that infection during pregnancy may cause aberrant macrophage activation, leading to switching from M2 polarisation (immunosuppressive) to M1 (pro-inflammatory) phenotype, with an increase in pro-inflammatory cytokine secretion; this would initially serve as a defence mechanism against invading pathogens but could in turn affect angiogenesis, trophoblast function and placental apoptosis resulting in a variety of pregnancy disorders (Renaud and Graham, 2008).

Macrophages are also capable of phagocytosis and decidual macrophages express specific phagocytic receptors which are able to bind with extracellular matrix products

(Repnik et al., 2008); high numbers of CD14$^+$ cells have been identified in proximity to spiral arteries suggesting phagocytosis and apoptotic cell clearance (Abrahams et al., 2004a). Furthermore, recent data demonstrates the identification of two unique human decidual macrophage populations; CD11c^{HI} and CD11c^{LO} both with pro- and anti-inflammatory functions (Houser et al., 2011) suggesting that these cells have the ability to facilitate immune responses and tissue remodelling events by the secretion of proteases and cytokines playing an important role at the maternal-fetal interface.

Macrophage derived TNF-α has been shown to restrict trophoblast invasiveness through inducing apoptosis and increased macrophage derived TNF-α levels have been associated with pregnancies complicated by pre-eclampsia, intrauterine growth restriction and early onset spontaneous abortion (Reister et al., 2001). However, overall there are few reports on decidual macrophage cytokine secretion and relatively little attention has focused on macrophage function in normal pregnancies, although various model systems have been used to characterise phenotype.

Despite the limited understanding of decidual leucocytes it appears that they are associated with one another and the factors they produce are likely to work together to provide an environment which is conducive for successful pregnancy. Hence, studies of isolated single leucocyte populations *in vitro* may not reflect the *in vivo* situation where interactions between the various cell types are likely.
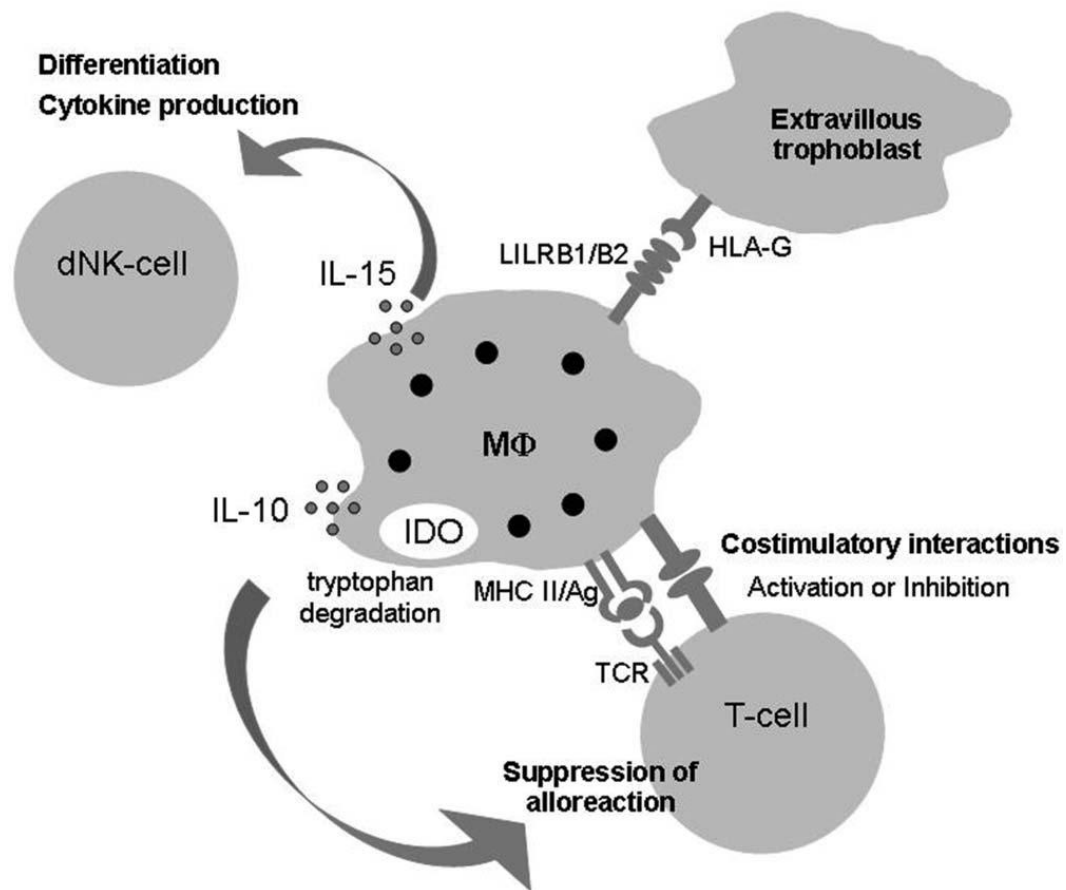
Figure 1.9 Molecular interactions of a decidual macrophage
Molecular interactions of decidual macrophages with cells of the decidua (Nagamatsu and Schust, 2010).

1.4.4 Decidual dendritic cells

Decidual dendritic cells account for 1-2% of the leucocyte population in early human pregnancy with numbers remaining constant throughout gestation. Dendritic cells are a unique cell population which induce primary immune responses. Following menstruation when oestrogen levels are highest, oestrogen stimulates the differentiation and maturation of dendritic cells to express costimulatory molecules including CD83 and CD86 and high levels of MHC class II (Paharkova-Vatchkova et al., 2004). Both immature (CD83$^-$/CD209$^+$) and mature dendritic cells have been identified in uterine tissues by expression of CD83 (Kammerer et al., 2000), suggesting that the presence of immature cells mediate tolerance to the developing fetus, whereas the mature dendritic cells protect the maternal-fetal interface from invading pathogens. Collins et al. (2009) recently presented evidence from studies in mouse agreeing with this; that the decidual environment prevents dendritic cell surveillance at the

maternal- fetal interface and that dendritic cell trafficking to the uterine lymph nodes from decidua is minimal; decidual factors play a role in sequestering the migratory capacity of mature dendritic cells whilst retaining the DC responsiveness to antigens. Kammerer et al. (2003) reported similar findings from isolated human decidual dendritic cells; these cells were unable to stimulate naïve T cells after antigen binding. The entrapment of decidual dendritic cells in the human endometrium remains to be understood and therefore migration and activation of T cell responses may contribute to pathological pregnancy. Uterine dendritic cells also secrete growth factors such as VEGF-A and TGF-β1 (Plaks et al., 2008) but they have no effect on trophoblast invasion (Huang et al., 2008), suggesting that their importance is for preparing the endometrium for embryo implantation. Decidual dendritic cells have been proposed to induce tolerance under normal physiological conditions acting as 'gatekeepers' of uterine immune environment (Blois et al., 2004).

Limited investigation of pathological pregnancies suggests that the function of these cells is in immunomodulatory control during pregnancy. Huang et al. (2008) reported infiltration of both CD83^{+} mature and CD209^{+} immature dendritic cells in decidua from women suffering pre-eclampsia suggesting that they may play a role in the pathogenesis of the disease; whether this is cause or effect is unclear.

1.4.5 Decidual stromal cells

Decidual stromal cells (DSCs) are the major component of the human decidua (Bulmer et al., 1995). Endometrial stromal cells are mainly comprised of fibroblast cells which are believed to originate from mesenchymal cells. Decidualisation of the endometrial stromal fibroblast cells is triggered by progesterone (Brosens et al., 2002, Gellersen and Brosens, 2003) and decidual stromal cells are considered to have a nutritional and endocrine function during pregnancy (Riddick and Kusmik, 1977). However, increasing evidence has demonstrated that decidual stromal cells orchestrate leucocyte differentiation, trafficking and recruitment into the endometrium during early pregnancy (Robertson et al., 2002). Hess et al. (2007) have also demonstrated that since EVT invade through the decidua, DSC are in close proximity to trophoblast cells *in vivo,* and these cells respond to trophoblast secreted products *in vitro*, enriching the

implantation site with cytokines and chemokines. DSCs can be identified within the decidua by their expression of CD10 and CD13 (Garcia-Pacheco et al., 2001).

The relevance of decidual stromal cells in pregnancy is that they exert immune functions and assist the immunomodulation of decidual factors ensuring a healthy environment for the developing fetus and these cells also produce a wide array of cytokines that may regulate EVT invasion and spiral artery remodelling.

1.4.6 Decidual epithelial cells

The human endometrium consists of glandular and surface epithelial cells. As well as the endometrial stroma, the glandular epithelium undergoes proliferation, differentiation and shedding throughout the menstrual cycle (Tabibzadeh, 1991). However, the functional role of glands post implantation has been sparsely studied.

There is increasing evidence that endometrial epithelial cells are important functional cells during the first trimester of pregnancy. Uterine glandular epithelial cells are present and highly active at 6 weeks of pregnancy, secreting carbohydrates, sugars and lipids providing a source of essential nutrients at the maternal fetal interface to drive the many processes (Hempstock et al., 2004). A variety of growth factors and cytokines have also been identified in glandular secretions including VEGF, LIF and epidermal growth factor (EGF) receptors for which are expressed by villous and extravillous trophoblast cells (Burton et al., 2007), suggesting that these secretory factors produced by decidual glands play roles in placental morphogenesis, trophoblast invasion and modulate immune responses at the maternal-fetal interface during the first trimester of pregnancy.

1.5 Interleukin-6

1.5.1 The role of IL-6

IL-6 is a small pleiotrophic cytokine which is an important mediator in acute-phase responses to injury and infection. IL-6 is part of the IL-6 family of cytokines which also includes LIF, IL-11, oncostatin M (OSM), ciliary neutrophic factor and cardiotrophin-1, all sharing the gp130 receptor subunit and involved in activation of target genes involved in cellular differentiation, proliferation, apoptosis and cell survival. This common signalling element results in overlapping functions of between these proteins.

IL-6 has pro and anti-inflammatory properties resulting in a wide array of functions besides host defence; IL-6 also has important roles in haematopoiesis, lipid metabolism and embryonic development. IL-6 is involved in the pathophysiology of many processes resulting in osteoporosis, rheumatoid arthritis and contributes to oncogenesis in many cancers. IL-6 has been reported to be expressed in a variety of malignant tumours (Kanazawa et al., 2007) and has been reported to increase the invasive potential of head and neck squamous cell carcinomas *in vitro* (Nishino et al., 1998) as well as reports of inducing morphological changes in a pleomorphic malignant fibrous histiocytoma cell line (Nakanishi et al., 2004). Walter et al. (2009) recently demonstrated that IL-6 secretion from adipose stromal cells in the breast promotes the migration and invasion breast tumour cells *in vitro*.

IL-6 is secreted by a range of cell types such as monocytes, lymphocytes and fibroblasts.

1.5.2 IL-6 cell signalling

1.5.2.1 IL-6 receptors

IL-6 achieves its biological effects by binding to plasma membrane receptor complexes consisting of an 80kDa protein IL-6Rα (gp80; CD126) and the common 130kDa signal transducing receptor protein gp130 (CD130).

Gp130 is ubiquitously expressed on a wide assortment of cells in the body, whereas distribution of membrane IL-6Rα is limited and is only expressed on a relatively small number of cell types such as hepatocytes, neutrophils and monocytes, indicating that the physiological effects of IL-6 may depend on the soluble form of the receptor sIL-6Rα, therefore allowing IL-6 to elicit its effects on a wide variety of cells. *In vivo* experiments have revealed sIL-6Rα is generated either by proteolysis of the membrane form of the receptor or as a result of alternatively spliced mRNA (Knupfer and Preiss, 2008). This soluble receptor - cytokine complex acts agonistically rather than antagonistically (Ancey et al., 2003) which is in constrast with most cytokine/soluble receptor systems where cytokine binding to the plasma membrane is prevented having an antagonistic effect. The soluble form of IL-6Rα is a 50-55k Da binding protein that binds IL-6 ligands with similar affinity to the membrane bound recpetor. Once IL-6 is

bound to sIL-6Rα, the protein-receptor complex is recognised by and associates with a homerdimeric transmembrane gp130 receptor which then initiates an intracellular response through activation of tyrosine kinases on the cytoplasmic tail of the receptor (Murakami et al., 1993). Some people view this complex of IL-6 and soluble IL-Rα as a cytokine complex rather than a receptor complex. This allows cells such as embryonic stem cells, neural cells, smooth muscle cells and endothelial cells which express gp130 but lack transmembrane IL-6R to respond to IL-6, widening the repertoire of cell types that are responsive to this cytokine giving IL-6 the potential to have a spectrum of functions such as pro and anti inflammatory activites and aberrant activity in disease states.

1.5.2.2 STAT3, SOCS, MAPKs

It has been established that most cytokines mediate their effects through two main signal transducing pathways; the signal tranducers and activators of transcription (STAT) pathway and the mitogen activated protein kinase (MAPK) pathway (Fitzgerald et al., 2005). Extensive studies have highlighted a pivotal role of STATs in IL-6 cytokine signalling, IL-6 type cytokines including IL-6 utilising the STAT pathway to mediate their effects (Fitzgerald et al., 2008). The STAT family is comprised of seven transcript variants; STAT1, 2, 3, 4, 5a, 5b and STAT6 and transient association with cytokine receptors causes their activation. STAT1 and STAT3 are potently activated by the gp130 receptor complexes of all the IL-6 type cytokines (Heinrich et al., 1998). IL-6 initiates its effects by binding to its receptor system on target cells which causes the gp130 receptors to aggregate. Dimerisation of gp130 leads to the juxtaposition of the Janus kinases (Jaks) (Fitzgerald et al., 2005), allowing the Jaks to cross-phosporylate one another leading to the phosphorylation of docking sites of the SH2 tyrosine phosphatase domain and STATs (Kang and Kang, 2008). The STATs then dissociate from the ligand where they form homo and heterodimers with other phosphorylated STATs. Activated STATs then translocate into the nucleus where they specifically bind to promoter regions of the DNA and upregulate the transcription of several genes which are involved in proliferation and cell differentiation (Corvinus et al., 2003, Fitzgerald et al., 2005) (Figure 1.10). This includes the upregulation of suppressors of cytokine

signalling (SOCS) which in an inhibitory feedback system stalls the transduction of the signal (Heinrich et al., 2003, Fitzgerald et al., 2005) (Figure 1.10).

A high degree of STAT3 activity is associated with malignancy of cells and can be activated through IL-6 ligand binding (Corvinus et al., 2003). The phosphorylation of STAT3 induced by IL-6 binding also leads to the activation of genes involved in chronic inflammation associated with arthiritis (Kishimoto, 2006). STAT3 deficient mice have demonstrated embryonic lethality: the embryos successfully implant but rapidly die due to placental defects (Takeda et al., 1997). STAT3 splice variants (STAT3α and β) have been observed in first trimester trophoblast cells with expression lost at term (Corvinus et al., 2003). This adds to the proposition for a role for STAT3, via cytokine signalling, in trophoblast invasiveness.
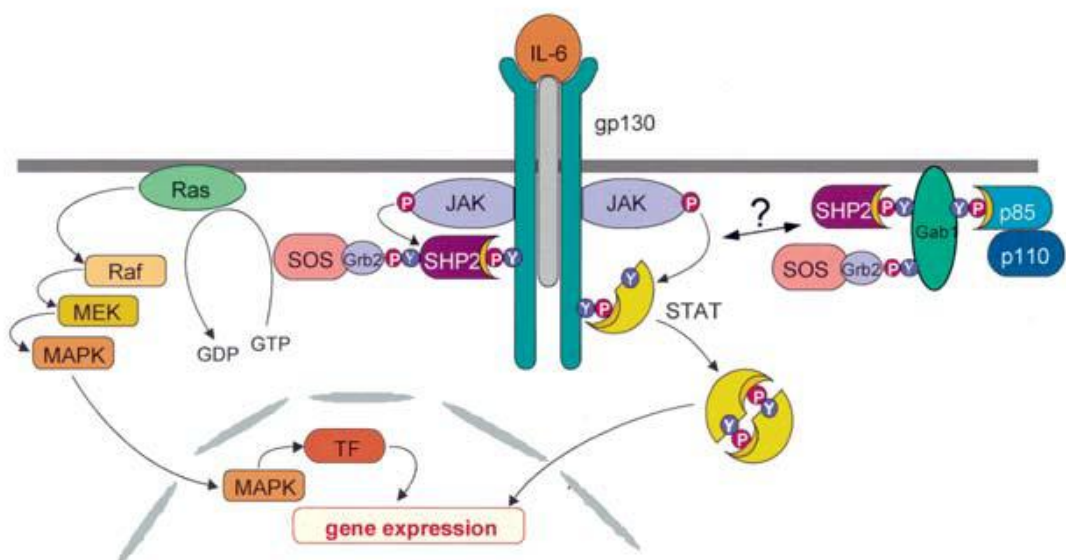
Figure 1.10 Representative diagram of IL-6 cell signalling pathways
Representation of the different pathways by which IL-6 can signal and stimulate differing cellular cascades. Activation of the Jak-STAT pathway by cytokine (IL-6) binding to its receptor (IL-6Rα and gp130). Cytokine-induced receptor aggregation leads to Janus kinase (Jak) phosphorylation and activation. Tyrosine residues within the cytoplasmic tail of the cytokine receptor are subsequently phosphorylated by the Jaks, providing docking sites for SH2 domain-containing signalling proteins including STATs. Tyrosine-phosphorylated STATs translocate to the nucleus where they bind to promoter regions of their respective target genes, including genes involved in acute phase protein synthesis, cell growth, cell differentiation and proliferation and the up-regulation of SOCS, creating a feedback regulatory system to dampen down the cytokine response. Adapted from (Heinrich et al., 2003).

Dimerisation of IL-6 gp130 complex also induces the MAPK cascade. MAPKs also play an important part in cellular migration and invasion (Pollheimer and Knofler, 2005). The activation of MAPKs includes activation of extracellular-regulated kinases (ERKs). The phosphorylation of ERK 1 and ERK 2 have been shown to increase cell growth and migration of the human trophoblast cell line, HTR-8/SVneo (Pollheimer and Knofler, 2005). In accordance with this, stimulated (phosphorylated) ERK 1 and ERK 2 have been detected in EVT of human placentae but only up to 12 weeks gestation (Kita et al., 2003), suggesting that ERK signalling plays a crucial role during early pregnancy.

1.5.3 IL-6 in pregnancy

IL-6 is present in human endometrium throughout the menstrual cycle as well as in early pregnancy decidua. IL-6 is secreted by various cell types during pregnancy, but production by specific decidual cells remain to be investigated. Aforementioned previously there appears to be a delicate balance between a Th1 and a Th2 immune response during the implantation window up to placental vascularisation (Chaouat et al., 2002, Chaouat et al., 2003, Chaouat et al., 2004). It has also been reported that EVT cells express IL-6, and it has been suggested this cytokine can influence trophoblast invasion by up-regulating MMPs (Salamonsen et al., 2007). Recent studies have localised IL-6 protein and IL-6 mRNA within mid-secretory phase endometrium, along with immunohistochemical evidence of IL-6 receptors (Tabibzadeh et al., 1995). Jauniaux et al. (1996) have demonstrated IL-6 expression in both syncytiotrophoblast and EVT from explanted placental villi and in various other cell types within decidual tissue using immunohistochemistry. Meisser et al. (1999) found that IL-6 induced trophoblast MMP-2 and MMP-9 activation, which are important mediators in trophoblast invasion, but IL-6 did not induce MMP synthesis. In addition, IL-6 deficient mice have shown a 48% reduction in implantation sites which has been linked to decreased fertility (Robertson, 2000) and IL-6 deficient mice displayed an increased rate of mid-gestation miscarriage, although the mice were still fertile (Kopf et al., 1994). The overall evidence from these studies therefore, suggests a role for IL-6 in successful early pregnancy, potentially being involved in regulating trophoblast invasion.

It has been suggested that IL-6 may contribute to trophoblast growth and placental development by stimulation of trophoblast derived human chorionic gonadotrophin (hCG) (Nishino et al., 1990), which is essential for the maintenance of pregnancy. Both endogenous IL-6 and exogenous IL-6 have been reported to increase migration and the invasive potential of the EVT cell line HTR-8/SVneo due to the up-regulation of the trophoblast integrin receptors α_1, α_5 and β_5 (Jovanovic and Vicovac, 2009), receptors which bind to components within the extracellular matrix. Arimoto-Ishida et al. (2009) have demonstrated that up-regulation of α_5-integrin under hypoxic conditions increased the invasiveness and migration of placental villous explants cells and BeWo cells (trophoblast-derived cell line). However, these are cell line data and may vary from isolated primary cells which have yet to be studied. Despite extensive documentation of the presence of IL-6 in implantation, little is known about the effects of this cytokine and how it mediates signalling in the trophoblast. Various reports exist on specific cell lines but the overall picture of IL-6 production and function in uteroplacental tissues is still unclear.

The effect of decidual IL-6 on uterine vascular remodelling has not been investigated. However, in different tissues IL-6 has been shown to be a potent pro-angiogenic cytokine during tumour progression and promotes vasculogenesis in murine brain development (Fee et al., 2000). Yao et al. (2007) have demonstrated the effect of IL-6 on the stimulation of human cerebral smooth muscle and endothelial cell proliferation and migration and Fan et al. (2008) have provided more recent evidence that cellular signalling cascades from blood derived endothelial cells are activated by IL-6 *in vitro* resulting in proliferation, migration and matrigel tubule formation.

1.5.3.1 IL-6 in pathological pregnancies

Although IL-6 has been widely associated with studies involving the progression of malignant tumours and is known to have an effect on the invasiveness of neoplastic cells, there is limited information on its role in normal pregnancy. Elevated levels of plasma IL-6 have been detected in women suffering from pre-eclampsia (Greer et al., 1994, Vince et al., 1995, Madazli et al., 2003, Freeman et al., 2004) and decidual cells have been suggested to account for increasing plasma levels of IL-6 (Lockwood et al., 2008). In addition, a recent study has demonstrated an increase in serum IL-6 levels in

women with symptoms of threatened pre-term delivery (Sorokin et al., 2010) indicating that the cause may be due to an immunological effect.

1.6 Interleukin-8

1.6.1 Role of IL-8

Interleukin-8 (IL-8) is classified as a pro-inflammatory multifunctional CXC chemokine, alternatively known as CXCL8. Chemokines are a large superfamily of structurally and functionally related cytokines with chemotactic activity targeted at leucocytes. The IL-8 gene encodes a 99 amino acid protein with a 77-72aa signalling component and expression of this gene is primarily regulated by nuclear factor (NF)-κB. However, a number of stimuli, including inflammatory signals (TNF-α and IL-1β) and a variety of steroid hormones, also up regulate expression of IL-8 (Brat et al., 2005). IL-8 is best characterised as a neutrophil chemoattractant at sites of inflammation and has the ability to cause de-granulation of neutrophils and invasion of tissues (Matsushima et al., 1988). In contrast to inflamed tissues, the level of IL-8 is relatively low in normal tissues. IL-8 also plays a chemotactic role for neoplastic cells. Chemotaxis occurs along a chemokine concentration gradient and changes in the morphology of leucocytes can be observed following chemokine binding (Dimitriadis et al., 2005). The biological activity of chemokines can be enhanced by MMPs, many of which are secreted by leucocytes following chemokine binding resulting in a positive feedback mechanism and thereby up-regulating the action of chemokines themselves.

1.6.2 IL-8 cell signalling

1.6.2.1 CXCR1 and CXCR2

The biological effects of IL-8 are mediated through the binding of two membrane bound G protein-coupled receptors, CXCR1 and CXCR2 (Holmes et al., 1991). These receptors are around 40KDa in size and share structural similarities but mediate different functional responses (Holmes et al., 1991, Rosenkilde and Schwartz, 2004). The structural similarity between the two receptors suggests that they arose from genetic duplication. Ligand binding to the receptors elicits a ligand induced conformational change resulting in coupling of the heterotrimeric G proteins (Gαβγ)

with the carboxy terminus of the 7 transmembrane receptor, which in turn activates the Gα subunit to dissociate from the Gβγ subunit. The signal is transduced mainly through Gαi which causes the rapid mobilisation of intracellular calcium inducing the chemotactic response and there is evidence that released βγ activates PI3Kγ pathways, inducing cellular migration (Arai et al., 1997, Neptune and Bourne, 1997). Depending on the Gα isoforms, the alpha subunit can mediate intracellular signalling indirectly by acting on effector molecules (Figure 1.11) or directly by regulating ion channel or kinase function (Figure 1.11). Because of the promiscuity of G-protein coupled receptors (GPCRs), activation of CXCR1 and CXCR2 induces diverse signalling pathways (Figure 1.11) resulting in a wide range of functions. Both CXCR1 and CXCR2 bind IL-8 with high affinity, but CXCR1 is only activated in response to binding with IL-8 and granulocyte chemotactic protein-2(GCP-2), whereas great promiscuity is found with CXCR2, which can be activated by a variety of CXC chemokines (Brat et al., 2005), increasing the redundancy of this receptor. CXCR1 and CXCR2 expression has been characterised on many cell types other than leucocytes including neurons, epithelial cells, endothelial cells, smooth muscle cells and stromal cells indicating that receptor activation by IL-8 binding is not solely responsible for leucocyte trafficking but has different functions including degranulation, angiogenesis, gene transcription and apoptosis (Thelen, 2001). IL-8 signalling stimulates the phosphorylation of Akt kinase (Figure 1.11) which is an important kinase with established roles in the modulation of cell migration and angiogenesis. The angiogenic effects of IL-8 are mediated primarily through CXCR2 binding; this is evident from human herpes virus-8 (HHV8) which expresses a mutated form of CXCR2 with absent CXCR1, resulting in highly vascularised tumours in the host (Rosenkilde et al., 1999).
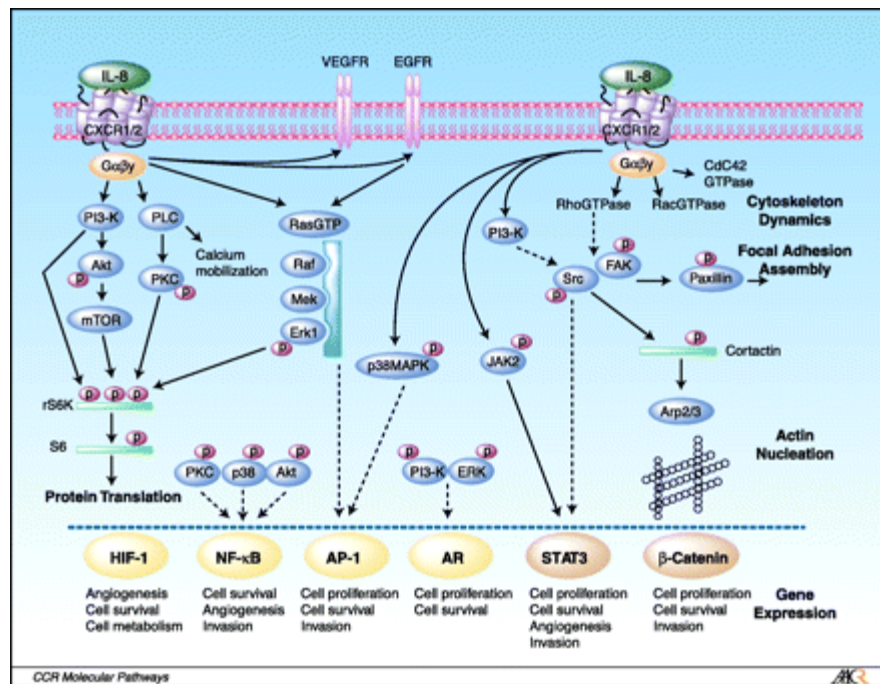
Figure 1.11 Representative diagram of IL-8 cell signalling pathways
Diagram showing the range of characterised IL-8 cell signalling pathways. Solid lines represent positive regulation by IL-8; dashed lines represent acknowledged pathways through which IL-8 mediates transcription factors. IL-8 signalling also activates Src and FAK which are non receptor tyrosine kinases (Waugh and Wilson, 2008).

1.6.2.2 Aminopeptidase N

The biological function of IL-8 is mediated through transmembrane G-protein coupled receptors that transmit a signal resulting in a cellular response (outlined in section 1.6.2.1).

Aminopeptidase N, alternatively known as CD13 is a Zn^{2+} dependent ectopeptidase membrane glycoprotein with a molecular weight of 150kDa (Hooper, 1994). It is a ubiquitously expressed broad functional protease that hydrolyses small peptides and preferentially cleaves neutral amino acids, removing N-terminus amino acids from their substrates. Ectoenzymes including aminopeptidase N have been recognised as moonlighting proteins due to their multifunctional capabilities (Jeffery, 2003). It has been proposed that an additional functional role of CD13 as well as its enzymatic activity is signal transduction in monocytes (Santos et al., 2000); CD13 does not contain a signalling motif due to its short cytoplasmic tail, but it is believed to associate with an auxiliary molecule giving the protein signalling capacity. Reports have demonstrated that cross-linking of CD13 in monocytes mediates the migration and aggregation of monocytes (Mina-Osorio et al., 2006) and induces the MAPK pathway leading to the

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influx of calcium effecting cytokine secretion and cellular adhesion; one such chemokine is IL-8 (Santos et al., 2000), further suggesting involvement of mechanisms other than enzyme activity. CD13 has been linked with the inactivation of IL-8 by degradation (Kanayama et al., 1995), along with degradation of CXCL11 (Proost et al., 2007) and causes down regulation of CXCR4 and inhibition of CXCL12 mediated migration in embryonic kidney cells (Wulfaenger et al., 2008) suggesting a regulatory role in inflammation, cell proliferation and invasion.

CD13 is expressed on the surface of many different cell types including macrophages, granulocytes, endothelial cells, synaptic membranes of the CNS, epithelial cells in kidney and intestines as well as a number of tumours (Look et al., 1989). CD13 has also been identified on placental villi, endometrial stromal cells and decidual stromal cells during the first trimester of pregnancy (Imai et al., 1992, Seli et al., 2001) and expression may be regulated by oestrogen (Seli et al., 2001), suggesting that CD13 may play an important role in the modulation of peptide metabolism to aid successful implantation and maintenance of pregnancy.

CD13 along with the other IL-8 receptors has not yet been studied in early pregnancy and the physiological roles of these enzymes and signalling components remains to be clarified. However, research to date suggests that these receptors may control the bioavailabilty of IL-8 and thereby contribute to the regulation of angiogenesis and leucocyte migration in pregnancy. Individual chemokine processing is complex due to redundancy of receptor binding and studies on IL-8 have mainly focused in endometriosis, chorioamnionitis, cervical ripening and parturition.

1.6.3 IL-8 in inflammation and angiogenesis

The chemokine system provides regulation and control of homeostatic leucocyte movement and inflammation. By their regulation of leucocyte activation, trafficking and differentiation, chemokines including IL-8 play a central role in immune defence. In addition IL-8 plays a role in other cellular functions (Section 1.5.2.1) including angiogenesis. Angiogenesis is described as the neo-formation of blood vessels and is essential for embryonic development, wound healing, inflammation and tumour growth. In 1992 IL-8 was the first angiogenic chemokine to be discovered (Koch et al., 1992) and there is increasing evidence of the potency of IL-8 angiogenic properties; IL-

8 signalling has been associated with the regulation of endothelial permeability by stimulating the phosphorylation of VEGFR-2 in endothelial cells by interaction with both CXCR1 and CXCR2 receptors (Petreaca et al., 2007). More recently it has been reported that IL-8 up regulates VEGF mRNA and protein levels in vascular endothelial cells by association of IL-8 with CXCR2; this further stimulates VEGFR-2 in an autocrine manner (Martin et al., 2009) inducing cellular proliferation, survival and migration of vascular endothelial cells. These reports highlight the potential importance of IL-8 in angiogenesis and raise the possibility of a role for IL-8 for the remodelling events in the uterine vasculature in normal pregnancy. In pathophysiology IL-8 is also important for tumour formation and growth, and many *in vitro* studies with cancer cell lines and *in vivo* studies with solid cancers have demonstrated that expression of IL-8 corresponds to the angiogenic and tumourgenic potential and hence the progression of cancers (Waugh and Wilson, 2008). To coincide with this hypothesis, there is evidence that IL-8 secretion is associated with glioma formation and malignant progression through the establishment of new blood vessels (Brat et al., 2005). Therefore, although the focus of investigating IL-8 has focused on its role in inflammation and chemotaxis, it is possible that the angiogenic effect of IL-8 is crucial in normal pregnancy.

1.6.4 IL-8 in pregnancy

The human endometrium is rich in a wide range of chemokines, including IL-8 which potentially regulates endometrial angiogenesis and leucocyte trafficking. Immunohistochemical studies have demonstrated expression of IL-8 on glandular epithelium during the menstrual cycle (Arici et al., 1993). With regard to early pregnancy, the recruitment and activation of macrophages and uNK cells are tightly regulated at implantation and this is likely to be achieved by the action of chemokines (Dimitriadis et al., 2005), although there is no direct evidence for IL-8 involvement. Uterine natural killer cells are themselves a major producer of IL-8 (Saito et al., 1994, De Oliveira et al., 2010). There are reports describing expression of IL-8 in the pregnany endometrium and IL-8 is likely to be involved in leucocyte trafficking to the spiral arteries during the first trimester of pregnancy (Hornung et al., 1997, Jones et al., 2004). IL-8 has been shown to be produced by cells of the placenta, including trophoblast, placental macrophages and fibroblasts, and it has been suggested that IL-

8 mediates the recruitment and accumulation of neutrophils at the maternal-fetal interface as a defence mechanism against invading bacteria (Shimoya et al., 1999). Although both IL-8 mRNA and protein have been identified in human gestational tissues and placenta little is known about its secretion and function in first trimester pregnancy, an area requiring exploration. We do know, however, that IL-8 contributes to uNK cell stimulation of trophoblast invasion (Hanna et al., 2006, De Oliveira et al., 2010).

1.6.4.1 IL-8 and pathological pregnancy

As mentioned in previous sections IL-8 plays an important part in inflammation, cell migration and tumourgenesis by the establishment of new blood vessels but the exploration of IL-8 involvement in spiral artery remodelling and trophoblast invasion has been sparsely studied and altered levels of uterine IL-8 may contribute to impaired remodelling and EVT invasion which are processes associated with preeclampsia and IUGR.

Several studies have associated IL-8 with preeclampsia; significantly elevated levels of plasma IL-8 have been observed in preeclamptic subjects compared with healthy pregnant and non pregnant subjects (Sharma et al., 2007), indicating that inflammation and endothelial dysfunction associated with IL-8 may contribute to the pathogenesis of preeclampsia. However, *in vitro* studies have demonstrated little alteration in endothelial gene expression in HUVECS after treatment with plasma from preeclamptic patients with only mild induction of IL-8 (Donker et al., 2005), suggesting that other factors are involved in endothelial dysfunction in preeclampsia. However, levels of IL-8 may be an effect of these alterations and not a cause.

1.7 Miscarriage

1.7.1 Prevalence of sporadic miscarriage

Miscarriage is the commonest gynaecological emergency (70000-90000 per year in England and Wales) (Everett, 1997)and has huge financial and personal implications. The vast majority of miscarriages occur in healthy women during the first trimester of pregnancy. Between 11 and 20% of all clinically recognised pregnancies are lost before

the 20th week of gestation. Most miscarriages are sporadic, that is they are non-recurring.

Approximately 50% of miscarriages are associated with chromosomal abnormalities (aneuploidy) (Jauniaux and Hustin, 1992). However, the cause in the remaining 50% is unknown and the mechanisms involved in sporadic first trimester miscarriages are poorly understood (Hustin et al., 1996).

Approximately 70% of early miscarriages have been associated with premature and continuous intervillous blood flow, evidence arising from flow patterns using gray scale Doppler. This was linked to oxidative stress in villous trophoblast, an effect of a thin and disrupted trophoblast shell (Jauniaux et al., 1994, Jauniaux et al., 2000). There is also evidence suggesting that deficient trophoblast invasion may be linked with first trimester miscarriage; using a morphological method (Hustin et al., 1990) demonstrated reduced trophoblast invasion in early aneuploid miscarriages. It was this observation that led to the suggestion that trophoblast invasion and spiral artery remodelling are primarily reduced in miscarriage, although reports are inconsistent. Ball et al. (2006b) assessed trophoblast subpopulations and spiral artery transformation in placental bed biopsies from sporadic miscarriage cases (n=73) compared with controls (n=179), using an immunohistochemical approach. In contrast to Hustin et al. (1990) there was no evidence of failure of trophoblast invasion and abnormal spiral artery remodelling in the decidua or myometrium in early ($\leq 12^{+6}$ weeks gestational age) miscarriage, suggesting that these processes do not have a pivotal role in the pathogenesis of early miscarriage. The varying results between the different studies may be due to the tissue sampled; the abnormalities in early miscarriages were detected in studies generally using aspirated gestational sacs which include cytotrophoblast cell columns and the cytotrophoblast shell (Hustin et al., 1990); whereas, placental bed biopsies extend deeper into the uterine wall (Ball et al., 2006b). However, reduced trophoblast invasion and spiral artery remodelling in late (≥ 13 weeks gestational age) miscarriage was observed (Ball et al., 2006a).

Increased production of Th1 type cytokines by peripheral blood monocytes, such as IFN-γ and TNF-α have been implicated in sporadic miscarriage (Zenclussen et al., 2001),

suggesting that an imbalance of the Th1:Th2 ratio may be detrimental to pregnancy. Although a later study involving mice, reported a reduction in IL-12 from decidual monocytes in SM compared with healthy pregnancy controls (Zenclussen et al., 2002), contradicting the Th1 hypothesis as a cause of SM.

1.7.2 IL-8 and IL-6 in sporadic miscarriage

There have been very few reports of IL-8 and IL-6 in miscarriage and results have been variable. Madhappan et al. (2003) reported significantly higher IL-8 levels in products of conception from women with two or more miscarriages (n=7) compared with women with normal pregnancy (n=4) or first miscarriage (n=12). Increased IL-8 levels in uterine cervical samples have also been associated with women suffering first trimester miscarriage compared with controls (Radulovic et al., 2010).

Jasper et al. (2007) reported *reduced* endometrial IL-6 mRNA expression in endometrial biopsies collected during the mid-secretory phase of the menstrual cycle from a group of women experiencing unexplained recurrent miscarriage (n=9) compared to women classified with proven fertility (n=12). In contrast, studies in mouse have demonstrated that *increased* levels of IL-6 at the maternal-fetal interface were associated with fetal loss (Zenclussen et al., 2003) and more recent data has demonstrated increased levels of serum IL-6 and IL-8 with women with second trimester abortion compared with levels from first trimester abortion (Galazios et al., 2011).

If IL-8 and IL-6 play a role in spiral artery remodelling, increased levels of IL-8 and IL-6 and loss of normal regulation of these cytokines in early normal pregnancy could lead to excessive and inappropriately early spiral artery remodelling, also leading to premature movement of the endovascular EVT plugs that sit in the mouths of the spiral arteries, hence resulting in premature blood flow into the intervillous space and oxidative stress to villous trophoblast. However, these cytokines may also affect the invasive potential of EVT and development of the placenta. Observations using immunohistochemistry and confocal laser microscopy show a significant increase in the translocation of NF-κB in chorionic villi and decidual tissues, with greater expression by decidual stromal cells, glandular epithelial cells and vessel endothelial

cells from women suffering sporadic miscarriage compared with viable pregnancies (Wang et al., 2010). These data suggest that an increase in pro-inflammatory cytokines such as IL-8 and IL-6, could possibly lead to the demise of pregnancy suggesting that the balance of cytokines relies on stringent regulation for pregnancy success. Therefore, IL-6 and IL-8 may play important roles in early pregnancy by establishing the necessary changes in the uterus. The few studies that are reported suggest that IL-6 and IL-8 may be altered in miscarriage but the receptor and signalling status of these cytokines have not been previously investigated in miscarriage.

Due to the pleiotrophy of IL-6 and its prevalent effect on invasion and growth of tumours and cancers decribed in section 1.5.1, its effect on the growth of hematopoietic precursor cells and its capability of inducing inflammation by upregulating acute phase reaction proteins and reducing inflammation by inhibiting neutrophil activation, along with receptor expression in glands of the endometrium and with altered levels in pregnancy pathology has led to the investigation of this cytokine in this study. Similarly the pro-angiogenic effects of IL-8 (described in section 1.6.3) and altered levels in miscarriage suggest that this cytokine could potentially play a role in early pregnancy processes and therefore has also been investigated in this study.

1.8 Hypotheses and aims

1.8.1 Hypotheses

The hypotheses to be tested were:-

1) IL-6, IL-8 and their receptors are expressed by cells in the placental bed in the first half of human pregnancy.

2) IL-6 stimulates EVT cell invasion in the first half of pregnancy.

3) Maternal leucocytes in decidualised human endometrium migrate towards and invade the VSMC wall of spiral arteries.

4) Maternal leucocytes release pro angiogenic growth factors which affect the integrity of vessel contributing to spiral artery remodelling at both 8-10 and 12-14 weeks gestation.

5) IL-6 and IL-8 have a crucial role in the control of spiral artery remodelling by disrputing the VSMC wall.

6) Decidual IL-6 and IL-8 levels are increased in sporadic miscarriage and dysregulation of their expression contributes to the pathophysiology of early sporadic miscarriage.

1.8.2 Aims

IL-6 and IL-8 may play a role in regulating the fundamental processes involved in the first half of pregnancy to ensure a successful outcome; therefore altered expression could be related to pregnancy complications, including early sporadic miscarriage. The aim of this study is to assess the roles of IL-6 and IL-8 in the first half of normal human pregnancy. This will clarify the specific role of these cytokines during pregnancy and improve understanding of how they affect the early processes both in normal and miscarried specimens. Subsequent to this an insight into the causes of sporadic miscarriage may become apparent and enhance our understanding of the pathogenesis, which in turn may lead to targeted therapies.

Specific aims:

1. To confirm the production and localisation of IL-6 and IL-8 and their receptors in normal pregnant decidua from 8-20 weeks gestation *in vivo* using immunohistochemistry.

2. To determine expression levels of these cytokines in decidual cell populations *in vitro* including uNK cells, macrophages, T cells, and decidual stromal cells.

3. To determine the effect of IL-6 on EVT invasion *in vitro* using primary cell isolates/ placental explants and an EVT- like cell line.

4. To explore the effects of IL-6 in EVT cell signalling pathways as a mechanism for biological activity.

5. To determine the effects of IL-6 and IL-8 on vascular smooth muscle cells by immunohistochemistry and by using a chorionic plate artery vessel model.

6. To confirm and compare the expression and localisation of IL-6, IL-8 and their receptors in samples obtained from karyotyped miscarriages up to 20 weeks gestation.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Tissues

All tissue specimens were obtained from surgical procedure, the nursing staff would then inform the laboratory for the samples to be collected fresh from theatre following extraction. Following surgical removal, tissue specimens were transferred to the laboratory all within a maximum of ten minutes and were immediately prepared for cell isolation, tissue preservation or experimental assays outlined in this chapter.

2.1.1 Normal early human pregnancy decidua

Normal pregnancy decidua was obtained from women undergoing elective surgical termination of pregnancy (TOP) at the Royal Victory Infirmary, Newcastle upon Tyne. Gestational age (GA) was determined by ultrasound measurement of fetal crown rump length (CRL) or biparietal diameter immediately prior to the procedure; specimens were taken at 7-14 weeks gestation. Following surgery aspirated tissue was collected into a sterile container and transported to the laboratory where under sterile conditions, within a class II laminar flow cabinet (Envair, Rossendale, UK) decidual tissue was identified macroscopically by its thick 'pink like' appearance and separated from the remaining products of conception. Decidual tissue was then washed several times in sterile phosphate buffered saline (pH7.4) (PBS; see Appendix) to remove excess blood and roughly chopped into 1-2 mm^3 pieces using two sterile scalpel blades. Chopped tissue was transferred into a sterile universal tube for cell fractionation/isolation (section 2.3)

2.1.2 Normal early pregnancy placenta

Normal pregnancy placenta was obtained from women (7-14 weeks GA) following surgical TOP (section 2.1.1). Placental tissue was identified by its branching villous appearance and separated from the aspirated products. The tissue was washed in sterile PBS several times to remove excess blood. Terminal chorionic villous tips (0.5mm^3) of the placenta were dissected using a sterile scalpel blade and suspended in either 20ml Hanks balanced salt solution for EVT isolation and culture (section 2.4.5) or 10ml sterile PBS for placental explant culture (section 2.4.6).

2.1.3 Placental bed biopsies

Placental bed biopsies (6-20 weeks GA) were taken transcervically under ultrasound guidance in women undergoing surgical TOP (section 2.1.1) as described previously (Robson et al., 2002). Biopsies were immediately transferred into a sterile container following surgical removal and transported to the laboratory where biopsies were either immediately snap frozen (section 2.1.8.1) or fixed in 10% neutral buffered formalin (section 2.1.8.2) for immunohistochemistry.

2.1.4 Sporadic miscarriage decidua

Decidua from sporadic miscarriage (6-20 weeks GA; calculated from last menstrual period (LMP)) was obtained from women admitted to the Royal Victory Infirmary, Newcastle upon Tyne, for evacuation of retained products of conception (ERPC). Diagnosis of 'missed abortion' was made on the basis of an identifiable fetal pole and the absence of fetal heart motion in all cases.

Patients who had suffered a miscarriage were divided into two different age categories: 'early' ($\leq 12^{+6}$ weeks gestation) and 'late' (≥ 13 weeks gestational age). Decidual tissue was subjected to enzymatic disaggregation (section 2.3.1) and $CD56^+$ and $CD14^+$ cells were isolated using MidiMACS (section 2.3.2 and 2.3.4), cultured for 24 hours after which cell supernatants were collected (section 2.3.2.2) and stored at -80°C until required.

2.1.5 Sporadic miscarriage placental bed biopsies

Placental bed biopsies were obtained from women undergoing ERPC (section 2.1.5) using the same technique as in section 2.1.3. Samples from patients who had suffered a miscarriage were divided into two different age categories: 'early' ($\leq 12^{+6}$ weeks gestation) and 'late' (≥ 13 weeks of gestation). Biopsies were either immediately snap frozen (section 2.1.8.1) for immunohistochemical analysis. These samples were collected as part of a previous morphological and immunohistochemical study of the placental bed in miscarriage (Ball et al., 2006a, Ball et al., 2006b).

2.1.6 Term placenta from Caesarean section

Term placenta (37-42weeks gestation) was collected from women undergoing elective Caesarean section at the Royal Victoria Infirmary, Newcastle upon Tyne. Following Caesarean section the placenta was transported to the laboratory and in a sterile class II laminutear flow cabinet (Envair, UK) was used for dissection of chorionic plate arteries (CpA).

2.1.6.1 Dissection of chorionic plate arteries (CpA)

Chorionic plate artery vessels were identified macroscopically by their white, thick, overlapping appearance on the fetal side of the term placenta. They were carefully removed from the placenta using forceps and scissors and immediately placed into a petri dish containing ice cold sterile PBS (pH 7.4). Connective tissue and blood were removed using a sterile scalpel blade and the arteries were chopped into 3mm intact rings and placed in sterile PBS until required.

2.1.7 Ethical approval and informed consent

Each patient agreeing to donate their tissue for research gave informed written consent. Consent was obtained by a team of Research Nurses. Collection of fresh samples was approved by the County Durham and Tees Valley Research ethics Committee. Collection of archival miscarriage samples was approved by the Joint Committee of Newcastle and Tyneside Health Authority and Newcastle University; these samples were incorporated into the Uteroplacental Biobank which was approved by Newcastle 1 Research Ethics Committee.

2.1.8 Tissue preservation

2.1.8.1 Freezing of tissue samples

Tissue to be frozen (placental bed biopsies, decidua, placenta or CpA segments) was placed onto a small piece of filter paper. A metal beaker containing isopentane (BDH Laboratory supplies, Poole, UK) was lowered into a flask containing liquid nitrogen. The metal beaker was removed from the liquid nitrogen after 1-2 minutes and the tissue on the filter paper was lowered into the cooled isopentane using forceps. To ensure that the tissue was frozen it was left in the isopentane for approximately 3-4 minutes

and was then placed into labelled freezing bags or a labelled cryotube (Corning, High Wycombe, UK) and stored at -80°C until use.

2.1.8.2 Preparation of frozen tissue sections

Serial tissue sections (5µm thick) were cut at -30°C using an automated cryostat (Reichert-Jung, 2700-Frigocut). The sections were applied onto aminopropyltriethoxysilane (APES) coated glass slides and allowed to air dry overnight at room temperature. APES coating aids the adherence of the tissue to the slide. Following overnight adherence the slides were fixed in acetone for 10 minutes at room temperature allowed to air dry and then wrapped in aluminium foil and stored at -20°C until use.

2.1.8.3 Preparation of formalin fixed paraffin embedded (FFPE) tissue sections and processing

Following tissue collection, placental bed biopsies, pieces of decidua, placenta and CpA segments were immediately fixed in 10% neutral buffered formalin (aqueous formaldehyde). Formalin fixes and preserves the tissue by forming cross-links between primary amino groups in proteins with other nitrogen atoms in proteins or DNA, delaying the effects of the cell cycle. Tissue was kept in formalin for a minimum of 24 hours and then embedded in hot paraffin wax. Once cooled serial 3µm sections were cut from the paraffin block, mounted onto APES treated glass slides, air dried in an oven at 150°C and stored in the laboratory at room temperature until required.

2.1.8.4 Haematoxylin and Eosin (H&E) staining

The haematoxylin and eosin (H&E) staining procedure was standard for the initial assessment of frozen and FFPE tissue, cell smears (2.4.2) and invasion assays (2.6.4). Frozen sections were brought to room temperature and rehydrated in TBS (pH7.6). FFPE tissue sections were dewaxed in xylene and rehydrated through descending concentrations of alcohol (99%, 95%, 70%, water) and then placed into TBS. Sections were stained in fresh Mayer's haematoxylin (see Appendix) for 1 minute to stain the cell nuclei blue and the slides were then washed in running tap water before being immersed into Scott's tap water substitute (see Appendix) for a further minute to develop the blue staining. The slides were then rinsed again in running tap water,

placed into eosin (see Appendix) for 1 minute to stain the cell cytoplasm pink and further washed in running tap water. Sections were dehydrated through ascending alcohol concentrations (70%, 95%, 99%) and cleared in xylene. The slides were mounted using glass coverslips and DPX mounting agent (Distryrene 80, dibutyl phthalate and xylene) (BDH).

2.2 Immunohistochemistry

Immunohistochemistry was performed on frozen (2.1.8.2) and FFPE tissue sections (2.1.8.3) using the avidin-biotin peroxidase complex (ABC) method (Hsu et al., 1981) with a mouse or rabbit Vectastain Elite Kit (Vector Laboratories Ltd, Peterborough, UK). Positive and negative controls were included in each staining run for each individual antibody. Positive controls included tissue from tonsil, small intestine, stomach and breast depending on the primary antibody being used. Negative controls were performed by omitting the primary antibody and replacing it with non-immune serum.

2.2.1 Avidin-biotin complex (ABC) method

FFPE tissue sections (section 2.1.8.3) were dewaxed in xylene, rehydrated though descending graded alcohols to running water as for the H&E stain (section 2.1.8.4). The sections were then immersed into a 1.5% hydrogen peroxide solution (H_2O_2) (Sigma) in distilled water for 10 minutes in order to block endogenous peroxidase activity. FFPE sections were rinsed in water and subjected to pre-treatment to aid antigen retrieval after the formalin fixation process; the type of pre-treatment was dependant on antibody used (see experimental design sections in results chapters for specific antibodies and their pre-treatments). The pre-treated slides were then placed into TBS (pH7.6) for 5 minutes at room temperature ready for immunostaining.

Pre-treatments were used for antigen retrieval. The majority of antibodies used required heat induced pre-treatment in citrate buffer pH 6.0 using a pressure cooker or microwave. Enzyme induced retrieval was used using trypsin working solution (see Appendix).

Acetone fixed frozen tissue sections were removed from -20°C and brought to room temperature. The tissue sections were outlined on glass slides using a wax

hydrophobic pen and immersed into TBS (pH7.6) for 5 minutes prior to immunostaining. Frozen tissue sections require no pre-treatment. The antibodies used for frozen tissue are listed in specific results chapters.

Immunolabelling was carried out using the avidin-biotin complex (ABC) method (Vectorstain Laboratories Ltd) and the reaction is outlined in Figure 2.1. In this method, an unlabelled primary antibody is recognised by a biotinylated secondary antibody, which then binds to a avidin-biotin enzyme molecule complex. Substrate is added and is converted from a colourless product into a coloured reaction product which is detectable in the tissue biopsy using light microscopy.

Non specific reactivity in tissue sections was first blocked by overlaying the sections with blocking serum (see Appendix) and incubating in a humid chamber for 10 minutes at room temperature. Following blocking the serum was tipped off and appropriately diluted primary antibody was added. The incubation period and temperature was dependant on the antibody used. After primary antibody incubation sections were immersed into TBS (pH7.6) and washed on a rocking platform for 2 x 5 minutes. Secondary antibody (see Appendix) was then added to the sections and incubated at room temperature for 30 minutes. The sections were washed again in TBS (pH7.6) for 2 x 5 minutes, incubated in tertiary antibody (see Appendix) for 30 minutes at room temperature followed by 2 x 5 minute washes in TBS (pH7.6). The reaction was then developed using DAB (see Appendix) for 2-3 minutes or until the reaction gave a brown product. The sections were rinsed in tap water and counterstained in Mayer's haematoxylin for 1 minute, further blued in Scott's tap water and dehydrated though ascending alcohol concentrations to be cleared in xylene. Slides were mounted using DPX synthetic mounting agent.

2.2.2 ImmPRESS™ Kit

The universal ImmPRESS™ kit (Vector Laboratories) was used for some antibodies to improve detection and reduce background staining. The reagents of the detection kit are biotin and avidin free (Figure 2.1A but without avidin-biotin complex). Secondary antibody is conjugated with a unique micro polymer containing active peroxidase enzyme and is reacted with peroxidase substrate for colour development, allowing sensitive and discrete antigen localisation.

Tissue sections were subjected to blocking in diluted normal horse blocking serum from the ImmPRESS™ kit for 20 minutes before being incubated with primary antibody then washed in TBS (pH7.6) for 2 x 5 minutes. Sections were then incubated with ImmPRESS™ reagent for 30 minutes, washed again in TBS (pH7.6) for 2 x 5 minutes and the reaction was developed using DAB peroxidase substrate (section 2.2.1). Sections were then lightly counterstained with Mayer's haematoxylin and mounted using DPX mountant (section 2.2.1).

2.2.3 Double immunohistochemical labelling

Double labelling allows the detection of two antigens expressed by the same cell, with the display of two separate chromogens for identification. This double labelling technique was performed on decidual CD14^{+} cell smears following isolation by MidiMACS positive immunomagnetic selection (section 2.3.4). The cells were initially labelled with a primary mAb during the MidiMACS protocol using anti-CD14 IgG conjugated microbeads and this was further developed in the cell smears using the ABC Vectastain Elite kit (section 2.2.1) which was developed using either Vector NovaRed (see Appendix) or DAB (see Appendix). Following the ABC method the cells were double labelled with a second primary mAb for 30 minutes; detection of the second antigen was performed using the alkaline phosphatase anti-alkaline phosphatase method (APPAP; section 2.2.3.1).

2.2.3.1 Alkaline phosphatase anti-alkaline phosphatase (APAAP) method

The APAAP method was devised by Cordell et al. (1984) and further developed by Hohmann et al. (1988). After terminating the enzyme reaction of the ABC method (section 2.2.1) by washing in tap water for 5 minutes the sections were then washed in TBS (pH7.6) and normal rabbit blocking serum (taken from Vectastain Elite goat IgG ABC kit) was added to the sections for 10 minutes to block non-specific binding and then replaced with second primary mAb diluted in TBS (pH7.6) for 30 minutes. Sections were washed in TBS (pH7.6) for 2 x 5 minutes and then incubated with secondary rabbit anti-mouse IgG antibody (see Appendix) for 30 minutes. The slides were washed in TBS (pH7.6) 2 x 5 minutes and then incubated in APAAP tertiary antibody (see Appendix) for 30 minutes. The slides were then washed in TBS (pH7.6) 2 x 5 minutes

and the reaction was developed using the alkaline phosphatase substrate kit III (see Appendix). The incubation proceeded in the dark at room temperature for approximately 20 minutes (development of the colour reaction was assessed microscopically). The reaction was stopped by washing the slides in running tap water. These sections were not subjected to any counterstain and they were not dehydrated as the alkaline phosphatase substrate is soluble in alcohol. Therefore, the sections were mounted using a permanent aqueous mounting medium (DAKO; S1964) and allowed to air dry overnight at room temperature.

2.2.4 Optimisation of antibodies

All primary antibodies were optimised before use. They were optimised using the recommended positive control tissue along with the tissue of interest with a number of different dilutions and pre-treatments (for FFPE sections). The pre-treatment and dilution for the given antibody that resulted in the strongest reactivity with reduced background staining was selected for use in the staining run.

2.2.5 Quantification of immunohistochemistry

All immunostained sections were blinded independently before assessment. Immunohistochemistry was quantified using a quick score method. The proportion of positive staining was measured using a 4 point scale; 1=<25%, 2=25-50%, 3=51-75% and 4=>75% and the intensity of positive staining was given a score of 1 - 3, with 1 being weak and 3 being strong immunoreactivity. To give a total score the intensity score was multiplied by the proportion score and added together to give a maximum of 12 (for example: if 40% of the cells were positively stained and the staining intensity was strong then this would have been giving a score of 2 x 3 = 6). Other examples include: 100% weak = 4 x 1 = 4; 100% strong = 4 x 3 = 12 and for variable staining: 40% weak and 60% strong = 2 x 1 + 3 x 3 = 2 + 9 = 11. The sections were then un-blinded following assessment.
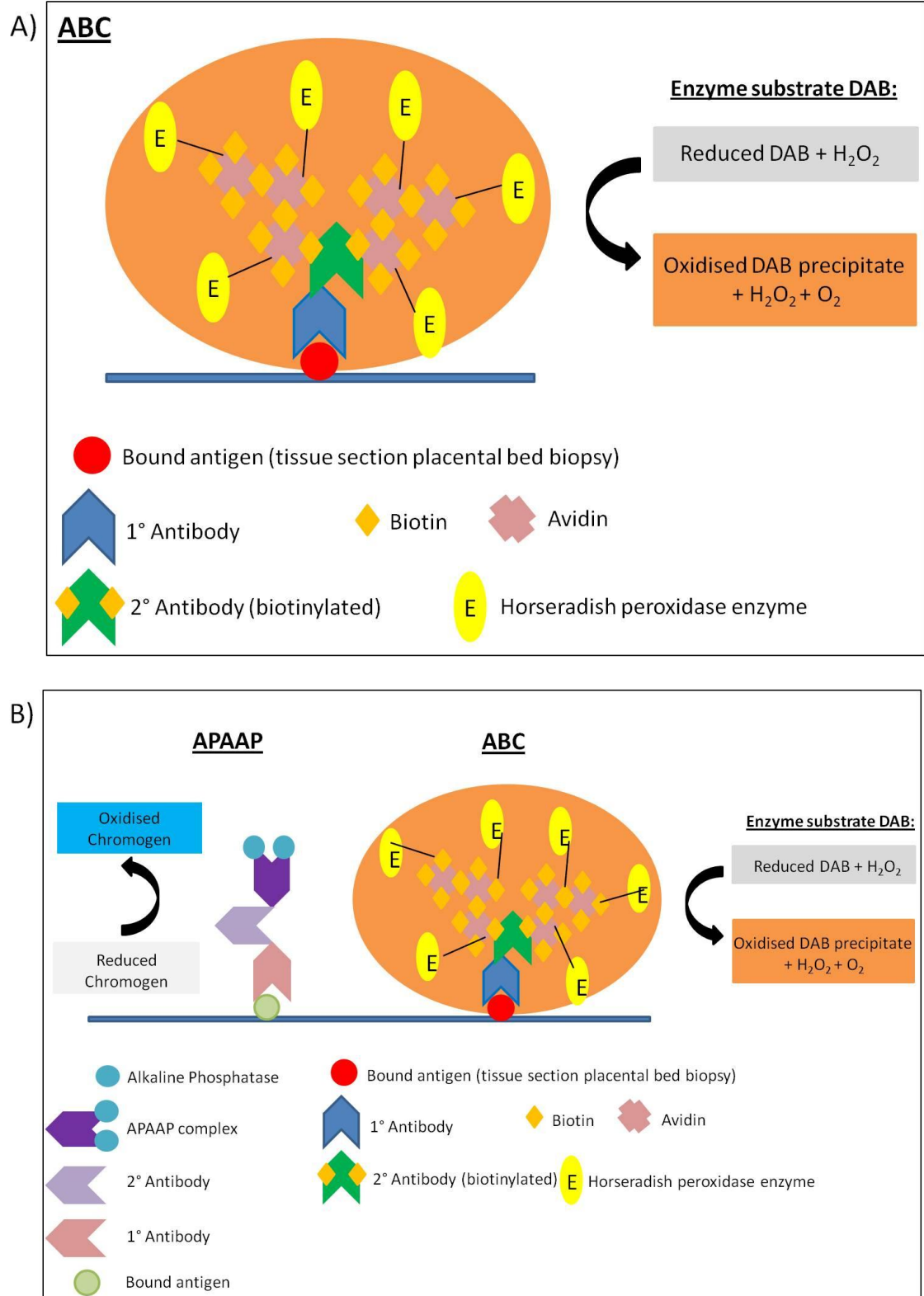
Figure 2.1 Schematic representation of immunohistochemical labelling
A) Single labelling with avidin-biotin complex (ABC) method; B) Double labelling combining the ABC and the Alkaline Phosphatase anti-Alkaline Phosphatase (APAAP) method. (Own diagram).

2.3 Tissue culture

2.3.1 Enzymatic disaggregation

A digest medium containing 1mgml^{-1} of collagenase type II and 0.1mgml^{-1} DNase type IV in incomplete RPMI 1640 medium was added to the chopped decidual tissue (section 2.1) and incubated for 40 minutes at room temperature with constant agitation. Following digestion disaggregated cells were removed, passed through a 40μm nylon cell strainer (BD Biosciences) and centrifuged at 300G for 5 minutes to pellet cells and aid the removal of any residual enzymes. The cell pellet was then resuspended in 1ml of complete RPMI 1640 culture medium (see Appendix). The remaining decidual tissue was subjected to a second 40 minute digest as above and the resulting cell suspension was pooled with the previous cell suspension.

The resuspended cells were added to a plastic T75cm^3 culture flask containing 20ml complete RPMI 1640 culture medium and incubated overnight at 37°C, 5% CO_2, in air to allow the adherence and therefore, removal of fibroblasts, epithelial cells, macrophages and decidual stromal cells, resulting in a cell suspension rich in leucocytes as they do not adhere to plastic (Oliver et al., 1999). The following day, the non-adherent cells were transferred from the flask into a sterile universal tube and the flask was rinsed in sterile PBS (pH7.4). The non-adherent cells were then centrifuged at 300G for 5 minutes and resuspended in complete RPMI 1640 culture medium to give a total concentration of 1x10^{6}cells ml^{-1}. 1x 10^5 cells (100μl) were plated out into wells of a plastic U bottomed 96 well culture plate and these were labelled 'total decidual fraction'.

2.3.2 Isolation of CD56+, CD8+ and CD10+ cells by MidiMACS

The remaining cell suspension (section 2.3.1) was centrifuged at 300G for 5 minutes and the cell pellet resuspended and incubated with either primary anti-CD56 (Coulter, High Wycombe, UK), anti-CD8 (DAKO) or anti-CD10 (Leica) monoclonal antibodies diluted 1:25 with incomplete RPMI 1640 culture medium for 30 minutes on an ice block. The antibody was then washed from the cells by addition of 20ml of ice cold sterile PBS (pH7.4) and centrifuged at 300G for 5 minutes. The cells were then incubated with MACS indirect goat anti-mouse IgG microbeads diluted 1:4 in ice cold

MidiMACS buffer (see Appendix) for 15 minutes. 800µl ice cold MidiMACS buffer was then added to the cells to give a total of 1ml. MidiMACS columns (Miltenyi) were prepared by placing the column in a magnetic field using a MACS separator and rinsing 500µl of ice cold MACS buffer through the column. The 1ml cell suspension was then applied to the column, the unlabelled cells washed through the column and collected as waste. The column was washed with 3x 500µl MACS buffer and the effluent collected. The column was removed from the separator and placed into a collection tube. Using the plunger provided, 1ml of MACS buffer was immediately flushed through the column to remove the magnetically labelled cells. The eluted cells were centrifuged at 300G for 5 minutes, the cell supernatant was removed and the pellet was resuspended in complete RPMI 1640 culture medium to give a total of $1x10^6 cellsml^{-1}$.

2.3.2.1 Preparation of CD56+, CD8+ and CD10+ cells for PCR

Following the MidiMACS cell separation technique (section 2.3.2) the resuspended cells were collected into labelled eppendorf tubes and microcentrifuged at 14.1rcf (relative centrifugal force) for 60 seconds. The supernatant was then removed and the cells were stored at -80°C until required.

2.3.2.2 Preparation of CD56+, CD8+ and CD10+ cell culture supernatants

Following the MidiMACS cell separation technique (section 2.3.2) the cells in suspension were seeded at 1×10^5 cells/well (100µl) into a 96 well culture plate and incubated at 37°C, 5% CO_2 in air for 24 hours. Following incubation the cell supernatants were collected in sterile eppendorf tubes and microcentrifuged at 14.1rcf for 60 seconds. The supernatant was collected into 100µl aliquots, placed into labelled freezer bags and stored at-80°C until required; the cell pellet was discarded.

2.3.2.3 Cell counting

Cells counts were performed using a haemocytometer and a light microscope magnification x200. 10µl cell suspension was applied to the counting grid of the haemocytometer and the cells counted. Cells in the outer 4 squares and the centre square of the grid were counted and the cell concentration was determined using the formula:

Cell concentration ml^{-1} = <u>number of cells in 5 squares</u>   x 10^6

<div align="center">20</div>

If there were few cells present (<1 cell per square), all 25 squares of the grid were counted and the cell concentration was calculated using the following formula:

Cell concentration ml^{-1} = total number of cells in 25 squares x 10^4

2.3.3 Preparation of cell smears

2.3.3.1 Cell viability and purity

Cell purity was assessed following MidiMACS positive immunomagnetic selection. Cell smears were prepared on APES-coated glass slides and left to air dry overnight at room temperature. Smears were then fixed in acetone for 10 minutes and immersed into TBS (pH7.6) for 5 minutes. The cell smears were then treated with secondary antibody from the Vectastain Elite ABC kit (section 2.2.1). After washing in TBS, cell smears incubated with tertiary antibody, washed again in TBS and the reaction was developed with DAB. The smears were counterstained with Mayer's haematoxylin, dehydrated, cleared in xylene and mounted with DPX synthetic resin for assessment of cell purity. Cells that had been positivity selected by MidiMACS were positively stained as brown cells (see Figure 2.2). The purity of the isolated cell populations were assessed microscopically in every 5th sample used; the population was considered 'pure' when purity was >95%.
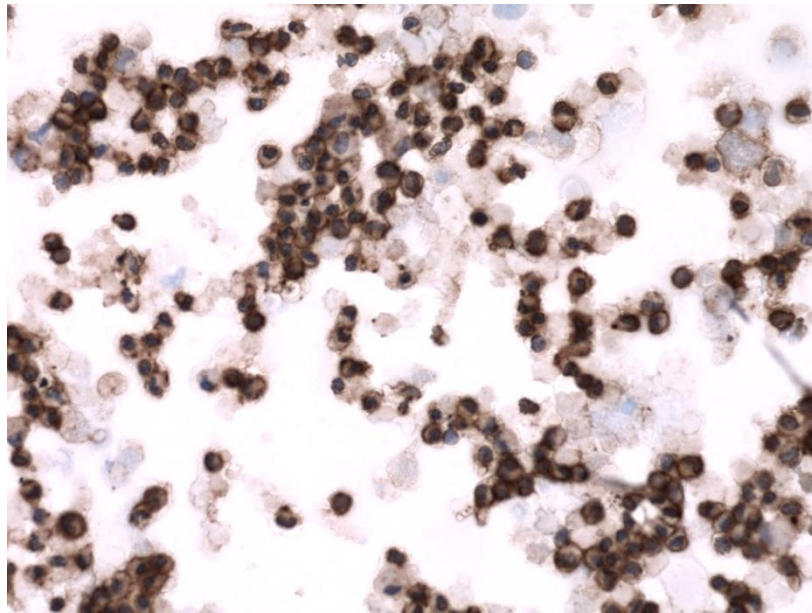
Figure 2.2 Purity examination of CD56$^+$ stained cells
Image of CD56+ stained cells which have been isolated by MidiMACS from first trimester decidua with a cell purity of >95% x200 magnification.

Cell viability was measured using Trypan blue (Sigma). Trypan blue is a stain which cannot be absorbed by healthy viable cells. Dead or damaged cells can readily absorb the stain and the cells appear blue when viewed with a microscope, allowing the dead cells to be counted. Cell viability was checked following cell isolation and cell culture for every 5th sample collected; the sample was discarded if viability and purity was too low. In sterile conditions, at T=0, 10µl suspended cells were added to 5µl Trypan blue and incubated for 5 minutes at room temperature. The cells were then smeared onto a glass slide and microscopically assessed. Samples were used for cell culture if the viability was >80%. After 24 hours of culture, once the cell supernatant was harvested, the remaining cells were tested for their viability by the addition of Trypan blue to the culture plate wells and assessed microscopically. Again the supernatants were deemed usable if the cell viability was >80%.

2.3.4 Isolation of CD14+ cells by MidiMACS

After enzymatic disaggregation of first trimester decidual tissue and miscarriage decidua, the cell suspension of was added to a T75 culture flask containing complete RPMI 1640 medium and incubated at 37°C, 5% CO_2 in air overnight. Following incubation cells remaining in the media suspension were discarded or used for CD56,

CD8 and CD10 cell isolation (see section 2.3.2). Cells that had adhered to the plastic base of the culture flask were then removed using a cell scraper and sterile PBS (pH7.4). The scraped cells were collected into a 50ml conical tube and centrifuged at 300G for 5 minutes. The cells were resuspended in complete RPMI medium to give a cell concentration of 1×10^6 cells ml^{-1} (total adherent fraction). These cells were plated into wells (2×10^5 cells per well) of a plastic 48 well culture plate. The remaining suspension was centrifuged for a second time at 300G for 5 minutes. The cell pellet was resuspended in 80µl MidiMACS buffer (see Appendix) and 20µl CD14 conjugated anti-goat IgG beads (Miltenyi Biotech) and incubated at 4°C for 15 minutes. Following incubation, the magnetically labelled CD14$^+$ cells were washed in 2mls MidiMACS buffer and centrifuged at 300G for 5 minutes. 500µl MidiMACS buffer was then added to the cell pellet and flushed through the prepared magnetic column (section 2.3.2). The eluted cells were centrifuged at 300G for 5 minutes and the cell pellet was resuspended in 1ml CD14$^+$ complete medium (see Appendix) to give a total of 1×10^6 cells ml^{-1}. The cells were seeded at 2×10^5 cells/well (200µl) into a 48 well culture plate and incubated at 37°C, 5% CO_2 in air for 24 hours; the supernatants were then collected and stored at -80°C (section 2.3.2.2).

2.3.5 Isolation of EVT from first trimester placenta

Placental samples from 7-14 weeks gestation were collected and prepared for isolation of EVT (2.1.2). EVT cells were isolated from placenta using a modification of the method described byTarrade et al. (2001), (Naruse et al., 2010). Briefly, placental chorionic villi were washed thoroughly in Hanks Balanced Salt Solution (HBSS, Mg/Ca free) to remove excess red blood cells. The chorionic villi were dissected from the placental membrane, minced to 0.5mm^3 and then enzymatically digested at 37°C using a digest media containing 0.025% trypsin (Roche Diagnostics Ltd, Burgess Hill, UK), 0.5mg DNase I and HBSS for 30 minutes without agitation. Supernatant containing a single cell suspension was removed and the remaining chorionic villi were subjected to a second 30 minute digestion. The single cell suspensions were mixed with 1ml FCS to inhibit any residual enzymes and centrifuged at 300G for 5 minutes. The cell pellet was then resuspended in incomplete DMEM:F12 (see Appendix). The cell suspensions were combined and layered onto a percoll gradient (10-55% percoll; diluted in HBSS) and

centrifuged (1200G, 30 minutes, no brake).The EVT cell layer (35-40% percoll) was removed, diluted in excess medium and centrifuged at 300G for 5 minutes. The cell pellet was resuspended in complete DMEM:F12 (see Appendix). The isolated EVT cells were cultured on 10µl/well of growth factor reduced Matrigel®(Becton Dickinson, Franklin Lakes, US) in a 24 well culture plate and placed overnight in an incubator set at 37°C, 5% CO_2 in air.

2.3.5.1 Preparation of whole cell lysates and EVT cell supernatants

Following overnight incubation EVT cells were washed in sterile PBS (pH7.4) to remove dead floating cells and fresh complete DMEM:F12 medium was added to the wells. The cells were then returned back to the incubator for 2-3 hours. Cells were treated with complete DMEM:F12 medium +/- 10ngml^{-1} IL-6 (Peprotech EC, London, UK) and returned to the incubator for 24 hours. Following incubation the conditioned medium was collected, micro centrifuged at 14.1 rcf for 60 seconds and the supernatants were collected and the cell pellets discarded. The cell supernatants were stored at -80°C until required. The adhered EVT cells were either lysed using 50mM HEPES protein extraction buffer containing 1% protease inhibitor cocktail (see Appendix) and a cell scraper or subjected to RNA extraction using the TRIZOL method (section 2.9.1). Cells were collected into an eppendorf tube and placed on a rocker at 4°C for 30 minutes. Cells were then micro centrifuged at 14.1 rcf for 5 minutes and supernatants were collected and stored at -80°C for future use.

2.3.6 Preparation of placental explants

Placental samples from 8-10 weeks gestation were used for preparing placental explants (section 2.1.2). Following sample collection placental tissue was prepared as previously described (Lash et al., 2006a). Briefly, the placental tissue was washed in sterile PBS (pH 7.4) to remove excess blood. Chorionic villous tips were dissected into 0.5mm^3 segments immersed in PBS (pH7.4) and centrifuged at 300G for 5 minutes. Tissue was resuspended in complete DMEM:F12 culture medium such that 15µl explant suspension contained 10mg tissue.

2.3.7 Chorionic plate artery culture

Following dissection of chorionic plate arteries (CpA) from term placenta (section 2.1.5.1), segments were either immediately snap frozen in liquid nitrogen (section 2.1.8.1) and stored at -80°C or fixed in formalin for embedding in paraffin (section 2.1.8.3); these tissues were labelled T=0. The remaining CpA segments were used for culture. The segments were placed into a 48 well culture plate containing complete RPMI 1640 culture medium (see Appendix) +/- 1 or 10ng ml^{-1} IL-6 or IL-8 (both Miltenyi), 5ng ml^{-1} sIL-6Rα (Miltenyi) and 10ng ml^{-1} IL-6 combined with 5ng ml^{-1} sIL-6Rα. Each experimental condition was performed in triplicate. The CpA segments were incubated at 37°C, 5% CO_2 in air for a total of 5 days; medium was changed every other day. Following 5 days incubation one segment per treatment was formalin fixed and the remaining 2 segments were snap frozen and stored at -80°C until required. Tissue that was fixed in formalin was paraffin embedded, processed into tissue sections (section 2.1.8.3) and immunostained using the ABC method (section 2.2.1) for VSMC differentiation markers (Myosin heavy chain, HCaldesmon, Calponin and α-smooth muscle actin), endothelial cells (CD31), proliferative cells (Ki67) and apoptotic cells (cleaved caspase 3). Tissue sections were also prepared from frozen tissue samples for immunohistochemistry (section 2.1.8.1) to investigate expression of IL-6 and IL-8 receptors on the VSMCs of CpAs. All antibodies used are listed in Chapter 5 Table 5.1.

2.4 Cell lines

2.4.1 HTR-8/SVneo cells

HTR-8/SVneo cells are a homogeneous human trophoblast cell line derived from first trimester trophoblast cells transfected with Simian virus 40 large T antigen (Graham et al., 1993). HTR-8/SVneo cells were kindly provided by Professor C.H. Graham, Queen's University, Kingston, Ontario, Canada. Cells were maintained at 37°C, 5% CO_2 in air in complete RPMI 1640 medium (see Appendix) and passages 10-50 were used for experiments. HTR-8/SVneo cells have been reported to be integrin α6 negative, but positive for integrin α1, cytokeratin 7 and HLA-G when grown on Matrigel® (Lash et al., 2005); this is similar to the phenotype of EVT. HTR-8/SVneo cells were grown to 100% confluence, trypsinised using 5mls trypsin/EDTA and resuspended in complete RPMI 1640 culture medium ready for use or to be frozen down and stored in liquid nitrogen.

2.4.2 Cryopreservation of cells

To preserve confluent HTR-8/SVneo cells, cells were trypsinised using Trypsin/EDTA and resuspended in 1ml complete RPMI 1640 culture medium which was then added to a cryovial containing 1ml DMSO freezing mix (see Appendix). The tube was slowly frozen in a vessel containing liquid nitrogen approximately at -210°C prior to storage in liquid nitrogen until further use.

2.5 Invasion assays

2.5.1 Matrigel® coating of cell culture membrane inserts

Tissue culture well membrane inserts with a pore size of 8.0µm (24 well format, Becton Dickinson, US) that sit into a 24 well culture plate were coated with 10µl chilled (4°C) growth factor reduced Matrigel® (Becton Dickinson, Oxford, UK). Coated inserts were then incubated at 37°C for at least 30 minutes to allow proteins in the Matrigel® to self assemble producing a thin film which the cells can attach to for cell culture. Matrigel® is a gelatinous protein mixture that resembles the extracellular environment found in tissues; it is liquid at 4°C but solid at 37°C.

2.5.2 Volume of tissue/number of cells per insert

Once the placental explants were prepared (see section 2.3.6). 15µl placental explant tissue (~10mg) was gently plated onto the Matrigel® coated membrane inserts. 400µl complete DMEM:F12 culture medium was added to the lower chamber and then incubated at 37°C overnight to allow adherence of the explants to the Matrigel®. The next morning (day 1) the medium was removed and 600µl fresh complete DMEM:F12 culture medium (see Appendix) was added to the lower chambers and 200µl of the treatment solution was added to the upper chamber of the corresponding well inserts (section 2.5.3). The tissue was then incubated at 37°C, 5% CO_2, in air for 6 days. Invasion assays were performed in duplicate for each sample and repeated on 5 separate occasions with 5 different placental explants (Figure 2.3).

HTR-8/SVneo cells were grown to 100% confluence and prepared for the invasion assay (see section 2.4.1). $1x10^5$ cells were added to each of the coated membrane inserts. 800µl complete RPMI 1640 culture medium was added to the lower chambers and 200µl of the treatment solution (section 2.5.3) was added to the upper chamber of

the well inserts. The cells were then incubated for 48 hours at 37°C, 5% CO_2, in air. Each invasion assay was performed in triplicate and the assay was repeated on 3 separate occasions (Figure 2.3).
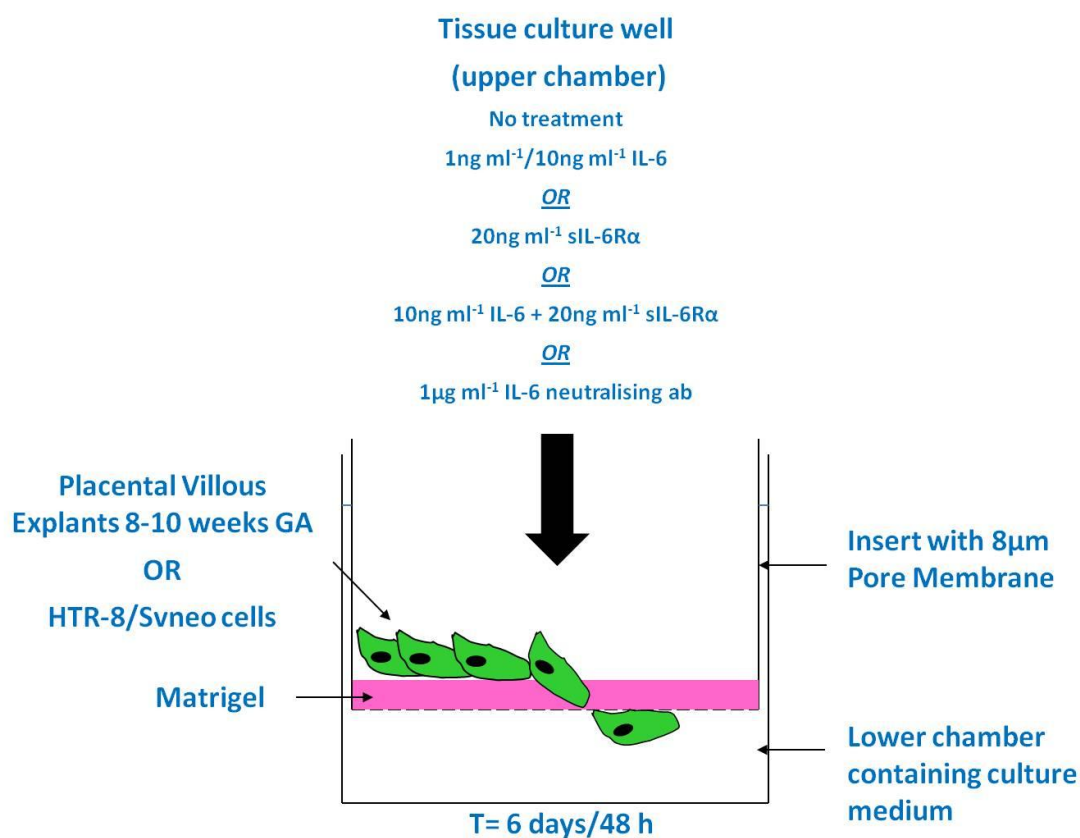
Figure 2.3 Diagrammatic representation of Matrigel® invasion assay
Trophoblast cells degrade the Matrigel® and migrate through to the underside of the pore membrane insert allowing invaded cells to be counted.

2.5.3 Concentration and type of treatments

The treatments added to both the placental explants and HTR-8/SVneo cells for the invasion assay were:

1. 1ng ml^{-1} or 10ng ml^{-1} human recombinant IL-6 (Peprotech EC, London, UK)

2. 20ng ml^{-1} human recombinant sIL-6Rα (Peprotech EC, London, UK)

3. 20ng ml^{-1} sIL-6Rα combined with 10ngml^{-1} IL-6

4. Medium control containing 10nM acetic acid (IL-6 and sIL-6Rα were reconstituted in acetic acid).

In addition placental explants were also treated with 1μgml^{-1} anti-human polyclonal IL-6 (Peprotech EC, London, UK) to inhibit any endogenous IL-6 that may be produced by the explants themselves and to see whether this abrogated the effect of EVT invasion.

2.5.4 Counting of H&E stained cells on chamber inserts

Following either 6 days or 48 hours incubation the tissue or cell supernatants were removed. The Matrigel® was removed and the upper side of the membrane inserts was cleaned with a cotton wool bud. For assessment of the number of invaded cells, the membrane filters were initially fixed in 99% alcohol for 5 minutes at room temperature and then stained with H&E (section 2.1.7.3). Since the membrane filters are plastic they were not cleared in xylene. The bottom of the filters was then cut away from the plastic and mounted onto untreated glass slides using DPX synthetic mounting resin (Raymond A. Lamb Ltd, London, UK). Each slide was blinded and the number of cells in the whole insert was counted using a microscope at x400 magnification (Figure 2.4). The mean of the duplicates or triplicates was calculated and then were normalised to the appropriate control values for each sample in order to facilitate comparison between experiments.
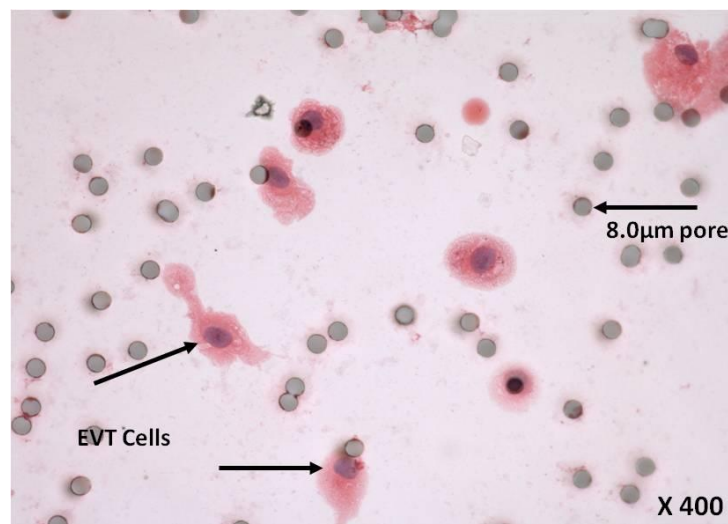
Figure 2.4 H&E stained invaded EVT through 8μm pore membrane filter insert
An H&E stained 8.0μm membrane pore insert illustrating the invaded extravillous trophoblast cells from a placental explant. The whole insert was counted.

2.6 Kinase phospho-array

The human kinase phospho array (R&D Systems Inc, UK) is a kit designed to detect the phosphorylation of 46 proteins involved in cell signalling and was used for comparison of cell lystates from untreated and treated cells.

2.6.1 Preparation of EVT cell lysates

Both isolated EVT from placenta at 7-14 weeks gestation (section 2.3.5) and 100% confluent HTR-8/SVneo cells (section 2.4.1) were grown on growth factor reduced Matrigel® (Becton Dickinson) overnight and then either treated with either 10ng ml^{-1} human recombinant IL-6 (Peprotech, EC, London, UK) or treated with 10nM acetic acid medium (control) for 10 minutes at 37°C, 5% CO_2 in air. The cells were then lysed to prepare whole cell lysates using the lysis buffer provided. A BioRad DC total protein assay (see section 2.11.2) was then performed to determine the protein concentration of the cellular extracts (100μg-500μg protein required for the assay). EVT cell lysates were pooled to give a total protein concentration of 250μg for each experiment. 300μg total protein concentration was used for each HTR-8/SVneo profile experiment. Following the protein assay, cellular extracts were stored at -80°C until required. The experiment was repeated on 3 separate occasions for both cell types.

2.6.2 Array protocol

Before the array was carried out all of the reagents, including the cellular extracts, were brought to room temperature. The array protocol was followed according to manufacturer's instructions (Human Phospho-Kinase Array Kit, R&D systems, Inc. Minneapolis, US). The nitrocellulose membranes were then developed using chemiluminescence (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions and exposed by X-ray KODAK film (Sigma) for 10 minutes.

2.6.3 Quantification of results using Adobe Photoshop™

The array signals from the X-ray film (Figure 2.5) were scanned and the images were analysed using Adobe Photoshop™ software package. The images were quantified by using a mean spot pixel density counting method for each kinase signalling protein and values were exported to Microsoft Excel for analysis. Corresponding signals were

compared to determine the relative change in phosphorylated kinase proteins between untreated and treated cells and between samples. Data from the array were normalised to control values to facilitate comparison between each experiment (n=3 in both EVT and HTR-8/SVneo cell lysates).
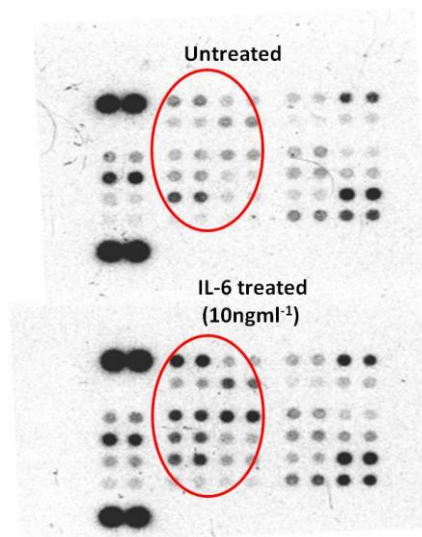
Figure 2.5 Representative phospho-array x-ray blot
Representative phospho-array x-ray blot after treatment with EVT cell lysates. The dots represent 46 kinase phosphorylation sites on nitrocellulose. A signal produced at each capture spot corresponds to the level of bound phosphorylated protein. Red circles highlight subtle differences in signal intensities between untreated and treated cells.

2.7 FASTQuant® multiplex cytokine array

The FASTQuant® array was used for the quantification of proteins secreted by first trimester EVT cells (Whatman).

2.7.1 Assay protocol

Three separate FASTQuant® kits were used; Human Th1/Th2, Human angiogenesis and Human II (see Appendix for types of proteins detected for each kit and solutions used). Each slide in the FASTQuant® kit contained 16 nitrocellulose arrays with the anti-cytokine monoclonal antibodies arrayed in triplicate. The assays were performed according to the manufacturer's instructions. Briefly, the prepared EVT cell supernatants (section 2.3.5.1) were removed from the -80°C freezer and allowed to defrost on ice. Whilst defrosting 70µl of 1x blocking solution (see Appendix) was added to each well and incubated for 15 minutes on an oribital shaker at room temperature.

Samples were then diluted 1:2 in diluent (see Appendix) and 70µl of each EVT cell supernatant was applied to the slides along with an 8 point standard curve (made by serial dilutions) after removing the blocking buffer. Samples and standards were incubated overnight on an oribital shaker with the FAST frame sealed in a plastic bag with a wet paper towel to prevent the FAST slides from drying out. Following overnight incubation, the samples were removed and the slides were washed by adding 90µl of 1x washing buffer (see Appendix) to each well and this wash step was repeated 5 times.70µl of biotinylated detection antibody cocktail (see Appendix) was then added to each well and incubated for 1 hour on an orbital shaker at room temperature. The wash step was then repeated and 70µl of streptavidin fluorescently tagged with Cy5 (see Appendix) (allowing visual detection of protein for quantification) was added to each well, the FAST plate was then covered in foil and incubated for 45 minutes on an orbital shaker at room temperature. The slides were then washed in dH_2O for 5 minutes and allowed to air dry before scanning.

2.7.2 Analysis of array raw data

2.7.2.1 Gene array vision

The labelled slides were visualised using an Axon GenePix® 4000B Microarray scanner and the images were transferred to Gene Array Vision software where the fluorescent images could be quantified. Data were exported to Excel for analysis.

2.8 Laser capture micro dissection of placental bed biopsies

Laser Capture Microdissection (LCM) is a method for obtaining individual homogenous cell populations, cell aggregates and morphological structures from heterogeneous samples such as tissue sections. Once the cells are captured they can be used for real-time RT-PCR and mRNA expression profiling.

Frozen tissue sections of placental bed biopsies were prepared (section 2.1.8.1 and 2.1.8.2) but the tissue sections required for LCM were stored at -80°C rather than -20°C and sections were placed onto polyethylene naphthalate (PEN) membrane glass slides (Leica Microsystems, Newcastle, UK) . In this study the LCM staining kit was used (Ambion). Tissue sections were removed from -80°C and immediately immersed into a chamber containing 95% ethanol for 30-40 seconds then the slides were transferred to

75% ethanol for 20-40 seconds and to 50% ethanol for 25 seconds before the addition of 300µl of cresyl violet staining solution (a basic stain that binds to negatively charged nucleic acids) directly to the tissue section. The tissue was stained for 1 minute and the cresyl violet solution was then drained away and the slides were incubated with fresh 50% ethanol for 30 seconds followed by 75% for 30 seconds then 95% ethanol for 30 seconds. The slides were then immersed in 100% ethanol for 40 seconds then added to another chamber containing 100% fresh ethanol for a further 40 seconds. The slides were rinsed in xylene and then placed in a staining chamber of fresh xylene for 5 minutes. The slides were then air dried at room temperature to allow the evaporation of xylene before microdissection.

Microdissection was performed using Leica Laser Microdissection Microscope. The laser used is a UV laser of 337 nm wavelength. The movement during cutting is done by the optics, while the stage remains stationary. The region of interest can be marked on the monitor and is cut out by PC control. An eppendorf tube is placed beneath the stage to capture the sample as it is cut away. The collected sample of interest is then subjected to RNA extraction (section 2.9.1).

2.9 RT-PCR

2.9.1 Total RNA extraction from cells and LCM samples

Total RNA was extracted from total decidual, leucocyte and EVT cell isolates (section 2.3.5.1) using the TRIZOL method (TRIZOL, Sigma). The protocol was followed according to the manufacturer's instructions. Briefly, 500µl TRI® reagent was added to isolated cells. The cells were removed using a cell scraper/pipette tip and the contents were added into 0.5ml eppendorf tubes. The transferred contents were then incubated at room temperature for 5 minutes. 100µl chloroform (Sigma) was added to each tube, which was inverted ~20 times and then incubated at room temperature for 5 minutes before being micro-centrifuged at maximum speed (14rcf) for 15 minutes. The colourless aqueous phase was collected into clean tubes containing 250µl isopropanol and incubated at -20°C for 1 hour. Following incubation the tubes were micro-centrifuged at maximum speed for 5 minutes, after which the supernatant was removed and the RNA pellet was washed in 500µl 75% ethanol (1500µl 100% ethanol in 500µl water). The tubes were then micro-centrifuged again for 5 minutes and the

RNA pellet allowed to dry at room temperature. Once the pellets were dry they were resuspended in 50µl nuclease free water and stored at -80°C prior to cDNA conversion (section 2.9.2)

RNA was extracted from tissue collected by laser capture microdissection using Micro RNeasy or RNAqueous-Micro kit (Ambion)

The method used followed the manufacturer's instructions Ambion:

RNAqueous®-Micro Kit; *RNA Isolation Procedure from LCM Samples*

1. Drop sample into 100 µL Lysis Solution and incubate for 30 minute at 42°C

Using sharp forceps peel off the thermoplastic film containing the captured cells and drop it into a 0.5 mL microcentrifuge tube containing 100 µL of Lysis Solution. Make sure that the sample is completely immersed in Lysis Solution.

a. Incubate the sample for 30 minute at 42°C.

b. Vortex briefly to mix. Briefly centrifuge to collect the fluid at the bottom of the tube.

2. Prewet the filter with 30 µL of Lysis Solution for ≥5 minute

a. Pre wet the Micro Filter Cartridge Assembly by applying 30 µL of Lysis Solution to the center of the filter and allow it to soak while performing the next two steps.

b. After completing steps 3 and 4 below, or after 5 minute, centrifuge the pre wetted filter for ~30 seconds at top speed to remove liquid.

3. Add 3 µL of LCM Additive to the lysate and mix

a. Add 3 µL of the LCM Additive to the lysate and mix well by briefly vortexing.

b. Briefly centrifuge to collect the fluid at the bottom of the tube.

4. Add 100% ethanol to recover either only large RNA species or all RNA

The amount of ethanol added to the lysate mixture determines what size of RNA will be captured on the filter. Instructions are provided below for recovering only RNA

species larger than ~75 nt, or for recovering both large and small RNA species, including tRNAs and microRNAs.

• To recover *only large* RNA species:

Add 0.5 volumes (52 μL) of 100% ethanol to the lysate mixture, and mix by pipetting up and down or by gently vortexing.

• To recover *large and small* RNA species:

Add 1.25 volumes (129 μL) of 100% ethanol to the lysate mixture, and mix by pipetting up and down or by gently vortexing. (Yields of total RNA using this method may vary from those obtained using the above method.)

5. Pass the lysate mixture through a prepared Micro Filter Cartridge Assembly

Be sure to remove the Lysis Solution used to prewet the filter by centrifugation (step 2.b) before applying the lysate mixture.

a. Load the entire lysate/ethanol mixture onto the prepared Micro Filter Cartridge Assembly and close the cap.

b. Centrifuge for 1 minute at 10,000 x g to bind the RNA to the filter.

6. Wash with180 μL of Wash Solution 1

a. Add 180 μL of Wash Solution 1 (working solution mixed with ethanol) to the filter and close the cap.

b. Centrifuge for 1 minute at 10,000 x g to pass the solution through the filter.

7. Wash the filter with 2 x 180 μL Wash Solution 2/3

a. Open the Micro Filter Cartridge, add 180 μL of Wash Solution 2/3 (working solution mixed with ethanol) to the filter and close the cap.

b. Centrifuge for ~30 seconds to pass the solution through the filter.

c. Repeat with a second 180 μL aliquot of Wash Solution 2/3.

8. Discard the flow-through and centrifuge the filter for 1 minute

a. Open the Micro Filter Cartridge assembly, remove the filter cartridge from the Collection Tube, and pour out the flow-through.

b. Replace the Micro Filter Cartridge into the same Collection Tube, close the cap, and centrifuge the assembly for 1 minute to remove residual fluid and dry the filter.

9. Elute the RNA in 2 x 8–10 µL of Elution Solution

a. Label a Micro Elution Tube (1.5 mL tubes provided with the kit) and transfer the Micro Filter Cartridge into it.

b. Apply 8–10 µL of Elution Solution, preheated to 95°C, to the *centre of the filter.* c. Close the cap and store the assembly for *5 minute* at room temperature.

d. Centrifuge the assembly for 1 minute to elute the RNA from the filter.

Tension from the hinge of the Micro Elution Tube can occasionally cause the cap to pop off during the elution spin. To minimize the chance of this happening, bend the cap hinge back and forth several times, then press the cap securely onto the Micro Filter Cartridge.

e. Repeat steps b–d with a second 8–10 µL of preheated Elution

Solution and collect the eluate in the same Micro Elution Tube.

2.9.2 cDNA conversion

The quantity of extracted RNA was assessed using a nanodrop machine (ND-1000 V3, Labtech Int, UK). 300-500ng extracted RNA was reverse transcribed using Superscript III (InVitrogen Ltd, UK) according to the manufacturer's instructions. Briefly, 2µl dNTP (10mM) and Oligo(DTs)$_{12-18}$ (0,5µgµl^{-1}) were added to the extracted RNA and topped up with 13µl nuclease free water. The reaction tubes were placed in a PCR thermo cycler (Eppendorf) and run at 65°C for 5minutes then cooled to 4°C. Meanwhile a master mix consisting of 5x RT buffer, 0.1M DTT, RNase OUT and Superscript III (all Invitrogen) with 4:1:1:1 ratio, respectively, was prepared. 7µl master mix was then added to the reaction tubes and run on the PCR machine at 50°C for 30-60 minutes. The reaction was terminated at 70°C for 15 minutes and the cDNA was stored at -20°C before PCR amplification (section 2.9.3).

2.9.3 Standard Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) amplification

RT-PCR was carried out in 0.2ml thin walled PCR tubes using 2µl DNA, 0.5µl 10mM dNTPs consisting of; dATP, dTTP, dGTP, dCTP (Invitrogen), 0.2µl 1x Taq polymerase, 2.5µl 25mM MgCl2, 2.5µl 10x Buffer IV (all from Invitrogen), 0.25µl 20µM forward primer, 0.25µl 20µM reverse primer and 17.8µl sterile filtered distilled water. A negative control containing sterile filtered water was included to ensure that the reaction mixture was not contaminated with another source of DNA. In addition to this, a positive control was used to ensure that all the reagents were still working appropriately. A thermo cycler (Eppendorf) was used and the steps of the PCR amplification are outlined below (Table 2.1).

Table 2.1 Conditions of a standard PCR reaction

1	$95^{o}C$ – 1 minute	Denaturation of cDNA
2	$95^{o}C$ – 30 seconds	Denaturation of cDNA
3	$62^{o}C$ – 30 seconds	Annealing of primers (IL-6, GAPDH)
4	$72^{o}C$ – 1 minute	Elongation
5	$72^{o}C$ – 3 minutes	Denaturation
6	$4^{o}C$	Temperature required to prevent degradation of PCR products prior to storage at -20°C
Cycles (steps 2-4)	39	Repetition of cycles

2.9.3.1 Agarose gel electrophoresis

The standard concentration of agarose was 2% in 1x Tris-acetate-EDTA (TAE) buffer (see Appendix) (2 grams agarose to 100mls 1x TAE buffer) and 0.1% (10µl) ethidium bromide. The agarose was boiled in a microwave for 3 x 1 minute to ensure that the agarose was fully dissolved. The solution was cooled with cold running tap water for

around 2 minutes to allow the temperature to cool to 55-65°C and then ethidium bromide was added. When cooled the solution was slowly poured into a gel tank containing a 14 well comb (which allowed 13 samples plus the 1Kb ladder to be run) and this was left to set for approximately 40 minutes. When solidified the comb was carefully removed and the gel fully immersed in 1x TAE buffer. The samples were prepared by adding 4µl PCR product to 1µl 5x DNA loading buffer and loading 4µl into the wells of the gel. A 1kb DNA ladder (InVitrogen) was also loaded onto the gel to analyse the size of DNA samples. Electrophoresis was performed at 100v for approximately 30 minutes allowing the fragments to completely separate. On completion, the agarose gel was immersed in Ethidium Bromide (1µg/ml) diluted in H$_2$O for 20 minutes. The DNA bands were visualised by placing the gel into a UV transluminator and the image was captured using Alpha Imager software.

2.9.4 Real time RT-PCR

A 20µl reaction mix was used; each sample contained 3µl cDNA, 10µl ABI Taqman Master Mix (Applied Biosystems, US) 1µl primer/probe (Table 2.2 outlines the different primers used) and 6µl nuclease free water. GAPDH primer was used as a reference and cDNA negative and positive controls (in house decidua control RNA) were included in each run. Real time RT-PCR was performed using an ABI7000 (Applied Biosystems) PCR machine and probes labelled with the fluorophore 6-FAM and quencher TAMRA. The reaction was performed in triplicate.

Table 2.2 Primers used for real time RT-PCR
Table showing real time primers used with assay ID and size of gene product. All primers were pre-designed by Applied Biosystems- TaqMan® Gene Expression Assays on Demand.

Primers	Assay ID	Amplicon length
GAPDH	Hs99999905_m1	122 bp
Gp130	Hs00174360_m1	72 bp
IL-6R	Hs01075667_m1	111 bp
CXCR2	Hs01011557_m1	91 bp
TIMP-1	Hs00171558_m1	104 bp
IL-1β	Hs01555410_m1	91 bp
RANTES	Hs00174575_m1	63 bp

2.9.5 Analysis of data

The amount of fluorescence being emitted increases exponentially in the PCR reaction, therefore the amount of cDNA doubles with every cycle of PCR. The number of cycles in which the gene is amplified is called the cycle number. If a gene requires a high number of cycles before amplification (increasing fluorescence) is detected there is less expression of this gene within the cells. The number of cycles of gene amplification is shown on the x-axis of the graph and the arbitrary fluorescence units are shown on the y-axis, this graph is produced by the ABI7000 software. The amplification of a gene eventually plateaus, therefore the cycle number at which the increase in fluorescence is exponential (linear) is the threshold and a horizontal line is placed onto the graph. The point at which the fluorescence crosses the threshold is called the cycle number threshold (Ct).

The data produced from the RT-PCR reactions in this study were exported to Microsoft Excel and the Delta-Delta Ct values were calculated from cycle number of the gene of interest compared with the housekeeping gene cycle number. Delta-Delta Ct method is an approximate method of gene quantification. The difference between the Ct value of GAPDH gene and Ct value of the gene of interest was calculated, this is Delta Ct:

$$\Delta Ct= [\text{Gene of interest Ct value} - \text{Reference gene (GAPDH) Ct value}]$$

In this study IL-6, IL-8 and GAPDH gene expression by the decidual leucocytes were quantified in relation to CD8^{+} T cell expression levels, therefore gene expression in CD8^{+} T cells was used as a control.

The difference between the Delta Ct value of each gene from each cell population with the Delta Ct value of gene expression from CD8^{+} T cells is the Delta-Delta Ct value:

$$\Delta\Delta Ct= [\text{Gene } \Delta Ct - \text{Control gene } \Delta Ct]$$

The fold change of Delta-Delta Ct of IL-6 and IL-8 gene expression in deciual CD10^{+}, CD14^{+} and CD56^{+} cell populations were then calculated in reference to gene expression levels in the decidual CD8^{+} T cells.

The Delta-Delta Ct method was also used when comparing IL-6 treated EVT cells with untreated EVT when investigating whether IL-6 altered the gene expression profiles of growth factors produced by EVT (Chapter 4).

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2.10 Enzyme-linked immunosorbent assay (ELISA)

2.10.1 Sandwich ELISA protocol

To detect and quantify the levels of secreted IL-6 and IL-8 from decidual cell supernatants at 8-10 weeks and 12-14 weeks gestation and from normal pregnancy and evacuated products of conception following spontaneous miscarriage (section 2.3.2.2), sandwich ELISAs were performed (Figure 2.6). Isolated decidual CD14$^+$ cell supernatants taken at 8-10 weeks and 12-14 weeks gestation were also measured for the presence of VEGF-C, ANG-1, TGF-β1 and PlGF (see section 2.10.1.1).

All ELISAs performed in this study were carried out using 96-well microplates (R&D Systems Ltd, UK). All reagents used were supplied by either Peprotech Ltd or R&D Systems unless otherwise stated. Initially capture antibody was diluted to a working concentration in PBS (pH 7.4) and 100μl of working capture antibody solution was coated onto the wells of the microplates, the plates were sealed using adhesive strips and capture antibody was incubated overnight at room temperature. Following overnight incubation any non-bound antibody was washed and aspirated by using a wash buffer (0.05% Tween 20 in PBS pH 7.4); this wash/aspiration step was further repeated for 3 times. The microplates were then blocked by adding 300μl of block buffer per well (1% BSA (Sigma) in PBS) and incubated at room temperature for 1 hour. The wash/aspiration step was repeated as previously mentioned for a total of 3 times. A stock standard solution was diluted to a working concentration in reagent dileunt (sections 2.10.1.1 and 2.10.1.2) and serial dilutions were made to make an 8-point standard curve. Samples were also diluted in reagent dileunt. 100μl of standards and samples were added to corresponding wells and incubated for 2 hours at room temperature. The wash/aspiration step was again repeated 3 times. 100μl of detection antibody was then applied to the wells and was incubated for a further 2 hours at room temperature. Following incubation the wash step was again repeated. See section 2.10.1.1 for R&D detection method and section 2.10.1.2 for Peprotech Ltd detection method.
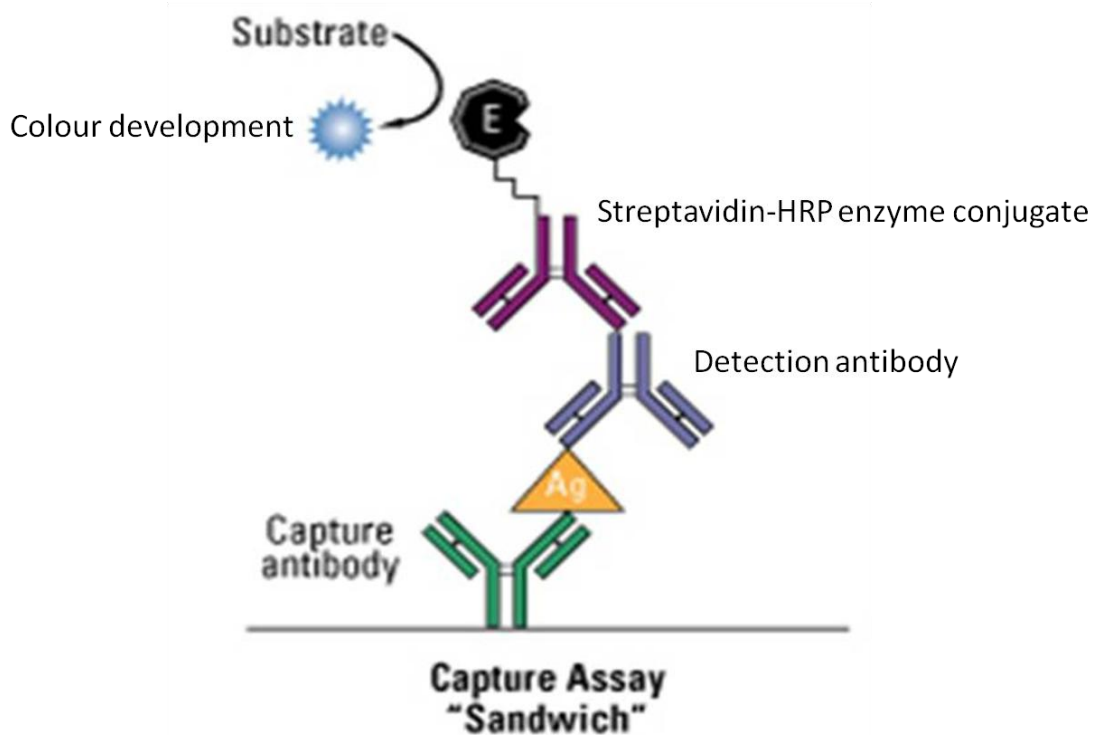
Figure 2.6 Schematic representation of a Sandwich ELISA
Image modified from ThermoScientific illustrating the mechanism of the sandwich ELISA.

2.10.1.1 Duoset R&D systems

The detection of IL-8, VEGF-C, ANG-1, TGF-β1 and PlGF were determined using human DuoSet® ELISA development kits (R&D systems, UK) and the protocol was performed according to the manufacturer's instructions. Samples were diluted 1:2 in reagent diluent (0.1% BSA (sigma), 0.05% Tween 20in TBS pH 7.4 sterile filtered). The streptavidin-HRP conjugate was diluted to 1:200 in reagent diluent; 100μl was added to each well and incubated for 20 minutes in the dark at room temperature. Following incubation the plate was washed and aspirated x 3 (as described in section 2.10.1) before addition of 100μl substrate solution (1:1 ratio of solution A and solution B) per well which was incubated for a further 20 minutes in the dark at room temperature. 50μl of STOP solution (2N H_2SO_4) was then added to each well until colour developed. Colour development was measured using an ELISA plate reader and the optical density was determined using Softmax pro with a wavelength setting of 450nm corrected at 540nm. The concentration of IL-8, VEGF-C, ANG-1, TGF-β1 and PLGF were determined with reference to an 8-point standard curve with top standard concentrations of

2000pg ml^{-1}, 6000pg ml $^{-1}$, 10000pg ml^{-1}, 2000pg ml^{-1}, 2000pg ml^{-1} respectively. All samples were run in duplicate and the mean value of the duplicates was used in the analysis.

2.10.1.2 Peprotech Ltd

The detection of IL-6 was determined using a human IL-6 ELISA development kit (Peprotech EC Ltd, UK) and the protocol performed according to the manufacturer's instructions. Samples were diluted 1:2 in diluents buffer (0.05% Tween 20, 0.1% BSA in PBS, sterile filtered). The avidin-HRP enzyme conjugate was diluted to 1:2000 in diluent buffer and 100µl was added to each well and incubated for 30 minutes in the dark at room temperature. Following incubation 100µl of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate solution (Sigma) was added to each well and was incubated at room temperature until colour developed. Colour development was measured using an ELISA plate reader (photospectrometer) and the optical density was determined using Softmax pro with a wavelength setting of 405-650nm. The concentration of IL-6 was determined with reference to an 8-point standard curve with a top standard concentration of 2000pg ml^{-1}. All samples were run in duplicate with the mean value of the duplicates was used in the analysis.

2.11 Western Blotting

IL-6 receptor expression was investigated by Western blot analysis.

2.11.1 Protein extraction

Protein was obtained from whole cell lysates of isolated EVT cells (section 2.3.5.1) and confluent HTR-8/SVneo cells (section 2.4.1).

2.11.2 Protein quantification

20µg protein was determined with BioRad DC protein assay which was followed according to the manufacturer's instructions:

1. Prepare working reagent A- 20µl of reagent S to 1ml reagent A.
2. Prepare serial dilutions of protein standards from 1.4mgml^{-1} of BSA (Sigma) to 0.2mg ml^{-1} in lysis buffer for standard curve.
3. Pipette 5µl/well of standards and samples into a 96 well plate in duplicate.

4. Add 25µl of working reagent A followed by 200µl of reagent B.

5. After 15 minutes the absorbance can be read at 750nm.

The protein concentration of samples was determined from the standard curve.

2.11.3 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For details of all buffers and gels see Appendix.

Gel preparation: The glass plate sandwich (BioRad assembly card) was assembled before adding separation gel. The separation gel was filled up to 1cm below from the top of the glass slide (for comb) and overlaid with isopropanol. The gel was allowed to polymerise for 45 minutes before pouring off the isopropanol and topped the set separating gel with the stacking gel and inserted the combs. The stacking gel was incubated for 45 minutes and the combs were removed, leaving behind wells in the gel. The size of gel was determined for each specific protein investigated (see Appendix and result chapters for more details).

5µl of protein loading buffer was added to 20µl cell lysates and heated at 95°C for 5 minutes. The reduced samples were loaded into the wells (total volume 25µl per well) along with 10µl protein marker ladder (Invitrogen) into an electrode tank filled with 1x electrode buffer and set to 100V and run until dye position has reached the bottom of the gel (1-2hours). Following electrophoresis, the gel slides were removed from the chamber, the stacking gel was removed and was allowed to equilibrate in cold transfer buffer for 5 minutes. The immunobilon P membranes (Millipore, Bedford, MA) were soaked in methanol for 15 minutes and then allowed to equilibrate in transfer buffer. The sponge and filter papers on plastic binders were prepared and immersed in transfer buffer before adding the gels and membranes. The plastic binders were closed and placed into an electrode tank filled with cold transfer buffer and blotted for 2 hours at 100V. Following blotting the membranes were removed and blocked in 5% milk in TBS-T at 4°C overnight ready for probing with antibody (section 2.11.4).

2.11.4 Immunodetection

After overnight incubation the membrane blots were removed from the blocking solution and washed in TBS-T (TBS-0.05%Tween20). The primary antibody (for list of specific antibodies and dilutions see experimental design in results chapters) was diluted in milk blocking solution. The membrane blot was then incubated in primary antibody for 1-2 hours depending on the antibody (see individual chapters), on a shaker at room temperature. The membrane blot was then washed in TBS-T for 3 x 5 minutes before addition of secondary antibody which was incubated for 1-2 hours at room temperature. The membrane blot was washed for a further 3 x 5 minutes before ECL detection (section 2.11.5).

2.11.5 Chemical detection of protein and developing signal

All antibodies were subsequently developed using chemiluminscence (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions and recorded by X-ray Kodak Scientific Imaging film (Sigma Chemical Co.).

2.12 Statistical analysis of results

Data are presented as the mean ± SEM. Data were statistically analysed using the commercial software package, StatView (Abacus Concepts Inc., Berkley, US). Differences were considered statistically significant at $P< 0.05$.

2.12.1 Student's *t*-test

Student's *t*-test was performed in order to compare two sets of continuous data with normal distribution.

2.12.2 One-way ANOVA test

To analyse the mean of two or more sets of samples a one way analysis of variance (ANOVA) followed by Fisher's post hoc analysis was used.

2.12.3 Non-parametric tests

For data that was found to have an irregular distribution (not normal) a non parametric test was performed. Mann Whitney U tests were applied for skewed data.

Chapter 3

Expression of interleukins-6 and -8, and their receptors in placental bed in early human pregnancy and miscarriage

3. Expression of IL-6 and IL-8 and their receptors in placental bed in early human pregnancy and miscarriage

3.1 Introduction

Cytokines are regulatory proteins that play a dominant role in immune responses. Although Th2 cytokines are abundant systemically, the role of these cytokines in early pregnancy is not well understood, although Th2 cytokines in general have been suggested to play an important role in development of successful pregnancy. However evidence suggests that Th1 inflammatory response cytokines are important proteins for successful pregnancy.

T-cells were originally hypothesised to be the main source of these cytokines in pregnancy (Piccinni, 2005) but this cell type exists as a minor leucocyte population in human decidua. The focus has been shifted onto uterine natural killer cells and uterine macrophages as being a rich source of cytokines.

The suggested roles of IL-6 and IL-8 during early human pregnancy have been reviewed in Chapter 1. They are thought to be important functional mediators during pregnancy but only a small number of studies have investigated these cytokines in early pregnancy and pregnancy complications. Although both cytokines have been implicated in pregnancy disorders, the production and source of these cytokines within uteroplacental tissues has not previously been thoroughly investigated. Studies to date have mainly focused on the effect of IL-6 and IL-8 in endometriosis (Ulukus et al., 2005, Luk et al., 2005), chorioamnionitis (Arntzen et al., 1998), cervical ripening and parturition (Robertson et al., 2010, Prins et al., 2012) but detailed examination of localisation and gestational age variation in normal pregnancy has not been performed.

Bowen et al. (2002) reviewed the production of cytokines in placenta and placental tissues and most cytokines have been localised to these tissues during normal pregnancy indicating proposed roles for cytokines in pregnancy. Placental tissue such as chorionic villi and trophoblast cells have been reported to produce IL-6 (Kauma et al., 1993) and IL-8 and its receptors have been localised to cells of the placenta (Saito et al., 1994, Dame and Juul, 2000). Despite these investigations in placenta, the production levels of IL-6 and IL-8 from specific decidual leucocytes, localisation and

distribution within the placental bed and decidual tissues in pregnancy remain limited and changes within protein levels at different gestations also remains to be investigated.

In the decidua uterine natural killer cells are a rich source of many different cytokines and growth factors including TNF-α, IL-10, IL-1β, LIF, GM-CSF and IFN-γ (Lash et al., 2006c). The functional role of these cytokines in pregnancy has been sparingly investigated and results suggest a potential role in regulating trophoblast invasion and the control of vascular remodelling but a function for uNK cells and the cytokines they secrete still remains unknown. IL-6 production by these cells has not yet been investigated in great detail.

Previous work in our laboratory had focused on the localisation of IL-8 in placental bed and its production by uNK cells and CD8^{+} T cells (Figure 3.1) (De Oliveira et al., 2010). The main reason for the study was to investigate the role of IL-8 on EVT invasion and IL-8 receptors (CXCR1 and CXCR2) were localised to EVT cells in placental bed (Figure 3.2). This study extends the investigation to include IL-8 production by other cell types of the placental bed and the localisation of IL-8 receptors elsewhere in the placental bed, especially focusing on receptor expression by SpAs. Aminopeptidase-N (CD13; see Chapter 1) relating to IL-8 signalling has also been investigated in this study.

The roles of IL-6 and IL-8 in early pregnancy remain largely understood but altered serum levels of pro inflammatory cytokines including IL-6 and IL-8 have been associated with first trimester sporadic miscarriage (Galazios et al., 2011, Calleja-Agius et al., 2012) and although altered levels of these cytokines and their receptors in specific pregnant tissues have not been investigated, these data suggest a possible importance for these cytokines in normal early pregnancy processes; their roles in angiogenesis, cell invasion and inflammation in other tissues and disease states suggest a potential role in regulating spiral artery remodelling and regulating trophoblast invasion.

In order to understand the potential role of IL-6 and IL-8 in pregnancy and reproductive complications, a detailed understanding of the production and function of these cytokines in normal pregnancy is essential. This chapter presents data on production and localisation of IL-6 and IL-6 receptor expression and further

investigates IL-8 and IL-8 receptor expression in normal early pregnancy as a basis for study of their function.
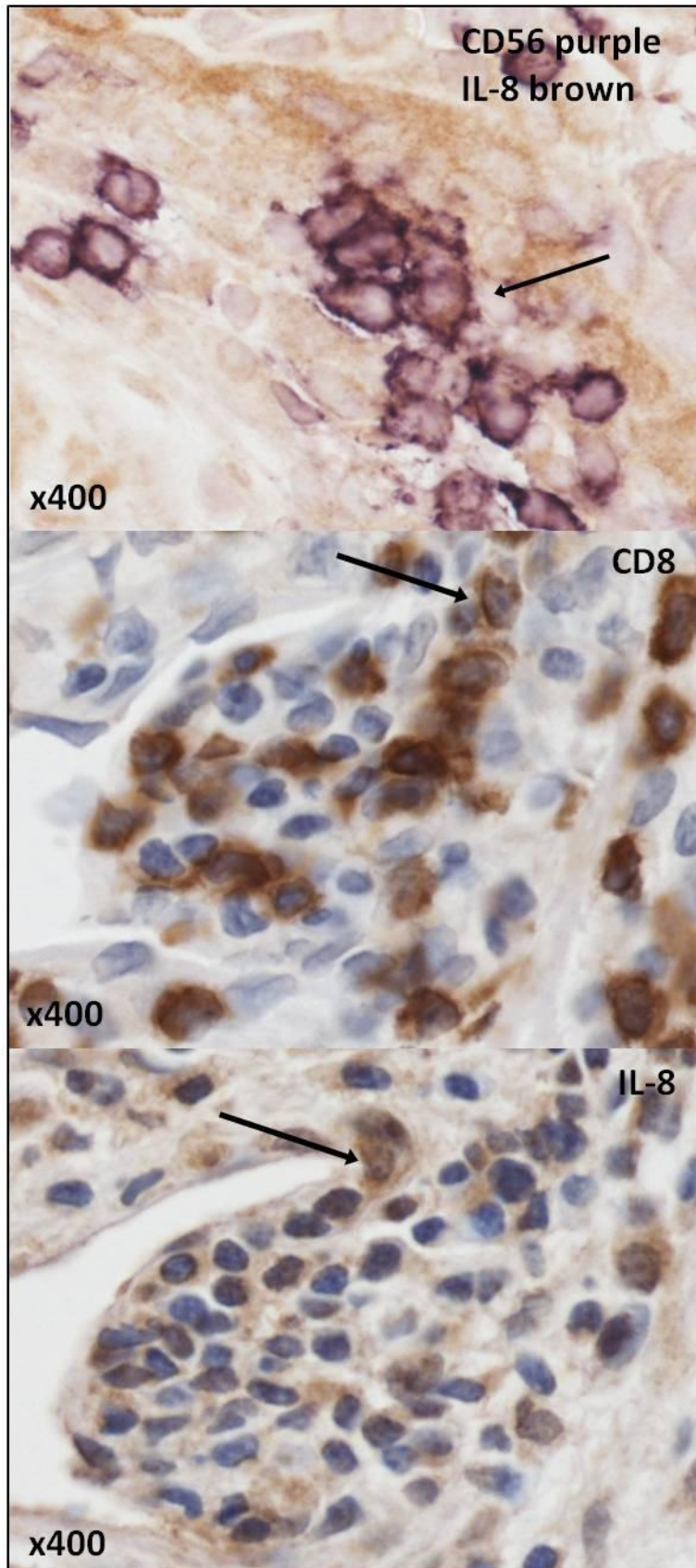
Figure 3.1
Immunohistochemistry of IL-8 staining on CD56+ and CD8+ cells in first trimester placental bed biopsies
Representative immunohistochemistry demonstrating CD56$^+$ cells (top) and CD8$^+$cells (centre) within FFPE placental bed biopsies of first trimester pregnancy. Double immunostaining for CD56$^+$ cells (Purple) also shows IL-8 expression (brown). Representative immunostaining for CD8$^=$ cells (centre) and a serial section demonstrates positive immunostaining for IL-8 in proximity to CD8$^+$ T cells. Original magnification x400.
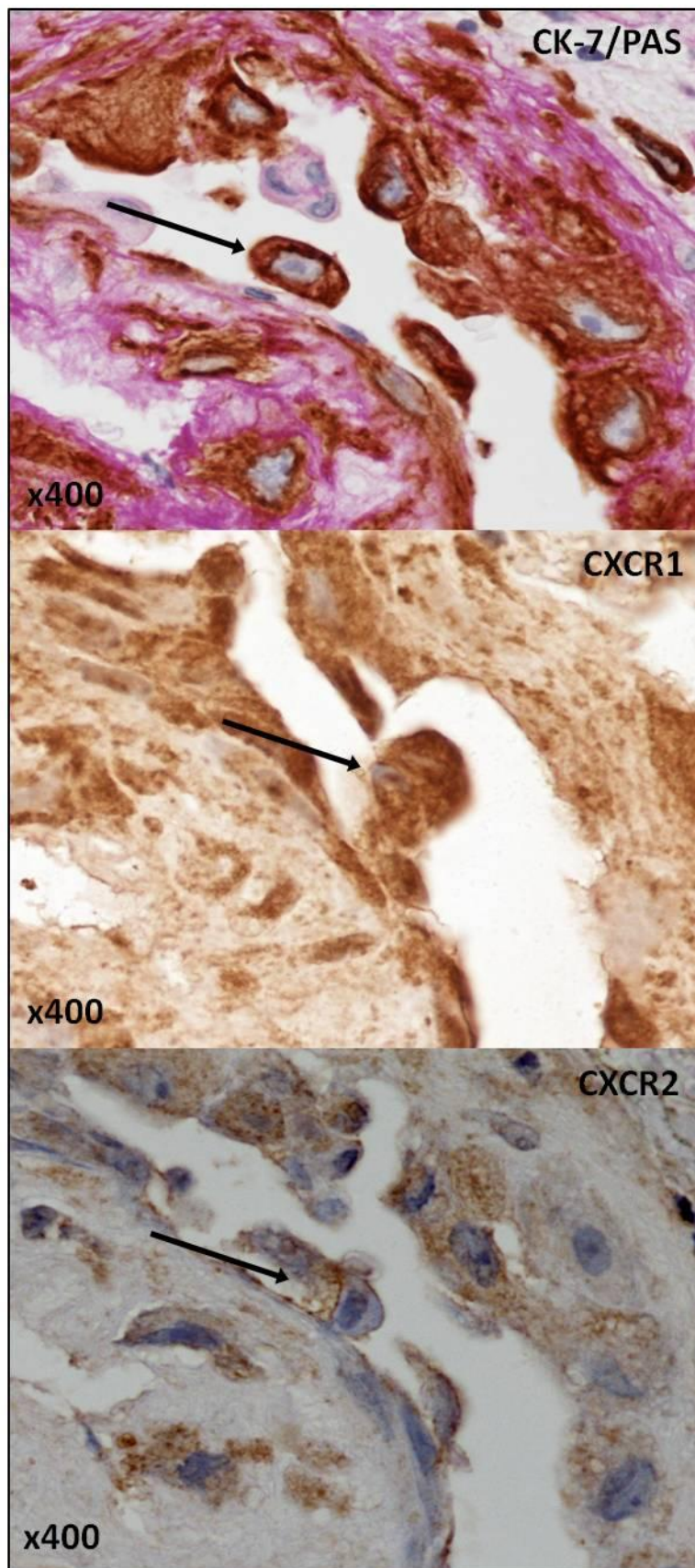
Figure 3.2
Immunohistochemistry of IL-8 receptor staining (CXCR1 and CXCR2) by EVT cells in first trimester placental bed biopsies
Representative immunohistochemistry demonstrating cytokeratin-7 immunopositive cells which identifies EVT in FFPE placental bed biopsies of first trimester pregnancy. Serial sections immunostained for IL-8 receptors (CXCR1 and CXCR2) show positive staining by EVT cells, confirming the expression of IL-8 receptors by trophoblast cells. Positive immunostaining of both receptors are evident on endovascular, intramural and interstitial EVT. PAS staining highlights fibrinoid deposition in remodelled SpA. Original magnification x400.

3.2 Aims and Hypotheses

3.2.1 Hypotheses

1. IL-6 and IL-8 are produced by decidual cells within the placental bed in early human pregnancy

2. Uterine natural killer cells, uterine macrophages and T cells are all sources IL-6 and IL-8 in early human pregnancy with increased expression levels at 12-14 weeks gestation compared with 8-10 weeks gestation.

3. Uterine natural killer cells are the major producer of both IL-6 and IL-8 within the placental bed in the first half of pregnancy.

4. IL-6 and IL-8 receptors are expressed by cells within the placental bed in early human pregnancy

5. Levels of IL-6 and IL-8 are increased in sporadic miscarriage.

3.2.2 Aims

1) Investigate the localisation of IL6, IL-8 and their receptors; IL-6Rα, gp130, CXCR1,CXCR2 using immunohistochemistry and laser capture microdissection

2) Measure IL-6 and IL-8 protein production from isolated decidual cell culture supernatants

3) Measure mRNA expression of IL-6 and IL-8 from isolated decidual cell populations

4) Investigate localisation of IL-6, IL-8 and their receptors between normal pregnancy and sporadic miscarriage using immunohistochemistry.

5) Compare IL-6 and IL-8 protein expression by decidual CD14^{+} and CD56^{+} cells isolated from early normal pregnancy and early sporadic miscarriage using ELISA.

3.3 Experimental design

3.3.1 Tissues

Placental bed biopsies were obtained from women undergoing elective TOP in first half of pregnancy (refer to section 2.1.3). Freshly obtained tissue from healthy pregnancy was snap frozen or formalin fixed and paraffin embedded (refer to section 2.1.8) and

used for immunohistochemical analysis (Table 3.1 for sample numbers and Table 3.2 for primary antibodies use).

Gestational age matched placental bed biopsies were also used from a former study obtained from women undergoing evacuation of retained products of conception (ERPC) following sporadic miscarriage (SM) and classified as either early euploid, early aneuploid, late euploid or late aneuploid, based on gestational age and cytogenetic analysis (Table 3.1) (Ball et al., 2006a, Ball et al., 2006b). All SM placental bed biopsies were snap frozen, fixed and cut into 3μm sections as previously described in section 2.1.8 for immunohistochemical analysis.

Placental bed biopsies obtained from healthy pregnancy were either snap frozen or formalin fixed and paraffin embedded and used for immunohistochemical analysis.

All samples were subjected to immunohistochemical analysis to identify EVT, SpAs and decidual cell leucocytes within the placental bed and IL-6, IL-8 and their receptor proteins were identified by positive staining on these cell types. Immunostaining was compared between SM tissue and normal pregnancy samples using a scoring method outlined in section 3.3.3.

Decidual tissue was obtained from women undergoing TOP at 8-10 and 12-14 weeks gestational age for positive immunomagnetic isolation of $CD56^+$, $CD14^+$, $CD10^+$ and $CD8^+$ cells for RT-PCR (T=0) and cell culture supernatants (T=24h; section 2.3.2) in order to investigate whether the cell types express IL-6 and IL-8 mRNA and quantify protein production by ELISA (sample numbers are listed in Table 3.1).

Decidual tissue was also obtained from women undergoing ERPC following early sporadic miscarriage (section 2.1.4) and used for positive immunoselection of $CD56^+$ (section 2.3.2) and $CD14^+$ cells (section 2.3.4) from which cell culture supernatants were harvested for analysis of cytokine production to compare levels of IL-6 and IL-8 in sporadic miscarriage and normal healthy pregnancy decidua (Table 3.1).

Table 3.1 Details of subject groups, sample number sizes and procedures performed in Chapter 3

Subject group	Gestational age range (weeks)	Number of subjects	Use in study
FFPE placental bed biopsies	8-10	5	Immunohistochemistry
FFPE placental bed biopsies	12-14	5	Immunohistochemistry
FFPE placental bed biopsies	16-20	5	Immunohistochemistry
Frozen placental bed biopsies	≤ 12	5	Immunohistochemistry
Frozen 2nd trimester placental bed biopsies	> 13	5	Immunohistochemistry
Frozen euploid SM placental bed biopsies	≤ 12	5	Immunohistochemistry
Frozen aneuploid SM placental bed biopsies	≤ 12	5	Immunohistochemistry
Frozen euploid SM placental bed biopsies	> 13	5	Immunohistochemistry
Frozen aneuploid SM placental bed biopsies	> 13	6	Immunohistochemistry
Decidua for CD56+ cell supernatants	8-10	10	IL-6 & IL-8 ELISA
Decidua for CD14+ cell supernatants	8-10	10	IL-6 & IL-8 ELISA
Decidua for CD10+ cell supernatants	8-10	10	IL-6 & IL-8 ELISA
Decidua for CD8+ cell supernatants	8-10	10	IL-6 & IL-8 ELISA
Decidua for CD56+ cell supernatants	12-14	10	IL-6 & IL-8 ELISA
Decidua for CD14+ cell supernatants	12-14	5	IL-6 & IL-8 ELISA
Decidua for CD10+ cell supernatants	12-14	5	IL-6 & IL-8 ELISA
Decidua for CD8+ cell supernatants	12-14	5	IL-6 & IL-8 ELISA
SM decidua for CD56$^+$ cell supernatants	7-12	8	IL-6 & IL-8 ELISA
SM decidua for CD14$^+$ cell supernatants	7-12	8	IL-6 & IL-8 ELISA
Decidua for CD56+ cells	8-10	5	Standard PCR (IL-6)
Decidua for CD56+ cells	12-14	4	Standard PCR (IL-6)
Decidua for CD56+ cells	8-10	10	RT-PCR
Decidua for CD14+ cells	8-10	10	RT-PCR
Decidua for CD10+ cells	8-10	10	RT-PCR
Decidua for CD8+ cells	8-10	10	RT-PCR
Decidua for CD56+ cells	12-14	10	RT-PCR
Decidua for CD14+ cells	12-14	5	RT-PCR
Decidua for CD10+ cells	12-14	5	RT-PCR
Decidua for CD8+ cells	12-14	5	RT-PCR

3.3.2 Immunolocalisation and specific antibodies used

For all FFPE placental bed tissue sections immunohistochemical labelling was performed as described in section 2.3.1 for the identification of the different cell populations including uNK cells, EVT cell populations, spiral artery VSMCs within the placental bed using mouse monoclonal antibodies. Details of primary antibodies, specificities and pre treatments are given in Table 3.2. Serial sections were then immunolabelled with anti-IL-6 and anti-IL-8 antibodies along with antibodies against their receptors; gp130, IL-6Rα, CXCR1, CXCR2 (Table 3.2 and 3.3). IL-6Rα, CXCR1 and CXCR2 were identified using frozen sections. Frozen sections of placental bed biopsies from both normal and miscarriage pregnancy were immunolabelled with antibodies detailed in Table 3.3 and immunohistochemistry was performed as described in Section 2.3.1. For any given antibody, all tissue sections were immunostained in the same staining run to avoid day-day variation. Positive controls were included for each antibody in each staining run; negative controls were performed for each sample by replacing primary antibody with appropriate non-immune serum.

Table 3.2 Primary antibodies used for immunohistochemistry on FFPE tissue sections

Antibody	Method	Species	Dilution	Incubation	Pre-treatment	Positive control
H-Caldesmon[1]	Elite Kit[5,6]	Mouse	1:100	RT, 60min	PC in Citrate buffer pH 6.0, 1min	Stomach
Myosin heavy chain[2]	Elite Kit[5,6]	Mouse	1:600	RT, 60min	PC in Citrate buffer pH 6.0, 1min	Stomach
Cytokeratin-7[2]	Elite Kit[5,6]	Mouse	1:20	RT, 60min	PC in Citrate buffer pH 6.0, 1min	Skin
CD31[2]	Elite Kit[5,6]	Mouse	1:20	RT, 60 min	PC in Citrate buffer pH 6.0, 1min	CpA
CD8[2]	Elite Kit[5,6]	Mouse	1:100	RT, 60min	PC in EDTA buffer pH 8.0, 1min	Tonsil
CD56[2]	Elite Kit[5,6]	Mouse	1:50	RT, 60min	PC in Citrate buffer pH 6.0, 1min	Placental bed biopsy
CD14[2]	Elite Kit[5,6]	Mouse	1:20	RT, 60 min	PC in Citrate buffer pH 6.0, 1min	Tonsil
CD3[2]	Elite Kit[5,6]	Mouse	1:20	RT, 30min	PC in Citrate buffer pH 6.0, 1min	Lymph node
gp130[3]	Elite Kit[5,6]	Mouse	1:50	RT, 60min	PC in EDTA buffer pH 8.0, 2mins	Breast
IL-6[4]	Immpress Kit[5,7]	Rabbit	1:800	RT, 60min	MW in EDTA buffer pH 8.0, 10mins	Lymph node

Source: [1]DakoCytomation, Denmark; [2]Leica Biosystems, (Novocastra) Newcastle LTD UK; [3]Santa Cruz Biochemicals, California, USA; [4]Abcam Cambridge, UK; [5]Vector Laboratories, Peterborough, UK; [6]ABC ELITE Kit- avidin-biotin peroxidase method (mouse monoclonal); [7]Immpress Kit- avidin-biotin peroxidase method (rabbit polyclonal); PC - Pressure Cook; MW - Microwave; RT - Room Temperature.

Table 3.3 Primary antibodies used for immunohistochemistry on frozen tissue sections

Antibody	Method	Species	Dilution	Incubation	Pre-treatment	Positive control
LP34[1]	Elite Kit[6,7]	Mouse	1:80	30mins	No pre-treatment (frozen)	placental bed
H-Caldesmon[2]	Elite Kit[6,7]	Mouse	1:100	RT, 30min	No pre-treatment (frozen)	Myometrium
IL-6[3]	Immpress Kit[6,8]	Rabbit	1:1500	RT, 30min	No pre-treatment (frozen)	Tonsil
IL-8[3]	Immpress Kit[6,8]	Mouse	1:20	RT, 60min	No pre-treatment (frozen)	Tonsil
CD13[3]	Immpress Kit[6,8]	Mouse	1:200	RT, 30min	No pre-treatment (frozen)	Tonsil
gp130[4]	Elite Kit[6,7]	Mouse	1:50	RT, 60 min	No pre-treatment (frozen)	Tonsil
IL-6Rα[4]	Immpress Kit[6,8]	Rabbit	1:50	RT, 60 min	No pre-treatment (frozen)	Tonsil
CXCR1[5]	Immpress Kit[6,8]	Rabbit	1:80	4°C, O/N	No pre-treatment (frozen)	Tonsil
CXCR2[5]	Immpress Kit[6,8]	Rabbit	1:40	4°C, O/N	No pre-treatment (frozen)	Tonsil

<u>Source:</u> [1] Leica Biosystems, Newcastle LTD UK; [2] Dakocytomation, Denmark; [3] Abcam Cambridge, UK; [4] Santa Cruz Biochemicals, California, USA; [5] R&D Biosystems; [6] Vector Laboratories, Peterborough, UK; [7] ABC ELITE Kit- avidin-biotin peroxidase method (mouse monoclonal); [8] Immpress Kit- avidin-biotin peroxidase method (rabbit polyclonal); RT - Room Temperature; O/N - Overnight

3.3.3 Immunohistochemical scoring

A quickscore was used to assess the immunohistochemical staining taking into account proportion and intensity of staining (Schiessl et al., 2009). A 4 point scale was used to determine the proportion of positively stained cells; 1<25%, 2= 25-50%, 3=50-75% and 4>75% and a 3 point scale used for staining intensity; 1= weak staining, 2= moderate staining and 3= strong staining. The scores for amount of staining and intensity of staining were multiplied together to give a final immune score to a maximum of 12 (high amounts of very strong staining). For example 50% of cells in the section analysed were positively stained and the intensity of that staining was moderate therefore, the score would be 6 (3x2).

3.3.4 ELISA

Decidual CD56^{+}, CD14^{+}, CD10^{+} and CD8^{+} cell culture supernatants (t=24h) isolated from normal pregnant decidua (8-10 and 12-14weeks GA) along with total decidual cells were subjected to ELISA to measure IL-6 and IL-8 protein concentrations to confirm

cytokine production by these cell types are producers of both cytokines and to quantify levels at different gestations (section 3.3.1). IL-6 and IL-8 protein levels were also measured from CD56$^+$ and CD14$^+$ cell culture supernatants isolated from early sporadic miscarriage decidua (≤12 weeks GA) and quantities were compared with normal pregnancy.

All decidual cell culture supernatants were isolated by midimacs positive immune selection (sections 2.3.2 and 2.3.4). Cells were checked for purity after isolation by preparing cell smears and performing immunocytochemistry (section 2.3.3).

A top standard of 2000pgml^{-1} was used for both IL-6 and IL-8 ELISAs (section 2.10) and a 7point standard curve was used as a reference (4PL curve). Sample numbers used for ELISAs are indicated in table 3.1

Briefly, samples were plated in duplicate, diluted by a factor of 2 in reagent diluents (see Appendix) and 100µl of sample was added to ELISA plates blocked with the appropriate capture antibody and steps were followed according to manufacturer's instructions (see section 2.10). ELISA plates were then read using a plate reader and protein concentrations were calculated.

3.3.5 RT-PCR

Total RNA was extracted using TRIZOL method (section 2.9.1) from decidual cell populations CD56$^+$, CD14$^+$, CD10$^+$ and CD8$^+$ at T=0 isolated from 8-10 weeks gestation decidua using immunopositive magnetic selection (n=5 for each cell population). CD56$^+$ cells were also isolated at T=0 from 12-14 weeks gestation decidua (n=4). 500ng of RNA was reverse transcribed into cDNA using Superscript III (section 2.9.2). 2µl of cDNA was used for PCR along with either IL-6, IL-8 or GAPDH primer pairs (IL-6, IL-8 and GAPDH Assay on demand, Applied Biosystems, USA) and real time RT-PCR was performed to 40 cycles (section 2.9.4). Data are presented as the ratio of the amount of IL-6/IL-8 to GAPDH.

To confirm amplicon length some of the IL-6 PCR products from CD56$^+$ cells were separated on 1% agarose gel soaked in ethidium bromide and bands were visualised at 95bp for IL-6cDNA and 250bp for GAPDH cDNA (section 2.9.3 for method).

For Laser capture microdissection of placental bed biopsies, they were initially stained and prepared (section 2.8). Spiral arteries were identified using immunohistochemistry on serial frozen sections (H-Caldesmon and CD31) alongside the prepared sections for LCM. The VSMC cell layer of the spiral arteries were the cells of interest and the remaining tissue and vessel endothelium were cut away. RNA was then extracted from the VSMCs and reverse transcribed (section 2.9.1). Primers for CXCR2, gp130 and IL-6Rα were used for real time RT PCR.

3.4 Results

3.4.1 Immunohistochemical characterisation of decidual cell populations in placental bed biopsies

The maternal-fetal interface contains numerous different cell populations (Figure 3.3) and in order to initially identify the different cell populations within the placental bed of early pregnancy FFPE tissue sections were subjected to immunohistochemical analysis (Table 3.2).

Positive cytokeratin-7 staining was observed in all tissue sections stained with the antibody. The cytokeratin-7 staining within the placental bed represents glandular epithelium and EVT cells; interstitial EVT cells observed embedded in the decidual tissue and inner third myometrium (Figure 3.3A); endovascular EVT observed in the lumen of spiral arteries (Figure 3.3B) and intramural EVT embedded in the VSMC wall of spiral arteries (Figure 3.3C). EVT cells were distinguished by their large appearance and unstructured nature embedded throughout the tissue whereas glandular epithelium was distinguished by their laminar, structured appearance, which surrounded the glandular wall. Positive CD56 staining within the placental bed represents uNK cells and these cells were only observed in the decidua and in superficial myometrium (Figure 3.3D). Positive CD14 immunostaining represents macrophages and dendritic cells within the placental bed (Figure 3.3E) and cells immunopositive for CD3 and CD8 (Figure 3.3F and 3.3G) represents T-cells and the cytotoxic T cell subset respectively. Uterine spiral arteries were identified in the placental bed by their thick muscular vascular wall which is demonstrated by positive H-Caldesmon immunostaining (Figure 3.3H) and SpA endothelium is demonstrated by CD31 positive immunostaining (Figure 3.3I) which lines the lumen of the arteries.
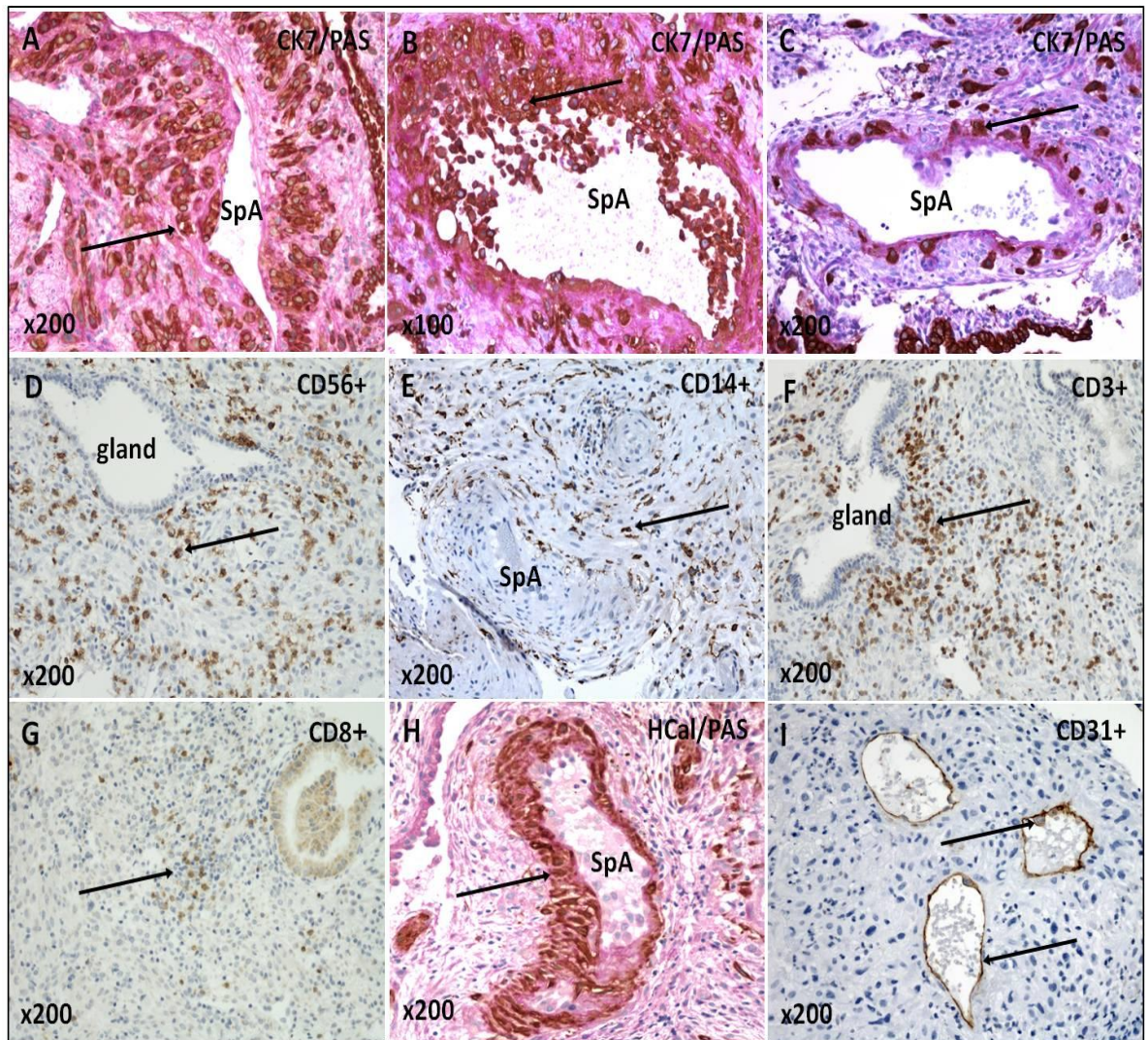
Figure 3.3 Immunohistochemistry demonstrating different cell types within the placental bed of first trimester pregnancy

Representative immunohistochemistry showing the different cell types in the placental bed in first trimester pregnancy and how they were identified in FFPE tissue sections. A: positive CK-7 staining demonstrating iEVT; B: positive CK-7 staining within SpA lumen demonstrating eEVT; C: positive CK-7 staining in VSMC wall of SpAs demonstrating inEVT; D: positive CD56 staining demonstrating uNK cells; E: positive CD14 staining demonstrating uterine macrophages and dendritic cells; F: positive CD3 staining demonstrating T-cells: G: positive CD8 staining demonstrating cytotoxic T cell subset; H: positive H-Caldesmon staining demonstrating VSMC wall of spiral arteries; I: positive CD31 staining demonstrating endothelium of SpAs (n=10 for both 8-10 and 12-14 weeks gestation). Image A, C, D, E, F, G, H, I original magnification x200; Image B original magnification x100.

3.4.2 Immunohistochemical analysis of expression of IL-6, IL-8 and their receptors in normal placental bed

An immunohistochemical study was performed to investigate whether the cell populations within the placental bed of early pregnancies express the cytokine IL-6. IL-6 receptor expression was also investigated on EVT and IL-6 receptor expression along with IL-8 receptor expression was investigated on spiral arteries to investigate whether these cytokines have the potential to play a functional role in the SpA transformation/ remodelling required for successful pregnancy.

3.4.2.1 IL-6 immunolocalisation

IL-6 immunostaining was observed throughout the placental bed and was not defined to one particular area. The majority of cells within the placental bed were in proximity to IL-6 immunostaining. The proportion of IL-6 staining in decidua was between 50-75% in all samples and the intensity of IL-6 staining was moderately expressed.

IL-6 expression was observed on CD56$^+$ immunostained cells in adjacent sections (Figure 3.4). CD56 immunopositive uNK cells were abundant in decidua in the placental bed at 8-10weeks gestation (Figure 3.4A) and roughly 50% of the CD56$^+$ cells were immunostained positive for IL-6 in the near serial sections (Figure 3.4B).

Representative immunostaining for CD8$^+$ cells and IL-6 expression is shown in Figure 3.5. CD8 immunopositive T-cells in the placental bed at 8-10 weeks gestation (Figure 3.5A) were immunopositive for IL-6 in near serial sections (Figure 3.5B).

IL-6 immunostaining in placental bed was quantified using a quickscore and compared between 3 gestational age groups (section 3.3.3) n=5 each gestational age group (Figure 3.6). There were no differences observed in the amount of CD56$^+$ (Figure 3.6A), CD8$^+$ (Figure 3.6B) and IL-6 positively stained cells in the different gestational age groups (n=5 all 3 age groups; 8-10, 12-14, 16-20 weeks gestation).

In addition to CD56$^+$ and CD8$^+$ cells, IL-6 immuno-positivity was also localised to other decidual cells, including stromal cells, glandular epithelial cells and EVT.
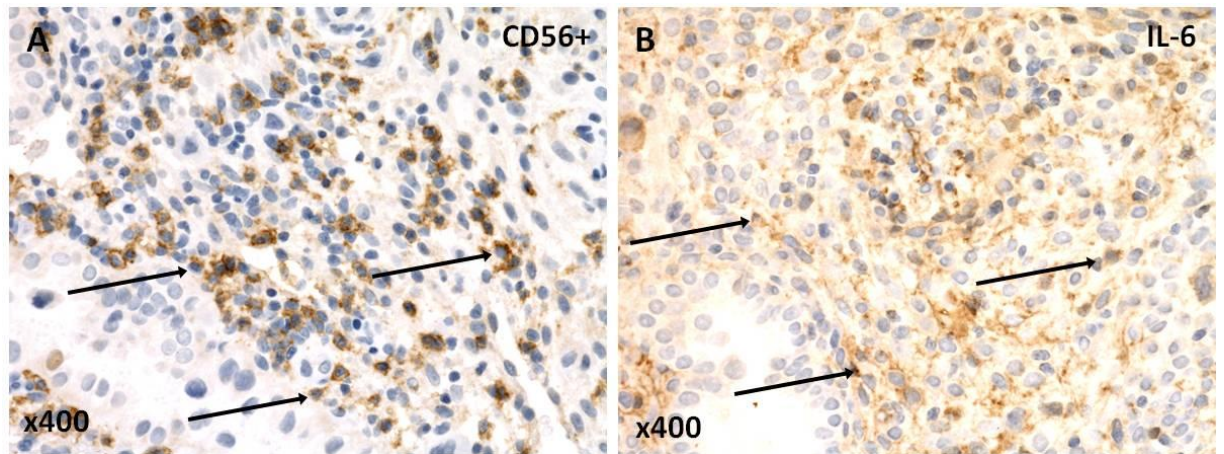
Figure 3.4 Immunohistochemistry demonstrating IL-6 localisation to uNK cells
Representative immunostaining of placental bed biopsies demonstrating localisation of IL-6 to uNK cells. A: CD56^{+} cells, indicating uNK cells in a 10 week placental bed biopsy. B: IL-6 positive cells localised in proximity of uNK cells in near serial section x400 magnification.
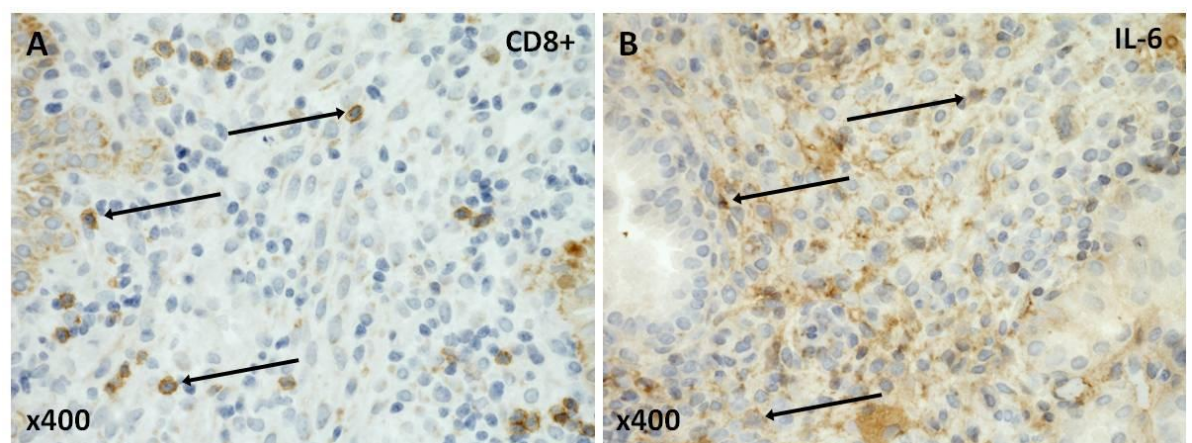
Figure 3.5 Immunohistochemistry demonstrating IL-6 localisation to CD8^{+} T cells
Representative immunostaining of placental bed biopsies demonstrating localisation of IL-6 to T-lymphocytes. A: CD8^{+} cells, indicating T-lymphocytes in a 10 week placental bed biopsy. B: IL-6 positive cells localised in proximity of T-cells in near serial section x400 magnification.
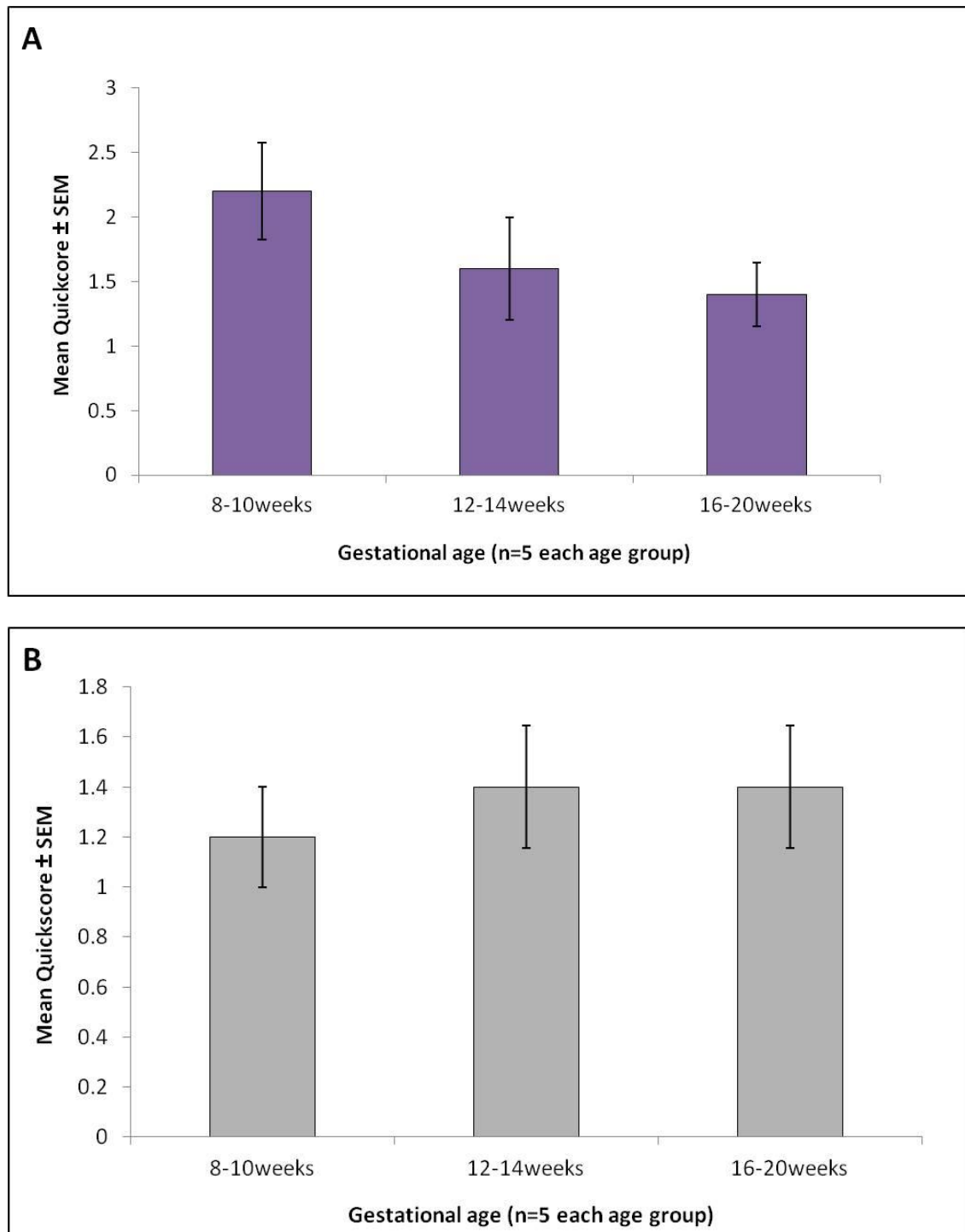
Figure 3.6 Graphical representation of the amount of CD56^{+} and CD8^{+} immunostaining in decidua in the first half of pregnancy

Quickscore assessment of immunostaining (1-12; % of staining x intensity) showing IL-6 positive staining expressed by A: CD56^{+} cells in decidua of placental bed biopsies and B: CD8^{+} T-cells in decidua of placental bed biopsies at 8-10, 12-14 and 16-20 weeks gestation (n=5 each age group). Data are presented as mean ± SEM.

3.4.2.2 IL-6 receptor expression on EVT

IL-6 receptors gp130 and IL-6Rα were both immunolocalised to EVT within the placental bed (Figure 3.7) at 8-10, 12-14 and 16-20 weeks gestational age. Immunohistochemistry demonstrates cytokeratin-7 immunopositive cells representing interstitial EVT cells in placental bed (myometrium and decidua) (Figure 3.7A) near serial sections represent positive immunostaining of gp130 expressed by EVT cells (Figure 3.7B). Approximately 75% of all EVT cells in each sample observed were immunopositive for gp130 receptor.

EVT cells identified in frozen tissue sections of placental bed biopsies were also immunopositive forIL-6Rα (Figure 3.7C). However, the proportion of IL-6Rα immunostaining by EVT was not a prevalent as gp130 immunostaining by EVT.

Quantification of positive gp130 immunostaining on EVT cells in placental bed between gestational age groups was assessed using quickscore assessment (Section 3.3.3) n=5 each gestational age group (Figure 3.8). IL-6Rα was not quantified due to IL-6Rα antibody being specific for frozen tissue sections only; therefore a qualitative assessment was only made.
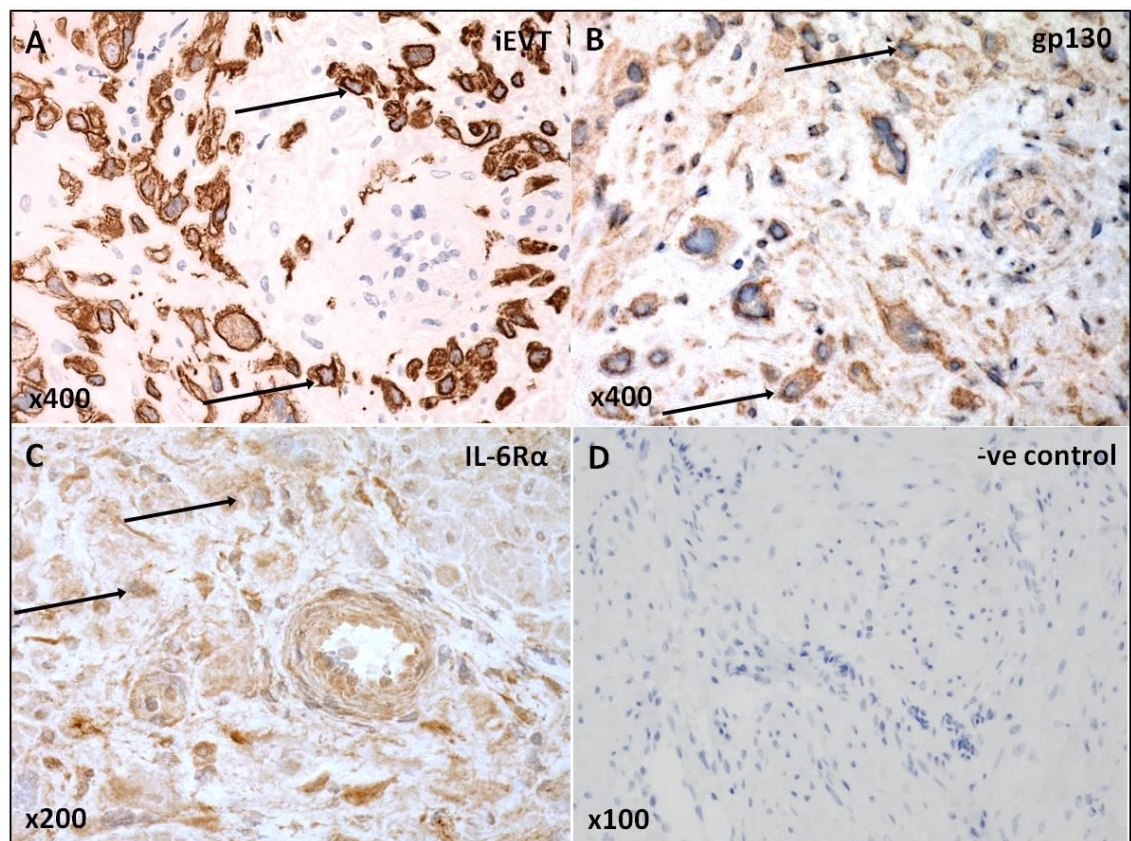
Figure 3.7 Representative immunohistochemistry of IL-6 receptor staining (gp130 and IL-6Rα) by EVT cells in a placental bed biopsy

Representative immunohistochemistry demonstrating EVT cells in an 18 week FFPE placental bed biopsy (A) and these cells are immunopositive for gp130 (B) and EVT cells are immunopositive for IL-6Rα in frozen tissue section at 19 weeks gestation (C) (n=5 each age group). Image of negative control included (non-immune serum) in FFPE placental bed biopsy at 16 weeks gestation (D). Magnification x400 for EVT and gp130; x200 magnification for IL-6Rα; x100 magnification for negative control.
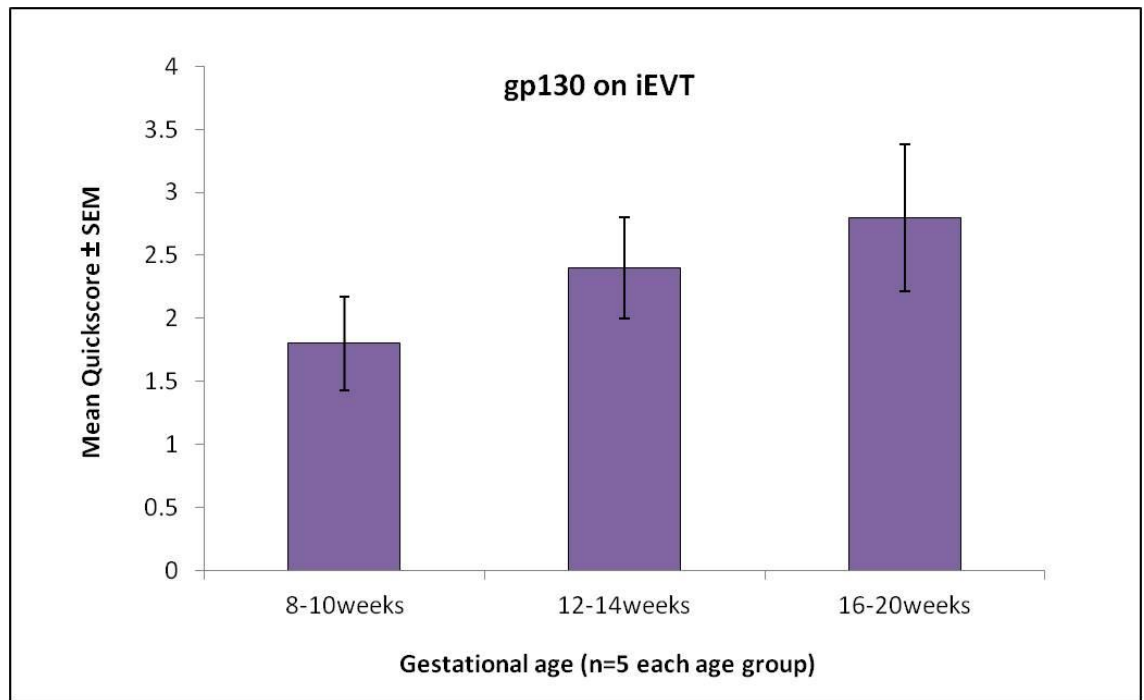
Figure 3.8 Graphical representation of the amount of gp130 immunostaining on interstitial EVT cells in the first half of pregnancy
Graph demonstrating immunohistochemistry data showing quickscore assessment of gp130 positive staining on iEVT cells in the placental bed at 8-10, 12-14 and 16-20 weeks gestation (n=5 each age group). Data are presented as mean ± SEM.

3.4.2.3 Expression of IL-6 and IL-8 receptors on spiral arteries

In order to investigate whether IL-6 and IL-8 may play a functional role in the remodelling processes of the uterine spiral arteries in the first half of pregnancy, cytokine receptor expression was assessed on decidual spiral arteries (SpAs) in placental bed of early pregnancy.

Both of the receptors for IL-6 (IL-6Rα and gp130) were expressed on decidual spiral arteries (Figure 3.9). Positive H-Caldesmon immunostaining in frozen tissue sections of placental bed biopsies was used to identify VSMCs within the wall of spiral arteries (Figure 3.9A) and these muscle cells were immunopositive for IL-6Rα in serial sections (Figure 3.9B). Positive immunostaining for myosin heavy chain was used to localise the VSMC wall of decidual spiral arteries in FFPE placental bed biopsies (Figure 3.9C). Positive staining for gp130 was evident on the luminal endothelium of the SpA in near serial sections, although the immunostaining was fairly weak; there were very few gp130 immunopositive cells within the VSMCs in the wall of the arteries (Figure 3.9D).

The receptors for IL-8; CXCR1 and CXCR2 were also expressed on spiral arteries in early pregnancy (8-14 weeks GA) shown by immunopositive staining in placental bed biopsies (Figure 3.10). Immunostaining for both CXCR1 and CXCR2 was localised to iEVT cells (1), SpA VSMCs (2) and endothelial cells of the spiral arteries (3) (CXCR1- Figure 3.10B; CXCR2- Figure 3.10C). Immunostaining of both receptors was more intense on endothelial cells of the spiral arteries compared to the other cell types investigated.

CXCR1 and CXCR2 immunostaining was also observed by other cells of the placental bed, with CXCR1 being very highly expressed by most cells.
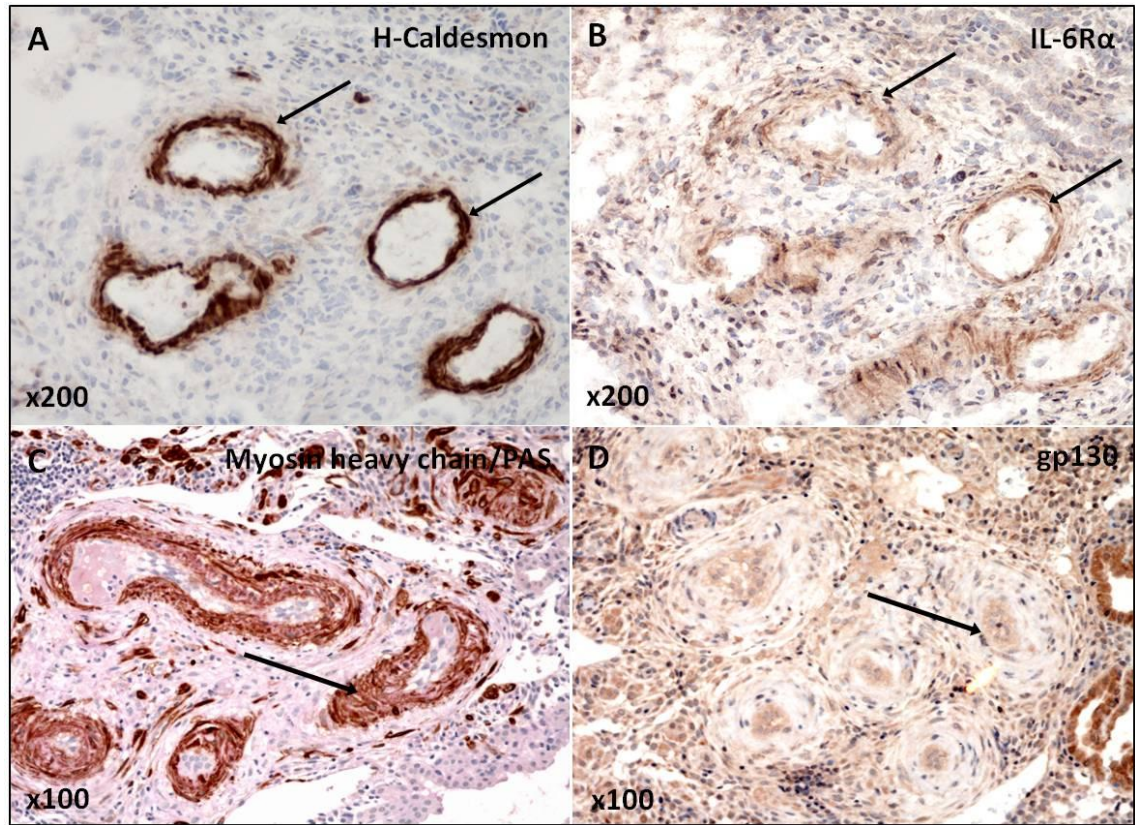
Figure 3.9 Immunohistochemistry demonstrating IL-6 receptor (gp130 and IL-6Rα) staining on decidual spiral arteries in first trimester pregnancy
Immunohistochemistry demonstrating decidual spiral arteries in early pregnancy (8-14 weeks GA) in frozen (A; x200 magnification) and FFPE (C; x100 magnification) placental bed biopsies. Serial sections demonstrate positive IL-6Rα expression on VSMC of spiral arteries (B; x200 magnification) and positive gp130 expression on endothelial cells lining the lumen of SpAs (D; x100 magnification).
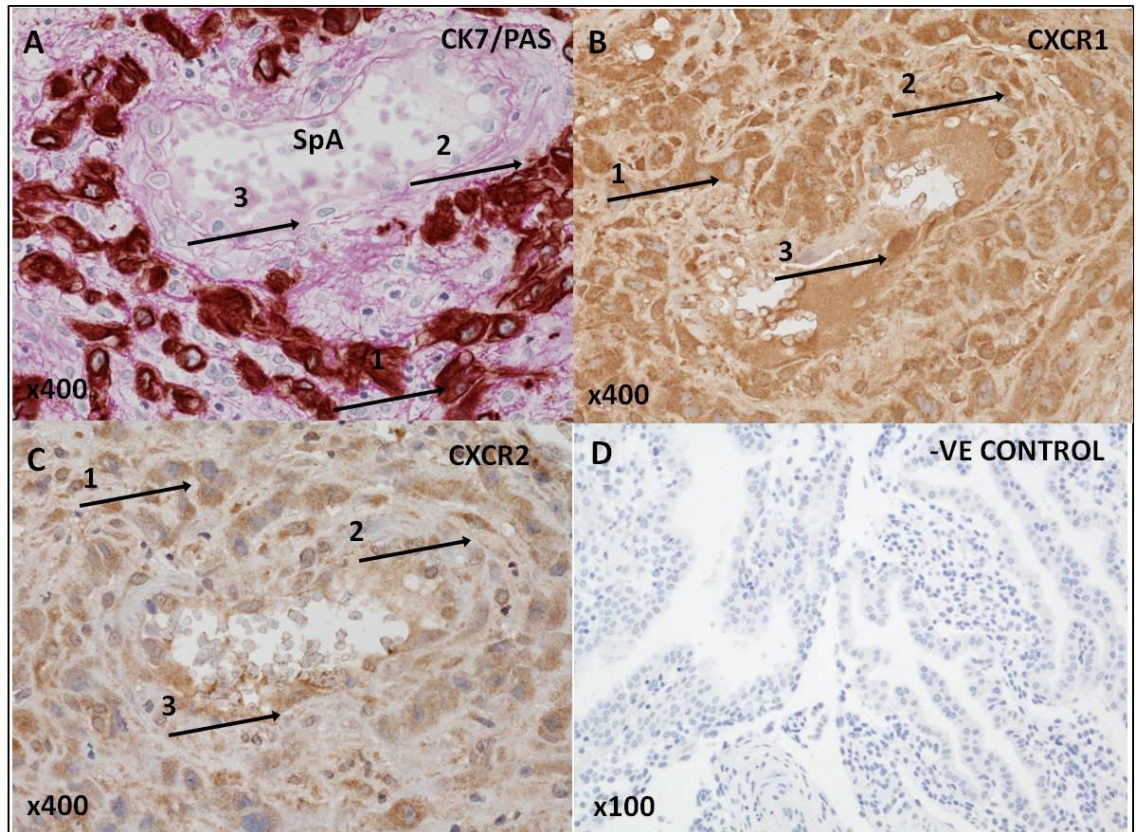
Figure 3.10 Immunohistochemistry demonstrating IL-8 receptor (CXCR1 and CXCR2) staining on decidual spiral arteries in first trimester pregnancy

Immunohistochemistry demonstrating a spiral artery in first trimester pregnancy (A) 1: EVT; 2 VSMC and 3: endothelial cells. Serial sections representing positive staining (brown) of CXCR1 and CXCR2 on EVT, the endothelium and VSMC of the spiral artery (B and C, respectively). Serial section (A) also represents CK7 immunopositive cells showing EVT. Original magnification x400. Negative control (non-immune serum) image included (D) magnification x100.

3.4.3 IL-6 and IL-8 protein production by decidual cell populations

Previous work by De Oliveira et al. (2010) have shown that CD56$^+$ cell culture supernatants (T=24h) secrete large amounts of IL-8 with a significant increase at 12-14 weeks gestation compared with 8-10 weeks gestation (Figure 3.11).

In this study secretion of IL-8 protein by total decidual cells and other decidual types including, CD8$^+$, CD10$^+$ and CD14$^+$ cells was investigated. As well as secretion of IL-6 by CD56$^+$ cells including total decidual cells and other decidual cell types at 8-10 and 12-14 weeks gestation (numbers of samples are indicated in Table 3.1) t=24h.

3.4.3.1 Secretion by CD56$^+$, CD10$^+$, CD8$^+$ and CD14$^+$ cell culture supernatants

IL-6 and IL-8 are secreted by all the decidual cell types investigated at both 8-10 and 12-14 weeks gestation in normal pregnancy (Figure 3.12A and B).

IL-8 was produced at higher levels within the decidua (Figure 3.12B) than IL-6 protein levels (Figure 3.12A). The total non adhered decidual cell population produced ~10000pgml^{-1} of IL-8 protein at 8-10 weeks gestation compared with ~6500pgml^{-1} IL-6 and at 12-14 weeks gestation there was 10 fold higher levels of IL-8 compared with IL-6 levels. The majority of IL-8 protein secreted by the total decidual cell populations was secreted by the cell types investigated, whereas other cell types within the decidua which have not been investigated also secreted IL-6 protein, along with the cell types investigated.

Decidual CD14$^+$ cell culture supernatants (full details of CD14$^+$ supernatant collection is reviewed in Chapter 6) expressed the highest amount of IL-6 compared with CD56$^+$, CD10$^+$ and CD8$^+$ cell supernatants, although all cell types produced this cytokine (Figure 3.12A). In addition, decidual CD14$^+$ and CD8$^+$ cell supernatants secreted the highest amounts of IL-8 compared with CD56$^+$ and CD10$^+$ cell supernatants (Figure 3.12B).
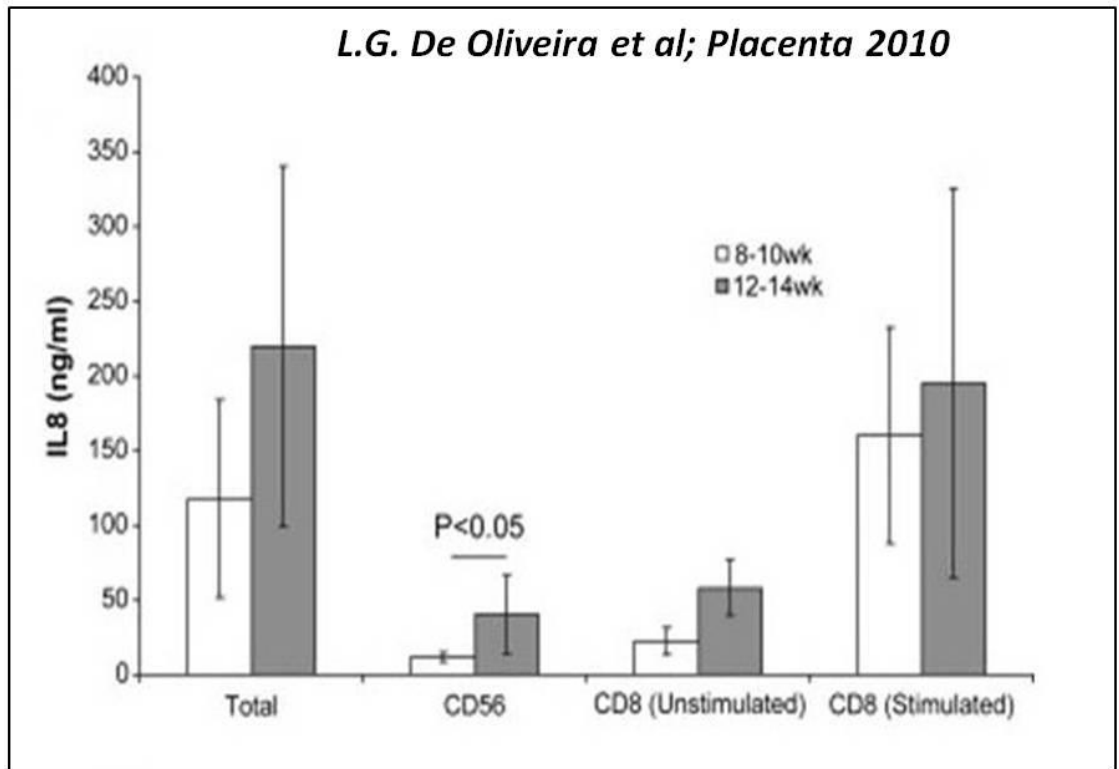
Figure 3.11 IL-8 protein secretion by decidual CD56^{+} and CD8^{+} cells at 8-10 and 12-14 weeks gestation

Image extracted from De Oliveira et al. (2010) demonstrating IL-8 protein secretion from decidual CD56 and CD8 cells. CD56 cells produce more IL-8 at 12-14 weeks gestation compared with levels at 8-10 weeks gestation. Data are expressed mean ± SEM (n=10 each GA group).
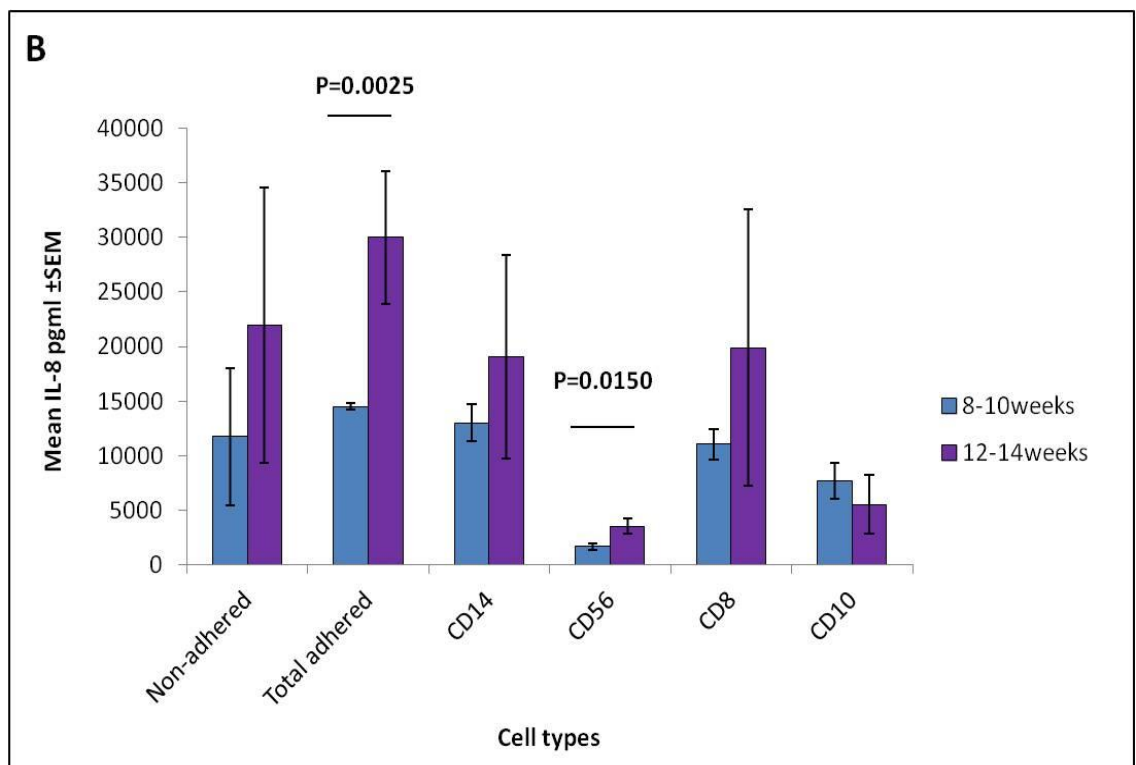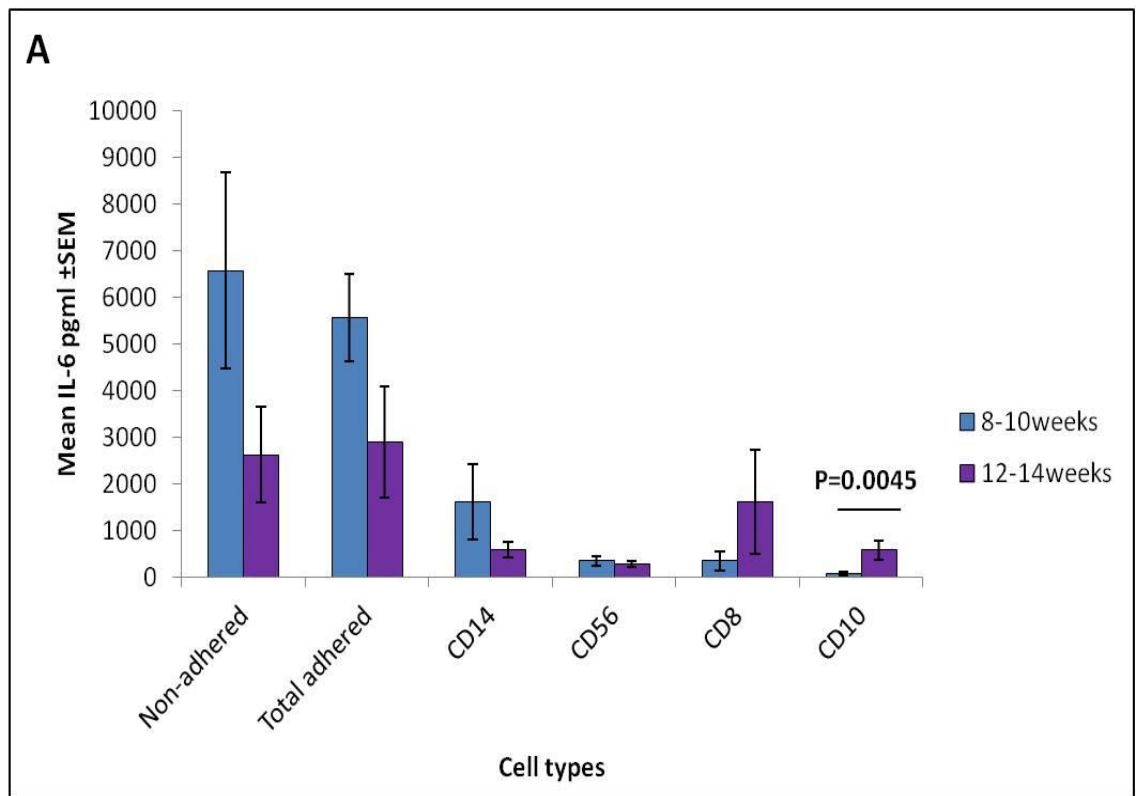
Figure 3.12 IL-6 and IL-8 protein secretion by decidual cells at 8-10 and 12-14 weeks gestation
Graphical representations of IL-6 (A) and IL-8 (B) protein levels secreted by decidual cells at 8-10 and 12-14 weeks gestation. Data are presented as mean ± SEM. Data also include total decidual cells prior to adherence (non-adherent) and total adhered decidual cells. n=10 samples per cell type at 8-10 weeks gestation and n=5 samples per cell type at 12-14 weeks gestation. All samples were run in triplicate in the ELISA.

3.4.3.2 Differences in protein production at 8-10 weeks and 12-14 weeks gestation

IL-6 protein levels by the total decidual cell fraction appear to be more abundant at 8-10 weeks gestation compared with 12-14 weeks gestation although this difference was not significant (Figure 3.12A). There were no gestational differences in IL-6 protein levels secreted by decidual $CD14^+$, $CD56^+$ or $CD8^+$ cells. However, $CD10^+$ stromal cells secreted significantly higher levels of IL-6 at 12-14 weeks than at 8-10 weeks gestation (P=0.0045).

IL-8 protein levels secreted by the total decidual cell fraction appear to be higher at 12-14 weeks gestation than 8-10 weeks gestation, but this was significantly different only for the total adhered decidual cell population (P=0.0025; figure 3.12B). There were no gestational differences in IL-8 protein levels secreted by $CD14^+$, $CD8^+$ or $CD10^+$ cells. However, $CD56^+$ cells isolated from 12-14 weeks gestation secreted increased levels of IL-8 than at 8-10 weeks gestation (P=0.0150), in agreement with previously published data (De Oliveira et al., 2010).

I further went on to investigate the production of IL-6 and IL-8 by $CD14^+$ and $CD56^+$ cells in miscarriage which is later reviewed in this chapter due to $CD14^+$ cells being the largest producer of both cytokines from the decidual cell types investigated and the gestational differences observed of cytokine levels produced by $CD56^+$ cells.

3.4.4 mRNA expression of IL-6 and IL-8 in decidual cell populations

To confirm IL-6 and IL-8 production by decidual cell populations CD56$^+$ uNK cells, CD14$^+$ macrophages, CD10$^+$ stromal cells and CD8$^+$ cytotoxic T-cells were isolated by positive magnetic selection from decidual tissue collected from women undergoing TOP at 8-10 weeks gestation. Cells were collected immediately following isolation (T=0; n=5 each cell type) and used for real time RT-PCR. CD56$^+$ cells isolated from decidua from a previous study (De Oliveira et al., 2010) had already shown to express IL-8 mRNA n=10 (Figure 3.13).

Each of the cell populations investigated expressed IL-6 and IL-8 mRNA transcripts (Figure 3.14A and B). A semi quantitative assessment of delta delta ct values demonstrated that CD14$^+$, CD10$^+$ and CD8$^+$ cells express higher levels of IL-8 than IL-6 (Figure 3.14A and B).

Fold change values also revealed that CD14$^+$ expressed ~20x more IL-6 than CD8$^+$ cells, (Figure 3.14A). CD10$^+$ cells expressed ~ 6x more IL-6 than CD8$^+$ cells and CD56$^+$ cells expressed ~3x more IL-6 than CD8$^+$ cells (Figure 3.14A).

Fold change values revealed that CD14$^+$ expressed the highest levels of IL-8 in relation to CD8$^+$ T cells: CD14$^+$ cells expressed ~10x more IL-8 than CD8$^+$ cells, and CD10$^+$ cells expressed ~5x more IL-8 than CD8$^+$ cells (Figure 3.14B).

Mean delta CT values are shown for IL-6 and IL-8 in each cell population in Table 3.4.

PCR gel was performed using total decidual cells and CD56+ cells (T=0) to confirm IL-6 mRNA expression by in decidua. Transcripts were detected at 95 base pairs which indicate IL-6 mRNA is expressed with no difference with GA (Figure 3.15).
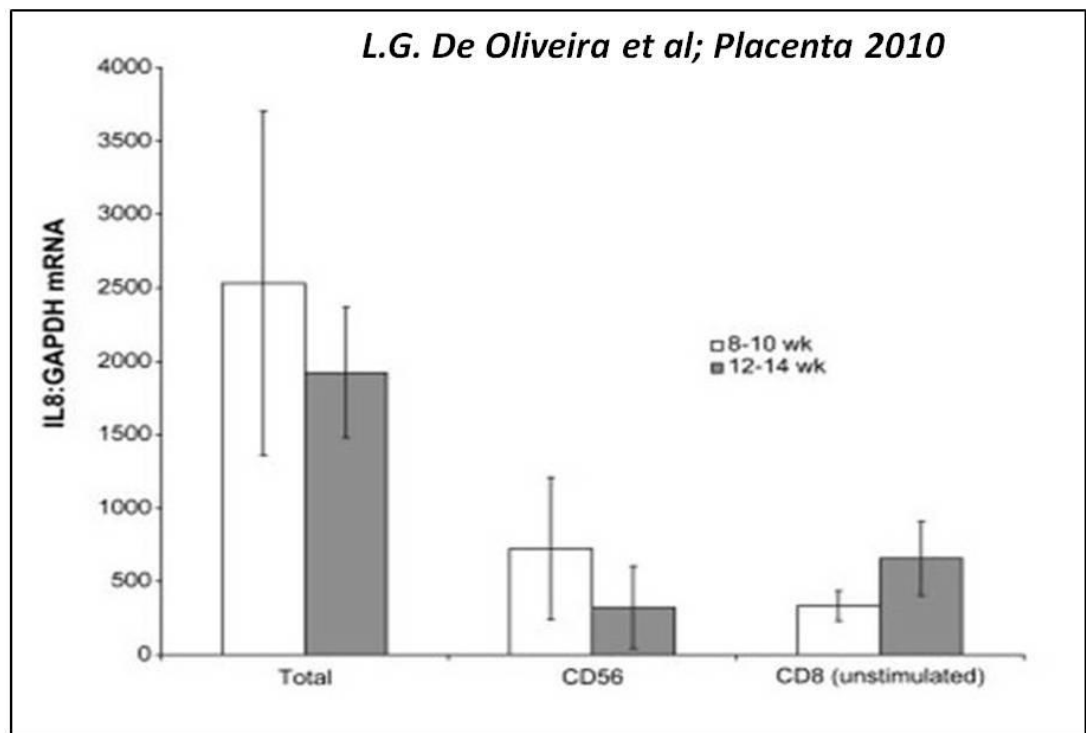
Figure 3.13 CD56^{+} IL-8mRNA expression at 8-10 weeks gestation
Image extracted from De Oliveira et al. (2010) demonstrating CD56^{+} IL-8 mRNA expression.
Data are expressed mean ± SEM (n=10 8-10 weeks GA).
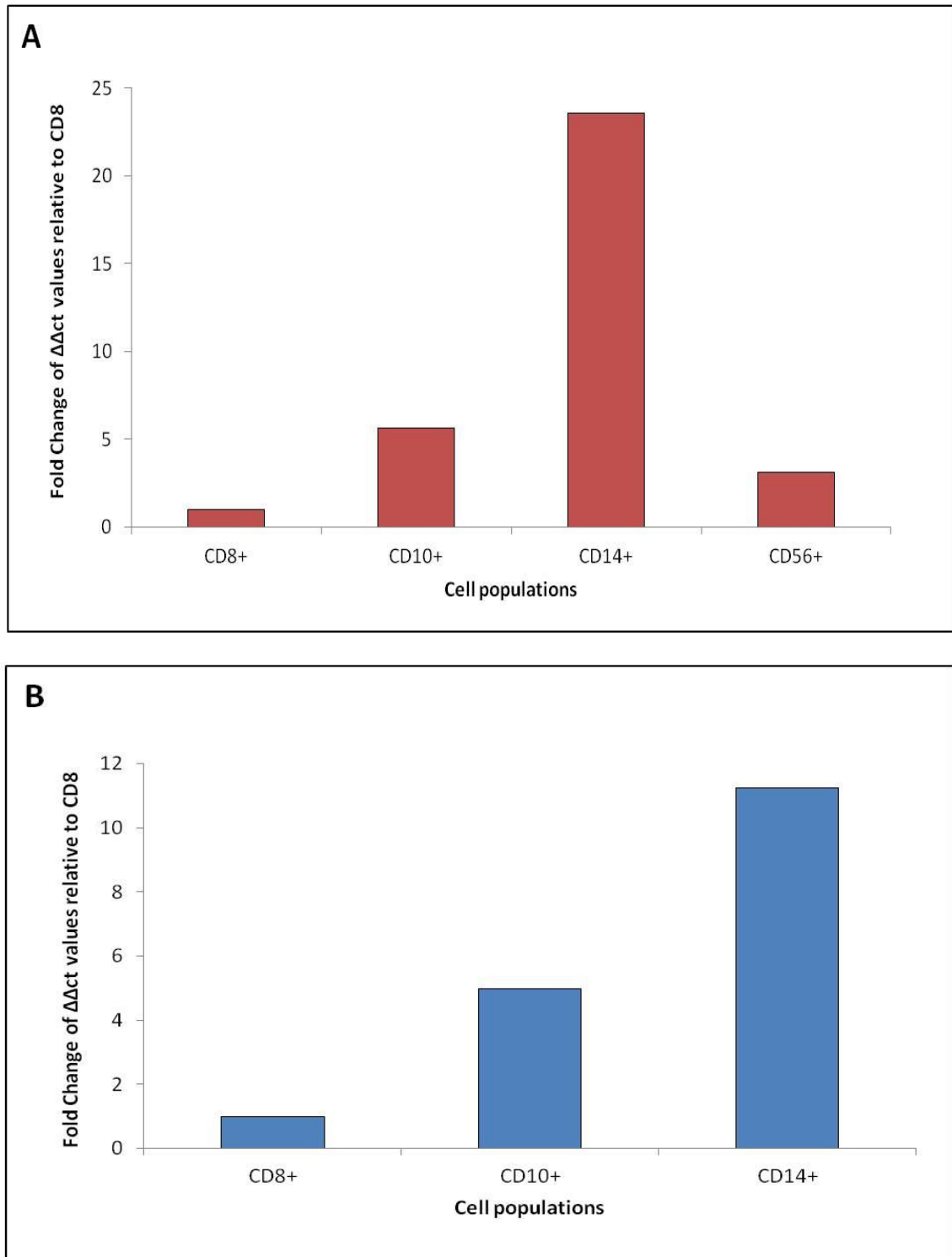
Figure 3.14 IL-6 and IL-8 mRNA expression by decidual cells at 8-10 weeks gestation
Graphical representation of fold change of IL-6 (A) and IL-8 (B) delta delta ct values of mRNA expression relative to CD8^{+} decidual cells (initial ct values with reference to GAPDH ct values; n=5 each cell type at 8-10weeks GA).

Table 3.4 Mean Δct values of IL-6 and IL-8 mRNA expression by decidual cells from 8-10 weeks gestation

Mean Δct values of IL-6 and IL-8 mRNA expression in reference to GAPDH in four decidual cell populations isolated from 8-10 weeks gestation n=5 each cell type

Cell populations	IL-6	IL-8
CD8+	5.45	-2.48
CD10+	2.95	-4.80
CD14+	0.89	-5.97
CD56+	3.81	

.
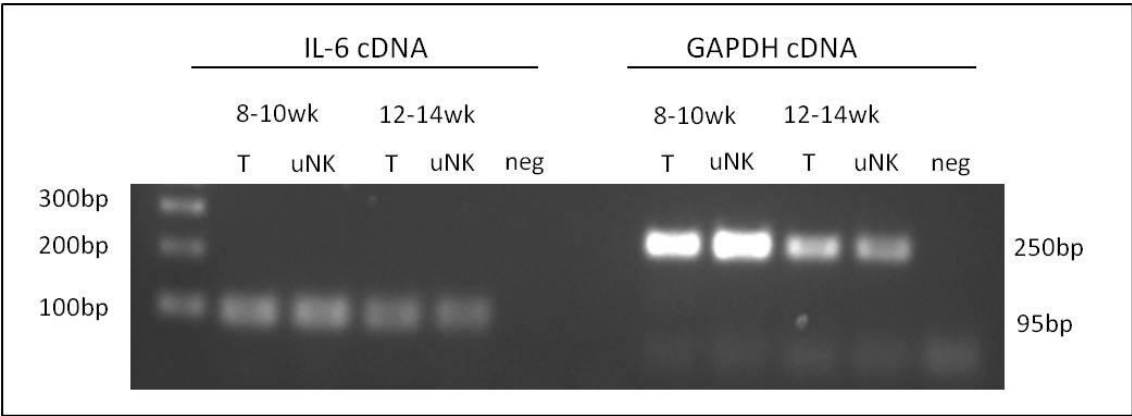
Figure 3.15 RT-PCR gel illustrating IL-6 mRNA expression by CD56+ cells at 8-10 and 12-14 weeks gestation

Representative RT-PCR illustrating IL-6 mRNA expression at 95bp (left) at 8-10 and 12-14 weeks gestational age (T= total decidual cell isolates; uNK= CD56$^+$ cell isolates). GAPDH mRNA expression at 250bp (right) is shown as a loading control for the experiment.

3.4.5 Expression of IL-6 and IL-8 receptors by spiral arteries

To further confirm the immunohistochemistry data of IL-6 and IL-8 receptors expression on spiral arteries, IL-6 and IL-8 receptor mRNA expression was investigated using laser capture microdissection of spiral artery VSMC cells (endothelium removed) from 8-10 week placental bed biopsies (n=5).

IL-6Rα, gp130 and CXCR2 receptor mRNA was detected in VSMCs of spiral arteries at 8-10 weeks gestation (Figure 3.16), this further verifies the immunohistochemistry results and confirms that gp130 expression is lower on SpA VSMC than IL-6Rα.
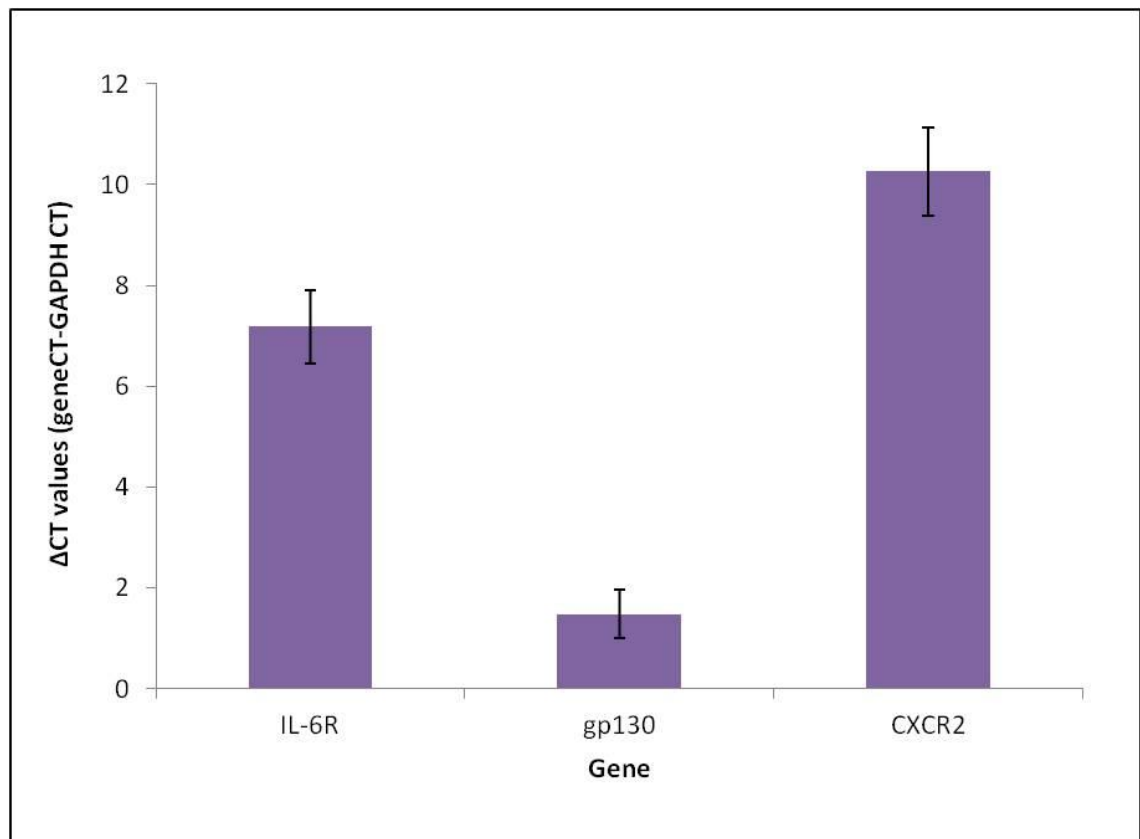
Figure 3.16 IL-6R, gp130 and CXCR2 mRNA expression by spiral arteries at 8-10 weeks gestation
Graph representing delta ct values of each receptor gene expressed on SpA VSMCs (8-10 weeks GA; n=5) from LCM.

3.4.6 Immunohistochemical analysis of expression of IL-6, IL-8 and their receptors in normal placental bed biopsies from miscarriage; comparison with normal early pregnancy

Placental bed biopsies from women undergoing ERPC and healthy TOP were snap frozen, fixed in acetone and immunostained with a collection of antibodies that detect IL-6, IL-8 and their receptors (Table 3.3).

The receptor ligands and cell types assessed are detailed in Table 3.5 and 3.6 and representative photomicrographs demonstrate staining patterns shown in figures 3.17 - 3.19.

Figure 3.17 demonstrates various cell types in the placental bed and how they were identified using immunostaining of serial sections. The immunostaining of the various cell types were assessed and compared between normal tissue sections and miscarriage tissue sections.

Interstitial EVT (iEVT), intramural EVT (imEVT) and endovascular EVT (eEVT) were identified by positive LP34 staining (Figure 3.17A). Decidual spiral arteries and myometrial spiral arteries were both identified by VSMCs positive immunostaining with H-Caldesmon (Figure 3.17B and 3.17C respectively).

IL-6 markers were identified by immunopositive staining of IL-6R on myometrial SpAs (Figure 3.18A), gp130 immunostaining was identified on glands and EVT (Figure 3.18B) and IL-6 immunostaining was observed on various cell types within the placental bed (Figure 3.18C).

IL-8 markers in placental bed in both miscarriage and normal specimens were identified by positive IL-8 immunostaining in placental bed biopsies with intense staining on the glandular epithelium and strong staining on SpA endothelium lining the lumen and weaker staining on VSMCs of the decidual SpAs (Figure 3.19A), CD13 expression was identified and localised to VSMCs of SpAs very strongly (Figure 3.19B), CXCR2 immunostaining was identified on giant cells and SpA endothelium (Figure 3.19C) and CXCR1 immunostaining was identified on SpA endothelium amongst most cells in the placental bed (Figure 3.19D).
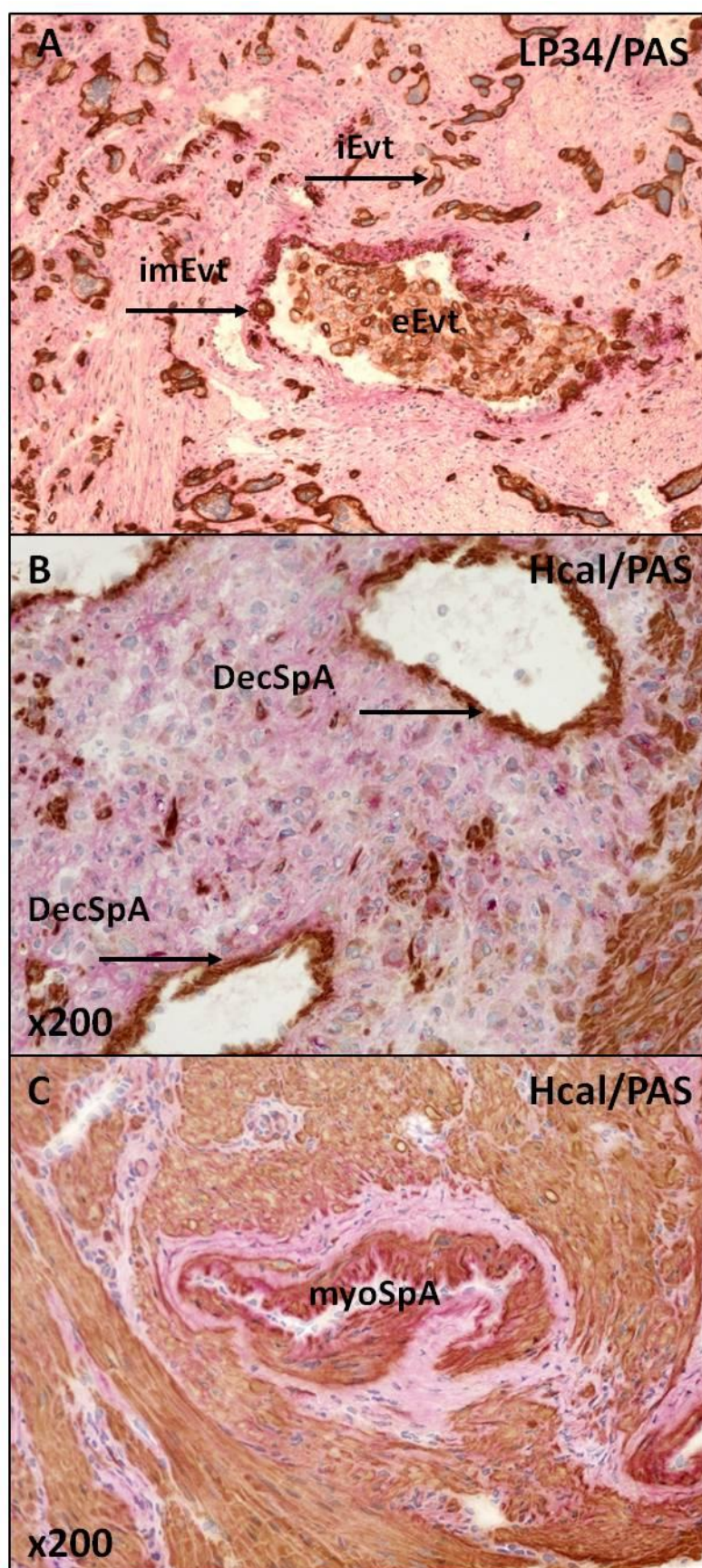
Figure 3.17 Immunohistochemistry demonstrating different cell types in frozen placental bed biopsies Immunohistochemistry demonstrating different cell types in the placental bed. A represents positive LP34 staining demonstrating EVT (Magnification x100); B represents positive H-Caldesmon staining in the decidua demonstrating decidual spiral arteries and C represents positive H-Caldesmon staining in the myometrium demonstrating myometrial spiral arteries. Original magnification x200.
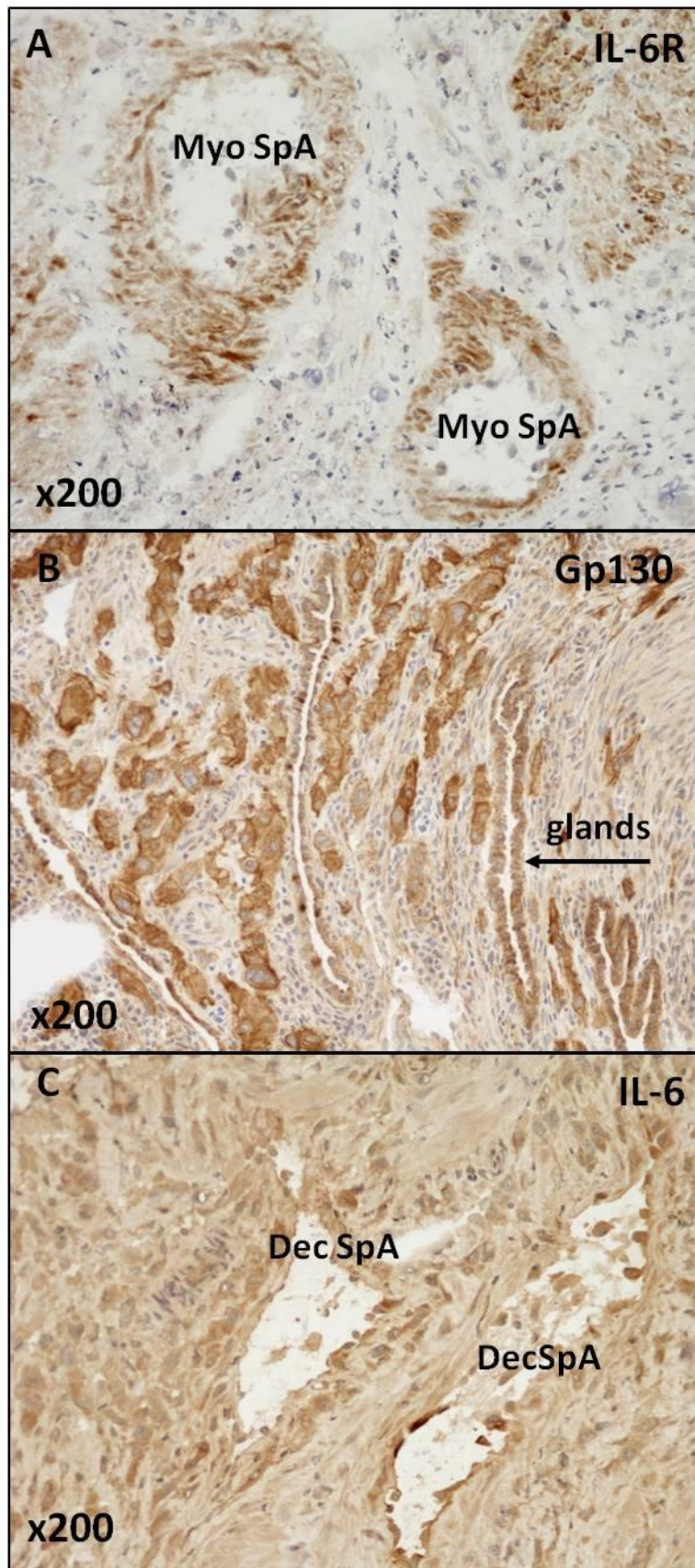
Figure 3.18 IL-6 and IL-6 receptor immunostaining in placental bed biopsies from early sporadic miscarriage Immunohistochemistry demonstrating immunostaining for IL-6 and its receptors in placental bed biopsies from early sporadic miscarriage (SM). Positive IL-6R staining (A) demonstrating the expression of IL-6 receptor on myometrial SpAs; Positive gp130 expression (B) on glands and EVT in decidua and positive expression of IL-6 (C) on a variety of cell types in the placental bed including endothelium on decidual SpAs. Original magnification x200.
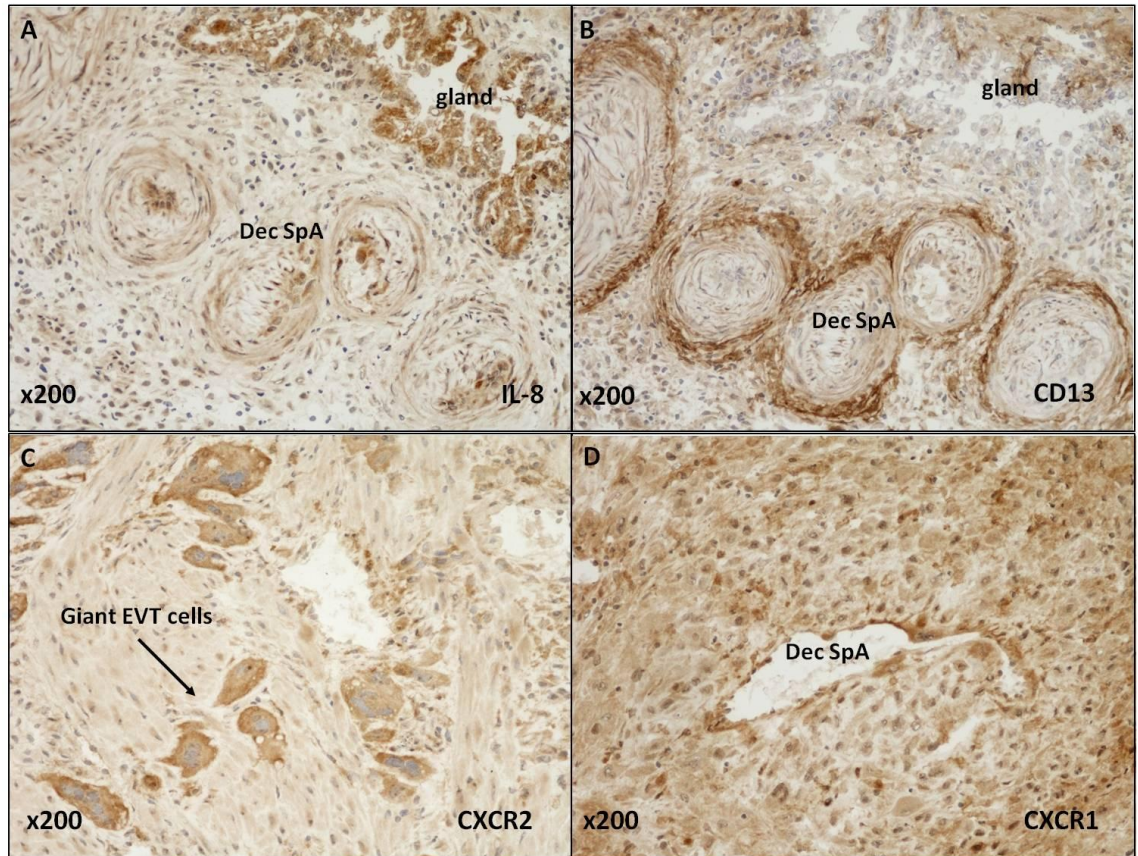
Figure 3.19 IL-8 and IL-8 receptor immunostaining in placental bed biopsies from early sporadic miscarriage

Immunohistochemistry demonstrating IL-8 and it's receptors in placental bed biopsies from early sporadic miscarriage. Positive IL-8 immunostaining (A) demonstrating the expression of IL-8 by decidual SpAs and glands; Positive CD13 immunostaining demonstrating the expression of amniopeptidase-N (B) on VSMCs of decidual SpAs; Positive CXCR2 immunostaining (C) demonstrating IL-8 receptor on giant trophoblast cells and positive CXCR1 immunostaining (D) demonstrating IL-8 receptor expressed on a number of cell types within decidua. Original magnification x200.

Immunoscoring was quantified using quickscore in normal pregnancy and sporadic miscarriage (SM) samples. There were no statistical differences between expression of IL-6 and IL-8 and their receptors on the different cell types investigated between euploid and aneuploid miscarriages, which was also observed in a study by Ball et al. (2006b). Therefore, the miscarriage specimens were categorised into early ($\leq12^{+6}$ weeks gestation) and late (≥13 weeks gestation) giving sample numbers of n=10 of each gestational age with respect to the miscarriage samples, data for euploid and aneuploid samples combined.

Tables 3.5 and 3.6 show all cell types and ligands analysed by immune quickscoring (n=10 each gestational age for SM and n=5 for each gestational age group for normal pregnancy). Lots of cell types and markers were investigated and from this the analysis revealed only a few statistical differences were observed between normal pregnancy and SM. Analysis from immunoscoring showed that the majority of statistical differences between normal pregnancy and SM were observed in the late gestations (≥13 weeks) with the majority of differences being increased immunostaining of markers in SM compared with normal pregnancy.

Immunostaining of IL-8 markers; CD13 and CXCR2 were ligands to be significantly different on certain cell types between SM and normal pregnancy, immunostaining of IL-8 and CXCR1 did not appear to change in miscarriage compared with normal specimens. Differences were observed in immunostaining of IL-6 and IL-6Rα in SM and normal pregnancy but gp130 immunostaining did not alter.

Table 3.5 Immunohistochemisty quickscore assessment of IL-6, IL-8 and their receptors on different cell types in early miscarriage
Table listing immunohistochemistry quickscore assessment of IL-6, IL-8 and their receptors in early MC samples (≤12+6 weeks GA; n=10) and early normal pregnancy (8-10 weeks GA; n=5) in different cell populations within the placental bed. Data expressed as Mean ± SEM.

Ligands >	gp130		IL-6Rα		CXCR1		CXCR2		CD13		IL-6		IL-8	
Cell types ∨	Normal (mean ± SEM)	Miscarriage (mean ± SEM)	Normal (mean ± SEM)	Miscarriage (mean ± SEM)	Normal (mean ± SEM)	Miscarriage (mean ± SEM)	Normal (mean ± SEM)	Miscarriage (mean ± SEM)	Normal (mean ± SEM)	Miscarriage (mean ± SEM)	Normal (mean ± SEM)	Miscarriage (mean ± SEM)	Normal (mean ± SEM)	Miscarriage (mean ± SEM)
Decidual stroma	3±1	4.5±0.89	1±0	0.88±0.3	7.5±4.5	9.22±0.8	5.5±3.5	5.88±0.76	4.5±1.5	5.25±1.26	10±2	7.56±1.22	1.67±0.33	2.13±0.67
Myometrium	3.6±0.75	2.5±0.45	1.2±0.2	2.22±0.6	7.4±1.03	6.3±0.99	3.8±0.8	5.44±1.04	4±0.63	4.33±0.73	3.8±0.8	2.7±0.58	2.8±0.74	2±0.44
Epithelium	12±0	10±1.07	0.6±0.25	3.13±1.03	1±0	8±1.15	10.5±1.5	8.83±1.56	6.2±0.8	2.13±0.58	4.5±4.5	8.14±1.16	10.67±1.33	7.88±1.49
iEVT	12±0	10.8±0.85	0±0	1.88±1.13	8±0	9.22±0.8	12±0	9.56±1.04	8±0	9.89±0.89	12±0	9.4±0.73	8±0	7.67±1.03
eEVT	8±0	5.5±2.5	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
imEVT	6±0	12±0	0±0	2.6±2.36	4±0	10.5±0.87	12±0	9.67±1.09	6±0	9±1.73	12±0	7.33±1.69	4±0	7±5
Giant cells	6±0	4±1	0±0	3.5±2.84	8±0	5.6±1.21	6±0	8.8±1.5	1±0	5.25±2.29	8±0	9.6±1.47	3±0	5±2.52
SpA VSMC wall (d)	2.5±1.5	1.14±0.51	6±0	7.33±1.41	12±0	11.57±0.23	1.5±1.5	2±0.78	10±2	11.14±0.86	4±0	1.13±0.52	4±2	2.5±0.96
SpA VSMC (m)	1.4±0.25	1.7±0.54	9.6±1.75	11±0.71	6.6±2.6	9.2±0.66	2.6±0.87	2.1±0.56	6.6±2.09	8±0.88	1.2±0.8	1.4±0.5	5.2±0.8	5.5±1
SpA endothelium (d)	6±2	6±1.07	1±1	1.5±0.96	1.5±1.5	2.57±1.78	10±2	6.67±1.91	0.5±0.5	4.29±1.77	12±0	10.13±1.49	5.33±2.67	6.14±1.32
SpA endothelium (m)	3.4±0.25	5.2±1.05	0.4±0.4	0±0	3.8±1.74	3.3±1.33	8±4	6.89±1.34	3±2.28	1.11±0.75	8.8±1.36	9.7±1.21	2.4±1.47	4.33±1.68

(iEVT= interstitial EVT; eEVT= endovascular EVT; imEVT= intramural EVT; d= decidua; m= myometrium)

Table 3.6 Immunohistochemisty quickscore assessment of IL-6, IL-8 and their receptors on different cell types in late miscarriage

Table listing immunohistochemistry quickscore assessment of IL-6, IL-8 and their receptor ligands in late MC samples (≥13 weeks GA; n=10) and late normal pregnancy (12-14 weeks GA; n=5) in different cell populations within the placental bed. Data expressed as Mean ± SEM.

Cell types ∨	gp130 Normal (mean ± SEM)	gp130 Miscarriage (mean ± SEM)	IL-6Rα Normal (mean ± SEM)	IL-6Rα Miscarriage (mean ± SEM)	CXCR1 Normal (mean ± SEM)	CXCR1 Miscarriage (mean ± SEM)	CXCR2 Normal (mean ± SEM)	CXCR2 Miscarriage (mean ± SEM)	CD13 Normal (mean ± SEM)	CD13 Miscarriage (mean ± SEM)	IL-6 Normal (mean ± SEM)	IL-6 Miscarriage (mean ± SEM)	IL-8 Normal (mean ± SEM)	IL-8 Miscarriage (mean ± SEM)
Decidual stroma	4.5±1.85	5.7±0.7	0.33±0.33	1±0.17	7.5±0.87	8.5±0.83	5.75±1.44	5.7±0.86	3.75±1.03	7±0.98	9±1.23	8.1±0.59	2.75±0.75	3.25±0.41
Myometrium	2.2±0.97	2.4±0.31	3.75±1.03	3.4±0.79	7.8±1.2	9.5±0.76	4±0.63	4.5±0.79	3±0.63	5.5±0.58	3±0.63	3.8±0.49	2.2±0.97	1.88±0.48
Epithelium	12±0	11.6±0.4	1±0	3.75±0.77	3.25±1.6	6.13±1.04	5.25±1.6	7.1±1.06	2.6±0.6	2.63±0.93	8.25±0.25	5.25±1.4	7.4±2.18	8.5±1.12
iEVT	12±0	11.6±0.4	0.75±0.48	1.44±0.41	8.4±1.6	10.1±0.82	10.6±0.87	10.5±0.62	5.8±1.11	10.22±1.22	11.2±0.8	8.6±0.99	5.8±1.11	6.67±1.16
eEVT	10±2	10.67±1.33	0±0	0.67±0.67	8±0	8±0	7±1	8±0	7±1	7.75±1.84	12±0	9.33±1.33	4±0	6.67±2.67
imEVT	8.8±1.5	10±0.98	1.33±1.33	0.17±0.17	9.8±0.92	9.14±1.14	8±0	9.86±0.77	5.6±1.03	9.57±1.41	12±0	9.43±1.29	6.5±1.89	6.33±1.5
Giant cells	12±0	12±0	0±0	1.6±1.19	5.2±1.96	8.2±0.8	9.4±1.17	10.2±0.76	3±1	5.1±1.49	9±0.78	9.6±0.83	3±1	3.9±0.67
SpA VSMC wall (d)	1±0	3±1.21	5±2.08	6±1.79	10±2	11.5±0.5	2±0.41	3.5±0.71	11.25±0.75	8.86±0.94	2.5±1.26	1±0.58	2.75±0.63	2.83±0.87
SpA VSMC (m)	1.2±0.37	1.6±0.34	11.25±0.75	11.2±0.8	9.6±1.12	11.3±0.47	4.2±0.49	2.9±0.91	12±0	8.6±0.7	2.4±0.75	0.5±0.4	4.2±1.24	3.75±0.84
SpA endothelium (d)	4.75±0.75	5.56±1.27	0±0	0.29±0.29	6.25±2.39	5.88±2	1.75±1.44	9.38±0.82	0.5±0.29	2±1.31	10.5±1.5	11.56±0.44	4.5±1.71	3.43±1.31
SpA endothelium (m)	5.8±1.43	5.3±0.92	0±0	0.2±0.2	5.6±2.11	4.1±1.38	5.4±0.23	7.9±1.25	0.6±0.25	0.9±0.61	10.8±1.2	11.3±0.47	3.8±2.33	2.67±1.04

(iEVT= interstitial EVT; eEVT= endovascular EVT; imEVT= intramural EVT; d= decidua; m= myometrium)

3.4.6.1 Altered immunoexpression of IL-8 markers between normal pregnancy and sporadic miscarriage

In the early gestational age groups there was more CD13 immunostaining on glandular epithelial cells in decidua in normal early pregnancy compared with early SM (P= 0.0015; Figure 3.20 A-C) and there were no other differences observed in early pregnancy. In the late gestational age group, CD13 expression was stronger on interstitial EVT (P=0.0343, Figure 3.21) and on myometrial stroma in SM compared with normal pregnancy (P=0.0199, Figure 3.22). A decrease in CD13 immunostaining was observed in myometrial spiral artery VSMCs in late SM compared with normal pregnancy (P=0.0051; Figure 3.23).

Immunostaining of CXCR1 receptor was not altered in any of the cell types investigated, however changes in CXCR2 staining patterns were observed. CXCR2 immunostaining on the decidual SpA endothelium was stronger and more apparent in miscarriage samples compared with normal pregnancy (P=0.0006; Figure 3.24). However, this receptor was not altered in any other cell type investigated.

3.4.6.2 Altered immunoexpression of IL-6 markers between normal pregnancy and sporadic miscarriage

IL-6Rα immunoscoring was increased on glandular epithelium in late SM compared with normal pregnancy (P=0.0184; Figure 3.25). However, immunostaining of the receptor was not significantly altered in any other cell types investigated.

In addition to changes in receptor immunostaining, a reduction in IL-6 immunostaining was observed on myometrial spiral artery VSMCs in late SM compared with normal pregnancy (P=0.0283; figure 3.26) but this difference was not observed on decidual artery VSMCs nor any other cell types. However, immunostaining was apparent on glandular epithelium in many samples but no differences were observed.

Immunostaining for gp130 receptor expression was apparent in all specimens investigated and staining was very intense on glands and iEVT. However, there were no differences in staining patterns between normal pregnancy and miscarriage.
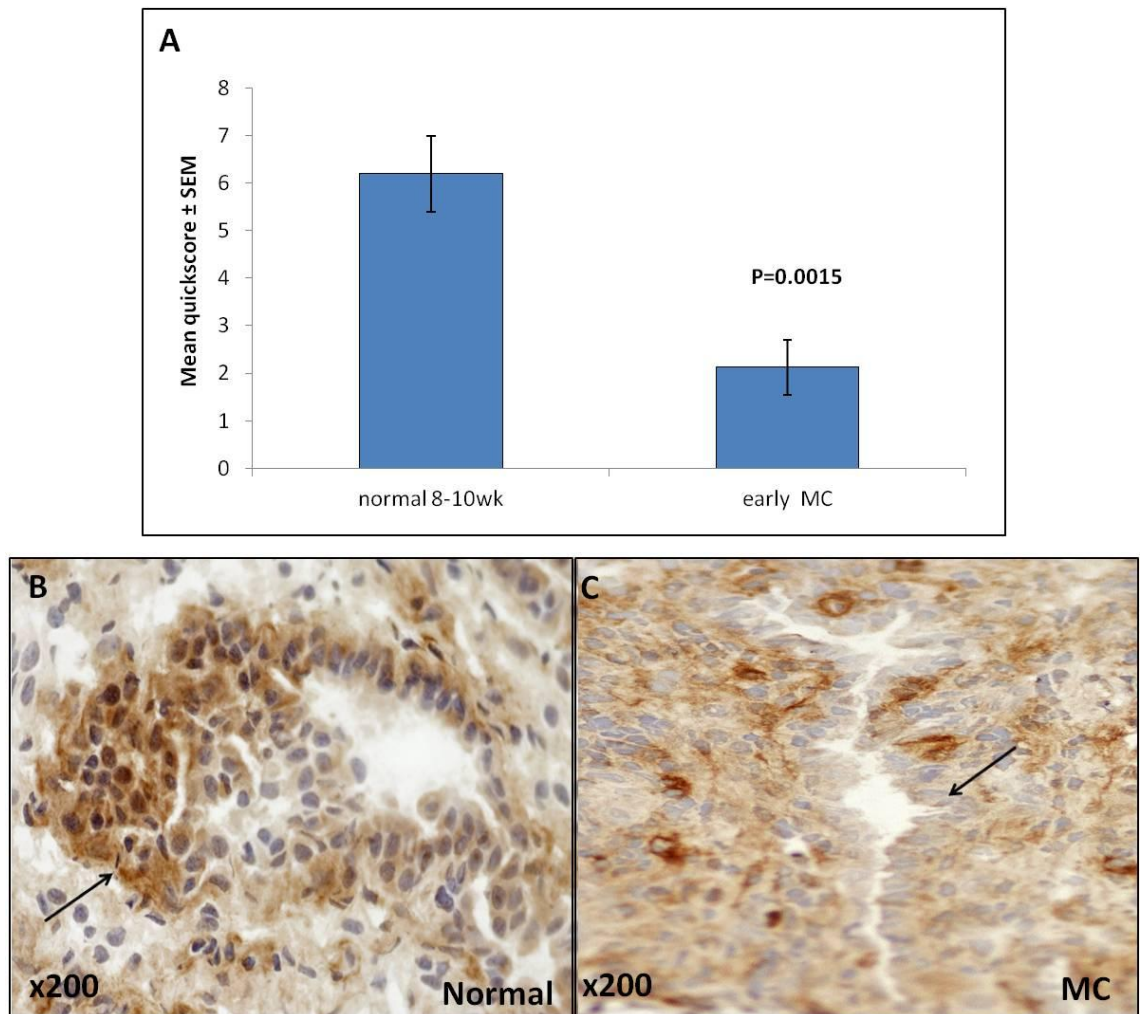
Figure 3.20 Differences in CD13$^+$ immunostaining on glandular epithelium in early normal pregnancy compared with early sporadic miscarriage

Graphical representation of immuno scoring comparison of CD13 immunostaining on glandular epithelium in normal pregnancy (n=5) and SM (n=10) early gestation (A). B: Immunohistochemistry representing CD13 positive staining on glandular epithelium in normal pregnancy and B: represents a loss of staining of CD13 denoted by the arrows. Data are presented as mean ± SEM where statistical significance is denoted by P<0.05. Magnification x200.
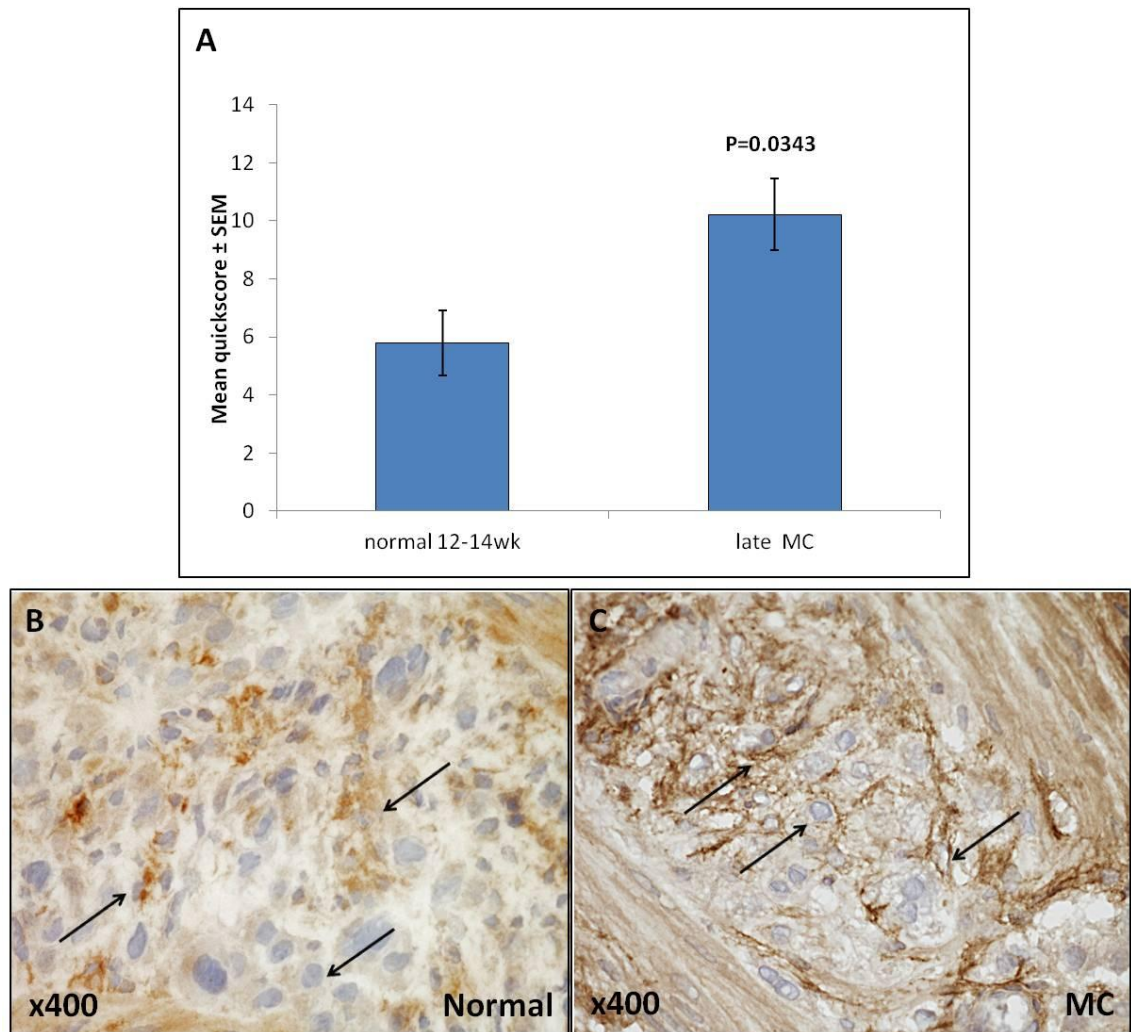
Figure 3.21 Differences in CD13$^+$ immunostaining on interstitial EVT in late normal pregnancy compared with late sporadic miscarriage

Graphical representation of immune scoring comparison of CD13 immunostaining on interstitial EVT in normal pregnancy (n=5) and SM (n=10) late gestation (A). B: Immunohistochemistry representing CD13 positive staining on interstitial EVT in normal pregnancy and C: represents CD13 staining on iEVT in SM. Arrows denote the differences in CD13 immunostaining of iEVT between normal and MC. Data are presented as mean ± SEM where statistical significance is denoted by P<0.05. Magnification x400.
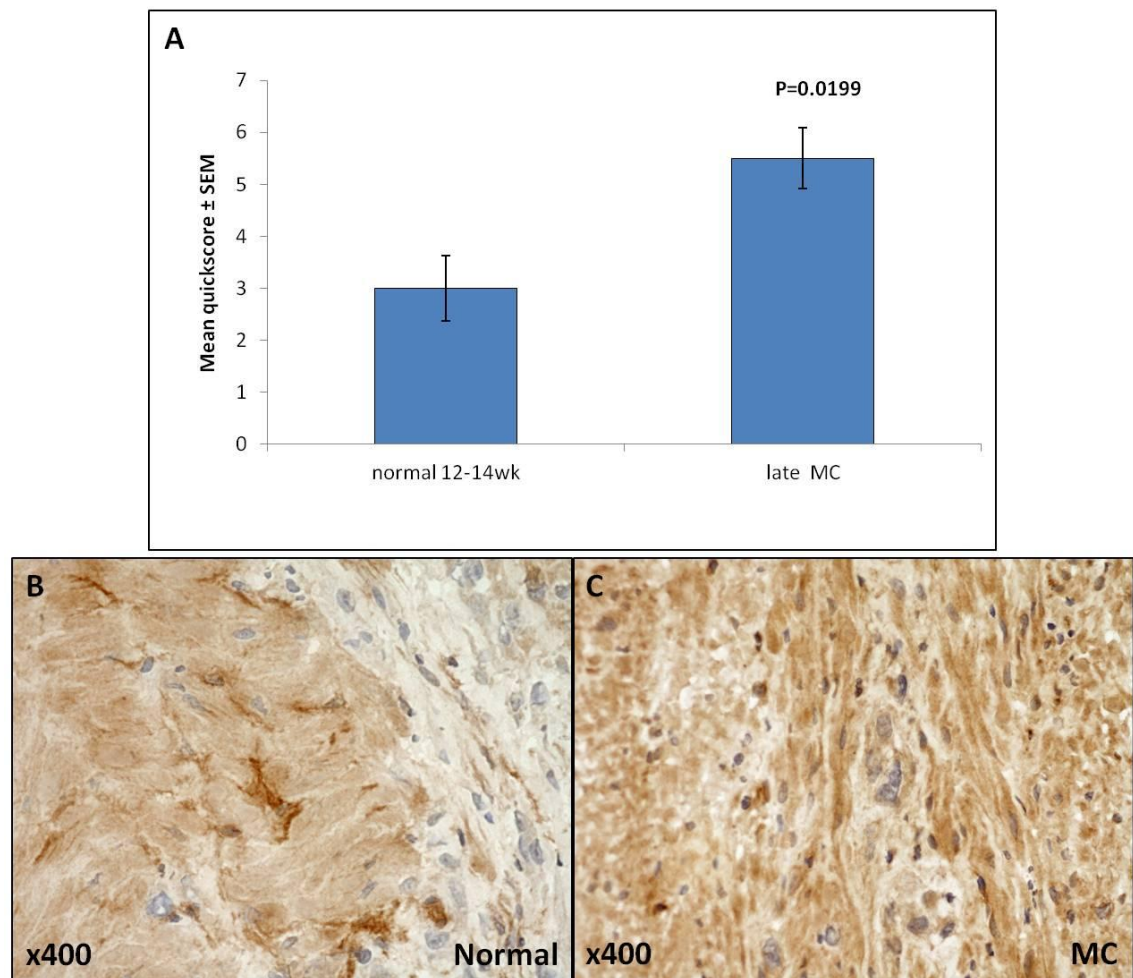
Figure 3.22 Differences in CD13⁺ immunostaining on myometrial stromal cells in late normal pregnancy compared with late sporadic miscarriage
Graphical representation of immunoscoring comparison of CD13 immunostaining on myometrial stromal cells in normal pregnancy (n=5) and SM (n=10) late gestation (A). B: Immunohistochemistry representing CD13 positive staining on myometrial stromal cells in normal pregnancy and C: represents CD13 staining on myometrial stoma in SM. Data are presented as mean ± SEM where statistical significance is denoted by P<0.05. Original magnification x400.
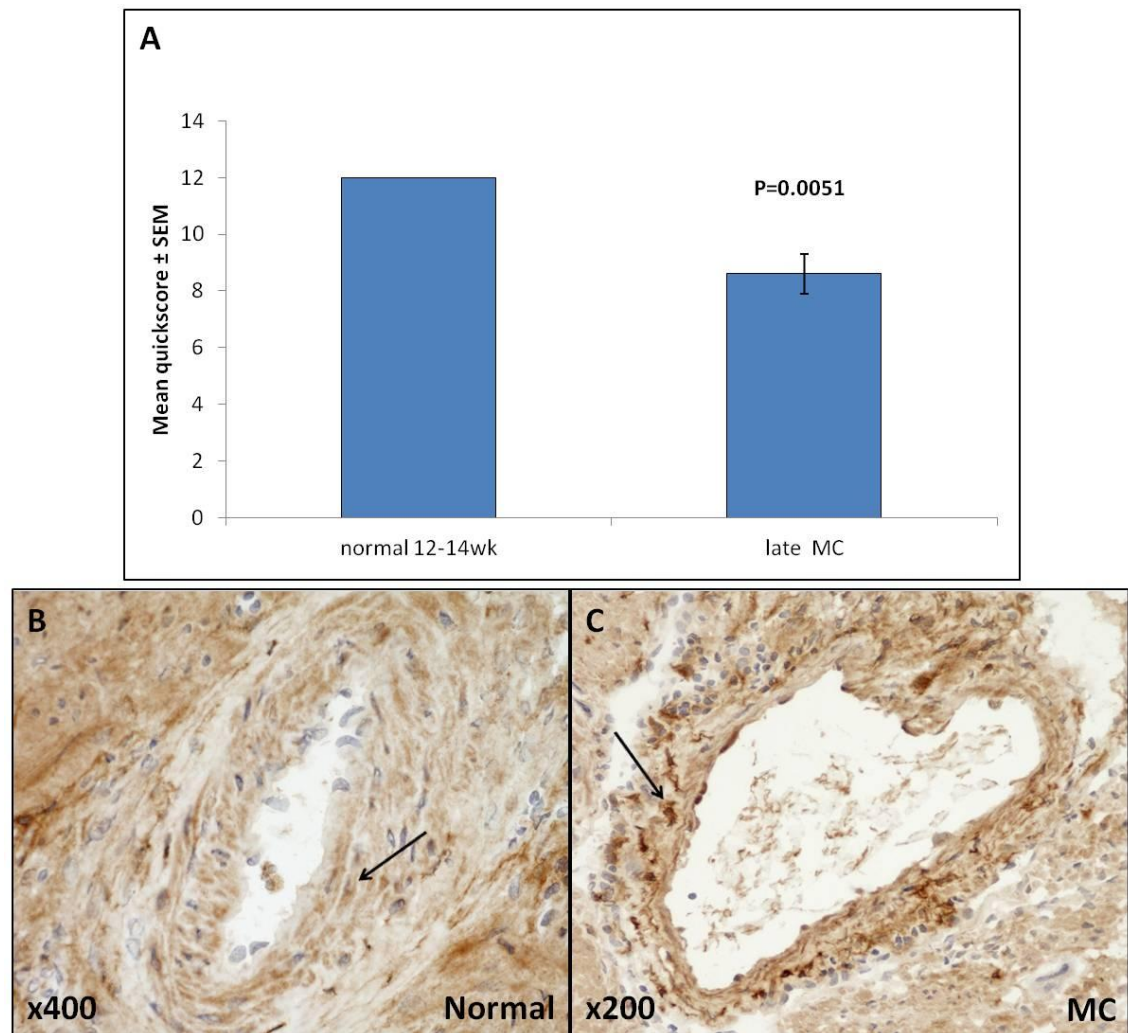
Figure 3.23 Differences in CD13^{+} immunostaining on VSMCs of myometrial spiral arteries in late normal pregnancy compared with late sporadic miscarriage

Graphical representation of immuno scoring comparison of CD13 immunostaining on VSMCs of myometrial SpAs in normal pregnancy (n=5) and SM (n=10) late gestation (A). B: Immunohistochemistry representing CD13 positive staining on myometrial SpA VSMCs in normal pregnancy and C: represents CD13 immunostaining on myometrial SpA VSMCs in SM. Arrows denote the difference in staining intensity of CD13 between normal and MC. Data are presented as mean ± SEM where statistical significance is denoted by P<0.05. Original magnification x200 (B) x400 (C).
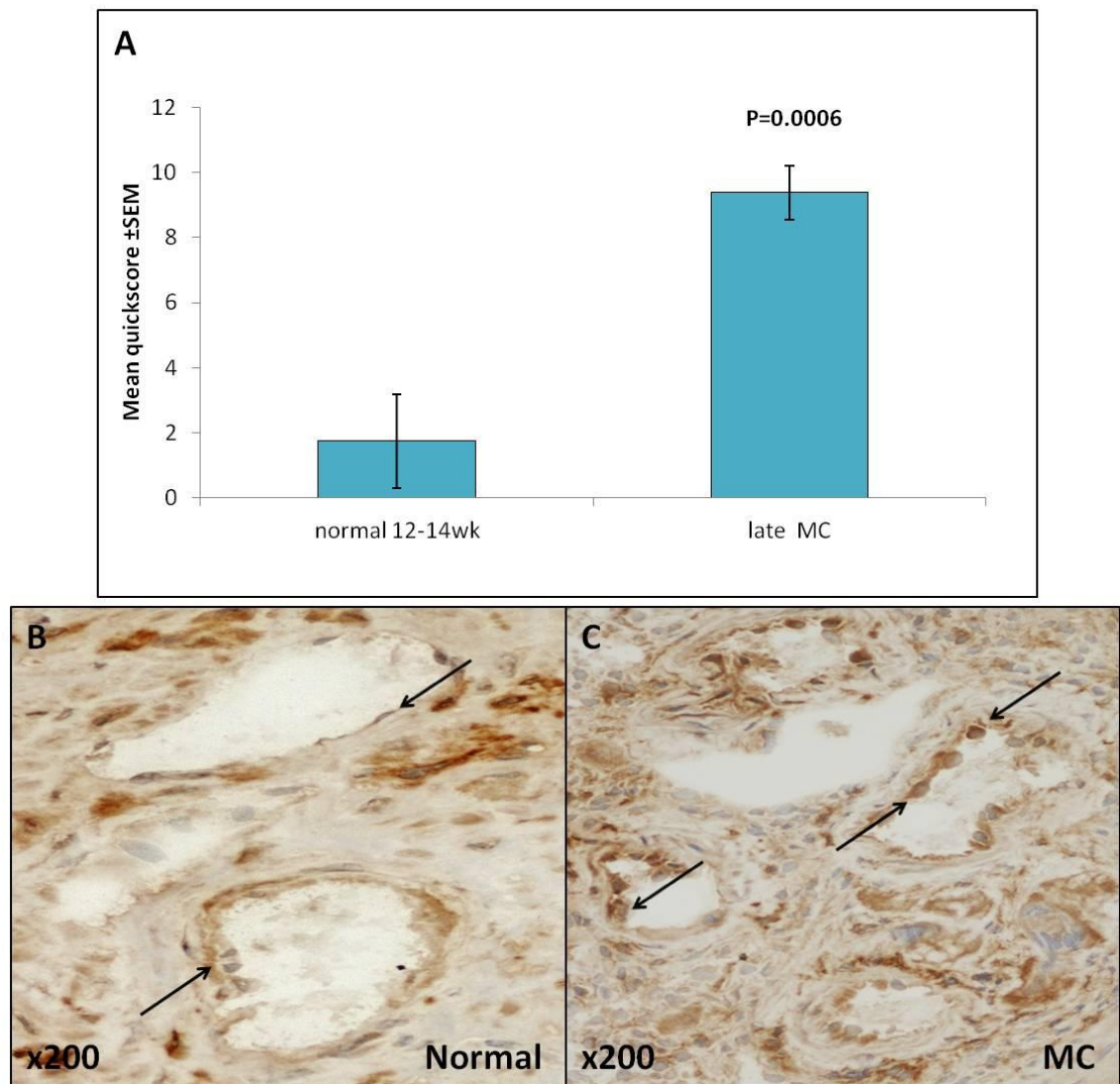
Figure 3.24 Differences in CXCR2 immunostaining on decidual SpA endothelium in late normal pregnancy compared with late sporadic miscarriage
Graphical representation of immunoscoring comparison of CXCR2 immunostaining on decidual SpA endothelium in normal pregnancy (n=5) and SM (n=10) late gestation (A). B: Immunohistochemistry representing CXCR2 positive staining on decidual SpA endothelium in normal pregnancy and C: represents CXCR2 immunostaining on decidual SpA endothelium in SM. Arrows denote the difference in staining intensity of CXCR2 between normal and MC. Data are presented as mean ± SEM where statistical significance is denoted by P<0.05. Original magnification x200.
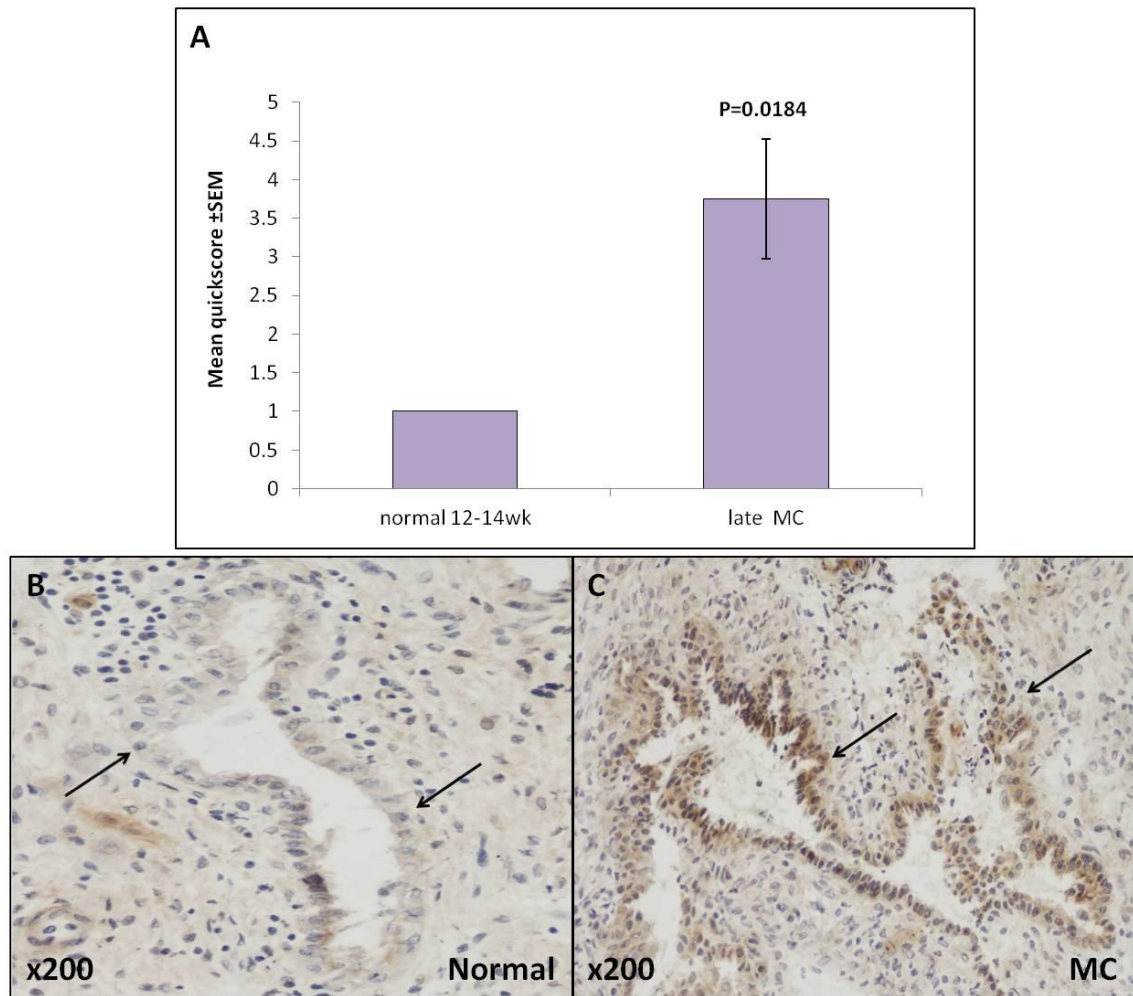
Figure 3.25 Differences in IL-6Rα immunostaining on glandular epithelium in late normal pregnancy compared with late sporadic miscarriage

Graphical representation of immunoscoring comparison of IL-6Rα immunostaining on glandular epithelium in normal pregnancy (n=5) and SM (n=10) late gestation (A). B: Immunohistochemistry representing IL-6Rα positive staining on glandular epithelium within decidua in normal pregnancy and C: represents IL-6Rα immunostaining on glandular epithelium within deciduas in SM. Arrows denote the difference in amount of immunostaining of IL-6Rα between normal and MC. Data are presented as mean ± SEM where statistical significance is denoted by P<0.05. Original magnification x200.
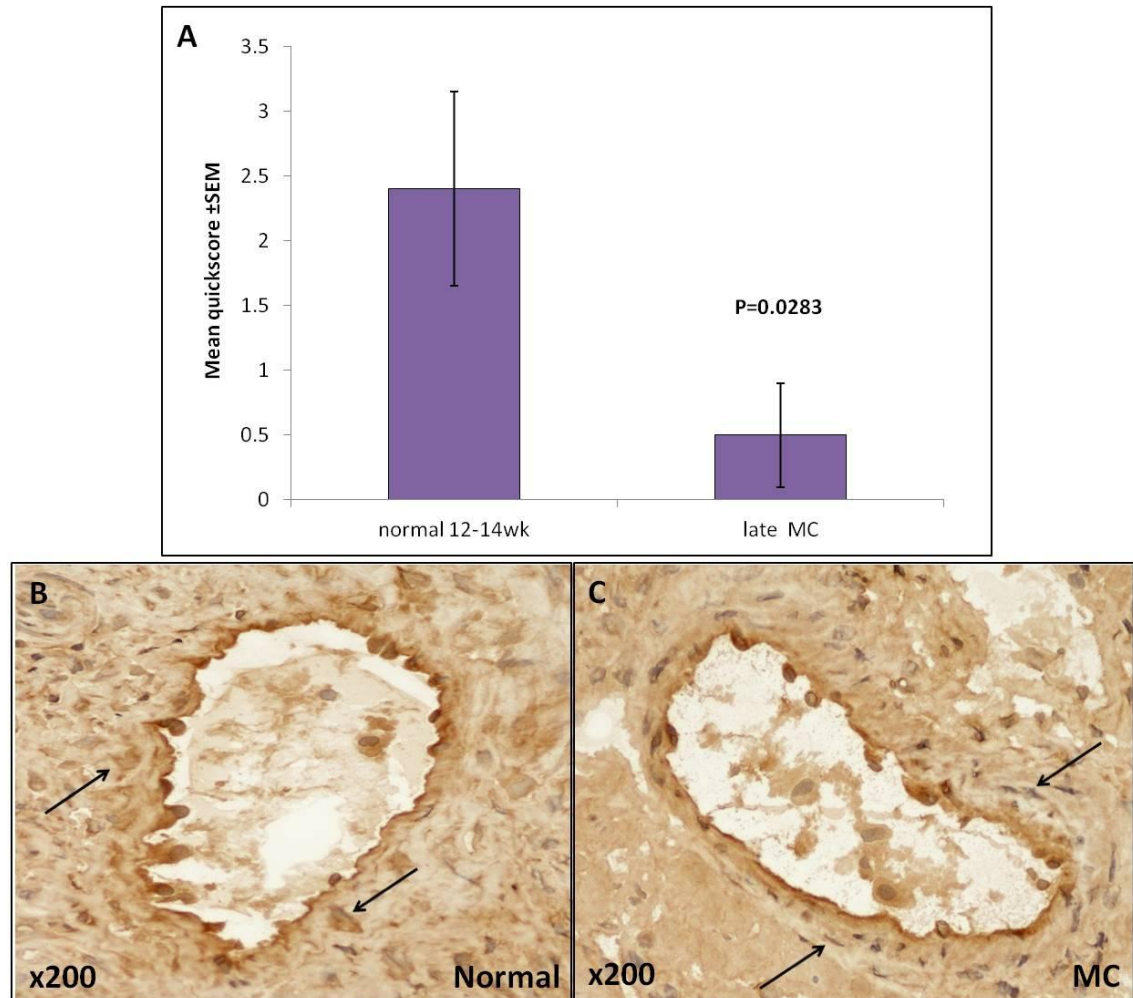
Figure 3.26 Differences in IL-6 immunostaining on VSMCs of myometrial spiral arteries in late normal pregnancy compared with late sporadic miscarriage
Graphical representation of immunoscoring comparison of IL-6 immunostaining on myometrial spiral artery VSMCs in normal pregnancy (n=5) and SM (n=10) in late gestation (A). B: Immunohistochemistry representing IL-6 positive staining on VSMCs of myometrial spiral artery in normal pregnancy and C: represents IL-6 immunostaining on VSMCs of myometrial spiral artery in SM. Arrows denote the difference in amount of immunostaining of IL-6 between normal and MC. Data are presented as mean ± SEM where statistical significance is denoted by P<0.05. Original magnification x200.

3.4.6.3 IL-6 and IL-8 protein production by decidual CD56$^+$ and CD14$^+$ cell culture supernatants isolated from miscarriage tissue; comparison with normal pregnancy

The cytokine protein analysis of decidual cell populations in normal pregnancy revealed that a major producer of IL-6 and IL-8 in early pregnant decidua is CD14$^+$ cells. There were also gestational differences in the levels of IL-8 secreted by decidual CD56$^+$ cells.

Therefore it was decided to compare the levels of IL-6 and IL-8 secreted by CD56$^+$ and CD14$^+$ cells isolated from SM decidual tissue with levels from healthy decidua obtained from pregnancies. Only specimens from ≤12^{+6} weeks gestation were analysed and compared due to difficulties in obtaining late miscarriage tissue (see Chapter 7).

There was a decrease in secretion of IL-6 protein from both CD14$^+$ and CD56$^+$ cells in SM compared with normal early pregnancy (P=0.0469; P=0.0206 respectively; Figure 3.27A). There was also a dramatic reduction in IL-8 secretion from both CD14$^+$ and CD56$^+$ cells in SM compared with normal early pregnancy (CD14$^+$: P<0.0001; CD56$^+$: P=0.003; Figure 3.27B).

Cells isolated from both healthy decidua and SM decidua secreted higher levels of IL-8 than IL-6.
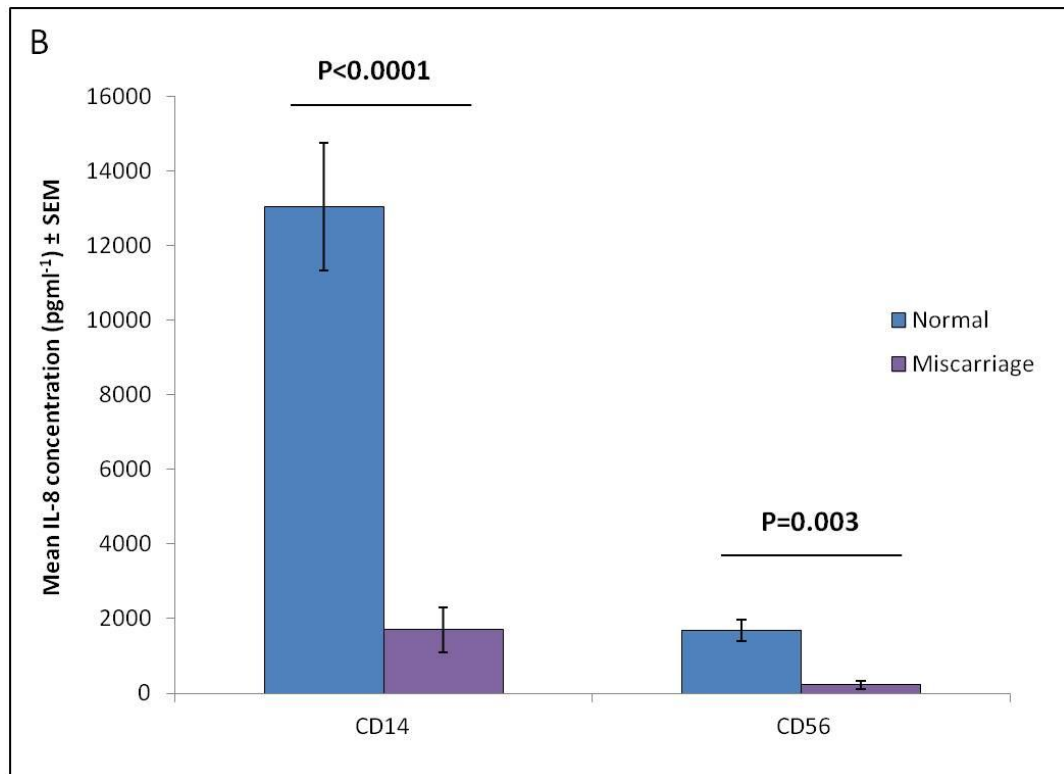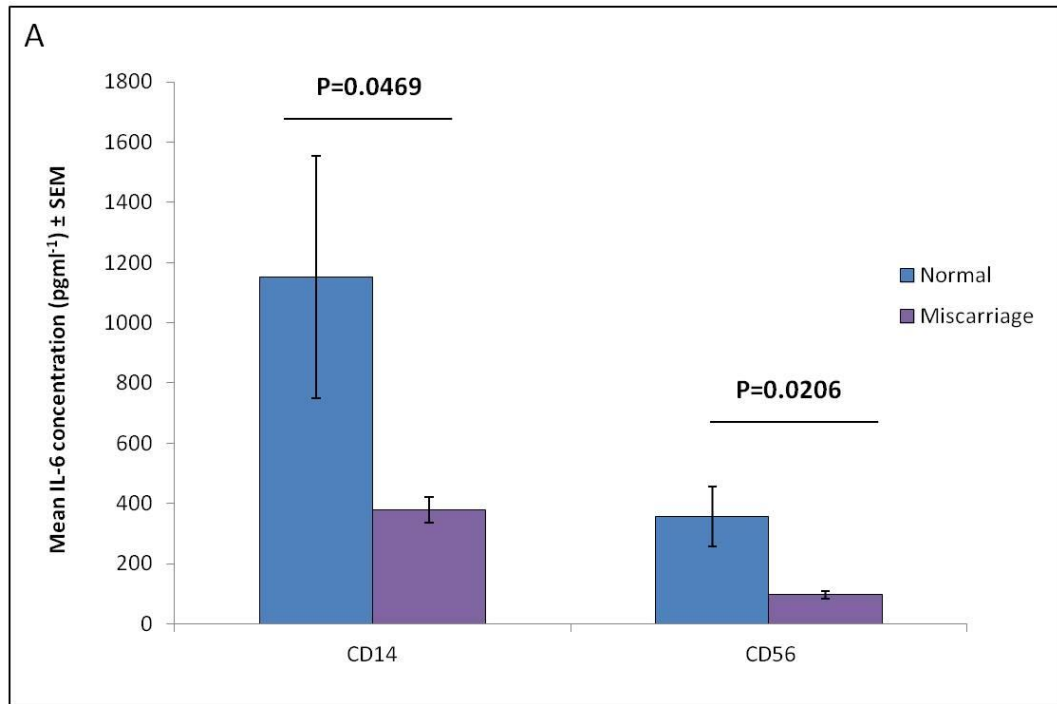
Figure 3.27 IL-6 and IL-8 protein secretion by decidual CD14$^+$ and CD56$^+$ cells in normal pregnancy and sporadic miscarriage
Graphical representation of IL-6 (A) and IL-8 (B) protein levels secreted by decidual CD14$^+$ and CD56$^+$ cells in normal healthy pregnancy (n=10) and early SM (n=8) both ≤12^{+6} weeks gestation. Data are presented as mean ± SEM.

3.5 Discussion

Using an immunohistochemical approach this present study provides evidence that IL-6 and IL-8 are expressed within the placental bed in the first half of normal pregnancy. IL-6 and IL-8 immunostaining was immunolocalised to lots of different cell types within the decidua including CD56$^+$ cells (uterine natural killer cells) and CD8$^+$ T cells, amongst other cell types in early human pregnancy. The immunolocalisation of IL-6 and IL-8 by cells of early decidua was further confirmed by *in vitro* studies demonstrating secretion and mRNA expression of these cytokines by cells of the decidua. Receptors for these cytokines were also immunolocalised to EVT cells as well mRNA detection by spiral arteries.

The immunolocalisation of IL-6, IL-8 and their receptors were altered on many cells of the decidua in miscarriage, in which *in vitro* studies further confirm altered IL-6 and IL-8 secretion in miscarriage by uterine CD56$^+$ and CD14$^+$ cells suggesting an importance for theses cytokines and decidual cells in the development of successful pregnancy.

3.5.1 Expression and production of IL-6 and IL-8 by decidual leucocytes

Decidual cell populations, including stromal cells, macrophages, uterine natural killer cells and T cells, all secreted both IL-6 and IL-8. IL-8 was produced in much higher concentrations than IL-6. We propose that these various cells within the decidua secrete these factors providing a unique immunological balance to facilitate in the processes required for successful pregnancy, beginning with implantation through to aiding invasion of trophoblast cells through maternal tissues and modulating remodelling of spiral arteries. The most abundant source of both IL-6 and IL-8 from the decidual cell types that were investigated was CD14$^+$ cells rather than CD56$^+$ cells as had originally hypothesised. Although macrophages account for 20% of the leucocyte population in the decidua they produce the vast majority of IL-6 and IL-8 in this tissue. Low levels of uNK secreted IL-6 and IL-8 compared with total amounts produced within the decidua suggest that these cells are secreting growth factors other than IL-6 and IL-8 such as IFN-γ, TGFB1 and VEGF (Schiessl et al., 2009) which play important roles in trophoblast invasion and remodelling of spiral arteries. The immunohistochemistry data shows IL-6 positive staining in glandular epithelium in decidua along with strong staining of gp130, although this cell type was not investigated in this study. Murine

luminal epithelial cells in cultures at estrous has been previously reported to be a major source of IL-6 in mouse uterus (Robertson et al., 1992) but this remains to be investigated in humans in pregnancy. IL-8 mRNA has also been detected in human epithelial cells cultured from human endometrium (Arici et al., 1993) but again there remains little investigation into IL-8 expression in pregnancy. However, this does suggest that decidual glandular epithelial cells are another potential source of both intrauterine IL-6 and IL-8. This evidence adds to the fact that cytokines do not work in isolation and lots of cells produce cytokines but a fine complex balance within the milieu is required for cytokines to fulfil their functional capabilities.

Although uNK cells produce a small amount of IL-8 in comparison with other decidual cell types, a dramatic increase in IL-8 levels secrete by uNK cells was observed at 12-14 weeks gestation; which is in concordance with recent data from our laboratory (De Oliveira et al., 2010). Other recent reports have demonstarted IL-8 production within the decidua from uNK cells (Hanna et al., 2006, Vacca et al., 2008) and decidual stromal cells (Engert et al., 2007), although these studies focused on IL-8 production in decidua they did not investigate IL-8 production by these cells isolated from first half of pregnancy and comparisons with different gestational ages. This evidence of very high levels in decidua and increased IL-8 levels at 12-14 weeks gestation suggests a functional importance for this cytokine as IL-8 exists in relatively low levels in normal adult tissues with levels elevating in oxidative stress and inflammation (Vlahopoulos et al., 1999). De Oliveira et al. (2010) have reported a possible involvement in the regulation of EVT invasion but a specific function has yet to be elucidated, although elevated serum IL-8 has been implicated in preeclampsia (Kauma et al., 2002) and is a result of shallow trophoblast invasion, suggesting a main role for IL-8. IL-8 may also play a role in SpA remodelling, a possibility investigated in this study in Chapter 5.

This study has demonstrated that there are differences in the secretion of IL-6 as well as IL-8 with gestational age, suggesting an importance for these cytokines in pregnancy development processes.

3.5.2 IL-6 and IL-6 receptors

IL-6 within the placental bed was immunolocalised to maternal leucocytes and that there were no differences in immunostaining throughout gestation up to 20 weeks.

However, gestational age changes were observed in IL-6 secretion by CD10$^+$ stromal cells and by CD56$^+$ cells along with a total cohort of decidual cells when secretion was quantified with ELISA. These data demonstrate that IL-6 is expressed in the decidua in early pregnancy with expression remaining constant up to 20 weeks gestation suggesting a functional role for this cytokine. IL-6 receptors (IL-6R and gp130) have been localised to EVT cells in situ by immunohistochemical staining providing evidence that IL-6 has the ability to target these cells and elicit a response which may contribute to trophoblast longevity, invasion or cellular events, (see Chapter 4). Expression of gp130 mRNA has previously been demonstrated in decidual (Dimitriadis et al., 2000) and placental tissues from first and third trimester pregnancies (Lee et al., 2011), although IL-6Rα has not been previously investigated; expression of IL-6Rα is required to assess and confirm the signalling capabilities of IL-6 as gp130 is a promiscuous receptor and can bind to many other ligands such as LIF, oncostatin, CNF and IL-11 (Bravo and Heath, 2000). IL-6 receptors were also immunolocalised to SpA with IL-6Rα staining strongly on myometrial SpA and expression being verified by RT-PCR which has yet to be demonstrated in any other study. This study provides evidence that IL-6 has the ability to bind to VSMC of the decidual SpA and therefore, may play a role in SpA transformation events making it an important mediator for successful pregnancy development (see Chapter 5 for further study).

3.5.3 IL-8 and IL-8 receptors

Previously published data by De Oliveira et al. (2010) demonstrated that intrauterine IL-8 is expressed by CD56$^+$ and CD8$^+$ T cells in the placental bed in human pregnancy with gestational age differences. This study further verifies this showing that IL-8 is immunolocalised to uNK cells, suggesting that these cells are a source of intrauterine IL-8. Localisation of IL-8 receptors; CXCR1, CXCR2 and DARC has not previously been investigated in human pregnant decidua, although CXCR1 and CXCR2 have been indentified in non-pregnant human endometrium (Mulayim et al., 2003) and in endometrial carcinoma (Ewington et al., 2012). IL-8 signals via both of the transmembrane receptors but each receptor system has different functional responses (Rosenkilde and Schwartz, 2004). Signalling through CXCR2 is suggested to mediate angiogenic responses (Addison et al., 2000) and this is reflected in mRNA expression on

decidual SpA observed from LCM in this study. Using immunohistochemistry data IL-8 receptors were also localised to EVT, coinciding with reports that IL-8 stimulates EVT invasion (De Oliveira et al., 2010).

CD13 (aminopeptidase N) has been reported to be expressed by decidual stromal cells and that its expression by these cells is regulated by oestrogen (Seli et al., 2001). DARC, the non-signalling decoy receptor for IL-8, was observed by immunohistochemistry in the placental bed of normal pregnancy at 8-10 weeks and 12-14 weeks gestation (n=3 each gestational age) and expression did not change between the gestational ages. Intense DARC staining was observed on EVT; especially on endovascular EVT with expression by endothelial cells in the lumen of SpAs and by decidual leucocyte cells where staining was very strong and intense. This intense staining was observed in areas where CD56$^+$, CD14$^+$ and CD3$^+$ cells were in proximity although this was not quantified. However, the immunolocalisation of DARC suggests an active role in the placental bed for this receptor and IL-8. DARC immunostaining was not compared in MC specimens due to the technical specificity of the DARC antibody used in this study. An antibody for use on frozen tissue sections does not exist and is only the one used in this study was only effective on FFPE sections, and therefore could not be used on the stored frozen MC sections that were used for this study. Immunostaining was attempted on frozen sections but with poor results. DARC immunostaining in normal pregnancy suggests that this receptor may regulate the amount of IL-8 available within the placental bed and decidua in early pregnancy, preventing local levels becoming dangerously high which could in turn cause pregnancy complications, as higher levels of IL-8 have been reported to increase cellular apoptosis, neutrophil activation and endothelial cell damage in pre-eclamptic women, although the source of IL-8 was from maternal serum (Laskowska et al., 2007). Localisation of DARC to EVT and SpAs suggest that IL-8 may elicit effects on these cells by regulating EVT invasion and playing a role in the remodelling of spiral arteries; expression of DARC on these cells may prevent IL-8 causing aberrant and excessive invasion and SpA remodelling, and thereby preventing the cause of pregnancy complications.

3.5.4 Receptor expression in miscarriage

Few reports suggest that IL-6 and IL-8 have an involvement in miscarriage and receptor expression of these cytokines has not yet been investigated in miscarriage compared with normal pregnancy.

We hypothesised that IL-8 levels within the decidua would increase in miscarriage; this would result in altered receptor expression on target cells. Euploid and aneuploid samples from early miscarriage ($\leq 12^{+6}$ weeks GA) (Ball et al., 2006b) and euploid and aneuploid samples from late miscarriage (≥ 13 weeks GA) (Ball et al., 2006a) were assessed and analysis revealed there were no differences in immunostaining, so therefore decided to combine both euploid and aneuploid samples together as discussed in section 3.4.4). CD13$^+$ immunostaining was stronger and more intense on interstitial EVT and myometrial stroma in miscarriage ≥ 13 weeks gestational age compared with interstitial EVT from normal pregnancy from 12-14 weeks GA. Conflicting reports exist on the role of CD13 (aminopeptidase N) and its association with IL-8. Reports from 1995 (Kanayama et al., 1995) state that aminopeptidase N was found to inactivate IL-8 by degrading it; therefore indicating that IL-8 is a substrate for this metalloproteinase. Seli et al. (2001) further concluded that CD13 is a degrading enzyme of IL-8 and reported expression in human endometrium, considered to be involved in regulating the bioavailability of IL-8. However, in contrast Wulfaenger et al. (2008) reported expression of CD13 on human embryonic kidney cells downregulated CXCR4 expression and inhibited CXCL12 stimulated migration but did not affect the activity of CXCL8 (IL-8). These authors argued that the mononclonal antibody WM15 (that binds with CD13 and used in the Kanayama study) causes varied results; WM15 stimulates calcium secretion which leads to phosphorylation of proteins involved in the cell signalling cascade which up further upregulates the expression of IL-8 mRNA in monocytic cells, so therefore claimed that CD13 had no effect on inactivating IL-8 and was a response from using WM15. The increased immunostaining for CD13 observed in miscarriage decidual specimens could be a possible explanation for the reduction in IL-8. The increase of cell surface CD13 seen on EVT could result in increased degradation of uterine IL-8 and this reduction could lead to insufficient invasion of trophoblast cells, being a causative factor in spontaneous abortion.

CXCR2 immunostaining was also increased on the endothelial cells of decidual spiral arteries in MC indicating that the angiogenic properties elicited by IL-8 may be altered in MC and contribute to aberrant SpA remodelling.

Expression of IL-6R was increased in the glandular epithelium of MC compared with normal pregnancy but gp130 was not altered. A decrease in IL-6 immunostaining was observed in myometrial spiral arteries in late MC compared with normal pregnancy suggesting that IL-6 may have some involvement in remodelling of deeper SpAs, thereby affecting intervillous blood flow which will further cause oxidative stress and may be a causative factor in pathology of MC, coinciding with reports of decreased myometrial SpA remodelling in relation to late miscarriage (Ball et al., 2006a).

A factor to be consider here is that the specimens obtained from normal pregnancy were collected throughout the duration of this study, whereas the decidua from miscarriage specimens had been collected years before the normal decidua was obtained. Therefore, the significant differences observed could have been a consequence of the differing timepoints of tissue collection, although the miscarriage specimens were stored at -20°C and tissue morphology appeared not to have altered when subjected to an H&E stain. Immunostaining of all tissue was performed at the same time also to prevent day to day variation in staining. However, it is likely that receptor expression would be altered in miscarriage as the data in this study provides evidence that the production of intrauterine cytokine levels is reduced compared to levels in healthy pregnancies.

3.5.5 Production of IL-6 and IL-8 in miscarriage

There are very few reports of IL-6 and IL-8 in miscarriage with the results varying significantly. Despite the varying results there is a correlation between altered expression and miscarriage. The results from this study support the hypothesis that altered cytokine expression is associated with miscarriage but the hypothesis stated that the cytokine levels would be increased in MC rather than being decreased.

 IL-6 and IL-8 production by $CD56^+$ and $CD14^+$ cells isolated from decidua from miscarriage was measured and compared with levels from normal pregnant decidua at comparable GA and the results were compelling. These decidual cell populations

produced significantly less IL-6 and IL-8 in MC compared with normal pregnancy. These data contradict findings from Madhappan et al. (2003) who reported higher levels of uteroplacental IL-8 in products of conception from women suffering recurrent MC compared with normal pregnancy controls. This contradicting data firstly can be explained by the source of IL-8, although the source has only been investigated and compared using two cell types, the cell types used were specific and both cell populations have been linked with successful pregnancy development, with macrophages being one of the main producers of IL-8 within the decidua and CD56^{+} cells being the most abundant leucocyte, making this evidence more feasible. Subject numbers in the study by Madhappan et al. (2003) may also account for the conflicting results; sample numbers were very low and the number of controls did not match that of the MC specimen numbers. In addition, the nature of the MC also needs to be taken into account. In the present study only specimens from women with sporadic miscarriages were examined, whereas Madhappan et al. (2003) investigated IL-8 levels in women suffering recurrent miscarriages (RM), a specific group of miscarriages where an immune cause is suggested which may differ from sporadic miscarriage and this is where the discrepancies may lie.

There are also conflicting reports of IL-6 in MC. In agreement with the present study Jasper et al. (2007) reported a significant reduction in endometrial IL-6 mRNA expression in women suffering from recurrent MC, although the source of IL-6 was not isolated to a specific cell type in pregnancy. Earlier studies in mouse disagree with both Jasper et al. (2007) and data from this study: Zenclussen et al. (2003) reported a significant increase in IL-6 at the fetal maternal interface, with increased expression by trophoblast cells observed by immunohistochemistry, increased production by monocyte cells of decidua and upregulated IL-6mRNA expression in decidual and placental tissue at day 18 of gestation which was associated with fetal loss. Also in agreement with this data is a report demonstrating increased levels of both IL-8 and IL-6 in women who had suffered second trimester abortions, although the levels of cytokines measured were taken from plasma (Galazios et al., 2011). Elevated levels of plasma IL-6 have also been demonstrated in women with euploid miscarriage (Calleja-Agius et al., 2012). The discrepancies between studies may be due to the difference in species, gestational time points and tissues investigated.

Since decidual CD14^{+} cells were the most abundant source of both IL-6 and IL-8 in early pregnancy and there was a dramatic reduction of the production/secretion of these cytokines in miscarriage subjects, this cell type appears likely to be an important mediator of normal pregnancy development as well as the uterine natural killer cells, which have been the focus of majority of studies of decidual leucocytes in early pregnancy to date. Further studies into mRNA expression in decidual leucocytes in particular uterine macrophages and functional studies of these cells are required to gain further insight into the mechanism of action of IL-6 and IL-8 in miscarriage and how these cytokines contribute to the pathology of sporadic MC. As stated before, the local cytokine milieu in uterus in early pregnancy is complex and other factors combined with these cytokines are likely to play a role, highlighting the challenge of unravelling the complexity of pregnancy complications. IL-8 has been reported to increase trophoblast invasion *in vitro* (De Oliveira et al., 2010) suggesting that it plays a role in the regulation of invasion. Reduced decidual IL-8 production could therefore result in disruption in this regulation and by reducing trophoblast invasion and this could lead to inappropriate increased SpA remodelling altering intervillous blood flow which could contribute to MC. Shallow trophoblast invasion and deficient SpA remodelling have been associated with numerous pregnancy complications (reviewed in chapter 1 and chapter 4). IL-6 could work in a coordinated manner with IL-8 to lead to pregnancy loss. Another possible function of these cytokines is that they contribute to the recruitment of other leucocytes to sites of SpA remodelling. However, in a large immunohistochemical study, Ball et al. (2006b) found no evidence of abnormal SpA remodelling and trophoblast invasion in early sporadic MC, indicating that these processes do not have an important role in the pathogenesis of early miscarriage. Carp (2004) reviewed the importance of cytokines and their involvement in recurrent miscarriage, although little evidence exists in humans. The studies that have been explored, immune cells and cytokine production in human RM do suggest differences in expression compared with healthy individuals; increased CD56^{+} cells in RM decidua, decreased IL-6 production in endometrium and a shift in cytokine production by peripheral blood monocytes are all results observed in human RM, although much of the evidence is contradictory and variations in data may be indicative of sample type, cells investigated and timing of miscarriage and pregnancy. However, the evidence

suggests that abnormal expression of immune cells and cytokines contribute to the disease but the underlying mechanisms remain unclear. The delicate balance of the cytokine milieu in pregnancy and disruptions may lead to altered immune surveillance/modulation resulting in increased apoptosis of paternal trophoblast cells through HLA interactions with uNK cells and macrophages which may be a possible explanation for pregnancy loss and is an area which requires further exploration.

3.5.6 Overall summary

This study demonstrates that the decidua produces significant amounts of IL-8 and IL-6 and that these cytokines are capable of signalling with target cells involved in processes of pregnancy. Immune cells are a source of both cytokines but uterine macrophages are a major source, suggesting that these cells contribute to successful pregnancy. There were dramatic reductions of cytokine production in miscarriage inferring that these cytokines contribute to successful pregnancy and therefore an importance of IL-6 and IL-8 in pregnancy development. However, this reduction does not determine whether the cytokines are dysregulated or simply not being produced; these are areas which will require further exploration. If these protein studies of miscarriage are confirmed in other studies, with increased knowledge on function and the mechanisms involved could be an area for targeted studies that will eventually translate this into clinical intervention.

Chapter 4

The effect of interleukin-6 on trophoblast invasion and function

in early human pregnancy

4. The effect of IL-6 on trophoblast invasion and function in early human pregnancy

4.1 Introduction

Trophoblast differentiation and importance of EVT invasion for successful pregnancy has been reviewed in chapter 1. Both interstitial and endovascular EVT invasion is tightly coordinated in a spatial and temporal manner and disruptions in this control are associated with various pregnancy complications including early and late miscarriage (Khong et al., 1987, Hustin et al., 1990, Ball et al., 2006a, Ball et al., 2006b), pre-eclampsia (Pijnenborg et al., 1991, Sibai et al., 2005) and fetal growth restriction (Khong et al., 1986). In contrast excessive deep EVT invasion is seen in placenta accreta (Norwitz, 2006, Rosen, 2008). The association of aberrant invasion with these diverse pregnancy disorders highlights the importance of controlled trophoblast invasion for successful pregnancy.

Despite the importance of this regulation, factors that control EVT invasion are poorly understood, although it is likely that decidual factors play an important role (Fitzgerald et al., 2008). Evidence is accumulating that the factors from decidua including cytokines, growth factors and oxygen tension all influence EVT invasion but the mechanisms that underpin regulation of the events of invasion remains to be elucidated. Cells within the decidualised endometrium of the uterus in pregnancy are believed to be major producers of growth factors and cytokines that are likely to be involved in control of EVT invasion (Lala and Chakraborty, 2003, Kharfi et al., 2003, Bischof and Irminger-Finger, 2005). Signals from trophoblast cells and maternal components in the decidua have been characterised recently (Dimitriadis et al., 2010) and it has been postulated that maternal immune cells within the decidua such as uNK cells, decidual macrophages and T-lymphocytes secrete many factors that may contribute in the regulation of EVT invasion and decidual stromal cells are also likely to have a role (Lash et al., 2011).

In the first half of pregnancy uterine natural killer cells have are the most abundant decidual leucocyte population and are in close contact with EVT at the maternal-fetal interface. Previous published data from our group have demonstrated that IL-8 stimulates EVT invasion *in vitro* (De Oliveira et al., 2010). Decidual cells were found to

secrete high levels of IL-8 with uNK cells as a major producer. In addition both CXCR1 and CXCR2 were localised to EVT *in situ* (see Chapter 3). These results are consistent with previous reports showing that soluble products of isolated uNK cells stimulate EVT invasion in a model using primary uNK cells and activated isolated EVT from early pregnancy (Hanna et al., 2006, Lash et al., 2010b, De Oliveira et al., 2010). This effect was partly dependent on IL-8, as an IL-8 neutralising antibody partially inhibited uNK stimulation of EVT invasion.

This present study has demonstrated that uNK cells, amongst many other cell types in the decidua, produce both IL-8 and IL-6 in early pregnancy (Chapter 3). IL-8 has been reported to stimulate EVT invasion *in vitro* (Hanna et al., 2006, De Oliveira et al., 2010), but the effect of IL-6 on trophoblast invasion has been sparsely investigated.

IL-6 has the potential to be an important regulator of EVT invasion since there is extensive literature demonstrating that this cytokine is able to increase the invasiveness of cancer cells, thus contributing to a variety of malignancies (Nishino et al., 1998, Kanazawa et al., 2007, Walter et al., 2009). Despite documentation of the presence of IL-6 in the human endometrium and expression by trophoblast themselves, little is known about the functional effects of IL-6 on trophoblast, precise sites of IL-6 production and mechanisms of IL-6 induced signalling in the trophoblast. Chapter 3 includes data of IL-6 localisation and production by maternal cells in pregnant decidua, along with evidence of IL-6 receptor expression (IL-6Rα and gp130) on decidual cells, but expression by EVT was not investigated. IL-6 signalling pathways involve STAT-3 and MAPK signalling pathways (reviewed in chapter 1.4.3) and evidence suggests that trophoblast cells mediate through the same pathways, suggesting a role for IL-6 in EVT signalling; activation of STATs through receptor mediated signalling is known to up-regulate genes which are involved in proliferation and cell differentiation (Corvinus et al., 2003, Fitzgerald et al., 2005) and MAPKs play an important role in the cellular processes of migration and invasion in human trophoblast cells (Pollheimer and Knofler, 2005). STAT-3 knockout mice studies have also demonstrated embryonic lethality (Takeda et al., 1997).

IL-6 is widely expressed in endometrium at implantation and knockout IL-6^{-0}/IL-6^{-0} mouse studies provide evidence that IL-6 plays an important role in implantation and

early placental development since the IL-6 KO mice showed a 48% reduction in implantation sites that has been linked to decreased fertility (Robertson, 2000).

Altered levels of IL-6 have also been implicated in human pregnancy disorders including pre-eclampsia and miscarriage. Increased levels of placental IL-6 have been detected in women suffering pre-eclampsia (Zhao et al., 2008) and Greer et al. (1994) reported an increase in IL-6 serum levels in pre-eclamptic women compared with normal pregnant women. Sera from women suffering recurrent miscarriage have been reported to have increased levels of soluble IL-6 receptor and IL-6 compared with controls (Arruvito et al., 2009). In contrast, inadequate IL-6 mRNA production has been reported in the endometrium of women suffering recurrent miscarriage (Jasper et al., 2007). As well as pregnancy pathology, IL-6 has also been implicated in infertility; recent data have demonstrated significantly increased serum concentrations of IL-6 in infertile compared with fertile women (Demir et al., 2009). These data suggest a possible role of IL-6 in the processes involved in early pregnancy.

A recent study by Jovanovic et al (Jovanovic and Vicovac, 2009) reported that IL-6 increased the migration and invasive potential of the EVT immortalised cell line HTR-8/SVneo *in vitro* due to the up-regulation of trophoblast integrins α_1, α_5 and β_5 that bind to components of the extracellular matrix. In addition, Dubinsky et al. (2010) reported that inhibition of endogenous IL-6 inhibited the migration and invasion of JEG-3 cells, indicating that IL-6 has role in the control of trophoblast invasion.

Our group has already reported stimulation of EVT by IL-8 and that EVT cells expressed both receptors for IL-8. In view of abundance of IL-6 in decidua in early pregnancy and reports in tumours and trophoblast cell lines, the aim of this part of the study was to investigate effect of IL-6 on invasion by primary EVT cells and the HTR-8/SVneo EVT cell line.

4.2 Aims and Hypotheses

4.2.1 Hypotheses

1) IL-6 receptors are expressed by isolated primary EVT cells

2) IL-6 stimulates EVT invasion and increases their invasive capacity within the placental bed

3) IL-6 plays a role in the stimulation of trophoblast cellular signalling proteins including STAT3 and MAPK.

4) IL-6 increases EVT secretion of growth factors involved in stimulating EVT cell invasion.

4.2.2 Aims

In order to test the hypothesis, the specific aims were

1) To localise cytokine receptor expression by trophoblast cells using immunocytochemistry and Western blotting

2) To measure the effect of varying concentrations of IL-6 on invasiveness by invasive potential of 1° EVT and HTR-8/SVneo cell line using a Matrigel invasion assay

3) Measure invasive potential of EVT using first trimester placental chorionic villous explants with varying concentrations of IL-6

4) To further investigate the effect of IL-6 on EVT by measuring stimulation of kinase signalling proteins, growth factors and cytokine secretion by EVT after IL-6 treatment using a kinase-phospho array and FASTQuant multiplex array

5) To verify the FASTQuant protein data by measurement of mRNA expression using Real Time RT-PCR

4.3 Experimental design

4.3.1 Immunocytochemistry

Primary EVT cells were isolated from first trimester placenta as previously described (section 2.3.5). The HTR-8/SVneo EVT cell line was cultured as described previously (section 2.4.1). Cell smears were prepared for immunocytochemistry as described in

Chapter 2 (section 2.3.3) and IL-6 receptor protein expression was investigated by immunostaining the cell smears with the antibodies shown in Table 4.1. The immunostaining was repeated on 3 separate occasions and all smears were subjected to LP34 and HLA-G staining as positive controls to identify EVT. Negative controls were assessed by omitting primary antibody by replacing with TBS buffer pH 6.5.

Table 4.1 Primary antibodies used for immunocytochemistry (trophoblast cell smears)
Primary antibodies used for immunocytochemistry

Antibody	Method	Species	Dilution	Incubation	Pre-treatment
LP34[1]	Elite Kit[4,5]	Mouse	1:80	RT, 60 min	No pre-treatment
HLA-G[1]	Elite Kit[4,5]	Mouse	1:200	RT, 60 min	No pre-treatment
gp130[2]	Elite Kit[4,5]	Mouse	1:50	RT, 60 min	No pre-treatment
IL-6Rα[3]	Elite Kit[4,5]	Mouse	1:50	RT, 30 min	No pre-treatment

Source: [1]Leica Biosystems, Newcastle LTD UK; [2]Santa Cruz Biochemicals, California, USA; [3]Millipore, CA, USA; [4]Vector Laboratories, Peterborough, UK; [5]ABC ELITE Kit- avidin-biotin peroxidase method (mouse monoclonal)

4.3.2 Western blotting of IL-6 receptor proteins and signalling proteins

IL-6 receptor expression was also investigated by Western blotting. Primary EVT cell lysates and cell lysates from HTR-8/SVneo cells were prepared (section 2.4; n=2). 20µg protein (determined with a BioRad DC protein assay- section 2.11.2) was subjected to SDS polyacrylamide gel electrophoresis using and resolved proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) and then blocked overnight in 5% milk blocking solution (0.05% TBS-Tween20 (TBS-T) and dried non-fat milk) at 4°C (section 2.11.3).

The membranes were probed separately with IL-6Rα antibody and gp130 polyclonal antibody (both from Santa Cruz, CA USA; Table 4.2). Protein signal for gp130 receptor was not detected in either HTR-8/SVneo cell or primary EVT cell lysates at the correct molecular weight (130kDa) using the specific antibody even using a positive control. This experiment was repeated on several occasions and different antibody

concentrations were used but the result remained negative. Western blotting of IL-6Rα was, however successful.

To assess the effect of IL-6 on STAT and MAPK signalling pathways in EVT cells, isolated primary EVT from pregnancies at 8-14 weeks gestation and HTR-8/SVneo cells were incubated with 10ngml^{-1} of IL-6 for 10 minutes at 37°C in 5% CO_2. Cells that were not exposed to IL-6 were used as controls. Following incubation the cells were lysed in lysis buffer-6 (included in the kinase profile kit; R&D systems, Inc. Minneapolis, USA). 20µg of each sample was subjected to SDS polyacrylamide gel electrophoresis and resolved proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Membranes were probed separately with anti-STAT-3, anti-p-STAT-3 (SantaCruz, CA, USA) antibodies and anti-MAPK1/2 and anti-p-MAPK1/2 (Millipore Corp. Temecula, CA, USA) antibodies (Table 4.2). All antibodies were subsequently developed using chemiluminescence (Thermo Scientific, Rockford, USA) according to manufacturer's instructions and recorded by X-ray Kodak Scientific Imaging film (Sigma Chemical Co.).

Table 4.2 Western blotting conditions and antibodies used

1° Ab	Dilution factor	Incubation solution	Incubation time	Temperature	2° Ab	Dilution factor	Incubation time	Temperature
IL-6Rα[1]	1:100	5% milk TBS-T	1 hour	RT	goat anti-rabbit[3]	1:3000	1 hour	RT
gp130[1]	1:100	5% milk TBS-T	1 hour	RT	goat anti-rabbit[3]	1:3000	1 hour	RT
STAT-3[1]	1:200	5% milk TBS-T	2 hours	RT	goat anti-mouse[3]	1:3000	2 hours	RT
p-STAT-3[1]	1:200	5% milk TBS-T	2 hours	RT	goat anti-mouse[3]	1:3000	2 hours	RT
ERK1/2[2]	1:2500	3% milk PBS-T	O/N	4°C	goat anti-rabbit[3]	1:5000	1.5 hours	RT
p-ERK1/2[2]	1:1000	3% milk TBS-T	O/N	4°C	goat anti-rabbit[3]	1:5000	1.5 hours	RT
Gβ[1]	1:1000	5% milk TBS-T	1 hour	RT	goat anti-rabbit[3]	1:1000	2 hrs	RT

[1]SantaCruz, CA, USA; [2]Millipore, CA, USA; [3]Sigma. RT= Room Temperature; O/N= Overnight; T= 0.05% Tween20

4.3.2 The use of placental villous explants rather than primary EVT cells in invasion assays

Initially in this experiment isolated EVT cells from first trimester placenta (section 2.3.5) were used in the invasion assays with aim of comparing results with those obtained using the trophoblast cell line HTR-8/SVneo cells. Although isolation of EVT

cells from first trimester placenta was successful and previous experiments in the laboratory had observed EVT invasion using the Matrigel invasion assay (section 2.5), invasion of these cells through the 8.0μm pore filters was not observed in these studies. It was necessary to see a minimum of 30 invaded EVT cells after the culture period but using isolated EVT only very small numbers if any had invaded through the growth factor reduced Matrigel onto the underside of the pore membrane filters both in controls and all other culture treatments. In addition insufficient cells were isolated to perform the number of wells required examine different IL-6 concentrations. We investigated different time points of the invasion experiment (12h, 24h, 48h and 72h) but failed to detect adequate invasion. The invasion assays using 1° EVT were repeated on 5 separate occasions and at the same time as those performed with HTR-8/SVneo cells (n=3). After isolation of EVT, cell smears were made and characterised for HLA-G and cytokeratin-7 expression; thus confirming isolation of EVT. The HTR-8/SVneo cells had invaded the pore membrane filters indicating that it was the nature of the EVT cells that prevented the invasive potential rather than any problem with the experiment design. The lack of invasion by primary EVT cells in this study is therefore unexplained; in order to complete the experiments the well described placental explants invasion model was used (section 2.3.6 and previously described in Lash et al. (2006a)).

4.3.3 Invasion assays with and without serum

Fetal calf serum within the media may affect the binding of exogenous IL-6 to EVT; the cytokine may bind to components of the serum including oestrogens, insulin and testosterone, thus preventing binding to the receptors on EVT thereby blocking the possible effects of IL-6 in these cells.

Using both isolated EVT cells and placental explants I investigated whether this would affect binding and cellular invasion by performing invasion assays using 0%, 0.5%, 5% and 10% FCS (Sigma) supplemented media +/- 10ngml^{-1} IL-6. In addition I also set up the same experiment with the same FCS concentrations but using charcoal stripped FCS (First Link, Birmingham, UK). Each experiment was repeated 3 times. However, cellular invasion was only observed using 10% FCS supplemented media; invasion was not observed at any other FCS concentration. Low numbers of cells had invaded using

10% charcoal stripped FCS media indicating that components of the normal FCS were essential for the invasion of these cells. Therefore, all invasion assays were performed in the presence of 10% FCS.

4.3.4 Invasion assays with addition of sIL-6Rα and IL-6 neutralising antibody

A combination of exogenous IL-6 and sIL-6Rα (both reconstituted in 10nM acetic acid; 10ngml^{-1} IL-6 + 20ngml^{-1} sIL-6Rα; n=5) was used in the invasion assays because of the agonist effects of sIL-6Rα; an amplified effect might have been observed rather than cytokine alone and this represented a more physiological effect. The role of endogenous IL-6 by EVT was investigated by addition of neutralising antibody (rabbit anti-human IL-6 1μgml^{-1}).

4.3.5 Kinase Array

EVT cell lystates and HTR-8/SVneo cell lystates were prepared (section 2.3.6) after treatment with 10ngml^{-1} recombinant IL-6. A BioRad DC total protein assay was then performed to determine the protein concentration of the cellular extract and EVT lysates were pooled to give a total protein concentration of 250μg for each experiment. HTR-8/SVneo cells were grown to 100% confluence ~ 1 x 10^{7} cells ml^{-1} and lysed using the lysis buffer provided (see Chapter2, section). 300μg total protein concentration was used for each HTR-8/SVneo profile experiment. The array protocol was performed as previously described in Chapter 2 (section 2.6). Signals from the X-ray film were scanned and the images were analysed using Adobe Photoshop software package and the images were quantified by using a mean spot pixel density counting method. Signal values were exported to Microsoft Excel for analysis. Corresponding signals were compared to determine the relative change in phosphorylated kinase proteins between untreated and treated cells and between samples. Data from the array were normalised to control values to facilitate comparison between each experiment (n=3 in both EVT and HTR-8/SVneo lysates).

4.3.5 FASTQuant® Array

Isolated EVT cells (8-14 weeks' gestation; n=4) were cultured on growth factor reduced Matrigel® (BD Biosciences) overnight, treated with 10ngml^{-1} human recombinant IL-6

or 10nM acetic acid medium (control) for 24 hours at 37°C, 5% CO_2. FAST Quant®
multiplex arrays (human II, TH1/2 and angiogenesis kits) were used according to
manufacturer's instructions (FASTQuant®, GE healthcare; section 2.7) to determine
whether IL-6 had any effect on EVT secretion of cytokines and angiogenic growth
factors. The slides were imaged using an Axon GenePix scanner and data analysis was
performed by the ArrayVision™ FAST® software.

4.3.6 Statistical analysis

Data are presented as mean +/- SEM. Data were analysed using StatView software
package (Abacus Concepts, Inc., Berkley, US). Statistical significance was determined
using one-way ANOVA, followed by Fisher's post hoc analysis. Wilcoxon non-
parametric tests were performed for non-normal skewed distribution of data and t-
test was used when only two sets of data were compared and data were normally
distributed. Differences were considered statistically significant at $P < 0.05$.

4.4 Results

4.4.1 Expression of IL-6 receptors expressed by trophoblast cells

To determine whether IL-6 may have an effect on trophoblast invasion, IL-6 receptor expression was initially studied to see whether this cytokine had the capability of binding with EVT and therefore, elicit an effect. Immunohistochemistry data (Chapter 3) indicated that EVT in the placental bed in first half of pregnancy (6-20 weeks gestation) were immunopositive for both gp130 and IL-6Rα and expression did not alter with gestational age. The expression of IL-6 receptors by HTR-8/SVneo cells and primary isolated EVT after isolation and culture therefore was investigated.

Immunocytochemistry of HTR-8/SVneo cells and isolated primary EVT cell smears demonstrated that both cell types express the IL-6 receptors, IL-6Rα and gp130 (Figure 4.1; n=3).

To further confirm IL-6 receptor protein expression by both trophoblast cell line HTR-8/SVneo cells and primary EVT cells and confirm IL-6Rα antibody specificity Western blot analysis was performed. The 80kDa full length IL-6Rα receptor was expressed in both isolated EVT and HTR-8/SVneo cell lysates (Figure 4.2; n=3 both cell types), confirming expression of IL-6Rα by trophoblast cells. The house-keeping gene Gβ (36kDa) was used as a loading control for the experiment. Gp130 expression was undetectable using the antibody used.
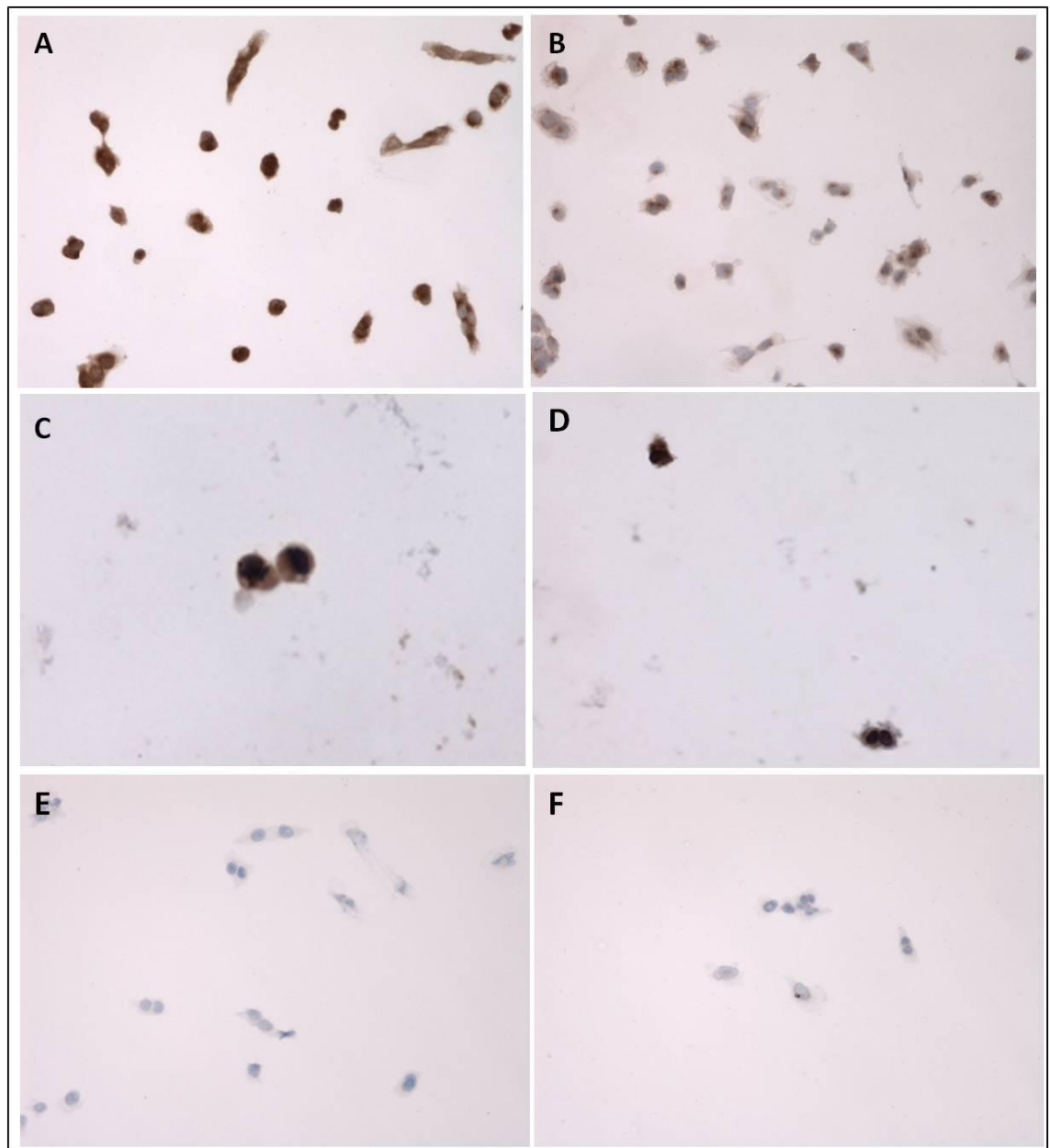
Figure 4.1 Immunostaining of IL-6 receptors (IL-6Rα and gp130) on primary EVT and HTR-8/SVneo cells

Representative immunocytochemistry illustrating expression of IL-6Rα and gp130 by trophoblast cells grown in chamber slides showing: A) positive IL-6Rα staining on HTR-8/SVneo cells; B) positive gp130 staining on HTR-8/SVneo cells; C) positive IL-6Rα on primary EVT cells; D) positive gp130 staining on primary EVT cells. E and F show negative primary antibody controls for IL-6Rα and gp130 with HTR-8/SVneo cells. A, B, E and F magnification x200; C and D magnification x400.
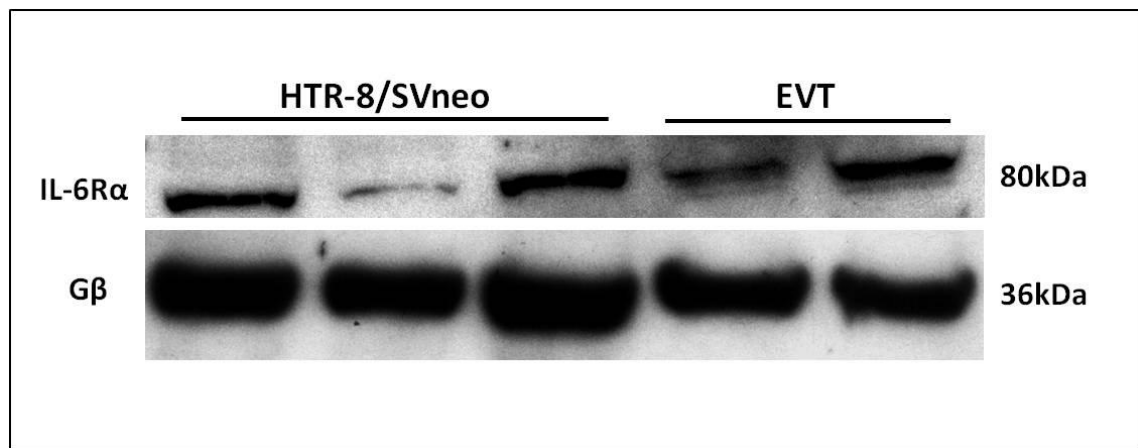
Figure 4.2 Western blot demonstrating expression of IL-6Rα on primary EVT and HTR-8/SVneo cells
Representative Western blot showing protein expression of the full length IL-6Rα (80kDa) on both HTR-8/SVneo (n=3) and EVT cell isolates taken from 8-14 weeks' gestation (n=2). The bands expressed at 36kDa demonstrate the expression of Gβ as a loading control.

4.4.2 Effect of IL-6 on the invasiveness of trophoblast cells

IL-6 receptor expression on trophoblast cells *in situ* and *in vitro* suggests that this cytokine has the ability to bind to these cells and may have a functional role. Therefore, the effect of IL-6 on trophoblast invasion was assessed using a Matrigel invasion assay.

Addition of exogenous IL-6 had no effect on the invasiveness of either HTR-8/SVneo cells (Figure 4.3A) or primary EVT cells from first trimester (8-10 weeks gestation) placental explants (Figure 4.3B). Addition of exogenous sIL-6Rα alone or combined with IL-6 also had no effect on the invasiveness of either HTR-8/SVneo cells or placental explants, demonstrating that endogenous IL-6 produced by the trophoblast cells themselves does not contribute to invasion. Moreover, the presence of IL-6 neutralising antibody had no effect on EVT cell invasion, suggesting that both endogenous IL-6 secreted by EVT cells and exogenous IL-6 plays no part in their invasiveness (Figure 4.3B).
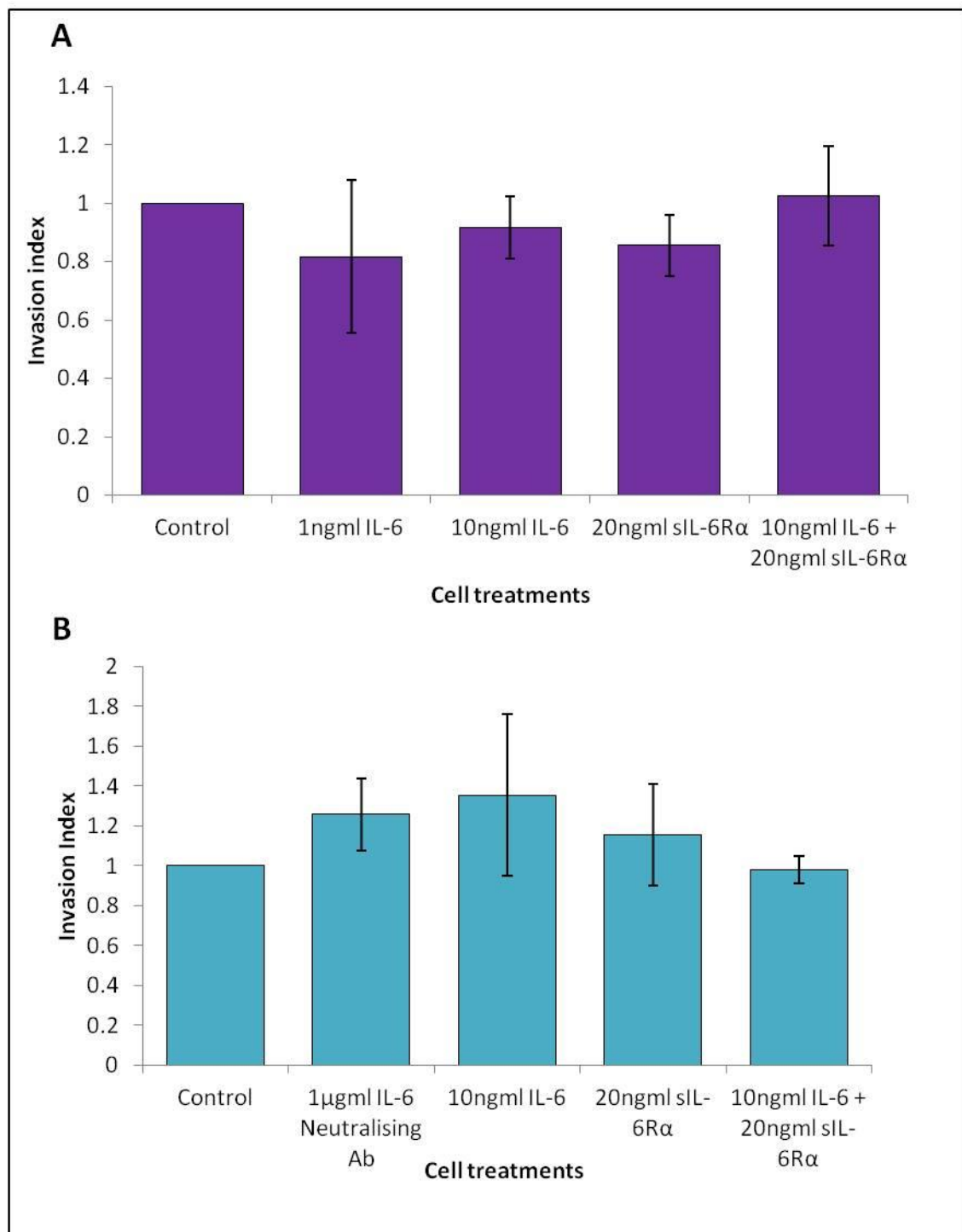
Figure 4.3 The effect of IL-6 on trophoblast invasion
A) The effect of exogenous IL-6 and sIL-6Rα on invasion of HTR-8/SVneo cells (n=3); **B)** the effect of exogenous IL-6, sIL-6Rα and IL-6 neutralising antibody on invasion of primary extravillous trophoblast cells from isolated villous explants at 8-10 weeks gestational age (n=5) in a Matrigel invasion assay. Data are expressed as invasion index normalised to the control for each experiment (mean ± SEM).

4.4.3 The effect of IL-6 on the stimulation of EVT signalling proteins

Although IL-6 receptors are expressed by trophoblast cells, IL-6 failed to stimulate invasion. Nevertheless, the expression of IL-6 receptors by trophoblast cells indicates a potential functional role for this cytokine. In order to investigate the functional importance of IL-6 for trophoblast signalling pathways in EVT after stimulation with IL-6 were investigated.

4.4.3.1 Western blot of STAT-3 and ERK1/2

Western blots for STAT-3 and p-STAT-3 were performed on HTR-8SVneo cell line lysates. However, there was no signal detected using the p-STAT-3 antibody even with the positive controls. A signal was produced for STAT-3 at 92kDa after incubation at 5, 10 and 30 minutes (Figure 4.4). With regards to MAPK1/2 both EVT isolates and HTR-8/SVneo cells produced these proteins: 44KDa ERK1 and 42KDa ERK2 (Figure 4.5 top). P-MAPK1/2 expression was also demonstrated by Western blot (Figure 4.5 bottom) in both HTR-8/SVneo and EVT cells. As the Western blots showed constitutive activation of MAPK1/2 signalling proteins in HTR-8/SVneo and EVT cells regardless of IL-6 treatment and time, we decided to carry out a kinase array to determine which signalling proteins were stimulated by IL-6 using 10 minutes as the incubation time (section 4.4.4.2).
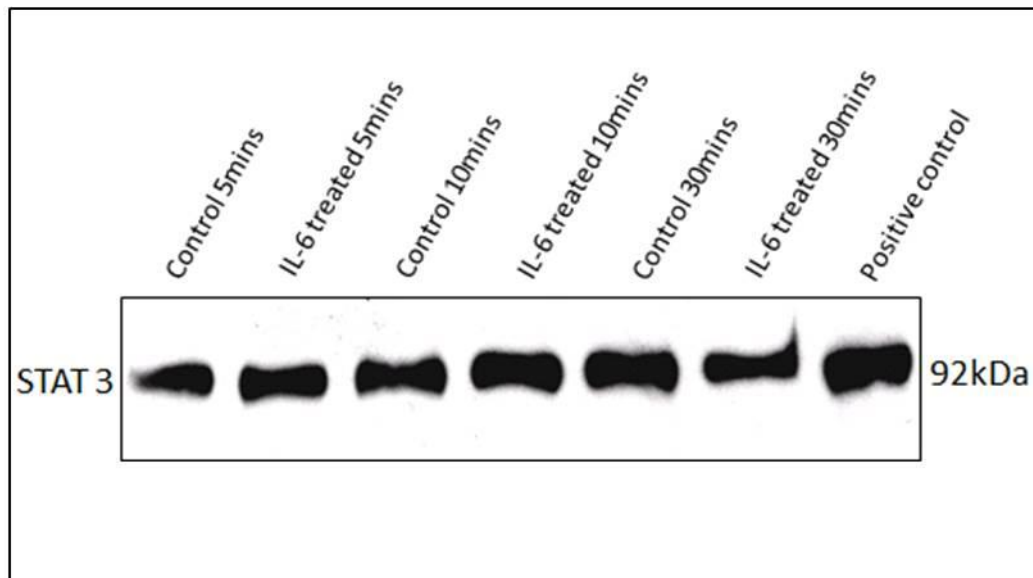
Figure 4.4 Western blot demonstrating the effect of IL-6 on trophoblast STAT-3 cell signalling
Western blot representing bands at 92kDa demonstrating STAT-3 protein expression in untreated and IL-6 treated HTR-8/SVneo cells. The cells were subjected to 10ngml^{-1} of exogenous IL-6 for different incubation times. The positive control used for the experiment was HeLa cells stimulated with IFN-γ.
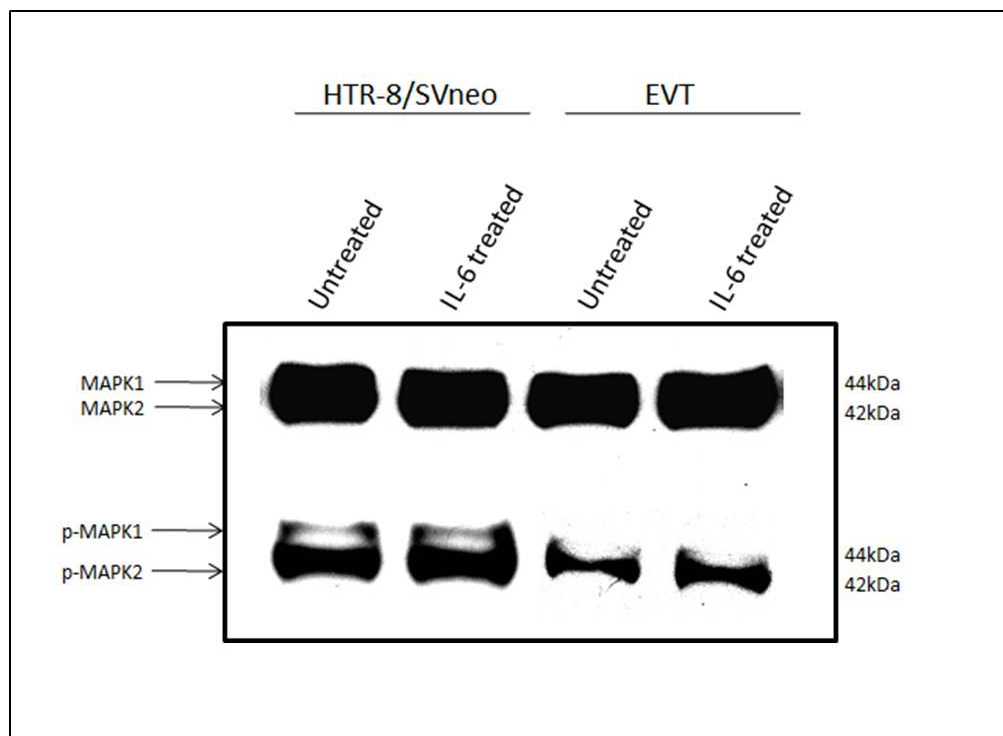
Figure 4.5 Western blot demonstrating the effect of IL-6 on trophoblast cell signalling via MAPK pathway
Western blot representing MAPK1/2 expression (44kDa and 42kDa) in untreated and IL-6 treated HTR-8/SVneo and EVT cells (upper blot). The lower blot represents the expression of stimulated MAPK1/2 (44kDa and 42kDa). 3T3/A31 cell lysate was used as a positive control (data not shown).

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4.4.3.2 Kinase-phospho array

A kinase array that measures phosphorylation status for 46 kinase proteins involved in cellular signalling cascades was performed to determine whether IL-6 signalled in trophoblast cells. IL-6 stimulated phosphorylation of 9 of the 46 signalling proteins investigated in isolated primary EVT (Figure 4.6; n=3). The proteins that were most highly stimulated upon treatment were Src; $P=0.01$; Jun $P=0.01$ and STAT-3; $P=0.02$. IL-6 significantly decreased stimulation of β-Catenin $(P=0.02)$, STAT1 $(P=0.04)$ and STAT4 $(P=0.002)$ and increased stimulation of STAT5a $(P=0.006)$ from the 46 different kinase proteins in the HTR-8/SVneo cell line (Figure 4.7; n=3). However, signal spot intensity was very high in all replicates of the experiment indicating that HTR-8/SVneo cell signalling proteins are constitutively stimulated regardless of cytokine treatment. When a Bonferroni correction $(P<0.001)$ was performed to take into account the multiple comparisons that were performed statistical significance was lost.
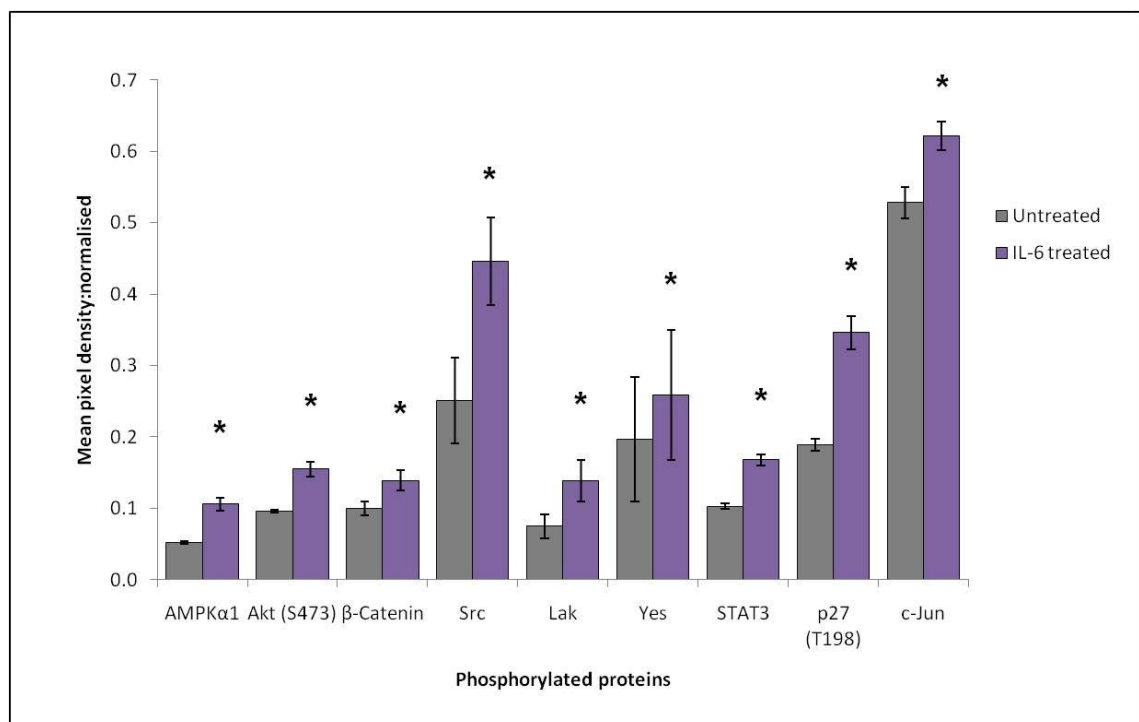
Figure 4.6 The effect of IL-6 on EVT kinase signalling proteins
The effect of IL-6 on stimulation of 46 kinase signalling proteins in isolated primary EVT cells 8-14 weeks gestation (n=3) Nine signalling proteins in EVT were stimulated with exogenous IL-6 treatment. A Students paired *t*-test was performed. * denotes significance between untreated and treated cells, (from left to right: $P=0.02$; $P=0.03$; $P=0.05$; $P=0.01$; $P=0.04$, $P=0.02$; $P=0.02$; $P=0.04$ respectively).
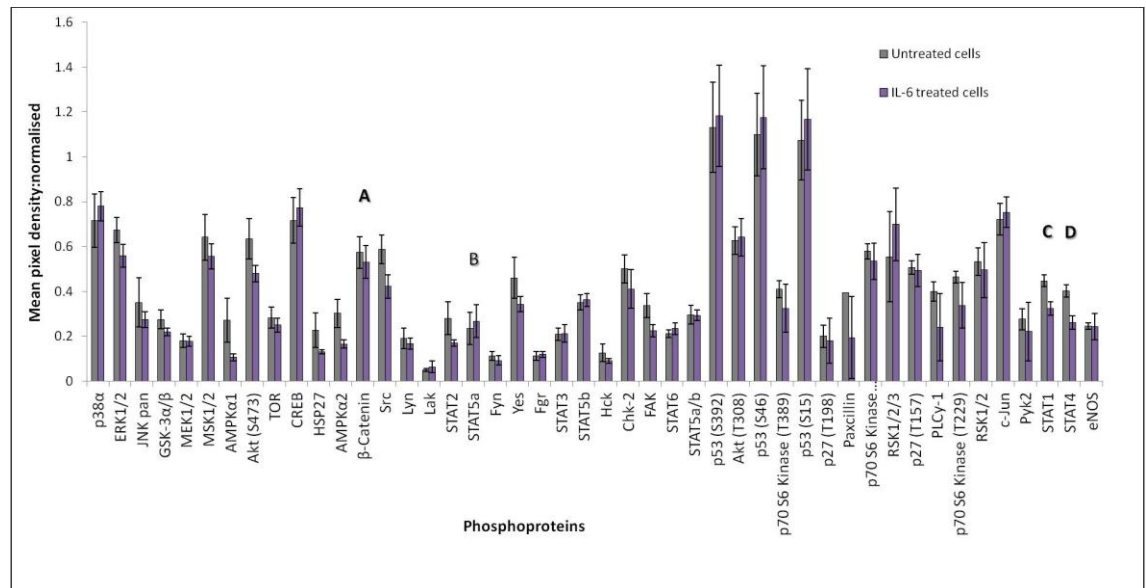
Figure 4.7 The effect of IL-6 on HTR-8/SVneo kinase signalling proteins

The effect of IL-6 on phosphorylation of 46 kinase signalling proteins in HTR-8/SVneo cells (n=3). Both untreated and IL-6 treated data were normalised to positive controls. A Students paired *t*-test was performed. Letters A-D denotes significant differences in phosphorylation of signalling proteins between treated and untreated cells. A, C and D demonstrates a significant decrease in stimulated proteins upon treatment (*P*=0.02; *P*=0.04; *P*=0.002 respectively), whereas B denotes a significant increase in protein phosphorylation after IL-6 treatment (*P*=0.006).

4.4.4 EVT production of angiogenic growth factors, Th1/Th2 and human II cytokines +/- IL-6

Having established that IL-6 could stimulate phosphorylation of EVT cells, the effect of IL-6 on EVT cytokine and angiogenic growth factor production was determined. Levels of secreted angiogenic growth factors and cytokines were measured in cell culture supernatants of isolated first trimester extravillous trophoblast cells.

After IL-6 treatment (10ngml^{-1}, 24h) EVT cells secreted similar levels of angiogenic growth factors and the majority of the cytokines measured to those detected in untreated EVT. Treatment of EVT cells with IL-6 decreased secretion of RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted) (P=0.03, Figure 4.8A) and there was also a trend towards a reduction in secretion of other proteins including angiogenin, Ang-2, PDGF-BB, VEGF-A, ICAM-1, IL-10 and IL-13 (Figure 4.8A).

Expression of RANTES mRNA was also reduced in EVT cells after treatment with IL-6 using real time RT-PCR (*P*=0.05, Figure 4.8B; n=4) further confirming the protein data.
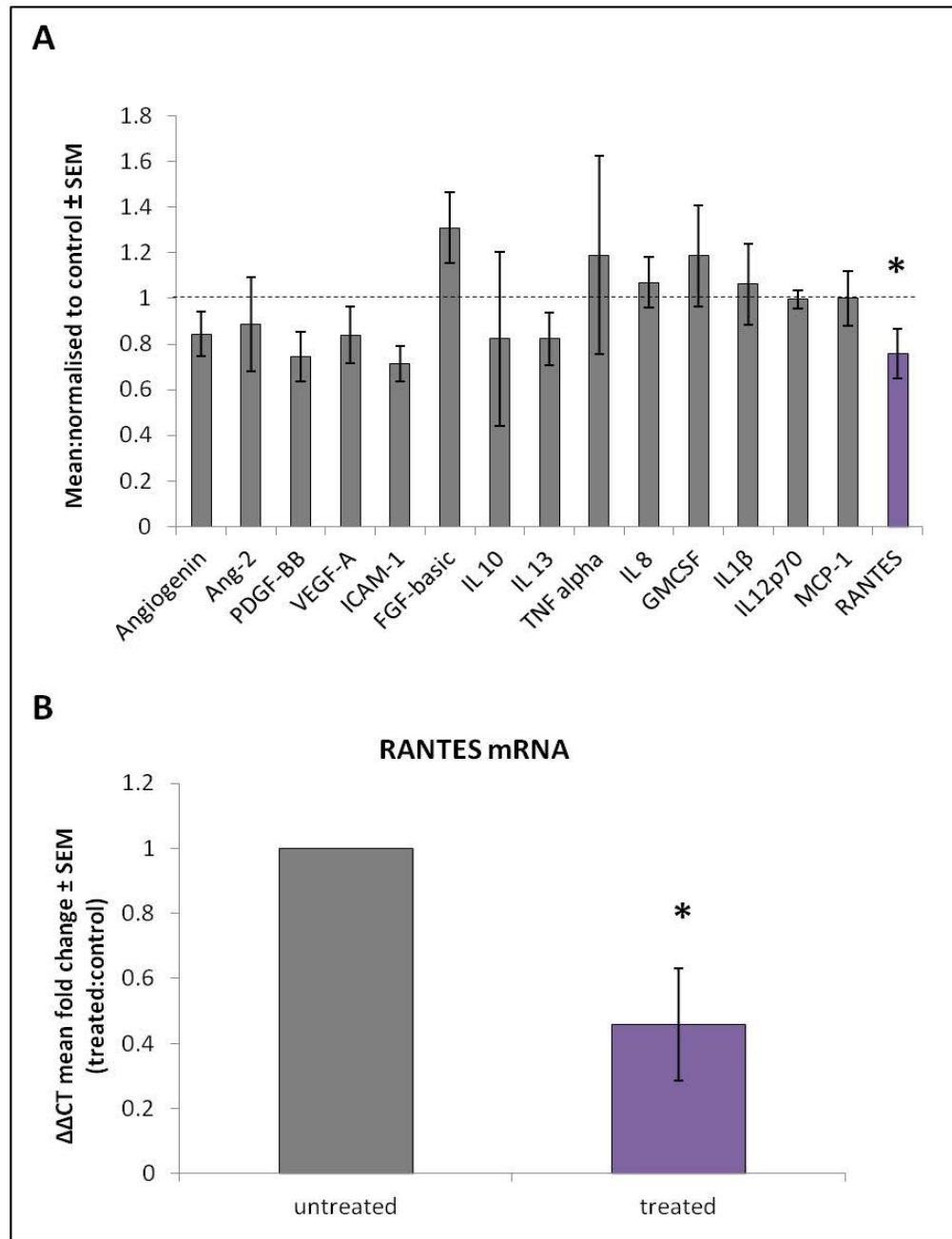
Figure 4.8 The effect of IL-6 on EVT protein secretion

Effect of exogenous IL-6 (10ngml^{-1}) on EVT protein secretion. **A** illustrates a significant reduction in EVT secretion of RANTES after treatment of IL-6, data from Human II FASTQuant (n=7). No significant difference was observed in the levels of EVT secreted proteins between untreated and IL-6 treated cells from TH1/2 and angiogenesis FASTQuant assays respectively (n=4). Protein levels measured from treated EVT were normalised to untreated control levels and data are shown as mean fold change. A non-parametric Wilcoxon Rank test was performed; * denotes significance compared with untreated cells P=0.03. **B** illustrates a significant reduction in RANTES mRNA expression after EVT cells were treated with IL-6. Data are normalised to control and are shown as mean fold change of delta-delta CT values. A Students paired t-test was performed; * denotes significance P=0.05 n=4.

4.5 Discussion

4.5.1 Expression of IL-6 and IL-8 receptors by EVT cells

IL-6 along with IL-8 is produced in high amounts within the decidua (reviewed in Chapter 3). IL-6 production has also been demonstrated in various choriocarcinoma cell lines, including BeWo, Jar and Jeg-3, as well as the primary extravillous trophoblast-like cell line HTR-8/SVneo (Jovanovic and Vicovac, 2009). IL-6 receptor expression by trophoblast cells has been identified in primary cytotrophoblast cells and HTR-8/SVneo cells (Jovanovic and Vicovac, 2009) but has not been previously explored using isolated EVT from first trimester pregnancies. Zhao et al. (2008) investigated the expression of both sIL-6Rα and gp130 in villous tissue from both normal and preeclampsia placenta from term pregnancies and reported that gp130 expression was increased whereas sIL-6Rα was reduced in preeclampsia. These reports led to the suggestion that IL-6 may play a functional role for IL-6 in the pathogenesis of this disease as well as in normal pregnancy, although this was observed in placental villous tissue rather than isolated trophoblast cells. Villous tissue may not be a good tissue source for investigating preeclampsia as villous trophoblast cells are not relevant in its pathogenesis and is related to invasion by EVT cells.

We have previously immunolocalised the IL-8 receptors CXCR1 and CXCR2 in first trimester EVT (De Oliveira et al., 2010) and CXCR1 has been reported to be expressed by EVT (Hanna et al., 2006) and by isolated cytotrophoblast (CTB) cells (Jovanovic et al., 2010). This present study and studies within our group have confirmed IL-6 and IL-8 receptor expression by trophoblast cells including isolated EVT both *in vitro* and *in situ* (Chapter 3) in early pregnancy, suggesting a functional role for both of the cytokines in the regulation of EVT invasion or other EVT function. They have the ability to bind and communicate with EVT to elicit a response. The signalling of IL-6 in EVT has not been investigated by other studies but has been explored in this study (section 4.5.3) EVT signalling effects were examined using IL-6 since IL-6 did not stimulate EVT invasion but we wanted to investigate other possible functions. IL-8 signalling in EVT was not investigated since an increase in EVT invasion was observed when EVT cells were treated with IL-8 but remains to be explored.

4.5.2 Effect of IL-6 on trophoblast invasion

It has been proposed that one of the main roles of IL-6 in uteroplacental tissues is regulation of trophoblast invasion. Although IL-6 is widely associated with studies involving progression of certain cancers and is known to have an effect on the invasiveness of cancer cells, there is limited information on its role in trophoblast invasion. Despite the expression of IL-6 receptors by primary isolated EVT and HTR-8/SVneo cells, neither exogenous nor endogenous IL-6 had any effect on trophoblast invasion in this study. Interestingly, the addition of sIL-6Rα with IL-6 did not increase the invasiveness of the trophoblast cells, nor did the addition of IL-6 neutralising antibody decrease invasion, further suggesting that the functional role of this cytokine is not related the regulation of EVT invasion. In contrast, a recent study has reported an increase in the invasive potential of first trimester villous cytotrophoblast cells and HTR-8/SVneo cells after IL-6 treatment *in vitro* (Jovanovic and Vicovac, 2009). This discrepancy could be explained by the differences in the cell type used; in the present study placental villous explants from which EVT are derived were used, whereas (Jovanovic and Vicovac, 2009) studied isolated villous cytotrophoblast cells. The varying results between the two studies with HTR-8/SVneo cells may be due to their differing passage number, which could reflect a change in cellular function. Quantification methods also differed between studies; rather than counting selected fields of the membrane inserts and including occupied pores within the cell quantification as reported by (Jovanovic and Vicovac, 2009), in this study only cells that had invaded completely through the pores were included and were quantified in the entire membrane. In addition, RNA silencing techniques have been used to inhibit expression of IL-6 in JEG-3 choriocarcinoma cells resulting in the inhibition of cellular migration and invasion (Dubinsky et al., 2010) suggesting that IL-6 does contribute to cell invasion in this cell type. Furthermore, Paiva et al. (2009) reported that IL-11 (a member of the IL-6 superfamily) reduced *invasion* of HTR-8/SVneo cells but, in contrast, promoted EVT *migration*.

There is increasing evidence that HTR-8/SVneo cells are an imperfect model for extravillous trophoblast as they respond differently to altered oxygen concentrations compared with primary EVT, and they do not express the specific integrin repertoire

and non-classical MHC class I molecules such as HLA-G that are typical of EVT (Kilburn et al., 2000) unless cultured on extracellular matrix (Apps et al., 2009, Lash et al., 2007). Recent data by Bilban et al. (2010) has demonstrated differences in gene expression profiles of integrins, proteases, hormones and growth factors between trophoblast cell lines and primary CTB and EVT. Indeed of the cell lines tested, HTR-8/SVneo cells were most distantly related to primary cells and therefore these cells are not an ideal model for extravillous trophoblast.

The effect of IL-6 on trophoblast invasion using primary EVT isolates or placental explants has not been previously reported but the results obtained from this study suggest that IL-6 does not play a role in regulating trophoblast invasion in early pregnancy; these results are also consistent with preliminary findings from other laboratories (Dr M. Knofler, personal communication). In accordance with this reports concluded that decidual cells, which we have shown to be producers of IL-6 (Chapter 3), do not play a major role in regulating CTB invasion *in vitro* using a decidual/EVT co-culture method (Cohen and Bischof, 2009). In contrast, CTB cells increased the invasiveness of decidual cells by increasing their secretion of MMPs. Recently Lash et al. (2010b) demonstrated that uNK cell supernatants from 12-14 weeks gestation pregnancies had more effect on the stimulation of EVT invasion compared with decidual supernatants from 8-10 weeks gestation. This coincides with findings from this study that more IL-6 is produced at 12-14 weeks gestation by uNK cells (Chapter 3) and suggests that IL-6 may possibly contribute to the invasive capacity of EVT at later gestation. However, EVT are more inherently invasive at 8-10 weeks gestation (Lash et al., 2005) and may require external stimuli from decidual components to proceed with invasion into the inner third myometrium after 8-10 weeks into later gestation.

4.5.3 Effect of IL-6 on the stimulation of cellular kinase proteins involved in EVT signalling

Although IL-6 had no effect on trophoblast invasion in the present study, the signalling effect of IL-6 on early pregnancy EVT was investigated, an area which has not been previously explored. The kinase profile array performed in this study demonstrated stimulation of nine signalling pathway kinase proteins in isolated primary EVT by exogenous IL-6, but not in the HTR-8/SVneo cell line. These signalling proteins were

constitutively phosphorylated in HTR-8/SVneo cells regardless of IL-6 treatment, suggesting constitutive state of activation and providing further evidence that this cell line is not an ideal model for EVT. Indeed, 3 out of the 4 proteins were less phosphorylated after treatment of HTR-8/SVneo cells with IL-6. In isolated EVT the proteins that were most highly phosphorylated by IL-6 were; Src protein, a proto-oncogenic tyrosine kinase which is reported to be involved in embryonic development and cell growth, STAT-3 a transcriptional activator of transcription of genes involved in cellular growth and proliferation and c-Jun an early transcription factor that interacts directly with specific target DNA sequences to regulate gene expression. STAT-3 is a major downstream protein involved in IL-6 signalling and molecular studies have shown that STAT-3 is a contributor to EVT invasive capacities (Fitzgerald et al., 2005). However, this provides conflicting evidence to the invasive capacities of IL-6 on EVT as no stimulation on EVT invasion was observed with IL-6 in this study and the absence of pSTAT protein in EVT after IL-6 treatment also is consistent with this finding, although the sensitivity of the kinase array may account for the detection of phosphorylated STAT-3. This indicates that signalling between EVT cells and IL-6 is a complex process and may include the involvement of other factors to achieve a functional role in EVT invasion. STAT-3 signalling is also involved in many cellular events and also plays a part in cellular development; IL-6 may help to up-regulate genes involved in trophoblast growth or differentiation, although further investigations are required to gain insight into the specific role for this cytokine in trophoblast function.

4.5.4 Effect of IL-6 on the secretion of growth factors and cytokines by EVT cells

Demonstration of IL-6 receptors on EVT and stimulation of signalling proteins in primary EVT after IL-6 treatment led to investigation of the effect of IL-6 on EVT secretion of angiogenic growth factors and cytokines since IL-6 had no effect on trophoblast invasion. Although, the majority of the secreted proteins analysed were reduced with IL-6 treatment, this was significant only for RANTES. RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) is a chemokine that is chemotactic for T-cells, eosinophils and basophils and has an active role in recruiting leucocytes to sites of inflammation. EVT cells penetrate the decidualised endometrium

and further migrate within the lumen of spiral arteries to remodel the uterine vasculature. RANTES has been shown to increase migration of isolated macaque trophoblast cells (Thirkill et al., 2005) and also promoted EVT invasion in EVT isolated from villous explants cultures *in vitro* (Fujiwara et al., 2005). Production of RANTES by both human primary CTB and EVT was also observed and production increased significantly between 8-10 and 12-14 weeks gestational age (Naruse et al., 2010). The evidence suggests that EVT RANTES is responsible in mediating trophoblast migration and may influence their invasive potential. IL-6 reduced the secretion of EVT RANTES in this study suggesting that EVT invasion would have been somewhat inhibited. However, this was not observed in this study, although trophoblast secretion of RANTES may play a role in mediating trophoblast migration and IL-6 could play a role in regulation of this function; a reduction in chemotactic factors may be required to support the formation of trophoblast plugs in spiral arteries in the first trimester, thus preventing EVT migration and favouring remodelling of the arteries (James et al., 2012).

4.5.5 Overall summary

An extensive literature reflects the importance of IL-6 in human implantation and has been implicated pregnancy complications but there is little understanding of the effect of IL-6 on trophoblast function in early pregnancy. It has been suggested that IL-6 may contribute to trophoblast growth and development by stimulation of trophoblast derived human chorionic gonadotrophin (hCG) (Nishino et al., 1990), which is essential for the maintenance of pregnancy and may play a role in the differentiation of cytotrophoblasts in blastocyst implantation. Indeed (Taniguchi et al., 1992), demonstrated that IL-6 enhanced secretion of hCG by Jar, HCCM-5 and BeWo choriocarcinoma cell lines. BeWo cells, in common with EVT and CTB, secrete high levels of RANTES and IL-1β which in turn increases the secretion of hCG. IL-6 may therefore control secretion of these factors by EVT, hence further increasing hCG secretion to maintain a healthy pregnancy. However, it should be noted that this has not yet been observed using primary EVT. In unpublished data studies (Dr Knofler et al personal communication) IL-6 did not cause stimulation of hCG in primary EVT in early pregnancy.

IL-6 is a pleiotropic cytokine with both pro-inflammatory and anti-inflammatory functions; many signalling pathways are affected by IL-6 resulting in a range of biological effects. The developmental processes that are essential for successful early human pregnancy, including EVT invasion, involve a complex network of multiple cytokines and growth factors, with both stimulatory and inhibitory functions. Due to the complexity of the cytokine milieu within the decidua it is difficult to determine the exact function of this individual cytokine. Furthermore functions of the range of different cytokines and growth factors present at the maternal-fetal interface may differ with gestational age.

In summary in the present study using both placental villous explants and a trophoblast cell line, HTR-8/SVneo, we have demonstrated that there was no effect of IL-6 on trophoblast invasion. Studies and understanding of physiological trophoblast invasion remains and the complex array of signalling cascades involved require clarification. We have shown that IL-6 signalling effects were able to contribute to the stimulation of signalling pathways in isolated EVT cells and affect production of cytokines that are involved in migratory function and which may have other roles in trophoblast biology such as regulating hCG secretion, EVT differentiation and influence EVT dependent SpA remodelling, areas which if altered could contribute to pregnancy complications and therefore require further exploration. The current study has therefore demonstrated that IL-6 has an effect on EVT, albeit the function remains unknown. Nevertheless these data signify a possible important role for IL-6 in early human pregnancy which may require both *in vivo* and *in vitro* approaches to unravel.

Chapter 5

The effect of interleukins-6 and 8 on the differentiation and morphology of vascular smooth muscle cells (VSMCs) in chorionic plate arteries (CpA) from term human placenta

5. The effect of interleukins-6 and 8 on the differentiation and morphology of vascular smooth muscle cells (VSMCs) in chorionic plate arteries (CpA) from term human placenta

5.1 Introduction

The importance of spiral artery remodelling in early pregnancy development has been reviewed in chapter 1. During early pregnancy once implantation has occurred, spiral arteries begin to transform from extensively coiled, narrow, thick muscular structures into highly dilated vessels ensuring that an adequate supply of blood is delivered to the fetus at an optimal pressure to ensure maximal nutrient exchange. This is achieved by the loss of their musculoelastic wall which is replaced by fibrinoid material and invading extravillous trophoblast (EVT) cells. Failure of remodelling during early pregnancy had been implicated in the pathogenesis of preeclampsia (Pijnenborg et al., 1991), intrauterine growth restriction (IUGR) (Khong et al., 1986) and late miscarriage (Ball et al., 2006a). Despite the importance of spiral artery remodelling in early pregnancy, the molecular mechanisms which trigger the process remain unclear but it is now more evident that there are both EVT-independent and EVT-dependent processes.

The process of spiral artery (SpA) remodelling is a unique process in human vascular biology which involves the transient loss of VSMC with complete restoration post partum. However, the events of SpA remodelling in early pregnancy are parallel to events of angiogenesis which occur during vascular injury or tumourgenesis. The early stages of SpA remodelling involve initial disruption of the vascular wall, characterised by endothelial cell swelling and loss, vessel dilatation and vascular smooth muscle (VSMC) separation and misalignment which occur before the presence of EVT. Mechanisms that underlie these processes have yet to be elucidated but VSMC migration and de-differentiation are suggested possibilities (Whitley and Cartwright, 2010, Bulmer et al., 2012). The differentiation of VSMCs is correlated to their behaviour and phenotype and they can switch between contractile and synthetic stages which makes them unique compared with other muscle cells (Kaplan-Albuquerque et al., 2005). The VSMCs in an established artery exhibit a contractile phenotype expressing proteins such as smooth muscle actin (α-SMA), calponin and myosin heavy chain and do not show signs of migration or proliferation (Owens et al.,

2004). However, extracellular signals can cause the VSMCs to adopt a synthetic phenotype resulting in increased migration and proliferation (Wilcox et al., 1997) and is associated with a reduction in the expression of contractile proteins (Halka et al., 2008) which could be evident in remodelling spiral arteries.

Maternal immune cells such as uNK cells and decidual macrophages have been proposed to play a role in the initiation of SpA remodelling (Lash et al., 2006c, Smith et al., 2009, Hazan et al., 2010, Robson et al., 2012). Uterine natural killer cells are a rich source of many cytokines and angiogenic growth factors and increasing evidence suggests a role for these cells in the SpA remodelling that ensures successful pregnancy. Studies in mouse pregnancies have demonstrated the importance of uNK cells in SpA modification via IFN-γ (Ashkar and Croy, 2001, Zhang et al., 2003). Human uNK cells also produce IFN-γ as well as Ang-1, Ang-2 and VEGF-C (Li et al., 2001, Lash et al., 2006c) which are all factors involved in vascular modification during angiogenesis events (Distler et al., 2003, Ferrara, 2004). Human uNK cell Ang-2 has been reported for the first time to be a significant factor in vascular modification using chorionic plate arteries (CpA) and non-pregnant myometrial arteries (Robson et al., 2012).

Uterine macrophages are also observed surrounding SpA undergoing remodelling although the function of these cells in SpA remodelling has not been examined (Chapter 6). In this study we have demonstrated that both uNK cells and macrophages are sources of the cytokines IL-6 and IL-8 (Chapter 3). IL-6 has been linked to the progression of tumour development in many cancers. However, the exact function of IL-6 in malignancy has yet been established. Ovarian carcinoma cells have been reported to secrete IL-6 which was shown to be a potent pro-angiogenic cytokine promoting the progression of the disease by increasing endothelial cell migration and therefore increasing vascularisation (Nilsson et al., 2005) as well as regulating the migration of VSMCs in rat aortas (Wang et al., 2007). Studies have also shown that over expression of IL-8 has been related to tumour progression and metastases (Terada et al., 2005, Bai et al., 2005, Reis et al., 2012) with expression shown to correlate with angiogenesis of many cancers (Huang et al., 2002, Karashima et al., 2003). Association of IL-8 with endothelial cells expressing CXCR2 up regulates VEGF mRNA resulting in cellular proliferation and migration increasing endothelial cell

permeability (Martin et al., 2009). These reports of IL-6 and IL-8 highlight the potential of their importance in angiogenesis and raise the possibility of a role for IL-6 and IL-8 in the regulation of the initial stages of SpA remodelling.

Human spiral artery remodelling during early pregnancy has remained difficult to study by the lack of an appropriate model. *In vivo* studies of uterine vascular changes are difficult due to the access of early normal pregnancy tissue; there are problems in collection of early decidual and placental bed tissue and the microscopic size and highly coiled structure of the spiral arteries make them difficult to dissect. Therefore, in an effort to address this, the present study used an *in vitro* vessel model using 5mm sections of chorionic plate arteries from term placenta of women undergoing Caesarean section in order to investigate the effect of both IL-6 and IL-8 on vascular changes relating to pregnancy. Previous investigation using this model within our research group has demonstrated that first trimester uNK cell supernatants disrupts VSMC organisation, suggesting a role for uNK cell decidual factors including IL-6 and IL-8. Furthermore effects of uNK cell derived cytokines and angiogenic growth factors were similar in CpA and myometrial spiral arteries, suggesting that is readily accessible vessel is a valid model for uterine SpA (Robson et al., 2012).

5.2 Aims and Hypotheses

5.2.1 Hypotheses

The hypotheses to be tested were:-

1) IL-6 and IL-8 receptors are expressed on chorionic plate arteries (CpA) from term human placenta
2) IL-6 and IL-8 disrupts VSMC organisation of CpA by increasing the separation between VSMC layers and increasing rounding of VSMCs within the vessel wall – displaying a more synthetic phenotype.
3) IL-6 and IL-8 alters the differentiation status of VSMCs in CpA and therefore, the cells treated will express markers typical of a synthetic phenotype compared with untreated cells that will express markers of a contractile phenotype.

5.2.2 Aims

The aims of the study were to:-

1) Investigate receptor expression using frozen sections of the CpA tissue at T=0 for immunohistochemical analysis

2) Investigate the effect of IL-6 and IL-8 on VSMC morphology and differentiation in CpA after culture for 5days

5.3 Experimental design

5.3.1 Chorionic plate artery culture - the *in vitro* vessel culture model

Term placentas (37-42 weeks gestation) were obtained from women undergoing Caesarean section and the chorionic plate arteries removed and dissected in the laboratory (section 2.1.6). Initially, dissected segments were immediately snap frozen in isopentane cooled in liquid nitrogen and stored at -80°C until required (section 2.1.8) and the frozen segments were labelled T=0. The frozen T=0 segments were then prepared on glass slides (section 2.3.7) and were immunostained with the muscle marker H-Caldesmon, endothelial marker CD34, IL-6 receptors; IL-6Rα and gp130 and IL-8 receptors; CXCR1 and CXCR2 (Table 5.1) n=3 to confirm receptor expression by the CpA.

Following confirmation of cytokine receptor expression by immunohistochemistry CpA segments that were going to be treated with exogenous IL-6 and IL-8 were prepared and the untreated control segments (T=0 days) were immediately fixed in 10% neutral buffered formalin.

Table 5.1 Primary antibodies used for immunohistochemistry on both FFPE and frozen tissue sections

Primary antibodies used for immunohistochemistry on both FFPE and frozen tissue sections of T=0 and T=5 CpA.

Antibody	Method	Species	Dilution	Incubation	Pre-treatment	Positive control
Myosin heavy chain[1]	Elite Kit[5,6]	Mouse	1:600	RT, 60min	PC in Citrate buffer pH 6.0, 1min	Stomach
H-Caldesmon[1]	Elite Kit[5,6]	Mouse	1:100	RT, 60min/ RT, 30mins (frozen)	PC in Citrate buffer pH 6.0, 1min/No pre-treatment for frozen	Small intestine
Calponin[1]	Elite Kit[5,6]	Mouse	1:80	RT, 30min	WB Trypsin/CaCl₂ pH7.8, 10min	Small intestine
α-SMA[1]	Elite Kit[5,6]	Mouse	1:75	RT, 30min	WB Trypsin/CaCl2 pH7.8, 10min	Small intestine
Ki67[2]	Elite Kit[5,6]	Mouse	1:200	RT, 60min	PC in Citrate buffer pH 6.0, 1min	Lymph node
CD31[1]	Elite Kit[5,6]	Mouse	1:20	RT, 60 min	PC in Citrate buffer pH 6.0, 1min	Lymph node
CD34[1]	Elite Kit[5,6]	Mouse	1:10	RT, 30min	No pre-treatment (frozen)	Tonsil
Caspase-3[2]	ImmPress Kit[5,7]	Rabbit	1:30	RT, 60 min	No pre-treatment (frozen)	Skin
CXCR1[4]	ImmPress Kit[5,7]	Rabbit	1:80	O/N 4°C	No pre-treatment (frozen)	Tonsil
CXCR2[4]	ImmPress Kit[5,7]	Rabbit	1:40	O/N 4°C	No pre-treatment (frozen)	Tonsil
gp130[3]	Elite Kit[5,6]	Mouse	1:50	RT, 60 min	No pre-treatment (frozen)	Tonsil
IL-6Rα[3]	ImmPress Kit[5,7]	Rabbit	1:50	RT, 60 min	No pre-treatment (frozen)	Tonsil

Source: [1]Leica Biosystems, Newcastle LTD UK; [2]Abcam, Cambridge, UK; [3]Santa Cruz Biochemicals, California, USA; [4]R&D Biosystems; [5]Vector Laboratories, Peterborough, UK; [6]ABC ELITE Kit- avidin-biotin peroxidase method (mouse monoclonal); [7]Immpress Kit- avidin-biotin peroxidase method (rabbit polyclonal); PC - Pressure Cook; WB - Waterbath; RT - Room Temperature; O/N - Overnight

5.3.2 IL-6 and IL-8 treatments

The dissected segments to be treated with exogenous IL-6 and IL-8 were placed into wells of a 48 well culture plate each containing 300μl of differing treatments; control RPMI complete medium (RPMI 1640, 10% fetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine); 1ngml^{-1} recombinant IL-6 or IL-8 (Miltenyi), 10ngml^{-1} recombinant IL-6 or IL-8, 5ngml^{-1} recombinant sIL-6Rα (Miltenyi); and 10ngml^{-1} IL-6 + 5ngml^{-1} sIL-6Rα. Each treatment was performed in triplicate (Figure 5.1). The CpA segments were cultured at 37°C in 5% CO_2 for a total of 5 days. The media was refreshed every other day. Previous investigation of this vessel type showed that 5 days was sufficient culture time and after 5 days loss of the vessel endothelium is apparent due to apoptosis.
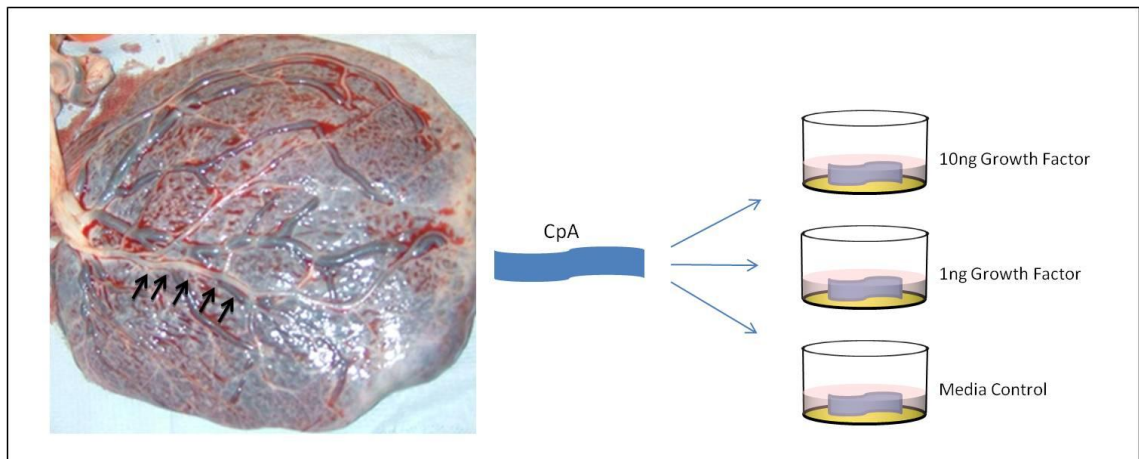
Figure 5.1 Identification of chorionic plate arteries (CpA) from term placenta
Image showing the chorionic plate of a term placenta and the identification of the chorionic arteries which were dissected into segments and cultured for 5 days.

5.3.3 Assessment of VSMC morphological differences

Following 5 days of incubation, the CpA segments were removed from culture and one of each of the segments was placed in 10% neutral buffered formalin (T=5 days). Both the T=0 and T=5 day segments were embedded and processed in paraffin wax and cut into 3µm sections ready for immunohistochemical analysis.

To investigate the effect of the cytokines on CpA VSMC morphology the prepared sections were immunostained for myosin heavy chain (Table 5.1). The slides were then assessed for changes in VSMC rounding, disorganisation of VSMC layers and the misalignment of VSMC layers using a four point scale (Figure 5.2).

In addition to using the four point scale to assess the degree of VSMC rounding, separation and disorganisation a generalised quickscore method was also applied; the vessels were given a score between 1 and 4; where 1 would score VSMCs having an overall contractile appearance (VSMC layers tightly compacted, VSMCs showing a spindle shaped appearance and VSMC showing little disorganisation) whereas 4 would score VSMC layers having being very separated, VSMCs showing a rounded appearance and the cells being highly disorganised.
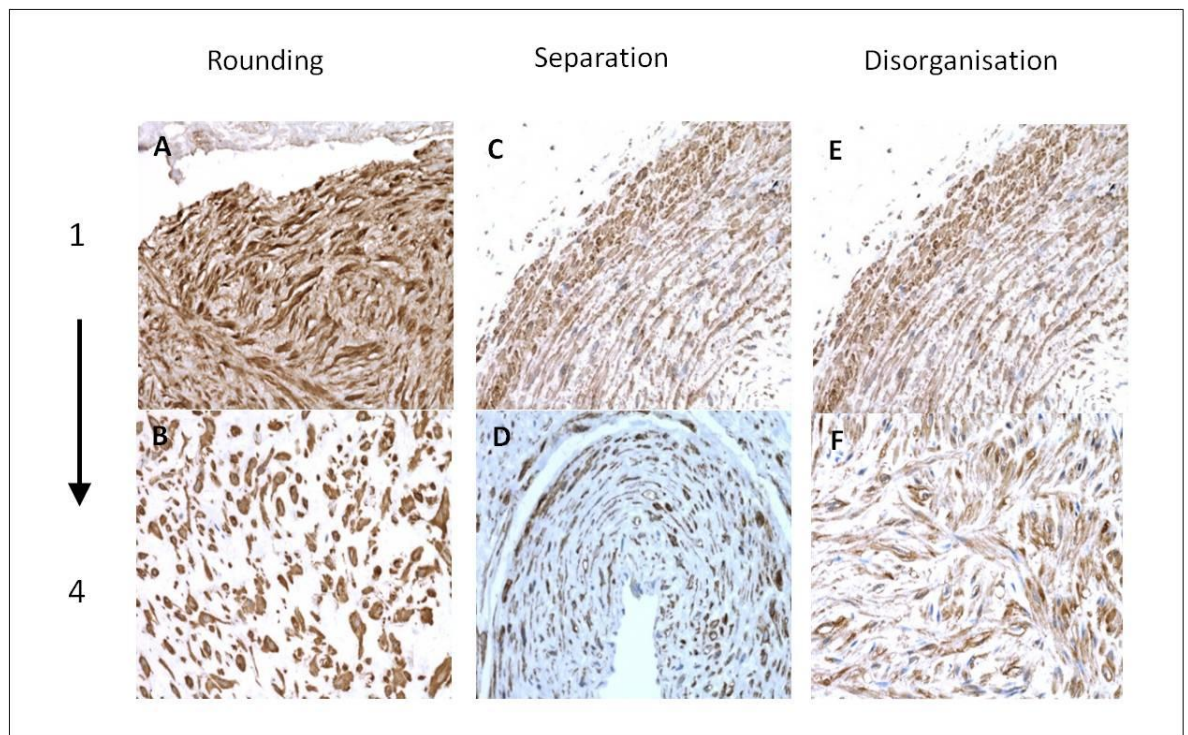
Figure 5.2 Scoring of vascular smooth muscle cell (VSMC) rounding, separation and disorganisation
Immunohistochemical representation of scoring of the changes in vascular smooth muscle cell morphology and organisation. A four point scale (1 <10%, 2= 10-25%, 3= 25-75%, 4= >75%) was used to assess the rounding of VSMCs; spindle like and elongated 0% rounding of cells (**A**) to rounded and spherical >75% of the cells (**B**), the degree of separation of VSMC layers (**C-D**) and the degree of disorganisation of VSMC layers (**E-F**). Sections were immunostained with anti-myosin heavy chain. Magnification x200.

5.3.4 Quantification of immunostaining using Adobe Photoshop™

In addition to myosin heavy chain immunostaining, tissue sections were also immunostained with markers of VSMC differentiation including H-Caldesmon (an established smooth muscle marker of muscle differentiation), calponin (an intermediate-early differentiation marker) and α-smooth muscle actin (an early differentiation marker). One characteristic feature of phenotypically modulated VSMCs is that they express markedly lower levels of differentiation markers that the smooth muscle cells change from a contractile to a synthetic phenotype.

The percentage of positive VSMC immunostaining expressed on each of the treated CpAs was assessed using a pixel counting method using Adobe Photoshop™ software. Microscopic images of the stained vessels were taken using (Nikon 80i microscope and NIS Elements software). The images were then uploaded onto Adobe Photoshop™. The number of positively immunostained pixels was divided by the total number of pixels in the entire image x100 to give a percentage. All sections were blinded. Following assessment the sections were un-blinded and mean values were used in analysis.

5.3.5 Fetal calf serum concentrations and charcoal stripped serum

To investigate whether fetal calf serum impaired binding of IL-6 and IL-8 to VSMC in CPA, by preventing specific binding to receptors, an additional experiment was performed using differing concentrations of fetal calf serum or charcoal-stripped fetal calf serum (First Link, Birmingham, UK). Charcoal stripped fetal calf serum is used instead of normal fetal calf serum to minimise the level of androgens and hormones which may compete with binding to the specific cytokine receptors, may prevent cytokines reaching their receptors or may inactivate cell signalling responses, therefore affecting the function of the cytokines. Charcoal stripped fetal calf serum is absorbed in activated carbon which aids the removal of non-polar compounds such as lipophilic material, growth factors, hormones and cytokines which may have impact on research results after culture (Herbert et al., 1965, Green, 1987).

Chorionic plate arteries were prepared as described in section 5.3.1.

Firstly, a time course experiment was performed. Following dissection a T=0 segment was immediately fixed in formalin. The remaining CpA segments were cultured in RPMI

medium with or without 10% FCS for 1 day (T=1), 2 days (T=2), 3 days (T=3), 4 days (T=4) or a total of 5 days (T=5) (n=3). Following each time point the corresponding vessels were fixed in formalin and embedded in paraffin for immunohistochemistry. The tissue sections were subjected to a haematoxylin and eosin stain to assess the orientation of each vessel and then immunostained for active caspase 3, CD31, myosin heavy chain and Ki67 to assess the degree of proliferation and apoptosis of VSMCs and endothelial cells (Table 5.1, n=3).

Following the time course experiment, arteries dissected from term placenta (n=5) were cultured with either standard RPMI media containing no FCS, 0.5% or 10% FCS versus charcoal stripped FCS supplemented media. CpA sections were cultured in media containing 10% of either normal FCS or charcoal stripped FCS with or without 10ngml^{-1} IL-6 for 5 days. After 5 days in culture the chorionic arteries were formalin fixed and paraffin embedded for immunohistochemistry (as above).

5.4 Results

5.4.1 Immunohistochemical localisation of IL-6 and IL-8 receptors on CpA VSMC

In order to investigate whether IL-6 or IL-8 had any effect on chorionic plate artery vascular smooth muscle cell morphology and differentiation, cytokine receptor expression was initially investigated to determine whether these cytokines were capable of binding to the CpA VSMCs and hence eliciting an effect.

Positive immunostaining for both IL-6 receptors, sIL-6Rα and gp130, was observed (Figure 5.3). Immunostaining for IL-6Rα showed strong expression of the receptor on VSMCs of chorionic plate arteries (n=3). Where there were several VSMC layers, IL-6Rα immunostaining was abundant and was stronger on VSMCs immediately beneath the artery lumen and slight staining was observed on endothelial cells. Gp130 was also expressed on VSMCs of CpAs (n=3), but gp130 immunostaining was more intense on the endothelial cells of the artery lumen.

Both receptors for IL-8, CXCR1 and CXCR2, were expressed on chorionic plate arteries with positive immunostaining observed on CpA VSMCs (Figure 5.4). CXCR1 immunostaining showed receptor expression on both CpA VSMCs and endothelial cells, with immunostaining being more intense on the endothelium. CXCR2 immunostaining also showed receptor expression on both VSMCs and endothelial cells with intense reactivity on endothelium compared with VSMCs. However, CXCR1 was more strongly expressed overall.
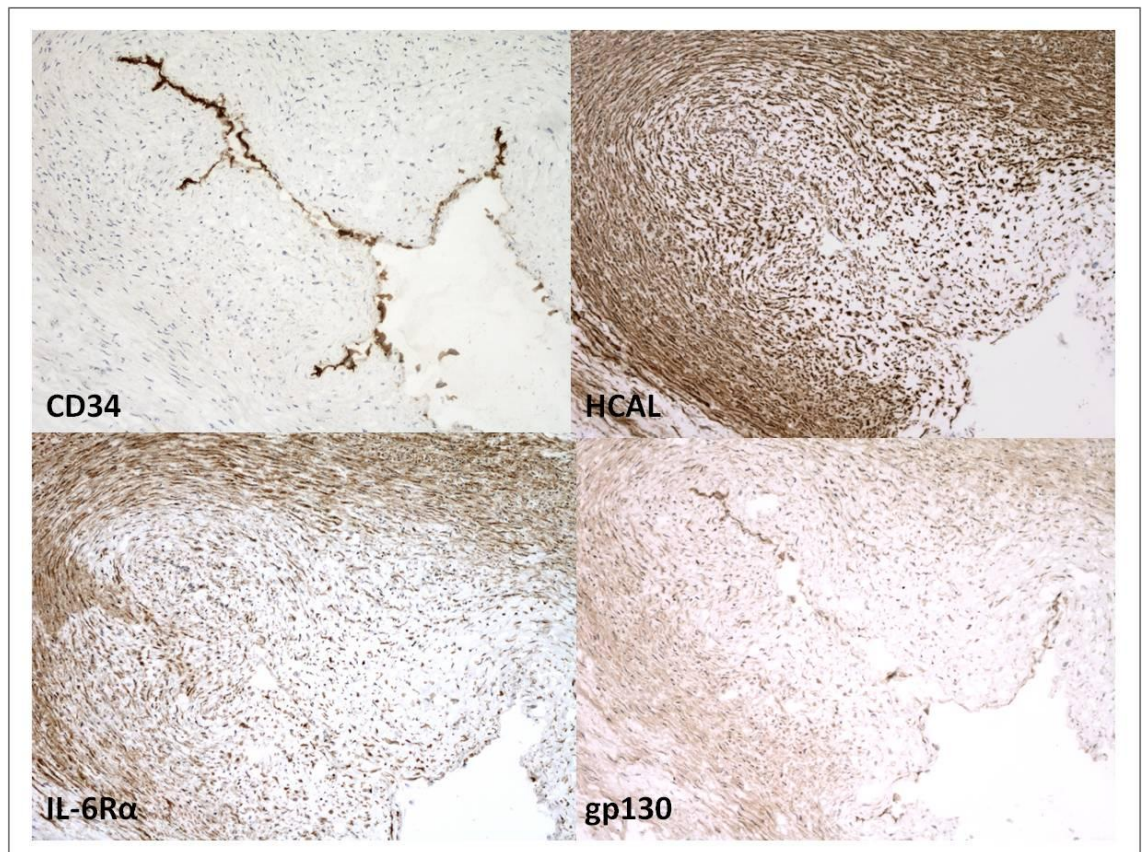
Figure 5.3 Evidence of IL-6 receptor immunostaining on CpA VSMCs and endothelial cell lining

Representative chorionic plate artery immunostained for CD34 showing endothelial lining; H-caldesmon showing VSMCs, IL-6Rα and gp130. Magnification x100 (frozen sections).
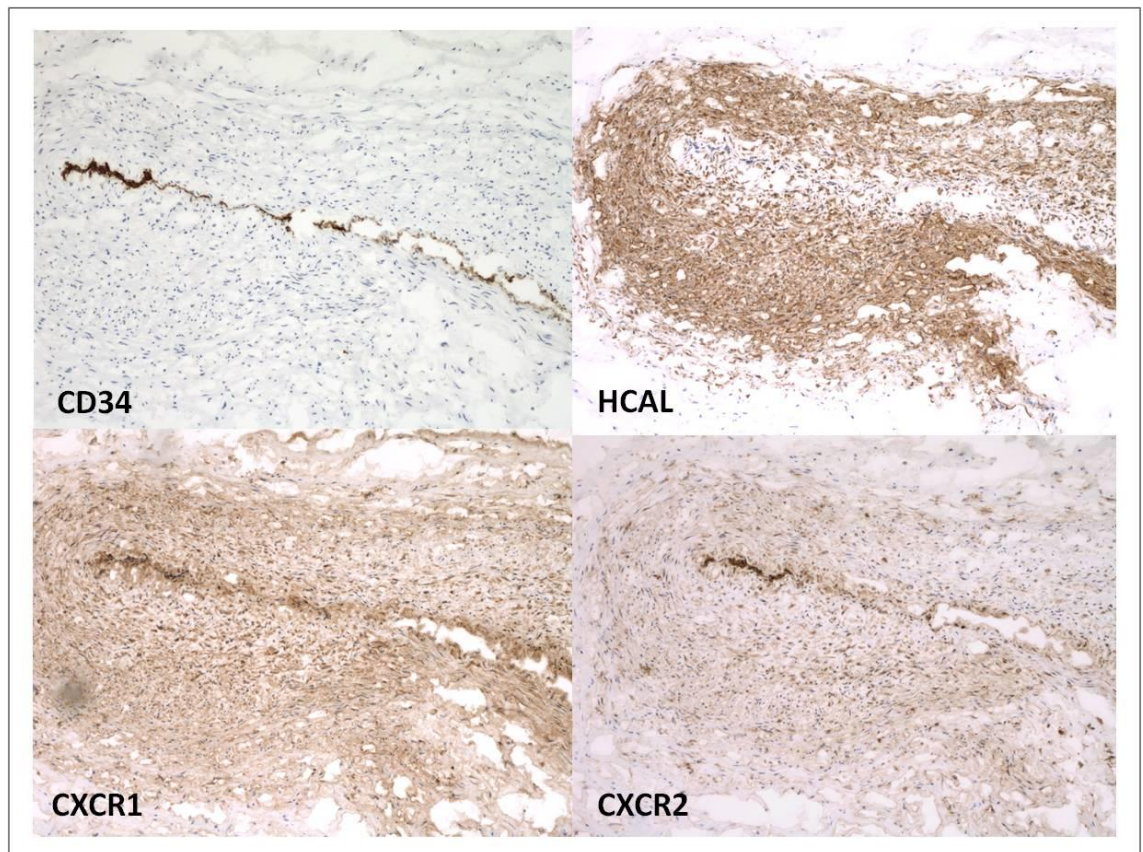
Figure 5.4 Evidence of IL-8 receptor immunostaining on CpA VSMCs and endothelial cell lining
Representative chorionic plate artery immunostained for CD34 showing endothelial lining; H-caldesmon showing VSMCs, CXCR1 and CXCR2. Magnification x100 (frozen sections).

5.4.2 Effect of IL-6 and IL-8 on the morphology of CpA VSMC using 10% fetal calf supplemented serum

Both IL-6 and IL-8 receptors were expressed on VSMC and/or endothelial cells of CpAs (section 5.4.1). Therefore, these cytokines have the ability to bind to these cells and may have an effect on the vascular changes in these vessels. Changes in CpA morphology were assessed on FFPE sections immunostained for myosin heavy chain using a four point scoring scale (Figure 5.2), after culture with exogenous IL-6 and IL-8 in RPMI complete medium (T=5 days). Using this scoring method no morphological changes were observed when the arteries were treated with 1ng ml^{-1} or 10ng ml^{-1} of IL-6 (Figure 5.5; n=6) or IL-8 (Figure 5.6; n=6).

To validate the four point scoring scale an overall quick score of VSMC rounding, separation of VSMC layers and the misalignment of VSMCs was also performed on all samples. Individual scores for rounding, separation and misalignment were added together and divided by 3 for each vessel. Again using this scoring method neither IL-6 (Figure 5.5) nor IL-8 (Figure 5.6) had any effect the morphology of CpA VSMC.
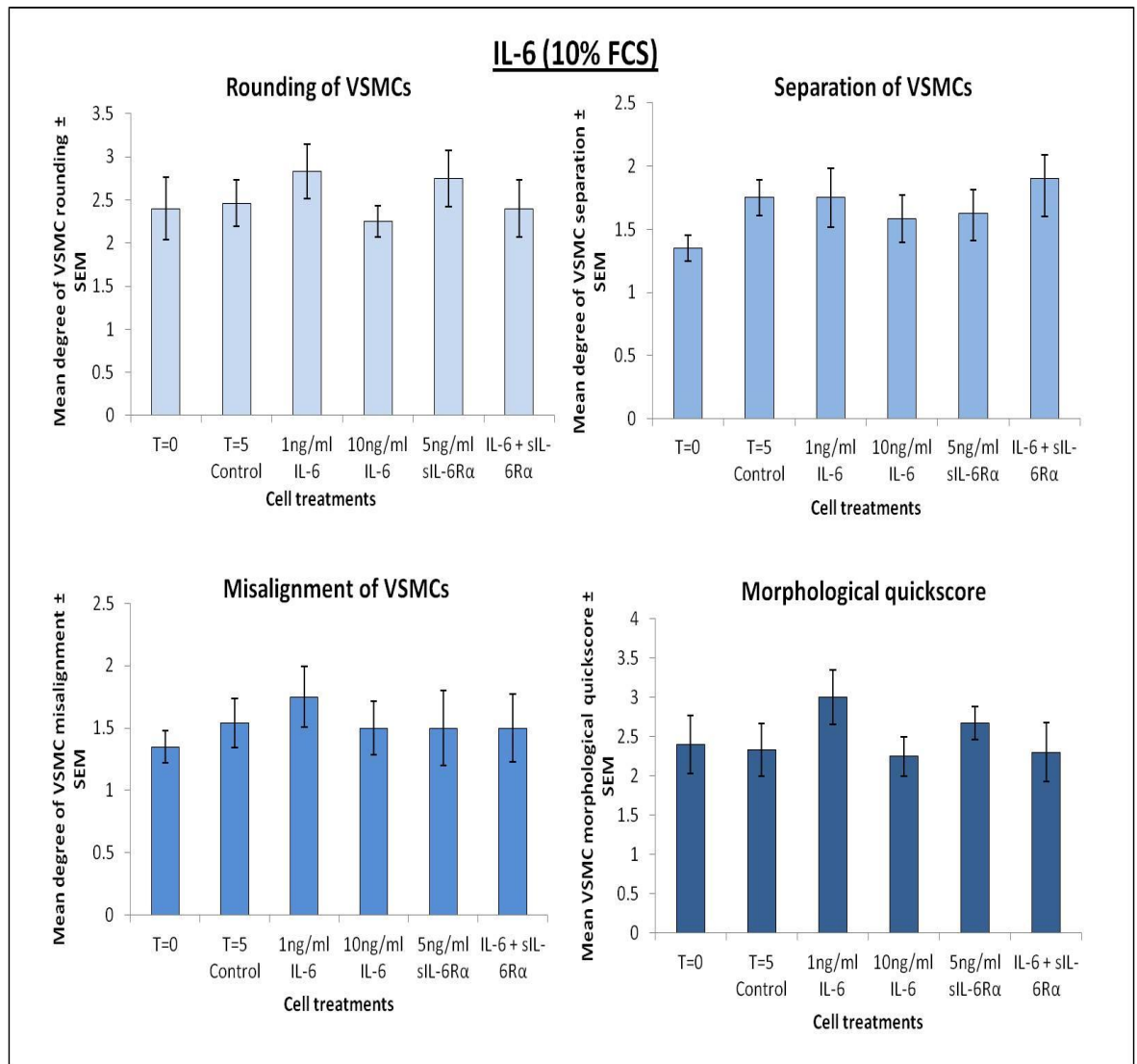
Figure 5.5 The effect of IL-6 on CpA VSMC morphology
Graphical representations showing the effect of differing concentrations of exogenous IL-6 on the morphological changes of VSMCs of chorionic plate arteries after 5 days of culture. Changes were assessed by myosin heavy chain immunohistochemical assessment with control treatments using a four point scale (n=6). An overall morphological quick score was also assessed. Data are expressed as mean ± SEM.
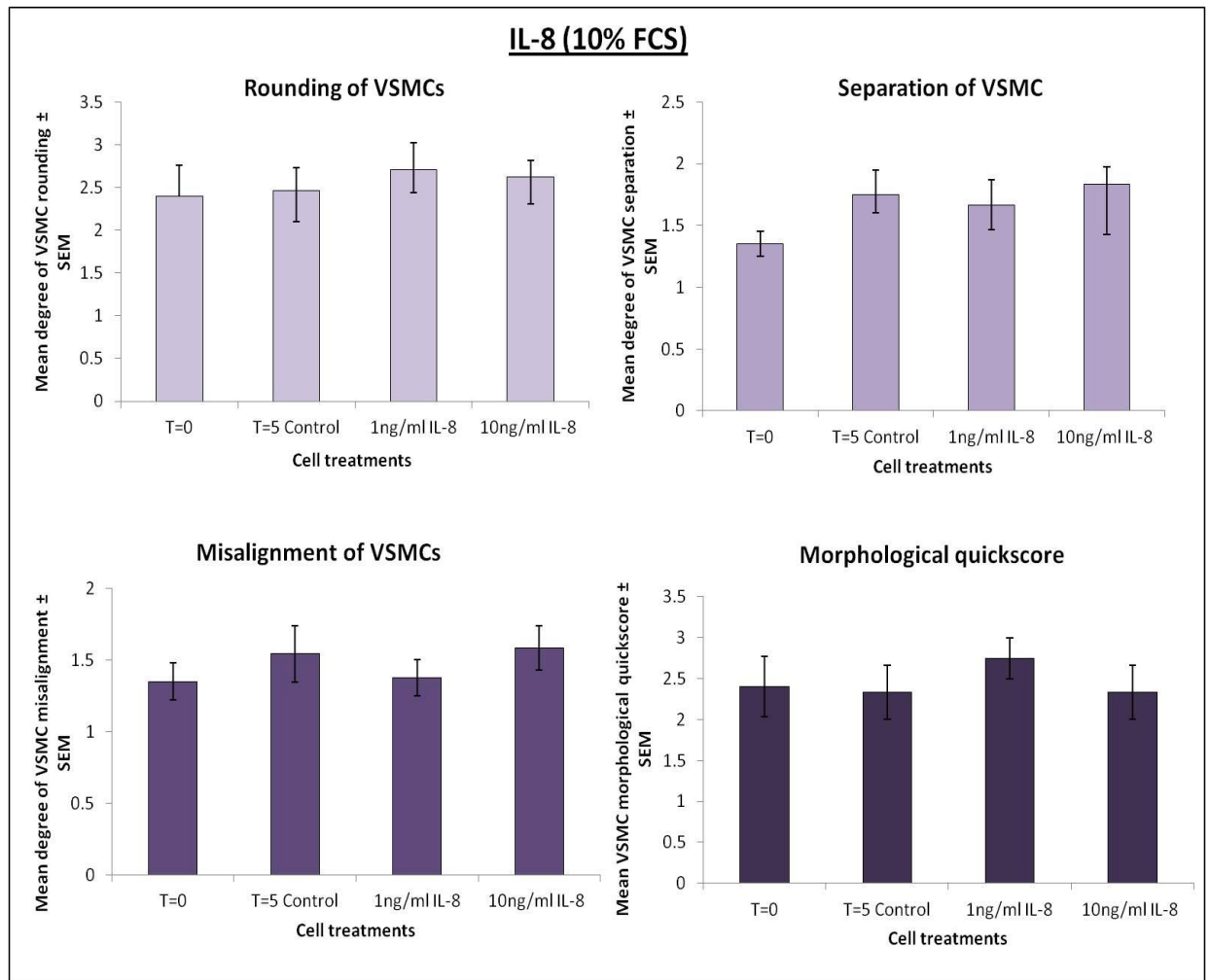
Figure 5.6 The effect of IL-8 on CpA VSMC morphology
Graphical representations showing the effect of differing concentrations of exogenous IL-8 on the morphological changes of VSMCs of chorionic plate arteries after 5 days of culture. Changes were assessed by myosin heavy chain immunohistochemical assessment with control treatments using a four point scale (n=6). An overall morphological quick score was also assessed. Data are expressed as mean ± SEM.

5.4.3 Effect of IL-6 and IL-8 on the differentiation of VSMC muscle markers using 10% fetal calf supplemented serum

A characteristic feature of phenotypically modulated VSMCs is that they express markedly lower levels of muscle differentiation markers; staining for markers of differentiated VSMC should therefore decrease if the vessels are transforming.

The percentage of positive immunostaining for the muscle differentiation markers expressed on the CpA was assessed using an Adobe Photoshop™ pixel counting method (section 5.3.4). The percentage of positive immunostaining for different muscle markers did not alter after IL-8 treatment compared with media controls (Figure 5.8). Similarily, expression of muscle differentiation markers H-caldesmon, myosin heavy chain and calponin did not alter in CpA treated with IL-6, sIL-6Rα alone and IL-6 + sIL-6Rα combined. However, arteries treated with 10ng ml^{-1} IL-6, 5ng ml^{-1} sIL-6Rα and 10ng ml^{-1} IL-6 combined with 5ng ml^{-1} sIL-6Rα showed a significant decrease in α-smooth muscle actin expression compared with media control treated arteries (Figure 5.7; a: P=0.05; b: P=0.02; c: P=0.03, respectively).
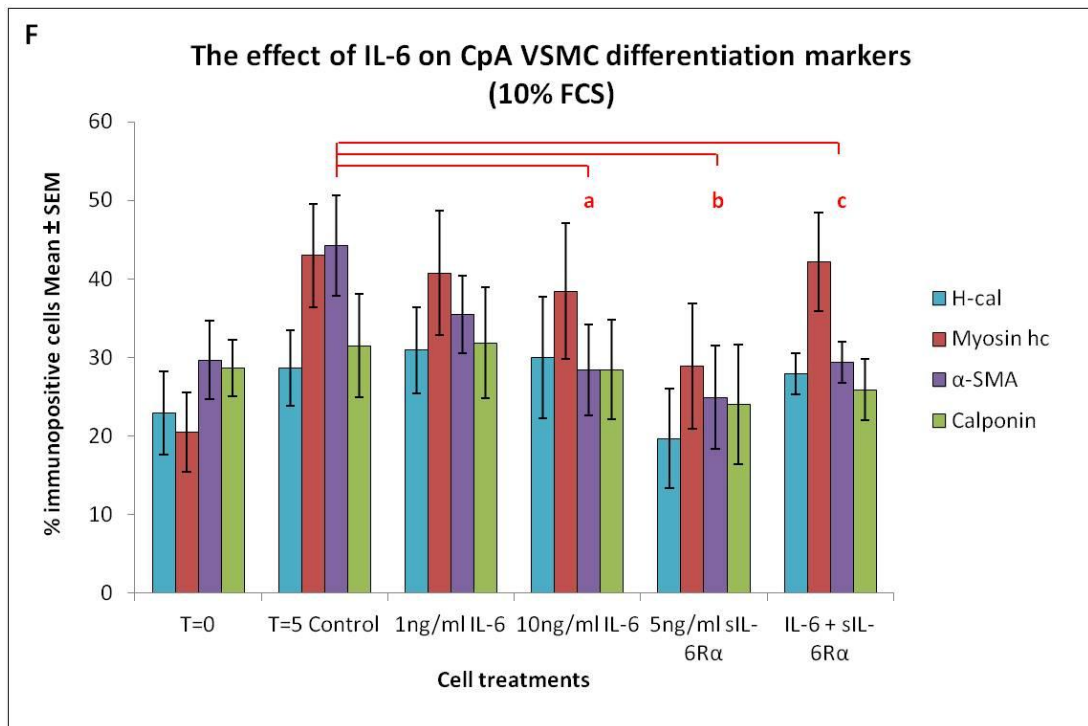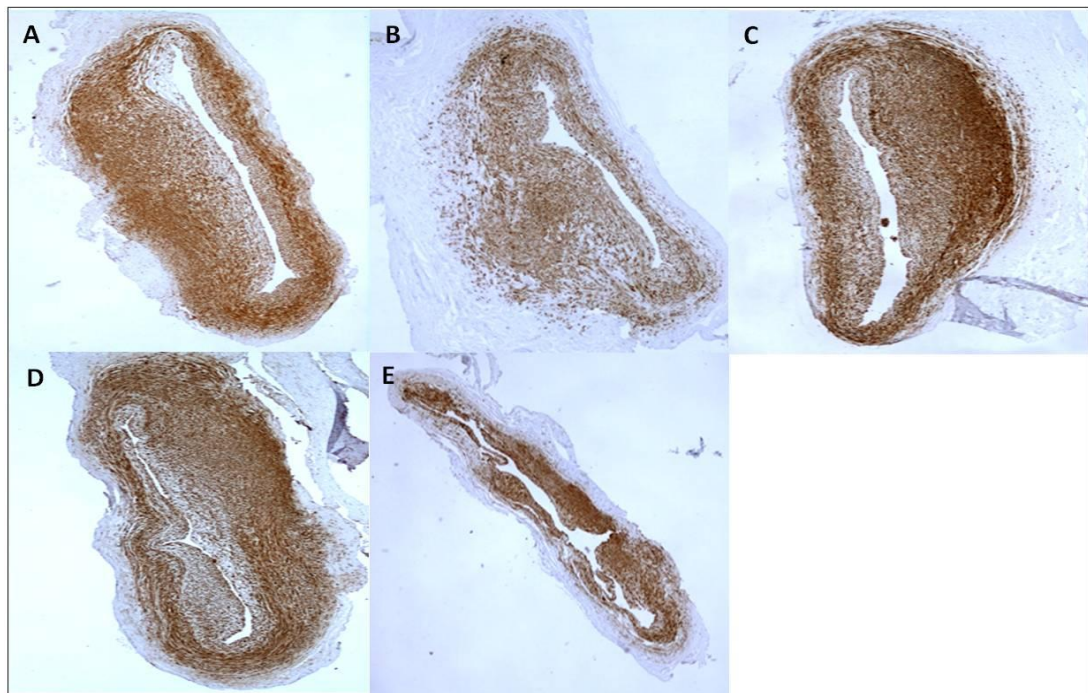
Figure 5.7 The effect of IL-6 on CpA VSMC differentiation when cultured with medium supplemented with 10% fetal calf serum (FCS)
Immunostaining for myosin heavy chain on VSMCs of chorionic plate arteries (CpA) from term placenta cultured for 5 days with (A): RPMI complete medium control; (B): 1ng ml^{-1} IL-6; (C): 10ng ml^{-1} IL-6; (D): 5ng ml^{-1} sIL-6Rα; (E): 5ng ml^{-1} sIL-6Rα + 10ng ml^{-1} IL-6 combined (magnification x40). (F): Graphical representation of the effect of IL-6 on muscle differentiation markers (H-caldesmon, myosin heavy chain, α-smooth muscle actin and calponin; n=6). Data are expressed by mean ± SEM; a, b, c denotes statistical significance compared with T=5 control P<0.05
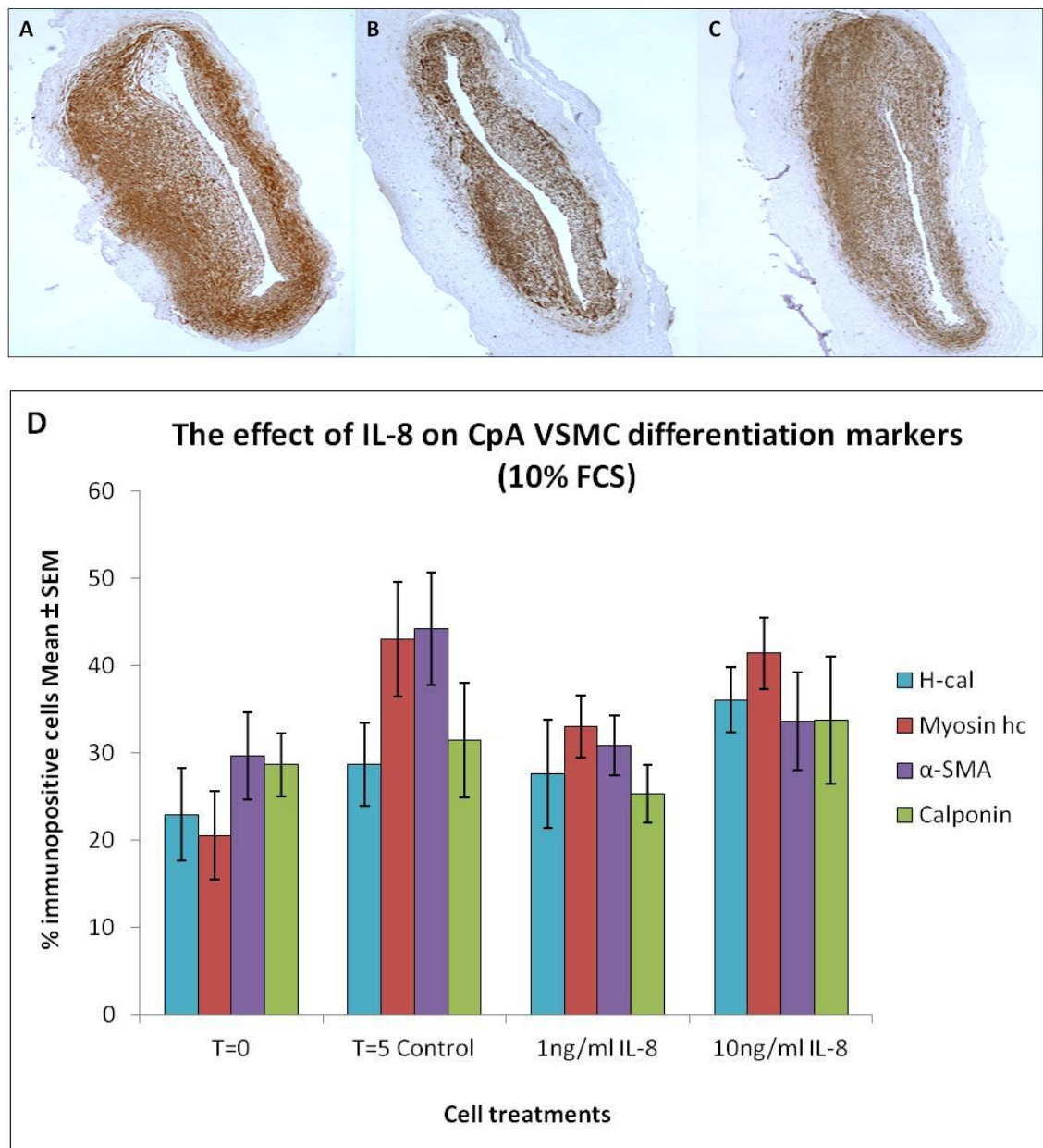
Figure 5.8 The effect of IL-8 on CpA VSMC differentiation when cultured with medium supplemented with 10% FCS

Myosin heavy chain immunostaining on VSMCs of chorionic plate arteries (CpA) from term placenta cultured for 5 days with (A): RPMI complete medium control; (B): 1ng ml^{-1} IL-8; (C): 10ng ml^{-1} IL-8 (magnification x40). (D): Graphical representation of the effect of IL-8 on muscle differentiation markers (H-Caldesmon, myosin heavy chain, α-smooth muscle actin and calponin; n=6). Data are expressed as mean ± SEM.

5.4.4 Dose response to sera- changes in VSMC morphology and degradation of endothelium

Following 5 days incubation arteries cultured in medium containing no fetal calf serum appeared to have lost the luminal endothelium as assessed by immunohistochemistry for CD34$^+$ cells compared with vessels treated with 10% FCS supplemented medium, regardless of cytokine treatment and there was also more apoptosis evident in the endothelium shown by the presence of positive immunostained active caspase 3 (Figure 5.9). The VSMC layers were more separated and cells in the connective tissue surrounding the lumen appeared vacuolated (Figure 5.9). These data suggest that substances within the fetal calf serum are important for vascular integrity. This was also observed using 0.5% fetal calf serum. VSMC morphology appeared similar using the same concentrations of the two different fetal calf sera (normal and charcoal stripped) without addition of IL-6. However, CpA cultured in 10ng ml^{-1} IL-6 with 0.5% carbon stripped fetal calf serum showed stronger Ki67 staining and hence more proliferation, compared with CpA cultured in normal fetal calf serum. Furthermore, arteries treated with 10ng ml^{-1} IL-6 and 10% charcoal stripped FCS showed differences in VSMC morphology from a rounded shape to a spindle shape with separation between VSMC layers indicating that the CpA VSMCs were more responsive to IL-6 compared to when treated with IL-6 cultured in normal FCS (Figure 5.10). No changes in apoptosis were observed using the different sera. We therefore, chose to use a concentration of 5% charcoal stripped fetal calf supplemented medium for the cytokine treatment experiments was sufficient for a morphological change as 0.5% charcoal stripped FCS had no effect on IL-6 whereas 10% charcoal stripped FCS did.
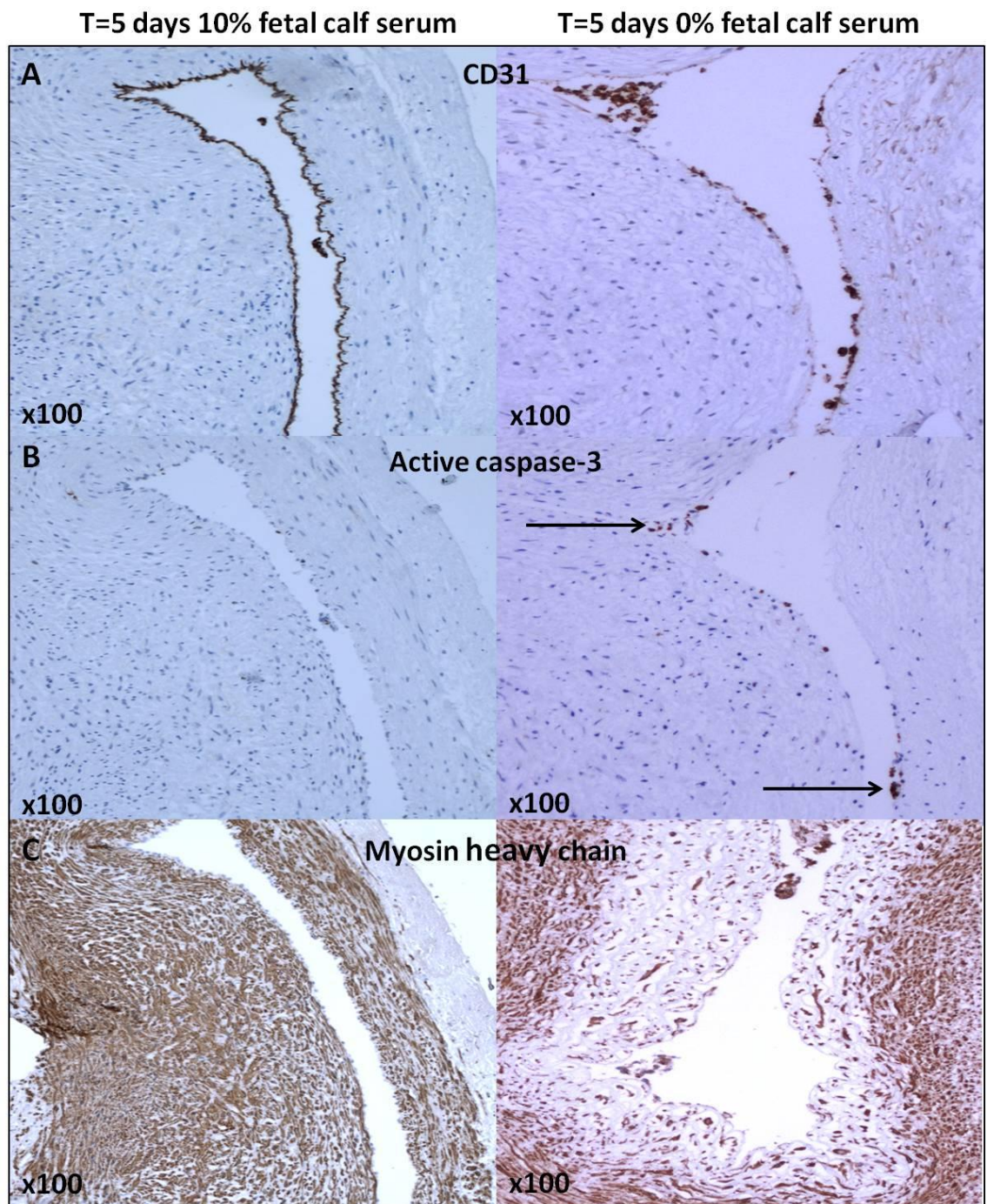
Figure 5.9 Comparison of culturing CpA in medium supplemented with 10% FCS compared with culturing CpA in medium without FCS
Photomicrograph illustrating immunostaining for CD31, active caspase-3 and myosin heavy chain demonstrating: A) The endothelium lining the lumen of term CpA; B) The level of apoptosis of the endothelium and C) VSMC layers surrounding the lumen after 5 days incubation (T=5). Left hand images represent CpAs cultured with 10% FCS supplemented medium and right hand images represent CpAs cultured in medium with no addition of FCS n=3. Original magnification x100.
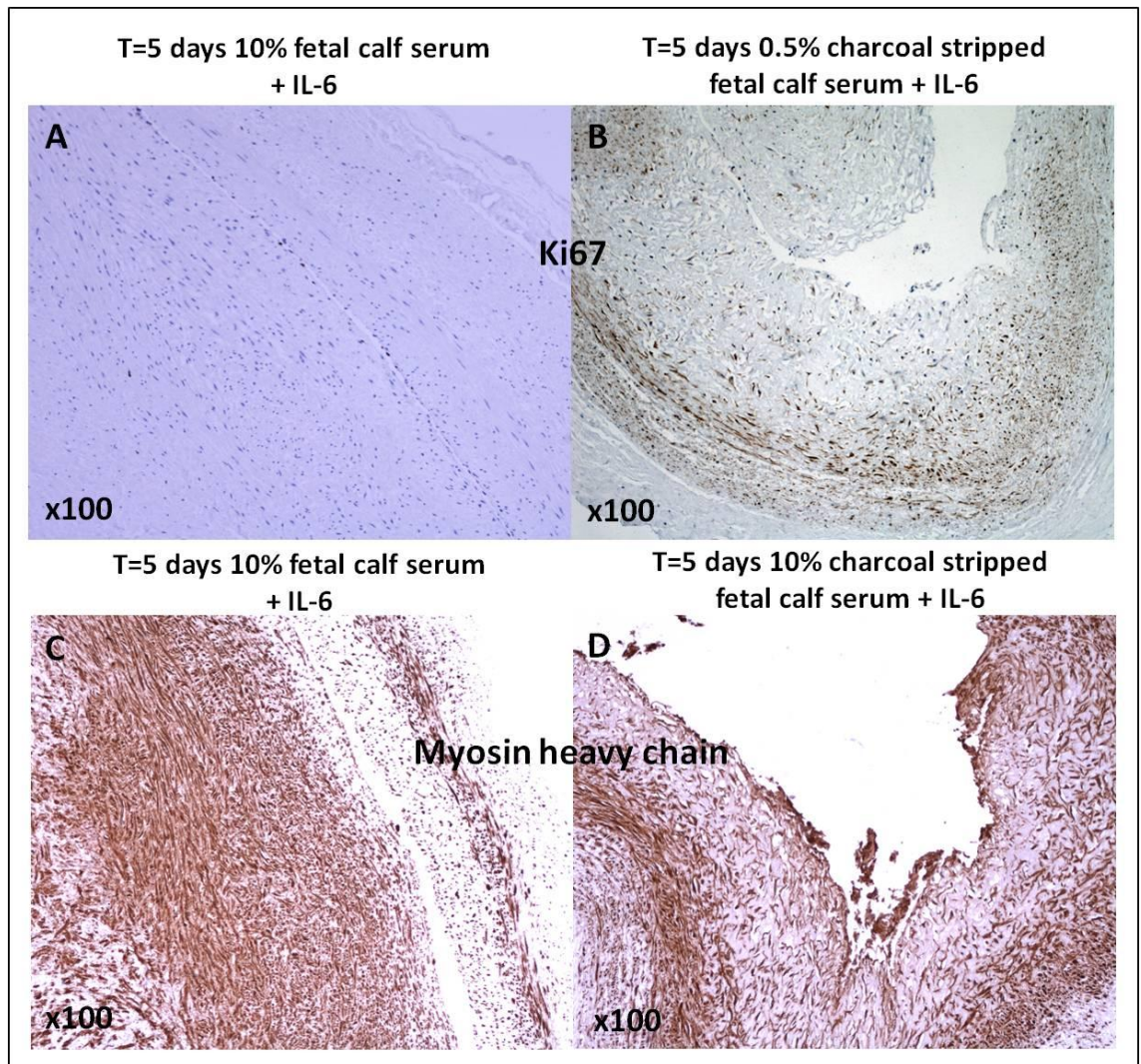
Figure 5.10 Comparison of culturing CpA in medium supplemented with 10% FCS compared with culturing CpA in medium supplemented with varying concentrations of charcoal stripped FCS with addition of IL-6

Photomicrograph illustrating Ki67 and myosin heavy chain positive immunostaining demonstrating: A and B) The degree of cell proliferation of term CpAs cultured in either medium containing 10% FCS + 10ng ml^{-1} IL-6 (A) or cultured in medium containing 0.5% charcoal stripped FCS + 10ng ml^{-1} IL-6 (B); C) VSMC morphology of CpA treated medium containing 10% FCS + 10ng ml^{-1} IL-6; D) CpA treated with medium containing 10% charcoal stripped FCS + 10ngml^{-1} IL-6 after 5 days incubation (T=5, n=3). Original magnification x100.

5.4.5 Effect of IL-6 and IL-8 on the morphology of CpA VSMC using 5% charcoal stripped fetal calf supplemented serum

Following the results from the dose response of CpAs to charcoal stripped fetal calf serum supplemented medium (section 5.4.4), chorionic plate arteries were cultured with exogenous cytokine made up in RPMI media supplemented with 5% charcoal stripped FCS (section 5.3.5) and VSMC morphology was assessed using a four point scale (Figure 5.2).

Treatment with IL-6 had no effect on CpA VSMC rounding but did, however, have an effect on the separation of VSMC layers and the alignment of VSMCs within the vessel (Figure 5.11A). Treatment of CpA with 10ng ml^{-1}IL-6 and 10ng ml^{-1} IL-6 + 5ng ml^{-1} sIL-6Rα significantly increased separation of VSMC layers compared with the 5 day media control (P=0.02; P=0.001 respectively). In contrast, only IL-6 + sIL-6Rα affected the misalignment of VSMC compared with the 5 day media control (P=0.04; Figure 5.11B).

Treatment of CpA with IL-8 had no effect on the rounding or separation of VSMC but did have a significant effect on the misalignment of VSMCs compared with the 5 day media control (P=0.001) (Figure 5.12).

A)
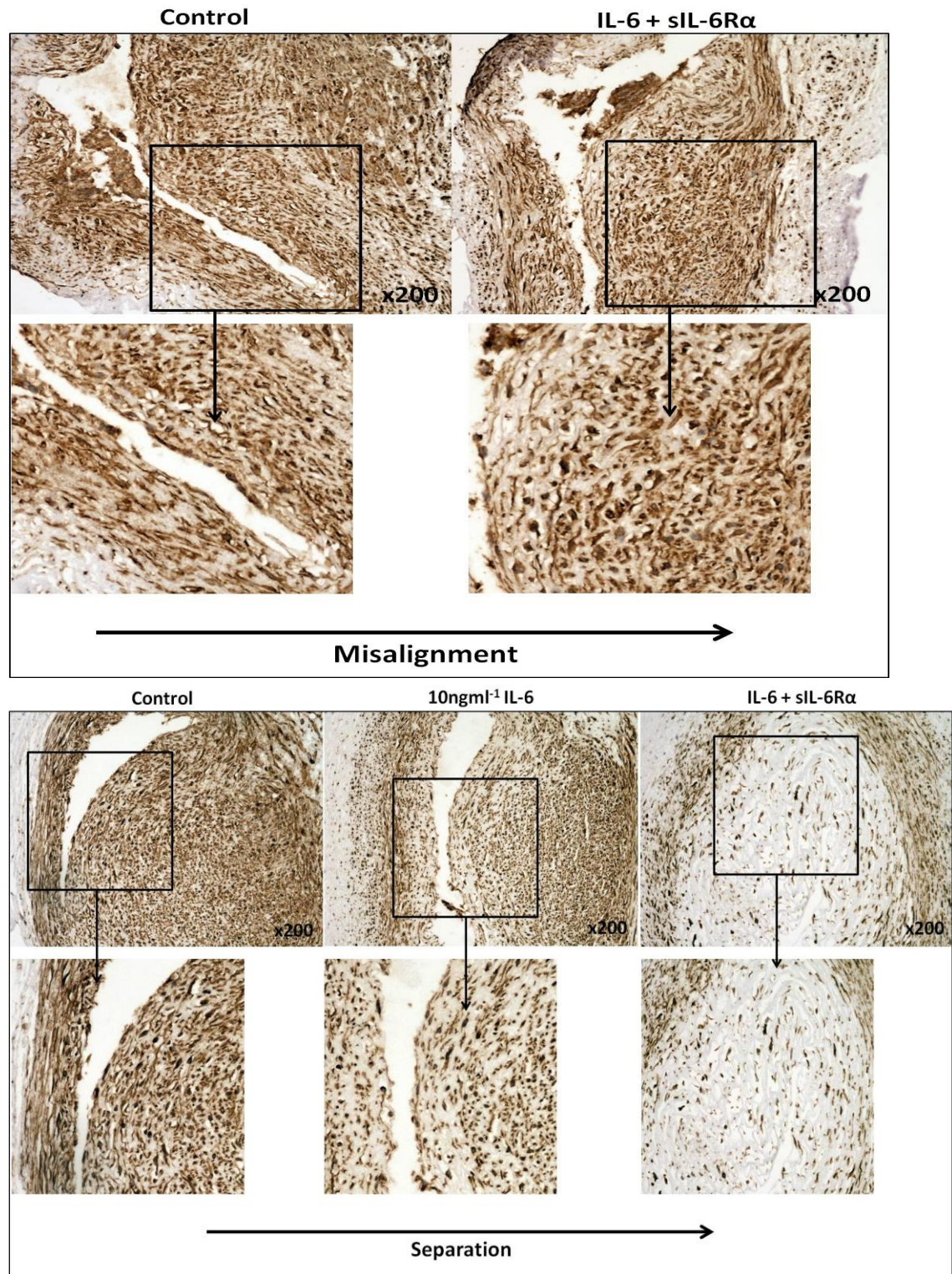
Figure 5.11 The effect of IL-6 on CpA VSMC morphology when cultured with medium supplemented with 5% charcoal stripped FCS
A) Photomicrogragh illustrating the effect of differing concentrations of exogenous IL-6 on the morphology of VSMCs of chorionic plate arteries after 5 days of culture using RPMI medium supplemented with 5% charcoal stripped fetal calf serum (cs FCS). Changes were assessed by myosin heavy chain positive immunostaining compared with control treatments using a four point scale (n=5). Original magnification x200.

B)
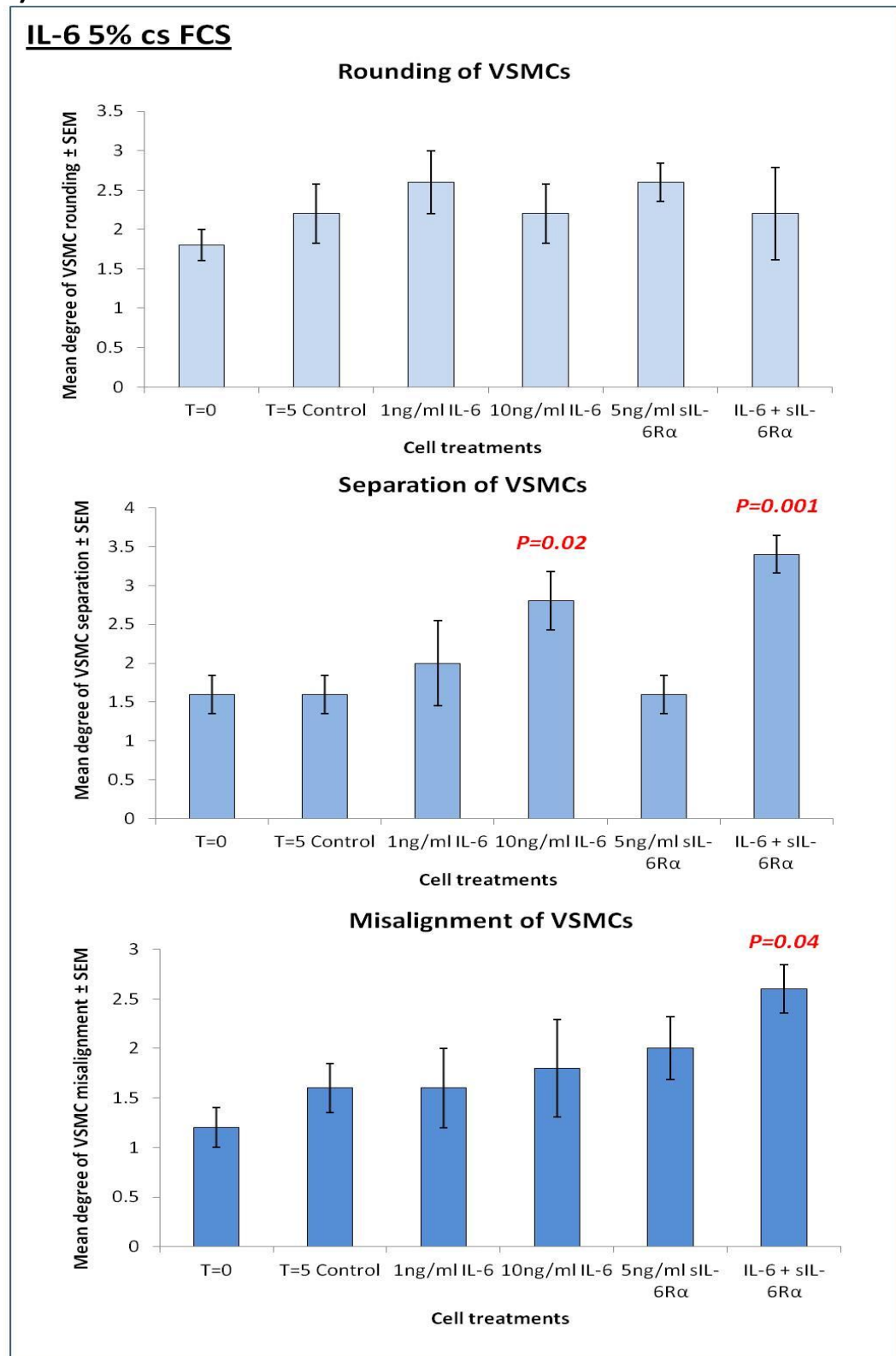
B) Graphical representation showing the effect of differing concentrations of exogenous IL-6 on the morphological features of VSMCs of chorionic plate arteries after 5 days of culture using RPMI medium supplemented with 5% cs FCS. Changes were assessed by myosin heavy chain immunohistochemical assessment with control treatments using a four point scale (n=5). Data are expressed as mean ± SEM.
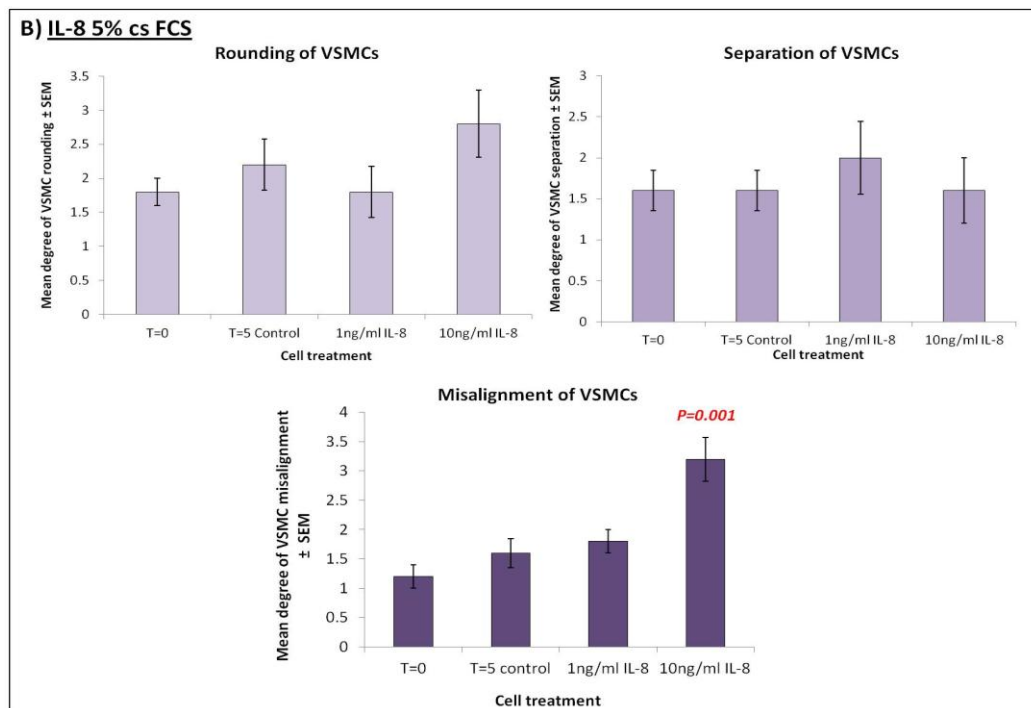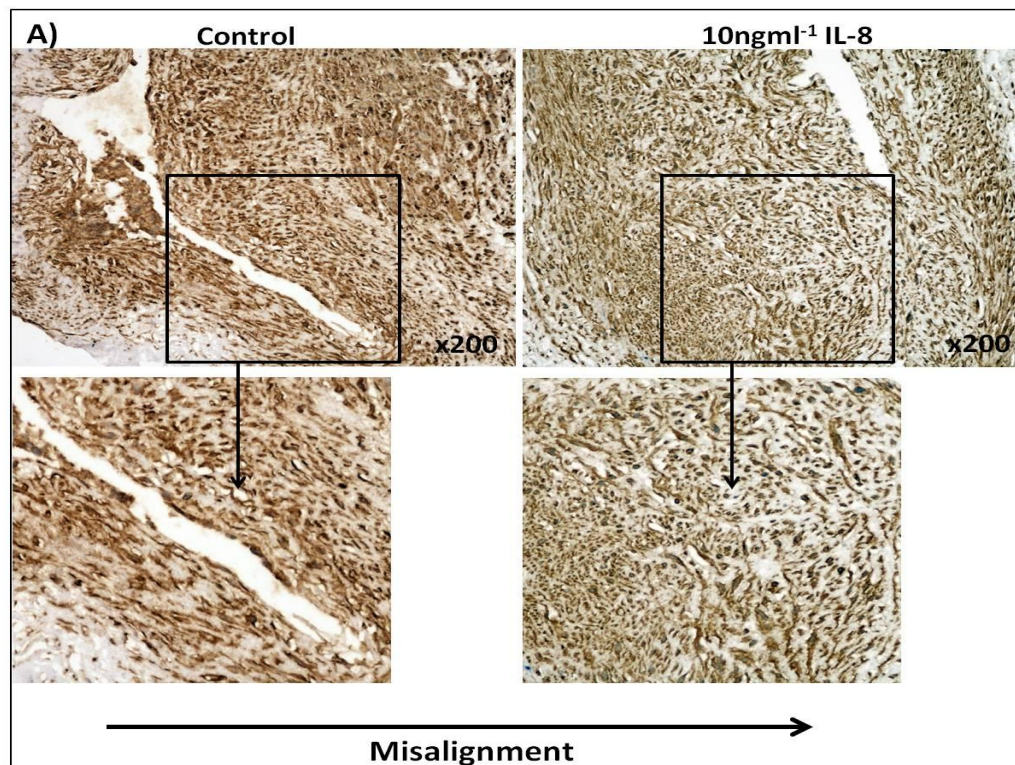
Figure 5.12 The effect of IL-8 on CpA VSMC morphology when cultured with medium supplemented with 5% charcoal stripped FCS

Immunohistochemical (A) and graphical (B) representation illustrating the effect of differing concentrations of exogenous IL-8 on the morphological features of VSMCs of chorionic plate arteries after 5 days of culture using RPMI medium supplemented with 5% cs FCS. Changes were assessed by myosin heavy chain immunohistochemical assessment compared control treatments using a four point scale (n=5). Data are expressed as mean ± SEM. Original magnification x200.

5.4.6 Effect of IL-6 and IL-8 on VSMC differentiation markers using 5% charcoal stripped fetal calf serum supplemented medium

The percentage of positive immunostaining for the muscle differentiation markers expressed on the CpA was assessed using an Adobe Photoshop™ pixel counting method (section 5.3.4).

The percentage of positive immunostaining of different VSMC markers did not differ after IL-6 treatment in 5% charcoal stripped FCS culture medium compared with medium controls (Figure 5.13). VSMC differentiation markers, H-caldesmon, myosin heavy chain and α-SMA expression did not alter in CpA treated with IL-8, although calponin expression increased in CpA VSMCs after treatment with 1ng ml^{-1} IL-8 compared with the T5 day medium control (P=0.02; Figure 5.14).
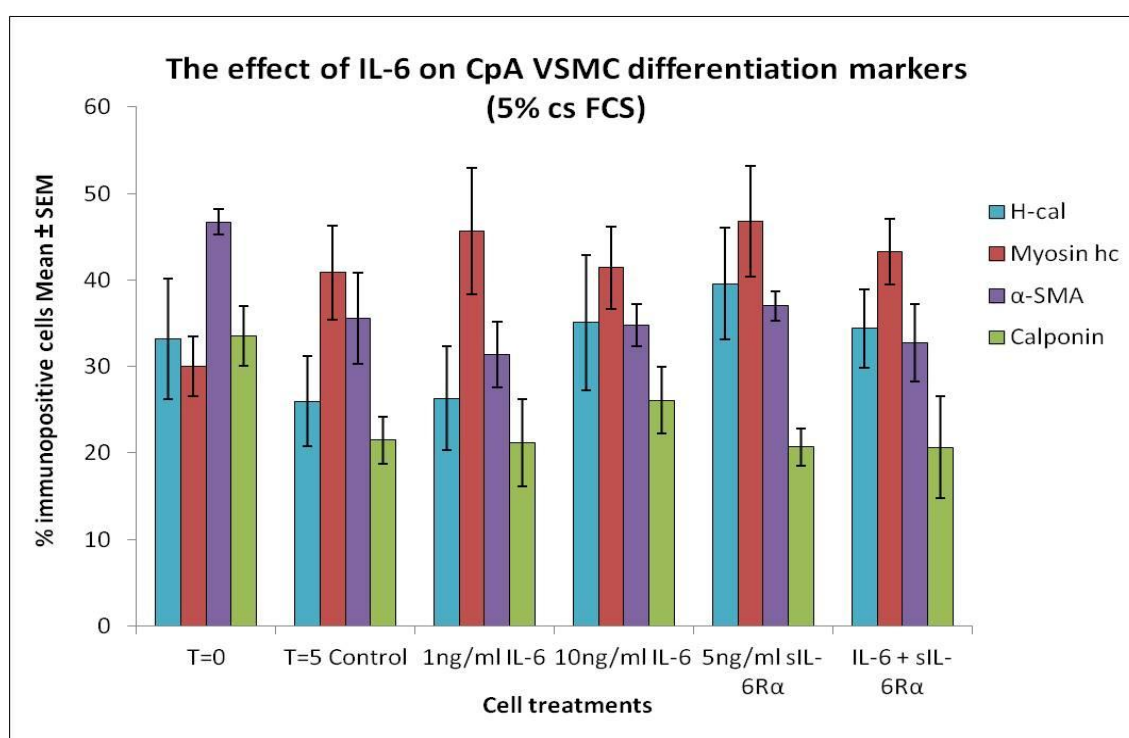
Figure 5.13 The effect of IL-6 on CpA VSMC differentiation when cultured with medium supplemented with 5% charcoal stripped FCS
Graphical representation of the effect of IL-6 (in RPMI medium supplemented with 5% cs FCS) on VSMC differentiation markers (H-caldesmon, myosin heavy chain, calponin and α-smooth muscle actin; n=5). Data are expressed as mean ± SEM.
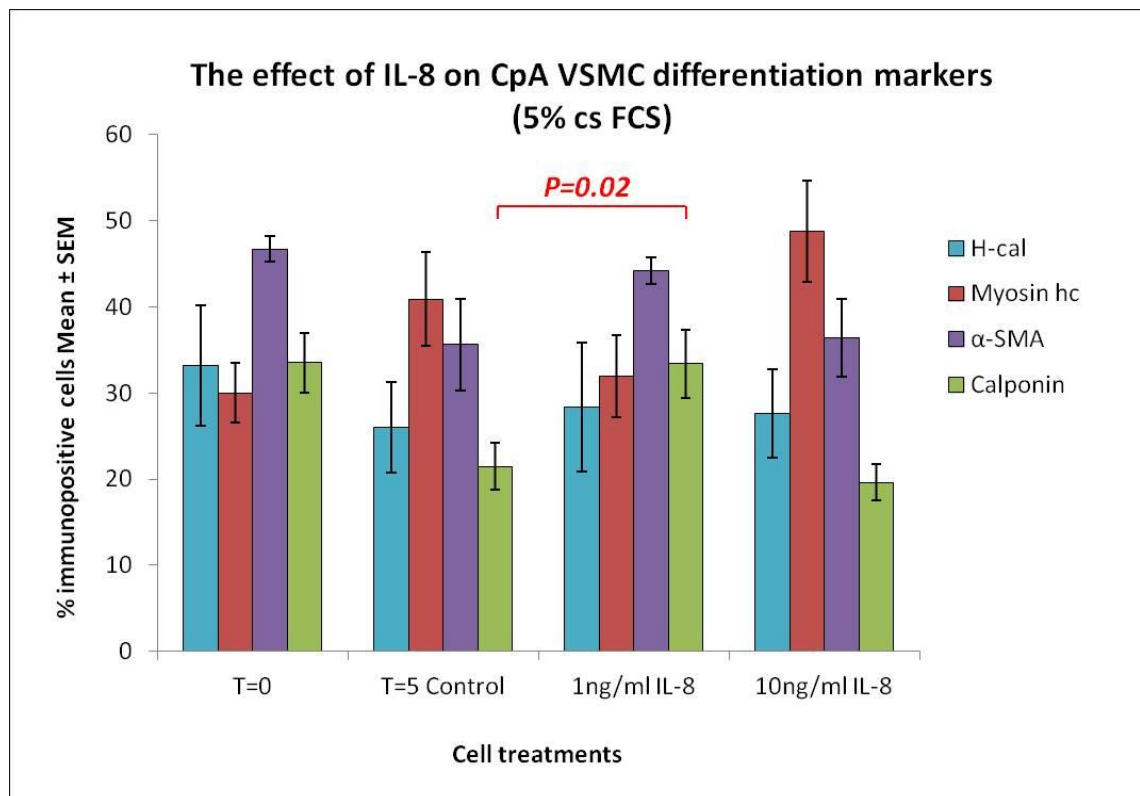
Figure 5.14 The effect of IL-8 on CpA VSMC differentiation when cultured with medium supplemented with 5% charcoal stripped FCS
Graphical representation of the effect of IL-8 (in RPMI medium supplemented with 5% csFCS) on VSMC differentiation markers (H-caldesmon, myosin heavy chain, calponin and α-smooth muscle actin; n=5). Data are expressed as mean ± SEM.

5.5 Discussion

5.5.1 IL-6 and IL-8 signalling in CpA VSMCs

The receptors forIL-6 (sIL-6Rα and gp130) and IL-8 (CXCR1 and CXCR2) were expressed by chorionic plate arteries of term human placenta. All of the receptors were expressed on the VSMCs; IL-6R and CXCR1 were predominantly expressed on VSMCs whereas gp130 and CXCR2 were mainly expressed on the vascular endothelium lining the lumen.

Therefore, both IL-6 and IL-8 have the ability to bind to both VSMCs and endothelial cells of chorionic plate arteries and elicit a response which may contribute to vascular changes within the vessel, hence disrupting integrity and stability of the artery. IL-6 is a potent pro-angiogenic cytokine and plays important roles in the progression of tumour neovascularisation, wound healing and vasculogenesis in the brain (Fee et al., 2000). IL-6 also stimulates circulating blood endothelial progenitor cell proliferation, migration and matrigel tube formation *in vitro* through binding to its receptors: IL-6Rα and gp130 (Fan et al., 2008); this could contribute to *de novo* vessel formation during wound healing.

IL-8 is known to be a potent mediator of angiogenesis. IL-8 signalling has been associated with the regulation of endothelial permeability by stimulating the phosphorylation of VEGF-R2 in endothelial cells by interaction with both CXCR1 and CXCR2 receptors (Petreaca et al., 2007) and more recently it has been found that IL-8 up regulates VEGF-A mRNA and protein levels in vascular endothelial cells by association of IL-8 with CXCR2, which further stimulates VEGF-R2 in an autocrine manner (Martin et al., 2009) inducing cellular proliferation, survival and migration of vascular endothelial cells. These reports suggest an important role for IL-8 in angiogenesis and a possible role for IL-8 in the physiological remodelling events of the uterine vasculature in pregnancy. In pathophysiology IL-8 is important for tumour formation and growth, and in agreement with reports of an angiogenic role in normal physiology many *in vitro* studies with cancer cell lines and *in vivo* studies with solid cancers have shown that IL-8 corresponds to the angiogenic and tumourgenic potential of the progression of cancers (reviewed by (Waugh and Wilson, 2008)) indicating the correlation of IL-8 with tumour angiogenesis and disease.

5.5.2 Charcoal stripped fetal calf serum vs. Normal fetal calf serum

In the present study fetal calf serum appears to be an important component of cell culture medium for the survival of chorionic plate arteries *in vitro*. Without it vessel integrity rapidly began to show signs of degradation. However, in our initial assessment, the effects of particular cytokines on this vessel in vitro were masked by the use of fetal calf serum. Different concentrations and types of fetal calf serum were therefore assessed. The addition of fetal calf serum provides vital growth factors required for cell culture, lowering the FCS concentration in the culture medium used in this study or omitting it altogether had a morphological effect on CpA VSMCs; the vessel integrity appeared to have been effected as the vessel lumen was highly vacuolated and vessel structure overall appeared different.

Charcoal stripped serum allowed both IL-8 and IL-6 to have an effect on VSMC morphology compared with using standard fetal calf serum. An explanation is that the stripping of hormones from the serum freed the exogenous cytokines to bind with VSMCs and therefore to elicit an effect not seen using standard fetal calf serum.

5.5.3 Effect of IL-6 on VSMC morphology

IL-6 was shown to have a significant effect on the separation of vascular smooth muscle cell layers and in the misalignment of these cells. IL-6 combined with its soluble agonist receptor sIL-6Rα, had the largest effect on VSMC separation and misalignment, suggesting that the binding of the cytokine coupled to its receptor elicits a more potent cell signalling response resulting in larger changes in the vasculature compared with controls, IL-6 or sIL-6Rα alone. This signalling response up-regulates the expression of STAT-3, which is known to enhance the transcription of the vascular endothelial growth factor (VEGF-A) gene. *In vitro* studies using gastric carcinoma tissues have shown IL-6 stimulates the production of VEGF-A in a dose/time dependent manner and that the JAK/STAT pathway is predominately involved in the transcription of VEGF-A in these cells (Huang et al., 2004) contributing to the tumourgenesis and progression of this disease.

Activation of the IL-6 signalling pathways (JAK/STAT and MAPK ERK1/2 pathways) plays a crucial role in endothelial proliferation, migration and tubule formation. However, in

circulating blood endothelial progenitor cells only a high level of IL-6 induced an angiogenic response (Fan et al., 2008). Similarly in the present study lower concentrations of IL-6 had little effect on VSMC morphology.

VEGF-A is one of the most potent angiogenic growth factors involved in vessel regrowth and neovasculogenesis. It promotes VSMC and endothelial cell migration, tubule formation and increases the permeability of blood vessels (Leung et al., 1989), characteristics also involved in successful spiral artery remodelling. Recent studies have also demonstrated that gliomas targeted with a combined treatment of anti-VEGF-A and anti-IL-6 showed markedly reduced growth as they were unable to undergo angiogenesis (Saidi et al., 2009), thus suggesting that these factors are essential for angiogenesis and that IL-6 stimulates VEGF-A production. IL-6 has the capacity to bind to blood vessels in vascularisation and angiogenesis through binding with IL-6Rα expressed by endothelial cells and VSMCs, which would potentially increase VEGF production resulting in vessel de-stabilisation by the migration of VSMCs and making vessels more permeable. In respect to spiral arteries, increased vessel permeability would allow the infiltration of essential factors such as leucocytes and trophoblast cells which are required for successful remodelling to ensure an adequate blood supply to the developing fetus.

5.5.4 Effect of IL-8 on VSMC morphology

IL-8 had a significant effect on the organisation of VSMC layers within CpAs but did not appear to have any other morphological effects on VSMC.

IL-8 is a chemotactic cytokine and a potent angiogenic agent as mentioned previously. IL-8 has been reported to induce the proliferation and chemotaxis of human umbilical vein endothelial cells (HUVECs) (Koch et al., 1992) and to stimulate prostaglandin secretion by human aortic and rat aortic VSMCs *in vitro* resulting in VSMC proliferation and migration (Yue et al., 1994). In the present study the observed disorganisation of VSMCs in CpA is the effect of IL-8 and the disorganisation may possibly be a sign of VSMC migration indicating that IL-8 acts as a chemoattractant for the VSMC. However, increased spacing between the VSMC layers was not observed; this may have been observed with increased concentrations of IL-8 since the misalignment of cells appeared to increase in a dose dependent manner. The results observed using CpAs

indicate that IL-8 has the potential to be major player in vascular changes in a variety of different blood vessels, which in turn suggests that physiological alterations of levels of IL-8 could also contribute to the pathogenesis of diseases such as atherosclerosis and a variety of cancers by aiding tumourgenesis. Together with the data from this study and its reported effects in tumourgenesis, uterine IL-8 may have an effect on uterine spiral arteries contributing to the vascular remodelling required for successful pregnancy. This could be achieved by VSMC and endothelial cell migration allowing the invasion of trophoblast cells and deposition of fibrinoid resulting in a low resistance high flow vessel. The molecular mechanisms whereby IL-8 achieves this in CpAs remain to be explored but IL-8 signalling could up-regulate other angiogenic factors including prostaglandins and recruit other cells to the vessel wall which secrete other growth factors which may contribute to vascular remodelling.

Recently, IL-8 has been shown to have a direct effect on EVT invasion (De Oliveira et al., 2010) by binding to its receptors expressed by EVT. The binding of IL-8 to EVT may also have an indirect effect of spiral artery remodelling by up-regulating the secretion of factors by EVT such as TRAIL (TNF-α-related apoptotic induced ligand); this is expressed by primary first trimester CTB cultured *in vitro* and by EVT *in situ*, and that CTB use the TRAIL pathway to induce smooth muscle cell apoptosis (Keogh et al., 2007) although there is little evidence of VSMC apoptosis *in vivo* (Bulmer et al., 2012).

5.5.5 Effect of IL-6 on VSMC differentiation

A property of smooth muscle cells is that during injury and remodelling their phenotype may alter to a de-differentiated state. A decrease in VSMC differentiation marker proteins is a characteristic of vascular changes following injury; changes in VSMC differentiation state allow cells to proliferate, migrate and synthesise extracellular matrix around them. The transition of VSMCs from a differentiated phenotype to a dedifferentiated phenotype assists in the pathogenesis of diseases such as atherosclerosis and hypertension (Owens et al., 2004).

It has been presumed that a reduction in VSMC differentiation markers in phenotypically modulated VSMCs *in vivo* is mediated at transcription level, although there is no direct evidence to support this. A recent report provides *in vitro* evidence that micro RNA 145 (miR-145), abundant in vascular walls, is selectively expressed and

VSMC differentiation marker genes such as α-SMA, calponin, and myosin heavy chain are upregulated by miR-145 and are downregulated by a miR-145 inhibitor (Cheng et al., 2009). However more studies are needed to elucidate the genetic factors involved and it is likely that changes may be induced post transcriptionally by growth factors and cytokines such as IL-6.

Chorionic plate arteries treated with 10ngml^{-1} IL-6, 5ngml^{-1} sIL-6Rα and IL-6 + sIL-6Rα combined in media supplemented with 10% FCS showed a significant decrease in α-SMA expression compared with media controls. This suggests that IL-6 contributes to the phenotypic modulation of VSMC and therefore contributes to changes in the vessel characteristics as a reduction α-SMA is indicative of differentiated VSMC. However, α-SMA is a marker of early VSMC differentiation and if IL-6 was mediating de-differentiation as a feature of remodelling the vasculature, alterations in expression of myosin heavy chain and H-caldesmon would have also been expected. Alternatively, IL-6 could play a role in preventing immature VSMCs from differentiating into mature cells; however, vascular smooth muscle differentiation is a complex process and many other factors may also be involved. In support of this complexity, the results using 5% charcoal stripped supplemented media differed and no loss of α-SMA expression was observed after treatment with IL-6. This indicates that components in FCS combined with IL-6 have some effect on VSMC differentiation whereas the lack of hormones and other factors in charcoal stripped FCS has none suggesting a role for serum proteins in the phenotypic modulation of VSMCs.

5.5.6 Effect of IL-8 on VSMC differentiation

Vessels treated with IL-8 medium supplemented with 10% FCS showed no significant changes in expression of differentiation markers compared with controls. However, calponin expression was significantly increased after treatment with 1ngml^{-1} IL-8 using 5% charcoal stripped FCS medium, although this was not observed using higher concentrations of IL-8. Calponin is an actin and calmodulin binding protein (Takeuchi et al., 1991) which is involved in the contractility of smooth muscle cells (Winder et al., 1993) and accumulates during smooth muscle differentiation (Gimona et al., 1990). However, if IL-8 was to cause VSMC differentiation it would be expected that calponin expression would increase with increasing concentration. However, low doses of IL-8

may increase calponin expression which binds Ca^{2+} allowing vascular smooth muscle to contract and may contribute to the misalignment of cell layers. However, this difference may be a direct result of multiple comparisons but a one way ANOVA with post hoc test was used which should correct this. Multiple comparisons within the analysis of data may account for the chance of a significant difference in the data. This may be apparent here as no differences in calponin expression were observed when CpAs were treated with higher concentrations of IL-8. The underlying mechanisms involved require further investigation with correctional statistics like a Bonferroni test.

5.5.7 Overall summary

The results from this study have indicated that both IL-6 and IL-8 have the ability to bind to blood vessels including chorionic plate arteries from term placenta, by the expression of their receptors on VSMC and endothelial cells. This further proposes that these cytokines have the ability to bring about vascular changes within this vessel which has been confirmed by changes in vessel morphology highlighted with immunohistochemistry. The results from this study and the fact that both IL-6 and IL-8 have potent angiogenic properties both in inflammation and disease suggests that these are important mediators of vascular remodelling maybe through the mechanisms of VSMC migration and vascular permeability, although, the molecular mechanisms need further exploration. This evidence suggests that these cytokines have the ability to contribute to spiral artery remodelling in the uterus by these proposed mechanisms. However, the limitation to this study is that chorionic arteries are derived from the fetus and are not maternal in origin. The vessels were used as an easy accessible source of vessels as the dissection of spiral arteries from early pregnant decidua and myometrium are very difficult to obtain. The use of non-pregnant myometrial vessels as an alternative was a suggestion in this study, but ethical approval was not feasible within the timescale. Nevertheless, investigation using maternal vessels would be a better model to explore the cytokine effects in relation to spiral artery remodelling and have been used in a study by Robson et al. (2012) whereby the effect of uNK cell factors on these vessels were investigated. Further validation of these results relating to the uterine arteries would have been to treat the CpA vessels using isolated CD56$^+$ and CD14$^+$ cell supernatants (known producers of

uterine IL-8 and IL-6, see chapter 3) from first trimester decidua and inhibiting endogenous IL-8 and IL-6 to investigate their endogenous effects in this vessel type. From the present study it is possible to conclude that IL-6 and IL-8 contribute to vascular changes in chorionic plate arteries *in vitro* indicating that cells of the decidua that secrete these factors may play a role in spiral artery remodelling in early pregnancy.

Chapter 6

The role of decidual leucocytes in spiral artery remodelling

6. The role of decidual leucocytes in spiral artery remodelling

6.1 Introduction

Changes in vascular smooth muscle cell (VSMC) disorganisation and separation of VSMC cell layers in chorionic plate arteries were observed after treatment with exogenous IL-6 and IL-8 (reviewed in Chapter 5). This study has demonstrated the production of intrauterine IL-6 and IL-8 by various cell types within decidua; predominantly by uterine macrophages but uterine natural killer cells, T cells and decidual stromal cells are all producers in early pregnancy (reviewed in Chapter 3).The data from chapters 3 and 5 combined have led to the hypothesis that decidual leucocytes play an important role in spiral artery remodelling in early pregnancy.

The prevalence and suggested roles of maternal leucocytes during pregnancy has been reviewed in Chapter 1. This chapter focuses on the decidual leucocytes; uNK cells, macrophages and CD3$^+$ T cells, and their early association with decidual spiral arteries, focusing on the possible roles of these leucocytes in the early remodelling events of the transformation of spiral arteries in early pregnancy prior to trophoblast invasion.

Understanding of the complexity of the stages of spiral artery remodelling is ever increasing but the cellular and molecular events remain yet to be defined. It is also apparent that compromised remodelling may lead to pregnancy complications such as pre-eclampsia and fetal growth restriction (IUGR), complications which appear clinically in the second trimester of pregnancy. However, it is likely that the aberrant processes occurring earlier in pregnancy play a role in complications seen later in pregnancy. Brosens et al. (1967) describe a physiological change in early pregnancy where spiral arteries transform from narrow, high resistance, low blood flow vessels into dilated, low resistance vessels capable of transmitting high blood flow to meet the increasingly demanding needs of the developing fetus. The process of delivering high volumes, of maternal blood at low resistance to the placental intervillous space is critical for successful healthy pregnancy. Remodelling of the arteries involves intricate steps from initial endothelial vessel vacuolation to loss of VSMCs and replacement of the vessel wall by fibrinoid material and invading extra villous trophoblast (EVT) cells.

For many years it was believed that invasive EVT cells were the main mediators of the spiral artery remodelling events by invading the vessel walls both interstitially from the surrounding decidua and myometrium and by invading the lumen of the arteries by an endovascular route (Pijnenborg et al., 1980). Some *in vitro* studies have suggested that EVT cells achieve such vascular destruction by enzymatic activity; they produce MMPs, actively cleaving components of the VSMC wall (Isaka et al., 2003, Jones et al., 2006). Isolated EVT cells have also been reported to be capable of inducing VSMC apoptosis (Harris et al., 2006, Keogh et al., 2007), although, *in situ* studies by (Bulmer et al., 2012) provide no evidence of VSMC apoptosis. Further evidence suggests that spiral artery remodelling events occur over many weeks and the initial stages involve mechanisms that are independent of trophoblast cell invasion. Prior to interstitial and endovascular trophoblast invasion (which occurs around week 8 of gestation) vascular changes that are characteristic to early SpA remodelling such as EC vacuolisation and VSMC hypertrophy have been observed in the absence of invading EVT (Craven et al., 1998, Kam et al., 1999). These trophoblast-independent changes have been described as decidualisation-associated vascular remodelling (Pijnenborg et al., 2006b) and it is now recognised that the initial stages of vascular remodelling may be regulated by immune cells within the decidua (King et al., 1998, Bulmer and Lash, 2005) and Smith et al. (2009) have observed high numbers of leucocytes in association with SpAs in the absence of EVT and have detected vascular changes *in vivo*. Uterine natural killer (uNK) cells and macrophages were the main leucocyte populations to be observed in association with SpAs. uNK cells produce a range of cytokines and growth factors and one cytokine that had been intially suggested to play a role in SpA remodelling was IFN-γ from mouse studies (Ashkar and Croy, 2001), more recently human uNK cells were found to secrete high levels of IFN-γ (Lash et al., 2006b) which up regulates genes involved in smooth muscle cell proliferation and cell adhesion suggesting that these cells may be important mediators in the remodelling process. This has been observed in mice studies where impaired uNK cells leads to aberrant formation of blood vessels via the IFN-γ pathway (Ashkar et al., 2000).

Smith et al. (2009) have reported high numbers of leucocytes in proximity to remodelling spiral arteries in first trimester decidua in the absence of EVT. They have also demonstrated the production of MMP-7 and MMP-9 by macrophages and uNK

cells within the vascular wall which may contribute to the degradation of the vessel wall, thereby affecting vessel integrity; similar changes are seen in tumour angiogenesis and metastases in relation to the production of MMP-7 and -9. A recent study using a novel placental-decidual co culture model (Hazan et al., 2010) have implicated leucocyte derived MMP-2 and MMP-9 in the remodelling events suggesting a participation of uNK cells and macrophages in VSMC disruption and clearance. However, the exact function of these leucocytes and remodelling events remains inconclusive. Elucidating the importance of maternal leucocytes in SpA remodelling will help in the understanding of impaired remodelling associated with pregnancy complications and may therefore lead to future therapies and prevention.

The aim of this part of the study was to further determine the association of uNK cells and macrophages with decidual SpAs and to investigate the effect of EVT cells on this association. This study will also explore ways of obtaining a pure population of decidual macrophages to investigate the factors that they secrete which may play an important role in spiral artery remodelling. Macrophages are the second most abundant leucocyte population within the decidua of early pregnancy and unlike uNK cells their numbers remain fairly constant throughout gestation. Decidual macrophages express a large variety of cell surface receptors and secrete a variety of cytokines suggesting that these cells play a homeostatic role in the decidua and may regulate trophoblast function, and unlike uNK cells, macrophages are also present in myometrium and SpA transformation extends to the inner myometrium but their role in pregnancy remains undefined. The importance of macrophages in successful early pregnancy is therefore becoming more apparent hence, the focus on this cell population in this study.

6.2 Aims and Hypotheses

6.2.1 Hypotheses

1) Decidual leucocytes play a role in the remodelling of the spiral arteries by penetrating the muscular wall causing VSMC disruption both in the presence and absence of EVT.

2) Decidual leucocytes are prevalent in and around the VSMC wall of SpAs in the decidua prior to trophoblast invasion.

3) Decidual leucocyte numbers are higher in the VSMC wall and adventitia of SpA at 8-10 weeks gestation compared with numbers at 12-14 weeks gestation

4) Decidual macrophages produce pro-angiogenic growth factors and cytokines that cause disruption of the VSMC wall aiding spiral artery remodelling at both 8-10 and 12-14 weeks gestation.

6.2.2 Aims

1. To assess leucocyte cell numbers in the musculoelastic walls of spiral arteries in decidua from placental bed biospsies at 8-10 and 12-14 weeks gestation in the presence or absence of EVT

2. To isolate a pure population of CD14$^+$ cells from first trimester decidua

3. To investigate which cytokines and growth factors are produced by uterine CD14+ cells using a FASTQuant multiplex cytokine array

6.3 Experimental design

6.3.1 Immunohistochemical labelling of decidual spiral arteries and decidual leucocytes

Placental bed biopsies were obtained from women undergoing elective TOP in early pregnancy at 8-10 and 12-14 weeks gestation (section 2.1.3) and tissue was prepared for FFPE sections for immunohistochemical analysis (section 2.1.8.3 and section 2.2), n=10 each gestational age. Table 6.1 outlines the primary antibodies used, with incubation times and pre-treatments, to identify SpA, EVT and decidual leucocytes within the spiral artery wall and surrounding areas of decidual spiral arteries at 8-10 and 12-14 weeks gestation.

Table 6.1 Primary antibodies used for immunohistochemistry on FFPE tissue sections

Antibody	Method	Species	Dilution	Incubation	Pre-treatment	Ab + control
H-Caldesmon[1]	Elite Kit[3,4]	Mouse	1:100	RT, 60min	PC in Citrate buffer pH 6.0, 1min	Stomach
Elastin[1]	Elite Kit[3,4]	Mouse	1:60	RT, 60min	WB Trypsin/CaCl$_2$ pH7.8, 10min	Umbilical cord artery
Cytokeratin-7[2]	Elite Kit[3,4]	Mouse	1:20	RT, 60min	PC in Citrate buffer pH 6.0, 1min	Skin
CD45[1]	Elite Kit[3,4]	Mouse	1:200	RT, 60min	PC in Citrate buffer pH 6.0, 1min	Tonsil
CD56[2]	Elite Kit[3,4]	Mouse	1:50	RT, 60min	PC in Citrate buffer pH 6.0, 1min	Placental bed biopsy
CD14[2]	Elite Kit[3,4]	Mouse	1:20	RT, 60 min	PC in Citrate buffer pH 6.0, 1min	Tonsil
CD3[2]	Elite Kit[3,4]	Mouse	1:20	RT, 30min	PC in Citrate buffer pH 6.0, 1min	Lymph node

Source: [1]DakoCytomation, Denmark; [2]Leica Biosystems, (Novocastra) Newcastle UK; [3]Vector Laboratories, Peterborough, UK; [4]ABC ELITE Kit- avidin-biotin peroxidase method (mouse monoclonal); PC - Pressure Cook; WB – Waterbath, 37°C; RT - Room Temperature.

6.3.2 Definition of SpA vessel wall

The structure of an artery is composed of three distinct layers; the intima, media and adventitia. The intima is the inner most layer that is in contact with blood in the lumen and includes an endothelial cell layer lining the lumen and an internal layer of elastic lamina. The media is the middle layer and consists mainly of compacted smooth muscle cells and elastic fibres. The outermost layer is known as the adventitia and is composed of fibrous collagen and connective tissue protecting the blood vessel and allowing anchorage to surrounding structures.

The purpose of this part of the study was to assess the number of leucocytes in the intima and media of SpAs. Definition of the outer limits (media) of the spiral artery musculoelastic wall proved difficult in this study and H-caldesmon staining was not sufficient to overcome the problems encountered. Defining the inner and outer limits of the vessel was difficult because the wall thickness often varied within any one vessel cross section; for example one side of the SpA would have a very thick medial layer, whereas the other side of the vessel wall would have lost the VSMC layers, making characterisation of the stage of vessel transformation hard to determine. The use of

standard histochemical staining techniques helped to overcome this problem and also helped to distinguish between arteries and veins.

6.3.2.1 Orcein and EVG staining techniques

In order to help to identify and define the limits of SpAs within the placental bed biopsies Orcein staining and Elastic Van Gieson staining were also applied to the FFPE tissue sections as well as immunohistochemistry for VSMC markers.

The Orcein staining technique is outlined in the Appendix (see Appendix); this a red-brown stain that is used to identify elastic layers in connective tissue. Decidual spiral arteries are known to lose their elastic lamina during early transformation processes in early pregnancy (Figure 6.1B) but deeper sections of SpAs within the myometrium appeared to have both internal and external elastic lamina present and intact (Figure 6.1A). This staining technique also helped to distinguish arteries from veins within the decidua. However, due to the loss of elastic layers in decidual SpAs it was difficult to determine vessel wall thickness using the Orcein stain.
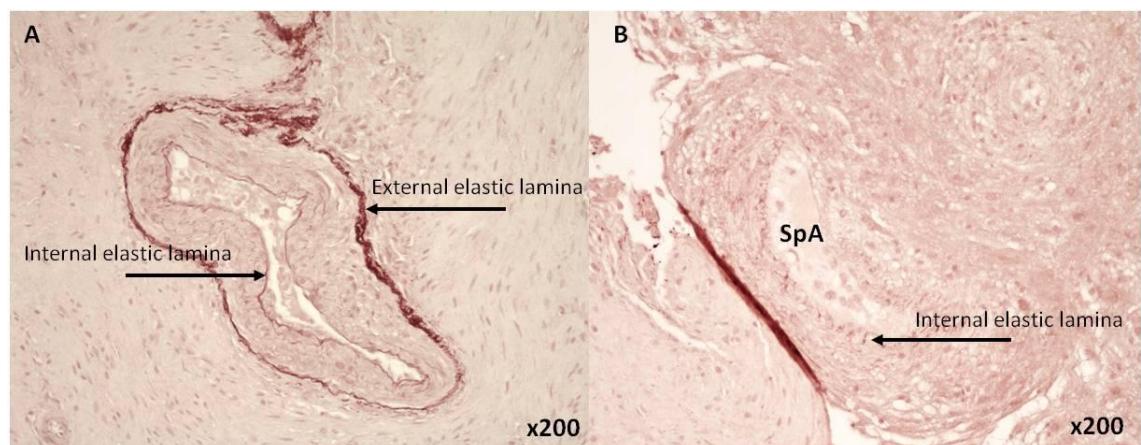
Figure 6.1 Orcein staining on placental bed spiral arteries to identify elastic layers
Image representing spiral arteries stained with Orcein staining technique in both FFPE tissue sections of myometrium (A; 8-10 weeks GA) and decidua (B; 8-10 weeks GA) from placental bed biopsies. Original magnification x200.

The Elastic Van Geison (EVG) staining technique is an alternative method to identify the connective tissue and elastic layers (see Appendix). Elastic layers are stained blue/black and muscle layers stain a yellow colour with an orange/red colour demonstrating connective tissue, the SpA adventitia. The identification of internal and external elastic laminae and adventitial connective tissue helped to identify the inner

and outer limits of the VSMC media wall and adventitia of the SpAs (Figure 6.2). This staining technique also provided evidence that there is no external elastic lamina present in decidual spiral arteries (Figure 6.2).
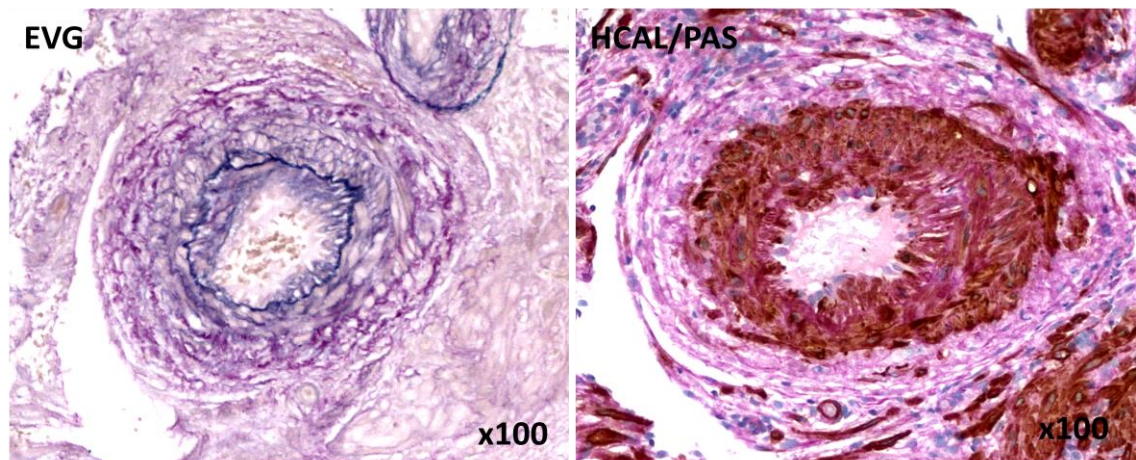
Figure 6.2 Elastic Van Geison (EVG) on placental bed spiral arteries for identification of internal and external elastic laminae and adventitial connective tissue
Image representing a non-transformed spiral artery. Left image illustrates a decidual SpA stained with EVG. Black staining indicates internal elastic lamina, yellowish/purple staining indicates muscle layers and reddish colour indicates connective tissue. The right image is a serial section of a decidual SpA in the placental bed positive for H-Caldesmon immunostaining demonstrating VSMCs (8-10weeks GA). Original magnification x100.

6.3.3 Assessment of leucocyte cell counts in SpA vessel wall and adventitia

Once a spiral artery was identified microscopically, the thickness of the musculoelastic wall and adventitia was determined and serial sections stained with leucocyte markers were assessed. Difficulties were encountered when trying to count leucocyte numbers within the vessel walls. Initially the sections were double labelled using immunohistochemistry with muscle markers and leucocyte markers or counterstained with muscle stains. However, this was unsuccessful because the staining with muscle markers masked the staining of the leucocyte markers. Therefore, serial sections were stained and immunolabelled separately which made it hard to identify exact areas within placental bed.

This problem was overcome by the use of a computer grid graticule applied to 3µm serial sections. An image of the area stained on the tissue section was taken using an image capture microscope (Nikon Eclipse 80i coupled with a DS-Fil camera) which was connected to the computer programme NIS Elements Software. Tissue sections that

were immunostained with H-caldesmon were assessed initially. The thickness of the spiral artery wall was determined by placing a graticule over the immunostained artery. A square of the graticule was placed so that the inner vessel wall (from the lumen) to the outer limit of vessel wall would fit the square and the width of the square was measured electronically (Figure 6.3).
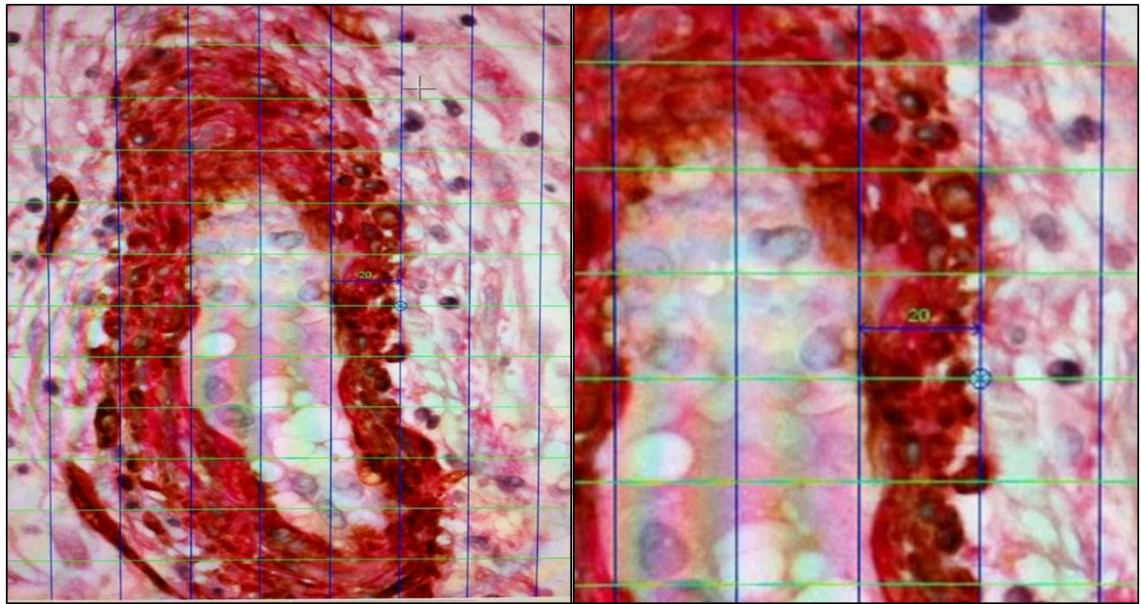
Figure 6.3 The use of a computer graticule in determining SpA vascular wall thickness
Image taken from computer screen to shown the use of the graticule grid and how VSMC wall thickness was determined. The squares on this graticule grid measure 20μm. However, parts of the vessel wall exceed this thickness (top of vessel ~60μm), and therefore an average thickness was measured for this dedidual SpA. Original magnification x200.

The thickness of the VSMC wall of the artery was assessed by measuring the various places within any one vessel and was then given an average thickness measurement. If an artery had a wall thickness that exceeded 30μm and the VSMC layers were very tightly packed with a swollen endothelium visually present it was characterised as a non-transformed vessel that was not going through the remodelling process (Figure 6.4A). Arteries which had a VSMC wall thickness of 20-30μm and showed slight separation of VSMC layers and migration of VSMC layers was characterised as an early partially transformed vessel (P1; Figure 6.4B). SpAs that had VSMC walls that were 10μm or less (one cell layer thick) were classed as late partially transformed vessels

(P2; Figure 6.4C) and arteries which had lost their VSMC wall completely or with one or two VSMC remaining were classified as fully transformed vessels (Figure 6.4D).

Initially difficulties did arise on the classification of vessel thickness and stages of transformation. Non-transformed and complete vessels were easy to define but intermediate stages were harder to assess. Also vessels that measured 10-20µm thick were in between P1 and P2 and had difficulty defining which stage of transformation they belonged. We attempted classification of the intermediate partial stages based on disorganisation and loss of muscle rather than VSMC thickness. The presence or absence of EVT was assessed separately and was not used initially to define transformation stage but in difficult specimens the presence of certain EVT populations helped on classification.

Serial tissue sections that were immunostained with leucocyte markers were assessed and counted by taking an image of the corresponding SpA, placing the grid graticule (measuring the same thickness of the SpA VSMC wall, e.g. 30µm thick) and counting the number of leucocytes within that 30µm area starting from the lumen. Cells in the adventitia were also counted. Adventitia was identified by EVG staining (section 6.3.2.1) and the thickness was measured using the computer graticule and was defined from the outer limits of the musculoelastic wall to the outer limits of connective tissue (where tissue anchored onto decidual tissue). This method was applied to all serial sections and all leucocyte markers.
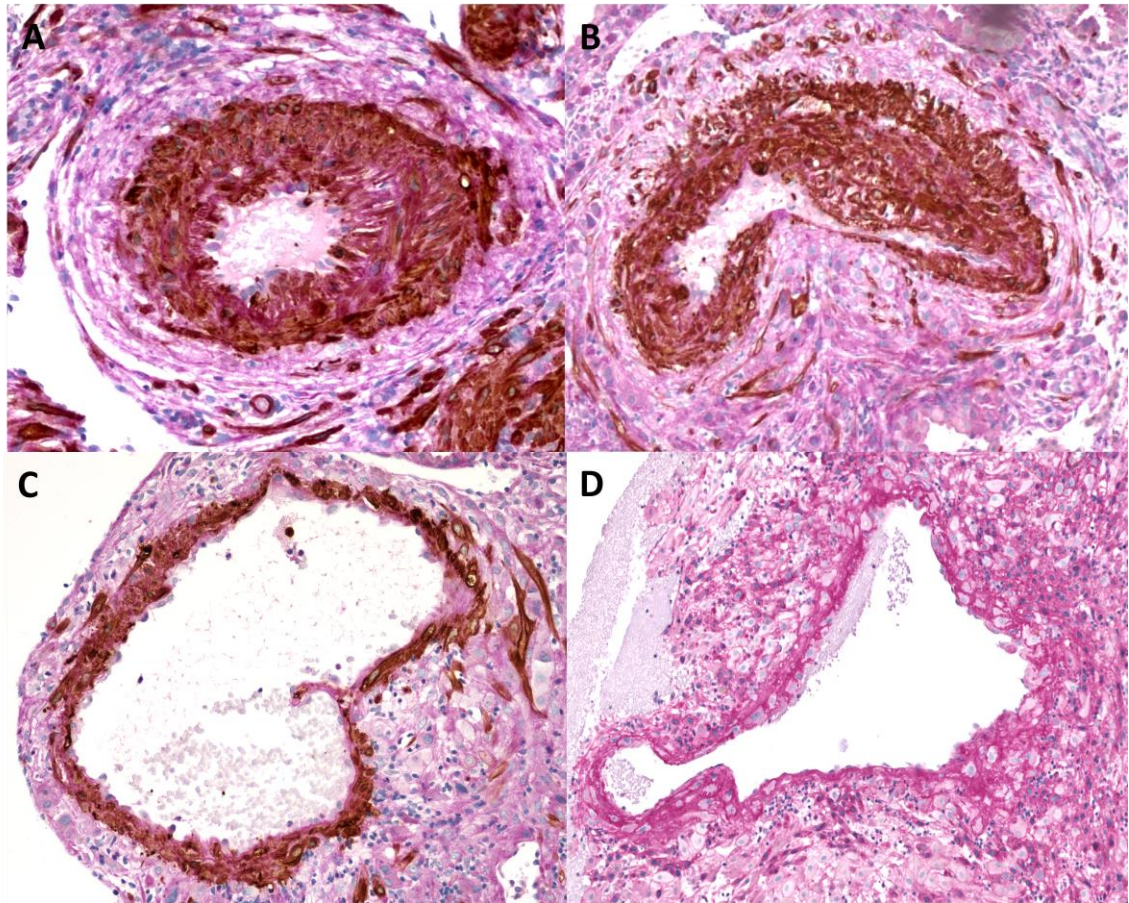
Figure 6.4 Identification of SpA vessel classification based on level of vessel transformation using immunohistochemistry with anti-H-caldesmon

Representative immunohistochemistry image demonstrating how SpA vessel classification was identified based on vessel transformation in placental bed biopsies. A: Non-transformed; B: P1 (early) partially transformed; C: P2 (late) partially transformed and D: Fully transformed. All tissue is immunostained with H-Caldesmon/PAS demonstrating VSMC wall of decidual SpAs with original magnification x100.

6.3.4 Isolation of decidual CD14$^+$ cell by MidiMacs

Isolation of a pure, viable source of decidual macrophages from first trimester decidua has not been previously described in literature. In this study we wanted to see whether it was possible to initially isolate CD14$^+$ cells from early decidual tissue and secondly to culture the decidual macrophages to pave the way in allowing to investigate their protein secretion and involvement in SpA remodelling.

Firstly, the proportion of CD14$^+$ cells in digested decidual tissue was investigated. Decidual tissue was subjected to enzymatic digestion (section 2.3.1) and incubated in a T75 tissue culture flask overnight at 37°C at 5% CO_2. Following overnight incubation the media was discarded and adhered cells were removed from the tissue culture flask using sterile PBS pH7.4 and a cell scraper. The removed cells were then centrifuged at

300G for 7mins, resuspended in RPMI complete medium and cell smears prepared (section 2.3.3).

The cell smears were then immunostained using antibodies against CD14, CD56, vimentin and LP34 (see Table 6.2). This was repeated 3 times.

Evaluation of these cell smears revealed that approximately 90% of the adhered cells in all 3 samples were positively stained with CD14, all the cells evaluated were positively stained with vimentin and none of the cells were CD56 positive or LP34 positive, indicating that the majority of the adhered decidual cells were a population of macrophages/dendritic cells which were mesenchymal in origin.

6.3.4.1 Preparation of CD14$^+$cell culture supernatants

The results showed that CD14$^+$ cells are present in adhered cells prepared from early pregnancy decidual tissue (8-14 weeks gestational age); the next stage was to isolate this population and collect cultured cell supernatants. Initially CD14$^+$ cells were isolated from early pregnancy decidua using the standard indirect Midimacs technique (section 2.3.4) and cell smears were prepared. The CD14$^+$ labelled cell smears were then stained with secondary and tertiary antibodies from the Elite ABC kit in order to check cell purity, none of the cells were positive for CD14$^+$ protein. This was repeated using another 2 decidual samples and results were the same each time.

Three more early pregnancy decidual samples were digested but for those samples isolation of cells was attempted using the Midimacs technique for macrophage isolation with labelled anti-CD14 microbeads; cell smears were again prepared to check cell purity.

Cells smears were also immunostained with anti-CD68 as well as checking CD14 purity (see Table 6.2). In all of the samples over 95% of the cells were CD68 positive and over 97% of the cells were CD14 positive. Indicating that this isolation procedure had worked and we were able to successfully isolate CD14 cells from early pregnancy decidual tissue.

6.3.4.2 Characteristion of decidual CD14^{+} cells

CD14 cells were isolated from decidual tissue (n=3) and CD14^{+} cell smears were prepared for double labelling with different antibodies (Table 6.2) using the APAAP method (see Appendix).

Frozen sections of decidual tissue from 9 weeks gestation was also immunostained with protein markers associated with the phenotype of macrophages (Table 6.2), along with the double labelling of these markers in CD14^{+} labelled cell smears. Frozen decidual tissue was used rather than FFPE tissue, to compare with immunostaining in cell smears because the antibodies used for cell smears were antibodies which were specific to tissue or cells that had not be formalin fixed so decidua that had been frozen was used to make ideal comparisons.

Table 6.2 Primary antibodies used in immunocytochemistry of CD14^{+} cell smears and used for immunohistochemistry on frozen decidual tissue sections
Primary antibodies used in immunocytochemistry (single and double labelling) of cell smears and immunohistochemistry of frozen tissue sections.

Antibody	Method	Species	Dilution	Incubation	Pre-treatment	Ab + control
CD14[3]	Elite Kit[9, 10]	Mouse	1:20	RT, 60min	No pre-treatment	Tonsil
HLA-DR[2]	Elite Kit[9, 10]	Mouse	1:500	RT, 60min	No pre-treatment	Tonsil
CD68[2]	Elite Kit[9, 10]	Mouse	1:50	RT, 60min	No pre-treatment	Tonsil
CD209[4]	Elite Kit[9, 10]	Mouse	1:20	RT, 60min	No pre-treatment	Tonsil
CD86[5]	Elite Kit[9, 10]	Mouse	1:50	RT, 60min	No pre-treatment	Tonsil
CD11b[1]	Elite Kit[9, 10]	Mouse	1:100	RT, 60min	No pre-treatment	Tonsil
Vimentin[1]	Elite Kit[9, 10]	Mouse	1:100	RT, 60min	No pre-treatment	Tonsil
LP34[1]	Elite Kit[9, 10]	Mouse	1:80	RT, 60min	No pre-treatment	Tonsil
A1AT[8]	Elite Kit[9, 10]	Mouse	1:1000	RT, 60min	No pre-treatment	Tonsil
MMP-9[3]	Elite Kit[9, 10]	Mouse	1:100	RT, 60min	No pre-treatment	Tonsil
CD56[7]	Elite Kit[9, 10]	Mouse	1:100	RT, 60min	No pre-treatment	Placental bed biopsy (frozen)
CD83[6]	Elite Kit[9, 10]	Mouse	1:100	RT, 60min	No pre-treatment	Tonsil

Source: [1]Leica Biosystems, Newcastle LTD UK; [2]DakoCytomation, Denmark; [3]Millipore, USA; [4]BD Biosciences, USA; [5]Abcam, Cambridge, UK; [6]AbD Serotec, Oxford, UK; [7]Cell Signaling Technologies, MA, USA; [8]Chemi-Con, USA; [9]Vector Laboratories, Peterborough, UK; [10]ABC ELITE Kit - avidin-biotin peroxidase method (mouse monoclonal); RT - Room Temperature.

6.3.4.3 Culturing isolated decidual CD14⁺ cells – viability optimisation

Following isolation of decidual CD14$^+$ cells, isolated fractions were tested for their cellular viability using the trypan blue method (section 2.3.3.1) to determine whether positive immunomagnetic separation affected cell viability. All samples checked had >97% viability of CD14 cells with 95% purity after midimacs with positive selected CD14 labelled microbeads. However, it was observed that after 24h in culture (in 96 well tissue culture plate) following midimacs isolation, cellular viability dramatically decreased to less than 20%.

To overcome this, the culture medium components were altered. The fetal calf serum concentration in the culture medium was increased from 10% to 20%. The isolated cells were again cultured for 24h at 37°C in 96 well culture plates. The 'total' (un separated) adhered decidual cell fractions were viable after culture but the CD14 cell fraction were all dead when using both 10% and 20% FCS supplemented medium; increased FCS concentration therefore did not improve viability.

The size of the culture plates was also up for debate as macrophages are large cells and it was suggested that the wells on a 96 well plate may be too small for the cells, causing them to die in culture. Therefore, instead of culturing the cells in 96 well plates after isolation the CD14$^+$ cells were cultured in 48 well plates. Although the cells appeared less compacted in a 48 well plate, viability was still low after 24h culture. The amount of time that the cells were in culture was also reduced from 24h to 12h but even after 12h the cells did not survive. It was therefore decided to continue using the 48 well plates but the culture medium used to culture the cells was changed:

One well of cells were cultured using DMEM media supplemented using 10% heat inactivated FCS (Sigma), another using DMEM media supplemented with 10% FCS that had been triple filtered and derived from American fetal bovine blood (ATCC) and another using DMEM media supplemented with 10% FCS containing IL-2 and LPS (dendritic cell FCS) all with/without the addition of 50ngml^{-1} granulocyte macrophage colony stimulating factor (GM-CSF). This was also run alongside cultures in RPMI medium using the same conditions. These experiments were repeated using 3 different decidual samples.

CD14⁺ cell smears prepared at T=0 were double labelled with the antibodies outlined in Table 6.2, using the APAAP method (section 2.2.3). This double labelling was also performed on cultured cells (T=24h) by fixing the remaining cells in methanol for ten minutes prior to immunostaining. Once the culture conditions were optimised, cell culture supernatant collection began and were harvested and stored at -80°C.

6.3.5 Measuring secretion of gowth factors by decidual CD14$^+$cells

Secretion of growth factors and cytokines by CD14$^+$ cells were measured using the CD14$^+$cell supernatants. Detection of these CD14$^+$ proteins at both 8-10 and 12-14 weeks gestational age was assessed using ELISA and FASTQuant assays.

6.3.5.1 ELISA

50µl of CD14$^+$ cell supernatant in 50µl reagent diluents (diluted 2 fold) was subjected to a sandwich ELISA (section 2.10) and ran in duplicate for detection of PlGF (2000pgml^{-1} top standard) , VEGF-C (6000pgml^{-1} top standard), TGF-β1 (2000pgml^{-1} top standard) and Ang-1(10000pgml^{-1} top standard) (Duoset R&D systems). An 8 point standard curve was used and generated using a computer 4-PL curve fit. The only detectable protein in the supernatants was Ang-1; the other proteins were at undetectable concentrations by CD14$^+$ cells. N=10 for each age group (8-10 and 12-14 weeks GA).

6.3.5.2 Th1/2 and angiogenesis FASTQuant analysis

Adhered decidual cell supernatants (total adhered cells) and CD14$^+$ cell supernatants subjected to FASTQuant assay (section 2.7) to detect angiogenic growth factors and cytokines secreted by these cells.

The angiogenesis kit detects antigens including angiogenin, Ang-2, KGF, PDGF-BB, FGF-basic, TIMP-1, ICAM-1 and VEGF. There were 24 wells of the angiogenesis FASTQuant kit available for this study; a standard curve was made from the standard cocktail specific to the angiogenesis kit and added to 8 wells, 8-10 and 12-14 weeks GA total adhered decidual cell supernatants (n=3 both gestational age groups) and 8-10 and 12-14 weeks GA CD14$^+$ cell supernatants (n=5 both gestational age groups) were used for the remaining wells.

The Th1/2 kit detects antigens including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ and TNF-α. There were 32 wells of the human Th1/2 FASTQuant kit available for this study; a standard curve was made from the standard cocktail specific to the Th1/2 kit and added to 8 wells, 8-10 and 12-14 weeks GA total adhered decidual cell supernatants (n=7 8-10 weeks GA and n=5 12-14 weeks GA) and 8-10 and 12-14 weeks GA CD14$^+$ cell supernatants (n=7 8-10 weeks GA and n=5 12-14 weeks GA) were used for the remaining wells.

Samples were diluted 2 fold (50μl of each sample was diluted with 50μl of standard diluent (0.1% BSA, 0.05% Tween20 in PBS pH7.4) to give a total volume of 100μl). After blocking the wells in blocking solution 70μl of each sample and standard was added to the corresponding wells and incubated in a moisture sealed bag overnight at room temperature and protocol was followed using manufacturer's guidelines. Antigen concentration and detection was visualised using a working solution of SA-Cy™5 fluorescent reporter and scanned using Genepix scanner (section 2.7.2).

6.4 Results

6.4.1 Leucocyte cell counts in vessel wall and adventitia of spiral arteries in decidua of placental bed biopsies

In order to initially identify the different cell populations within the placental bed of early pregnancy FFPE tissue sections were subjected to immunohistochemical analysis (Table 6.1, section 6.3). Sections were immunostained to allow identification of CD3$^+$ T cells, CD14$^+$ macrophages and CD56$^+$ uNK cells.

All three leucocyte populations were identified in the vessel walls and adventitia of SpAs at both gestational age groups (8-10 and 12-14 weeks GA) both in the presence and absence of EVT (Figure 6.5).

6.4.1.1 Leucocyte cell counts in vessel wall at 8-10 and 12-14 weeks gestational age

CD14$^+$ leucocytes were observed to be the most prominent leucocyte population within the vessel wall of partially transforming and fully transformed decidual SpA at 8-10 weeks gestational age compared with CD3$^+$ and CD56$^+$ leucocyte populations

(Figure 6.6). A high number of CD3$^+$ and CD14$^+$ leucocytes were observed in non-transformed vessels with numbers diminishing as the vessels underwent transformation. CD56$^+$ cell numbers remained fairly consistent from non-transformed to partially transformed vessels but numbers declined in fully transformed vessel walls.

There were no significant differences observed in the numbers of CD56$^+$ cells in SpA VSMC walls throughout each stage of vessel transformation. However, the number of CD14$^+$ cells significantly declined from the numbers observed in non-transformed vessels to fully transformed vessels (a: P=0.0193) and there were also significantly decreased CD14$^+$ cell numbers observed in late partially transformed (P2) SpA compared with fully transformed SpA (b: P=0.0155). There was also a dramatic reduction in CD3$^+$ cell numbers from non-transformed to early partially transformed (P1) SpA, P2 and fully transformed SpA (c ,d, e: P=0.0001).
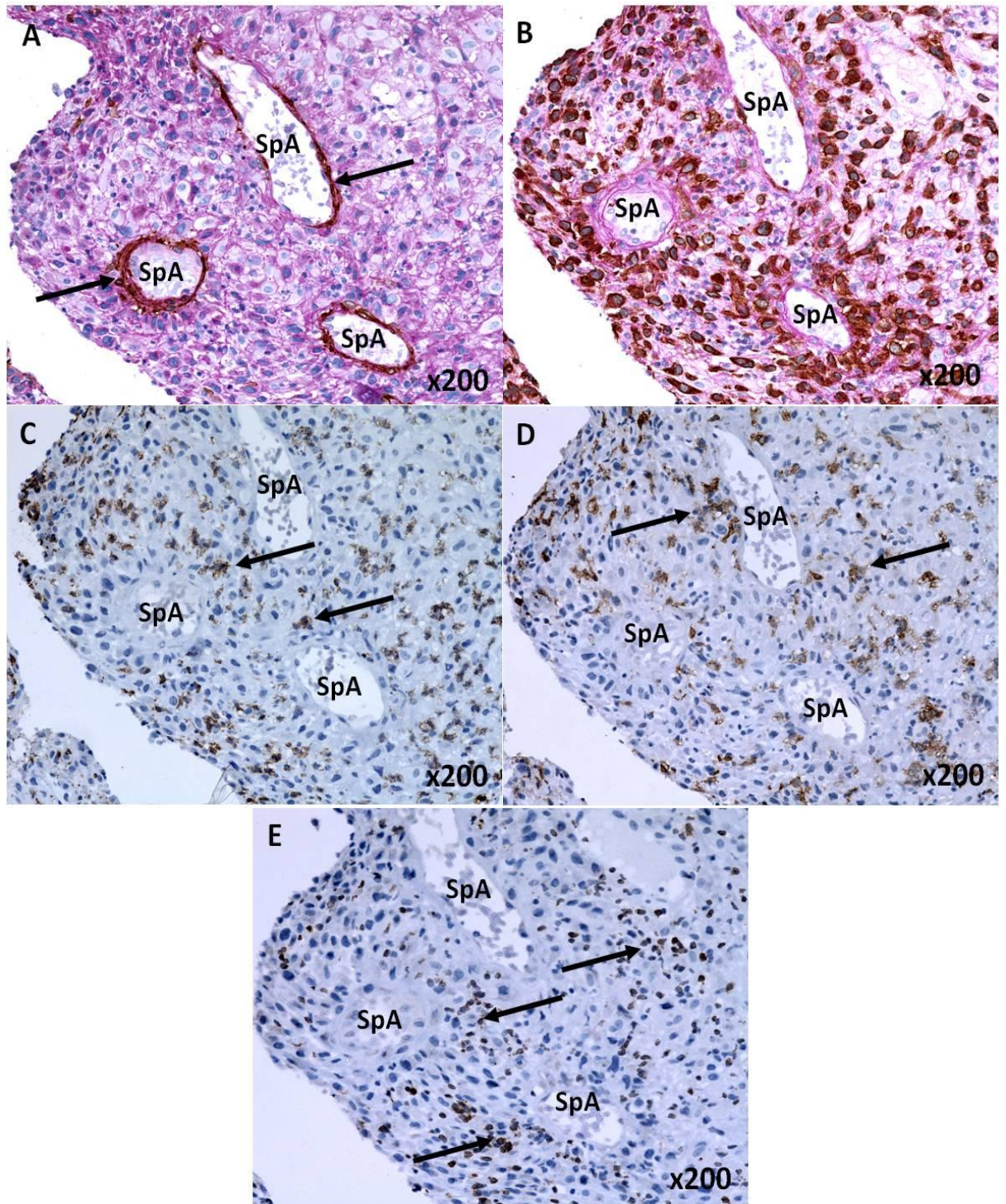
Figure 6.5 Immunohistochemistry images representing the different leucocyte cell populations in placental bed of early pregnancy
Immunohistochemical representation of P2 partially remodelled SpA within the placental bed at 8-10 weeks GA associated with maternal leucocytes. A: H-Caldesmon/PAS staining demonstrating VSMCs; B: Cytokeratin-7/PAS staining demonstrating EVT cells; C: Positive CD56 staining demonstrating uNK cells; D: Positive CD14 staining demonstrating macrophages; E: Positive CD3 staining demonstrating T-cells. Original magnification x200.
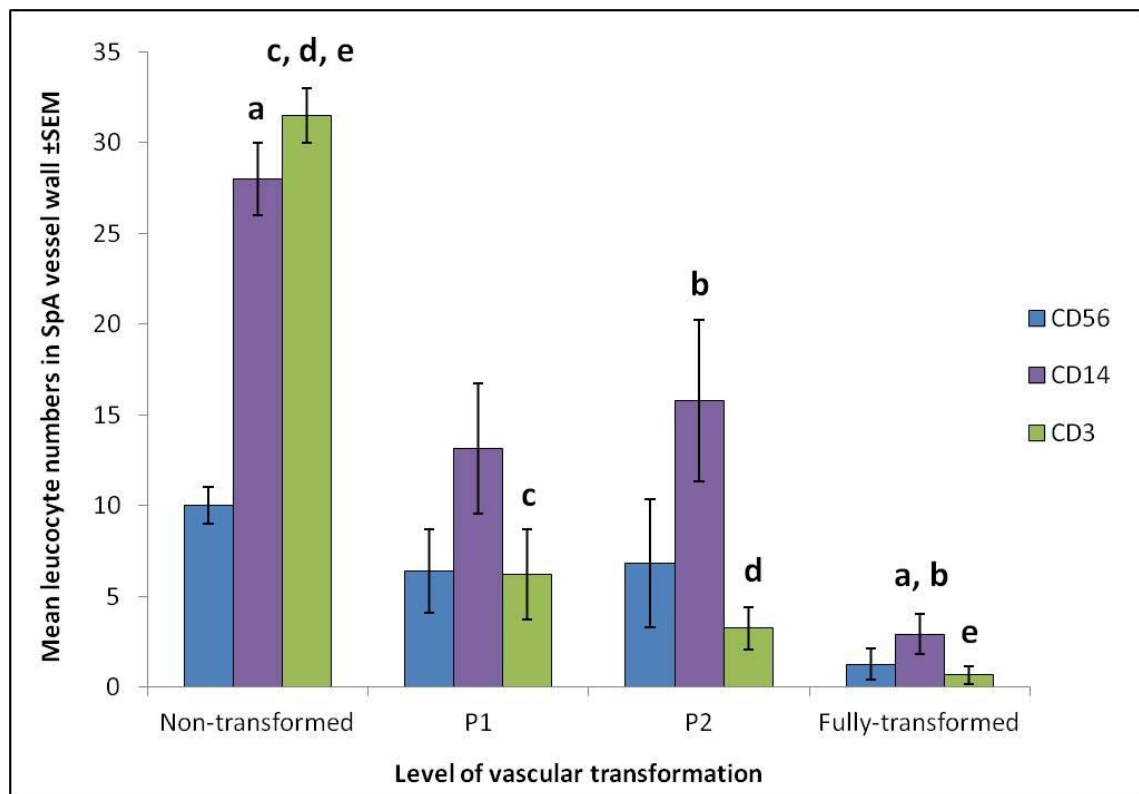
Figure 6.6 Mean leucocyte cell numbers in SpA vessel wall at each stage of vascular transformation at 8-10 weeks gestational age

Mean numbers of CD56^{+}, CD14^{+} and CD3^{+} cells observed within the wall of decidual SpAs at four different stages of transformation at 8-10 weeks gestation. Non-transformed (n=3): no transformation, thick VSMC layers; P1 (n=8): early partial remodelling of the VSMC, separation of VSMC cell layers; P2 (n=17): late partial remodelling of vessel wall, VSMC loss/displacement and increased vessel size; Fully-transformed (n=12): complete loss/displacement of VSMC layers, no evidence of VSMC. Data are expressed as Mean ± SEM and statistical significance is defined P=0.05 using one-way ANOVA with Fishers post hoc analysis.

CD14^{+} cells were also the most prominent cell type observed in SpA vessel walls at 12-14 weeks GA, with CD56^{+} cells being the second most prominent and CD3^{+} cells were observed in low numbers only in partially transformed SpAs at 12-14 weeks GA. The highest counts of CD14^{+} leucocytes were observed in early partially transformed (P1) SpA with numbers decreasing with level of transformation (Figure 6.7).

CD14^{+} leucocytes were the only cell population observed in the wall of non-transformed SpA at 12-14 weeks gestation; CD56^{+} or CD3^{+} cells were not detected in the wall of non-transformed SpA in the samples assessed (Figure 6.7). CD14^{+} cell numbers were increased in vessels showing early partial transformation (P1) compared with non-transformed SpA (a: *P*=0.0409) but numbers significantly decreased later in the transformation process with reduction in CD14^{+} cells in late partially transformed

(P2) and fully transformed SpA compared with P1 SpA (b: *P*=0.0332; c: *P*=0.0030 respectively). There were no differences in the number of CD56$^+$ leucocytes observed in the vessel wall throughout each stage of transformation, although CD56$^+$ cell numbers did slightly decline.
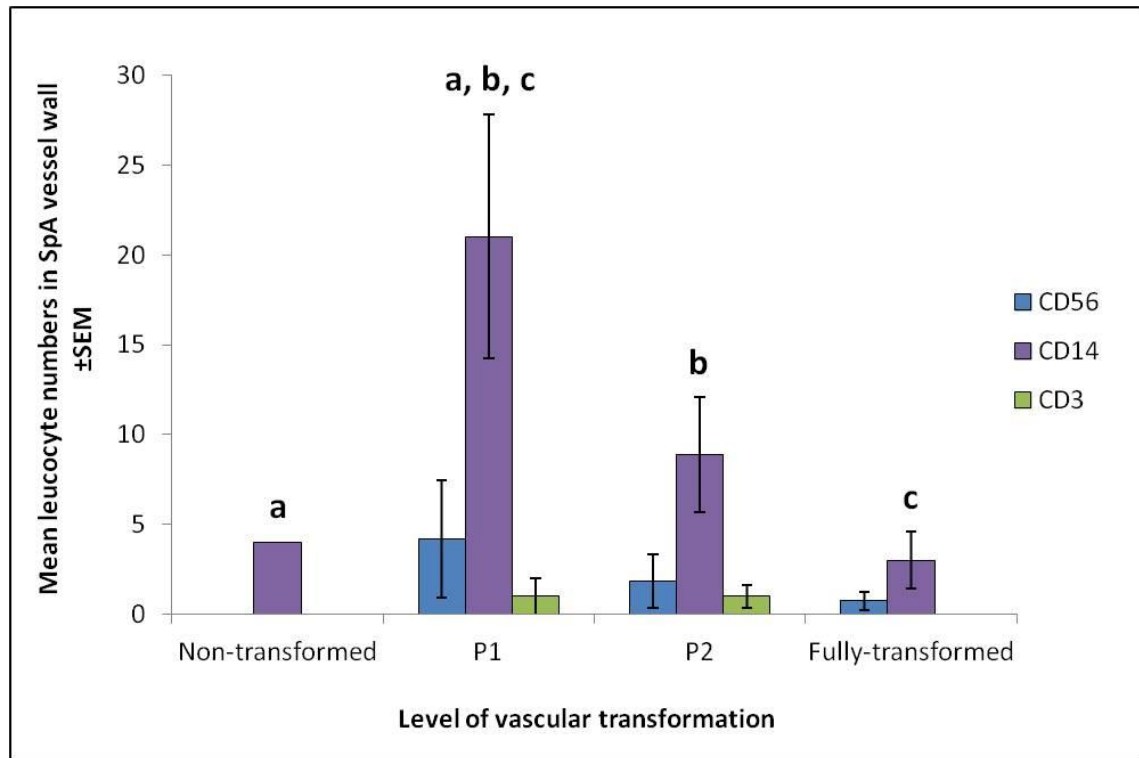
Figure 6.7 Mean leucocyte cell numbers in SpA vessel wall at each stage of vascular transformation at 12-14 weeks gestational age

Mean numbers of CD56$^+$, CD14$^+$ and CD3$^+$ cells observed within the wall of decidual SpAs at four different stages of transformation at 12-14 weeks gestation. Non-transformed (n=3): no transformation, thick VSMC layers; P1 (n=5): early partial remodelling of the VSMC, separation of VSMC cell layers; P2 (n=8): late partial remodelling of vessel wall, VSMC loss/displacement and increased vessel size; Fully-transformed (n=8): complete loss/displacement of VSMC layers, no evidence of VSMC. Data are expressed as Mean ± SEM and statistical significance is defined P=0.05 using one-way ANOVA with Fishers post hoc analysis.

6.4.1.2 Leucocyte cell counts in adventitia at 8-10 and 12-14 weeks gestational age

Higher numbers of all the leucocyte populations studied were observed in the adventitial layer of SpAs compared with numbers observed in SpA vessel wall at 8-10 weeks gestation. Leucocytes were clustered around the vessel; $CD14^+$ cell clusters appeared to be most striking and again were the most abundant compared with $CD56^+$ and $CD3^+$ cell numbers (Figure 6.8). $CD14^+$ and $CD3^+$ cell numbers were highest in non-transformed vessels and numbers decreased with vessel transformation. Compared with non-transformed SpA there were significant decreases in the number of $CD14^+$ cells of early partially transformed (P1) SpA (a: $P=0.0439$) and fully transformed vessels (b: $P=0.0409$). Differences were also observed between the numbers of $CD3^+$ cells within the adventitia; $CD3^+$ cell numbers observed in the adventitia decreased significantly from non- transformed to early partially transformed (P1) SpA (c: $P=0.0063$), P2 (d: $P=0.0032$) and fully transformed vessels (e: $P=0.0016$). Despite reducing numbers within the vessel adventitia of $CD14^+$ and $CD3^+$ leucocytes as SpA remodelling progresses, $CD56^+$ cell numbers remained consistent at each stage of SpA transformation.
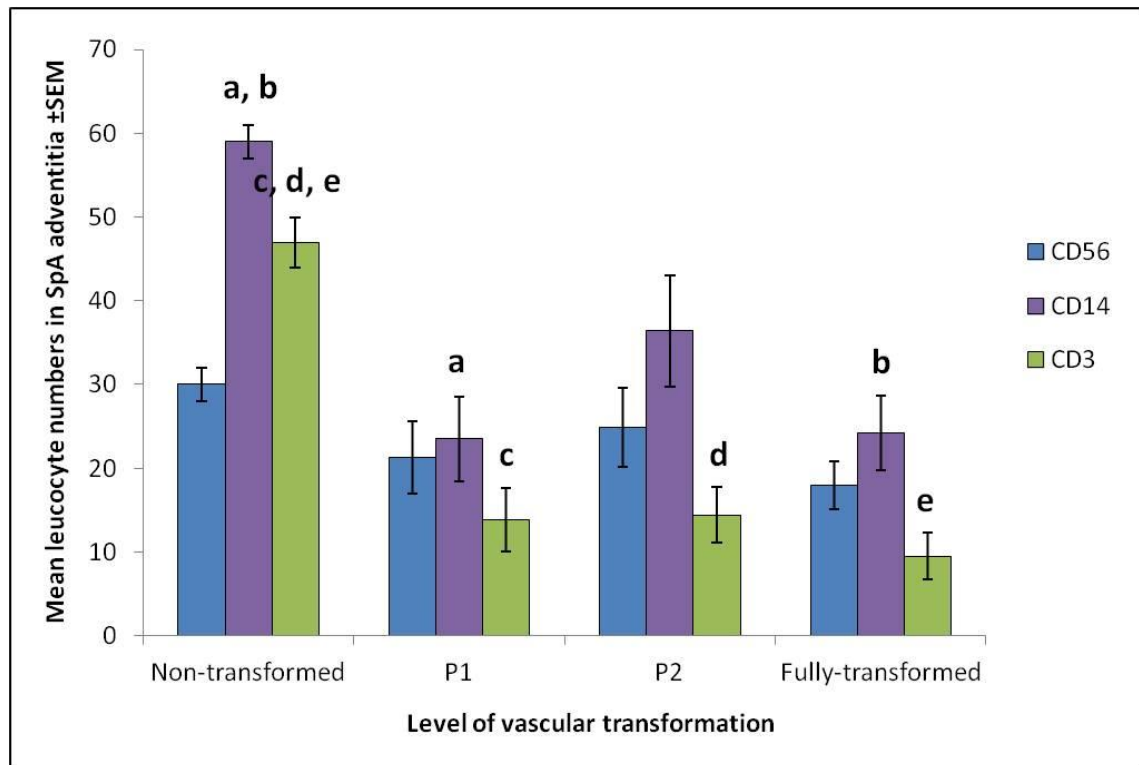
Figure 6.8 Mean leucocyte cell numbers in SpA vessel adventitia at each stage of vascular transformation at 8-10 weeks gestational age
Mean numbers of CD56^{+}, CD14^{+} and CD3^{+} cells observed within adventitia of decidual SpAs at four different stages of transformation at 8-10 weeks gestation. Non-transformed (n=3): no transformation, thick VSMC layers; P1 (n=8): early partial remodelling of the VSMC, separation of VSMC cell layers; P2 (n=17): late partial remodelling of vessel wall, VSMC loss/displacement and increased vessel size; Fully-transformed (n=12): complete loss/displacement of VSMC layers, no evidence of VSMC. Data are expressed as Mean ± SEM and statistical significance is defined P=0.05 using one-way ANOVA with Fishers post hoc analysis.

More leucocytes were observed in the adventitia of spiral arteries at 12-14 weeks gestation compared with numbers observed within the vessel walls themselves (Figure 6.9). CD14^{+} cells were the most prominent leucocyte population with highest numbers observed in the adventitia of non-transformed and early partially transformed (P1) SpAs, with numbers decreasing from P1 to P2 (a: *P*=0.0051) and fully transformed vessels (b: *P*=0.0007). CD56^{+} cell numbers again remained consistent throughout each stage of vessel transformation. Very few CD3^{+} cells were observed at 12-14 weeks gestation both in the vessel walls and adventitia and CD3^{+} cells were only identified in non-transformed and partially transformed vessels, with none detected in fully transformed SpA.
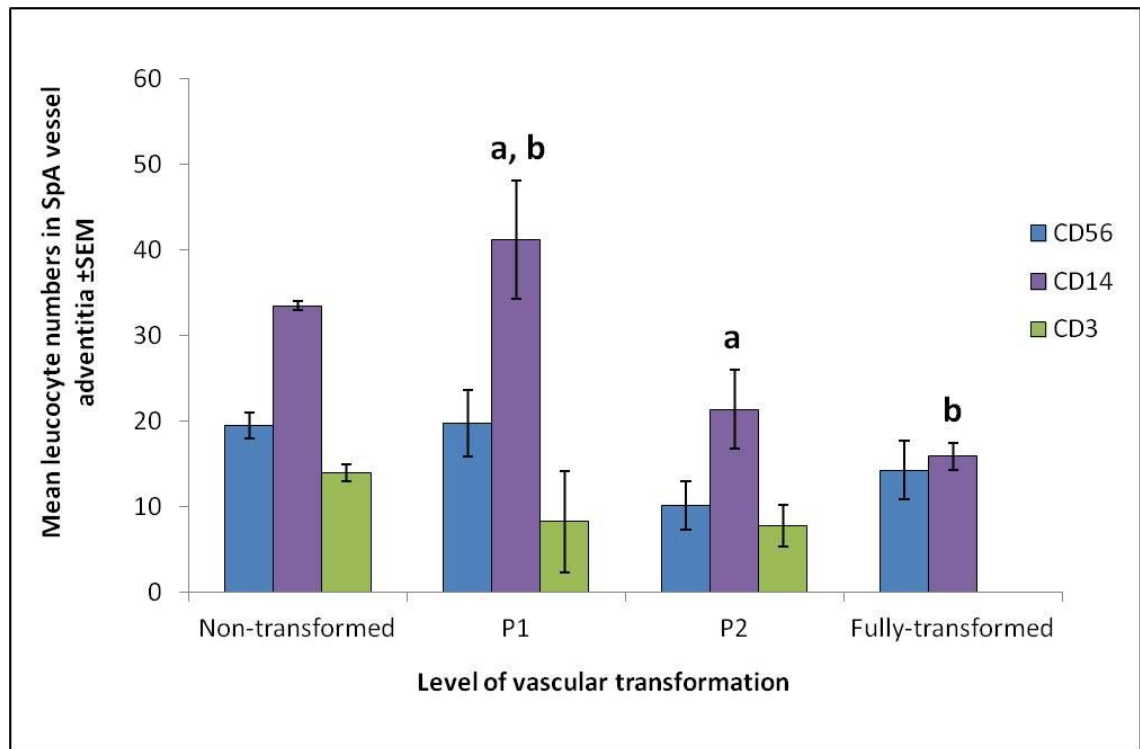
Figure 6.9 Mean leucocyte cell numbers in SpA vessel adventitia at each stage of vascular transformation at 12-14 weeks gestational age

Mean numbers of CD56$^+$, CD14$^+$ and CD3$^+$ cells observed within adventitia of decidual SpAs at four different stages of transformation at 12-14 weeks gestation. Non-transformed (n=3): no transformation, thick VSMC layers; P1 (n=5): early partial remodelling of the VSMC, separation of VSMC cell layers; P2 (n=8): late partial remodelling of vessel wall, VSMC loss/displacement and increased vessel size; Fully-transformed (n=8): complete loss/displacement of VSMC layers, no evidence of VSMC. Data are expressed as Mean ± SEM and statistical significance is defined P=0.05 using one-way ANOVA with Fishers post hoc analysis.

6.4.2 Comparison of leucocyte cell numbers in vessel wall and adventitia between 8-10 and 12-14 weeks gestation

6.4.2.1 Leucocyte cell numbers in vessel wall and adventitia between 8-10 and 12-14 weeks gestation

Reduced numbers of all three leucocyte populations were observed in the SpA wall and adventitia at 12-14 weeks compared with 8-10 weeks gestation.

It appeared that there were more CD56$^+$ cells observed in the walls and adventitia of SpA at 8-10 weeks compared to 12-14 weeks GA at all stages of vascular transformation (Figure 6.10). However, CD56$^+$ cell numbers were only higher in the vessel walls of non-transformed vessels at 8-10 weeks gestation compared with cell numbers observed in these vessels at 12-14 weeks gestation (Figure 6.10A; P=0.0099) and only higher in the adventitia of non-transformed and late partially transformed (P2) vessels at 8-10 weeks gestation compared with numbers observed at 12-14 weeks gestation (Figure 6.10B: P=0.0523 and P=0.0527 respectively).

CD14$^+$ cell numbers observed in both the adventitia and SpA wall declined at 12-14 weeks gestation compared with numbers observed at 8-10 weeks gestation (Figure 6.11). Non-transformed vessels at 8-10 weeks gestation had the highest number of CD14$^+$ cells observed in the walls of the vessels compared with 12-14 weeks gestation (Figure 6.11A; P=0.0069) and were also higher in the adventitia (Figure 6.11B; P=0.0065). However, early partially transformed vessels appeared to have higher numbers of CD14$^+$ cells at 12-14 weeks gestation compared with 8-10 weeks gestation SpA but this is not as apparent as vessel transformation increased (Figure 6.11).

It appeared that there were more CD3$^+$ leucocytes observed in both SpA vessel walls and vessel adventitia at 8-10 weeks gestation at each stage of vessel transformation compared with 12-14 weeks gestation (Figure 6.12). However, the differences in CD3$^+$ cell numbers observed were only significant in the vessel walls of non-transformed SpA at 8-10 weeks gestation compared with numbers observed at 12-14 weeks gestation (Figure 6.12A; P=0.0023) and in the adventitia of non-transformed SpA and fully transformed SpA at 8-10 weeks gestation compared with 12-14 weeks gestation (Figure 6.12B; P=0.0040 and P=0.0126 respectively). The number of CD3$^+$ leucocytes declined in later stages of transformation, with reduction of CD3$^+$ numbers in both the

wall and adventitia of fully transformed vessels compared with non-transformed SpA (Figure 6.12).
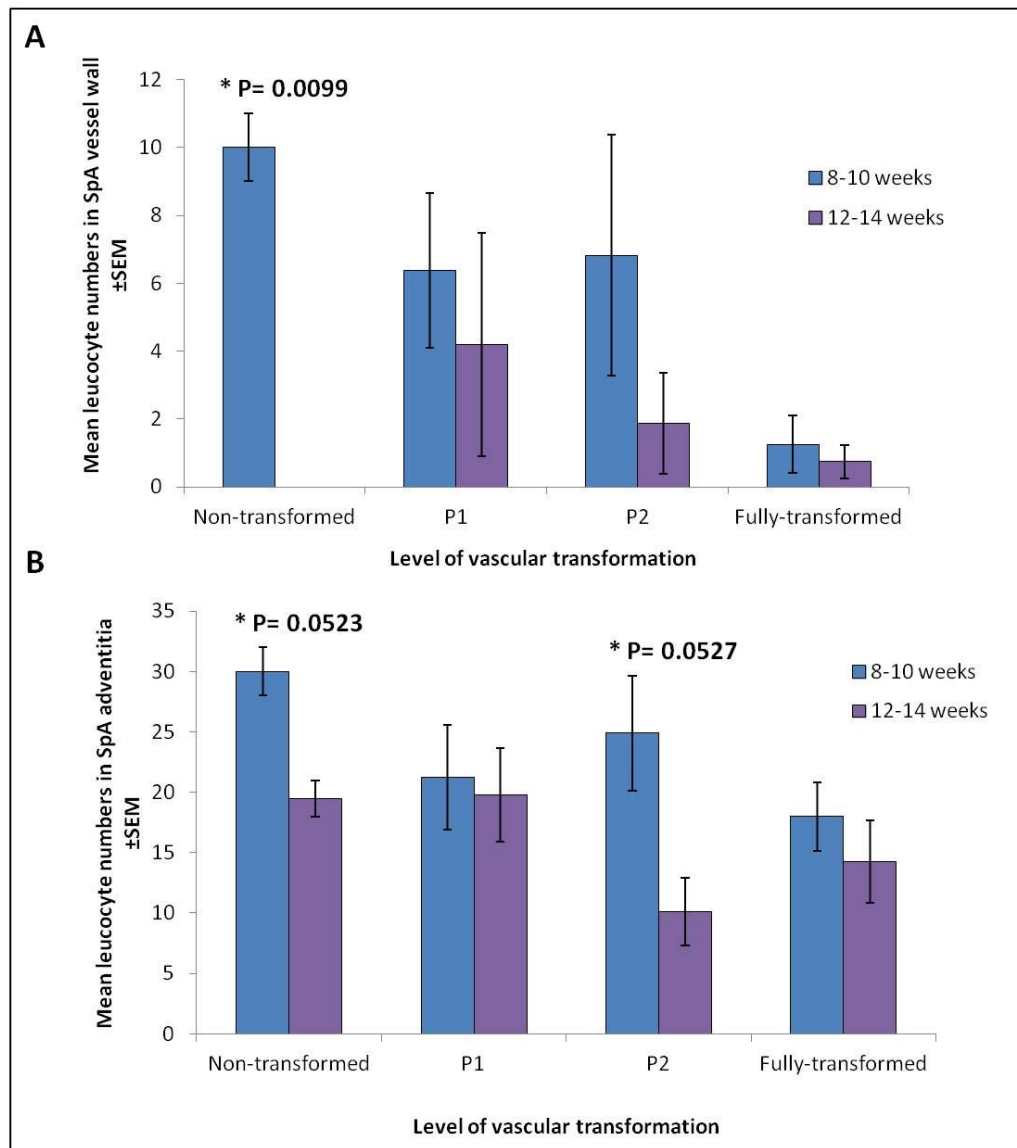
Figure 6.10 Comparison of CD56^{+} cell numbers in both SpA vessel wall and SpA adventitia between 8-10 weeks and 12-14 weeks gestation
Graphical representation of CD56^{+} cell numbers observed in SpA walls (A) and in SpA adventitia (B) in placental bed biopsies at both 8-10 (non-transformed n=3, P1 n=8, P2 n=17, fully transformed n=12 vessels) and 12-14 (non-transformed n=3, P1 n=5, P2 n=8, fully transformed n=8 vessels) weeks gestation. Data are expressed as Mean ± SEM. Significance is denoted by P≤0.05.
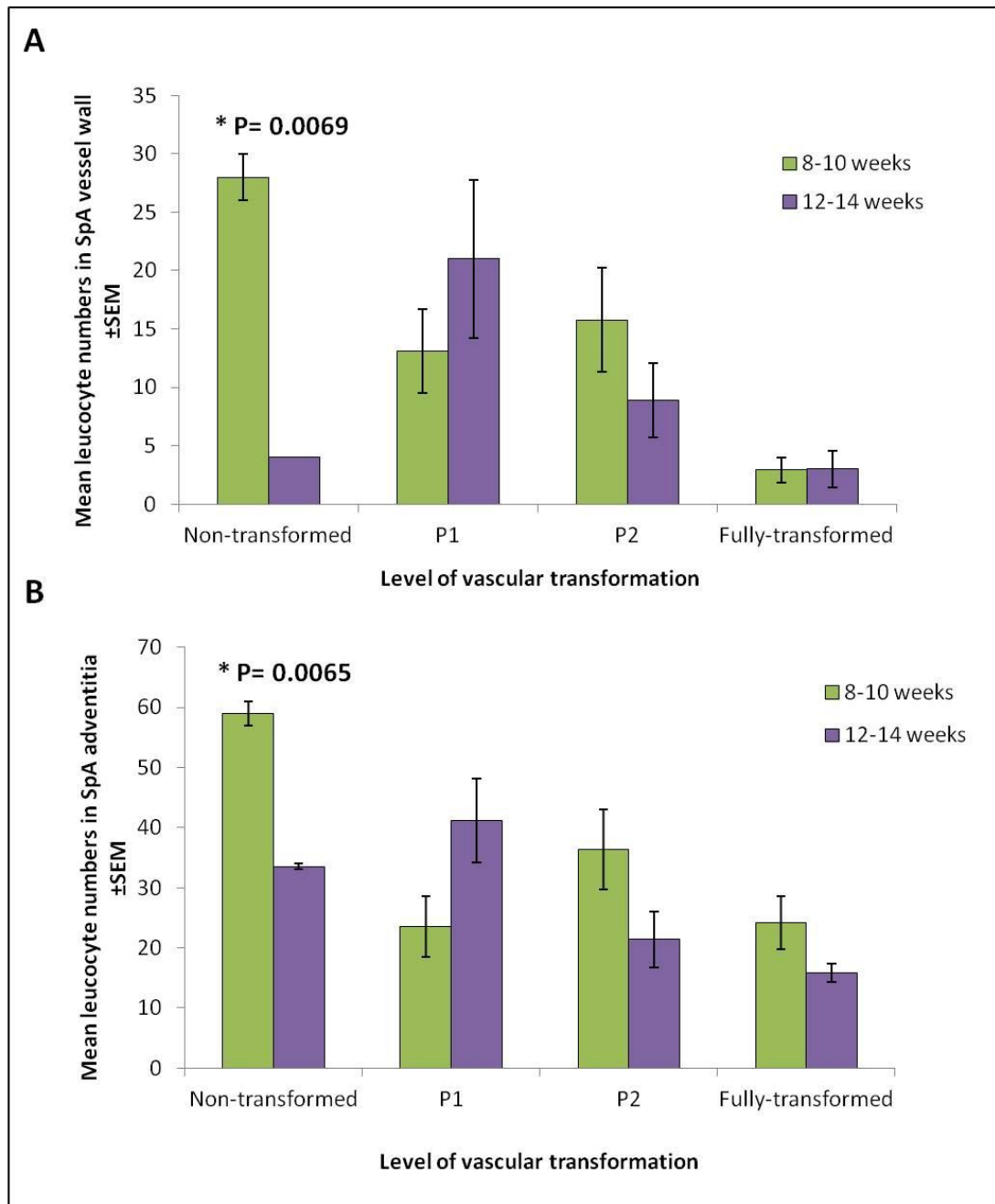
Figure 6.11 Comparison of CD14^{+} cell numbers in both SpA vessel wall and SpA adventitia between 8-10 weeks and 12-14 weeks gestation
Graphical representation of CD14^{+} cell numbers observed in SpA walls (A) and in SpA adventitia (B) in placental bed biopsies at both 8-10 (non-transformed n=3, P1 n=8, P2 n=17, fully transformed n=12 vessels) and 12-14 (non-transformed n=3, P1 n=5, P2 n=8, fully transformed n=8 vessels) weeks gestation. Data are expressed as Mean ± SEM n=10. Significance is denoted by P≤0.05.
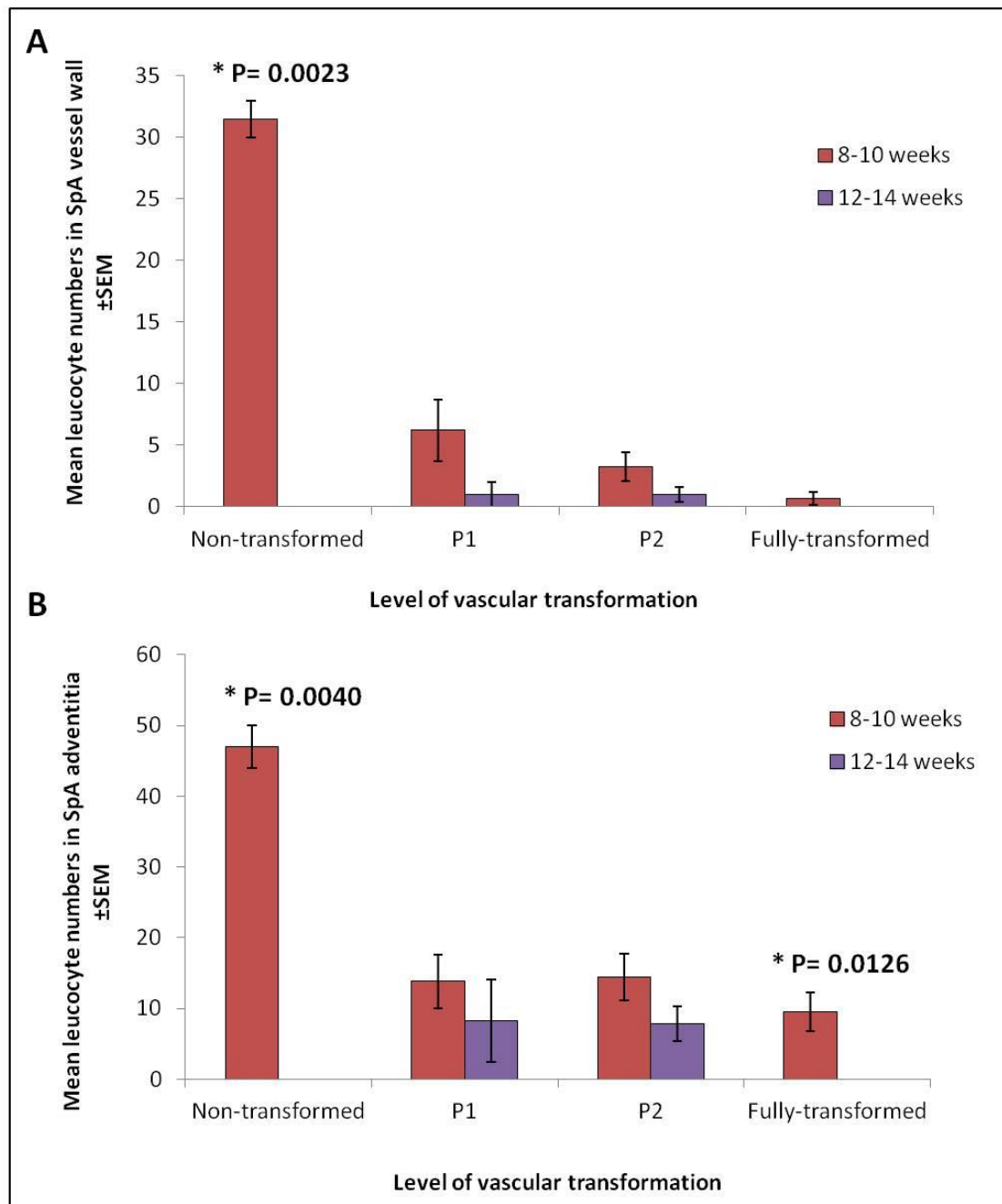
Figure 6.12 Comparison of CD3^{+} T lymphocyte numbers in both SpA vessel wall and SpA adventitia between 8-10 weeks and 12-14 weeks gestation
Graphical representation of CD3^{+} cell numbers observed in SpA walls (A) and in SpA adventitia (B) in placental bed biopsies at both 8-10 (non-transformed n=3, P1 n=8, P2 n=17, fully transformed n=12 vessels) and 12-14 (non-transformed n=3, P1 n=5, P2 n=8, fully transformed n=8 vessels) weeks gestation. Data are expressed as Mean ± SEM. Significance is denoted by P≤0.05.

6.4.2.2 Leucocyte cell numbers in vessel wall compared with leucocyte cell numbers in adventitia at 8-10 and 12-14 weeks gestation

At 8-10 weeks gestation significantly higher numbers of CD56$^+$ cells were present in the adventitia at each stage of vascular transformation compared with numbers observed within the vessel walls (Figure 6.13A; non-transformed: P=0.0123; P1: P=0.0089; P2: P=0.0045; fully transformed: P<0.0001).

At 12-14 weeks gestation no CD56$^+$ cells were observed in the vessel wall in non-transformed SpA, but were observed in low numbers at the other stages of transformation (Figure 6.13B). There were significantly higher CD56$^+$ cell numbers observed in the adventitia at each stage of vessel transformation compared with cell numbers observed in the vessel wall at 12-14 weeks gestation (Figure 6.13B; non-transformed: P=0.0059; P1: P=0.0153; P2: P=0.0213; fully transformed: P=0.0015).

At both 8-10 weeks and 12-14 weeks gestation there were significantly more CD14$^+$ cells observed in the adventitia compared with cell numbers observed in the vessel wall (Figure 6.14A and B respectively). CD14$^+$ cells numbers were significantly higher in the adventitia in non-transformed (8-10 weeks: P=0.0082; 12-14 weeks: P= 0.0003), P2 (late partially transformed; 8-10 weeks: P=0.0148; 12-14 weeks: P=0.0428) and fully transformed SpA at both gestational ages, with the biggest difference observed in fully transformed vessels (8-10 weeks: P=0.0001; 12-14 weeks: P<0.0001). Although, CD14$^+$ cell numbers observed in P1 (early partially transformed) vessels at both 8-10 weeks and 12-14 weeks gestation appeared to be higher in the adventitia compared to cell numbers observed in the vessel wall, the difference was not significant.

Higher numbers of CD3$^+$ cells were also observed in the adventitia compared with cell numbers observed in the vessel wall at both gestational age groups (Figure 6.15). At 8-10 weeks gestation CD3$^+$ cell numbers were highest in the adventitia in non-transformed (Figure 6.15A; P=0.0438), P2 (Figure 6.15A; P=0.0048) and fully transformed vessels (Figure 6.15A; P=0.0104) compared to cell numbers observed in the vessel wall. However, CD3$^+$ cell numbers observed in the adventitia and the vessel wall in P1 vessels did not differ.

There were no CD3$^+$ cells observed in either the vessel wall or adventitia of non-transformed and fully transformed SpA at 12-14 weeks gestation (Figure 6.15B). There

were a few CD3^{+} cells observed in the partially transformed vessels (P1 and P2) with higher cell numbers observed in the adventitia in P2 vessels compared with cell numbers observed in the vessel wall (*P*=0.0214).
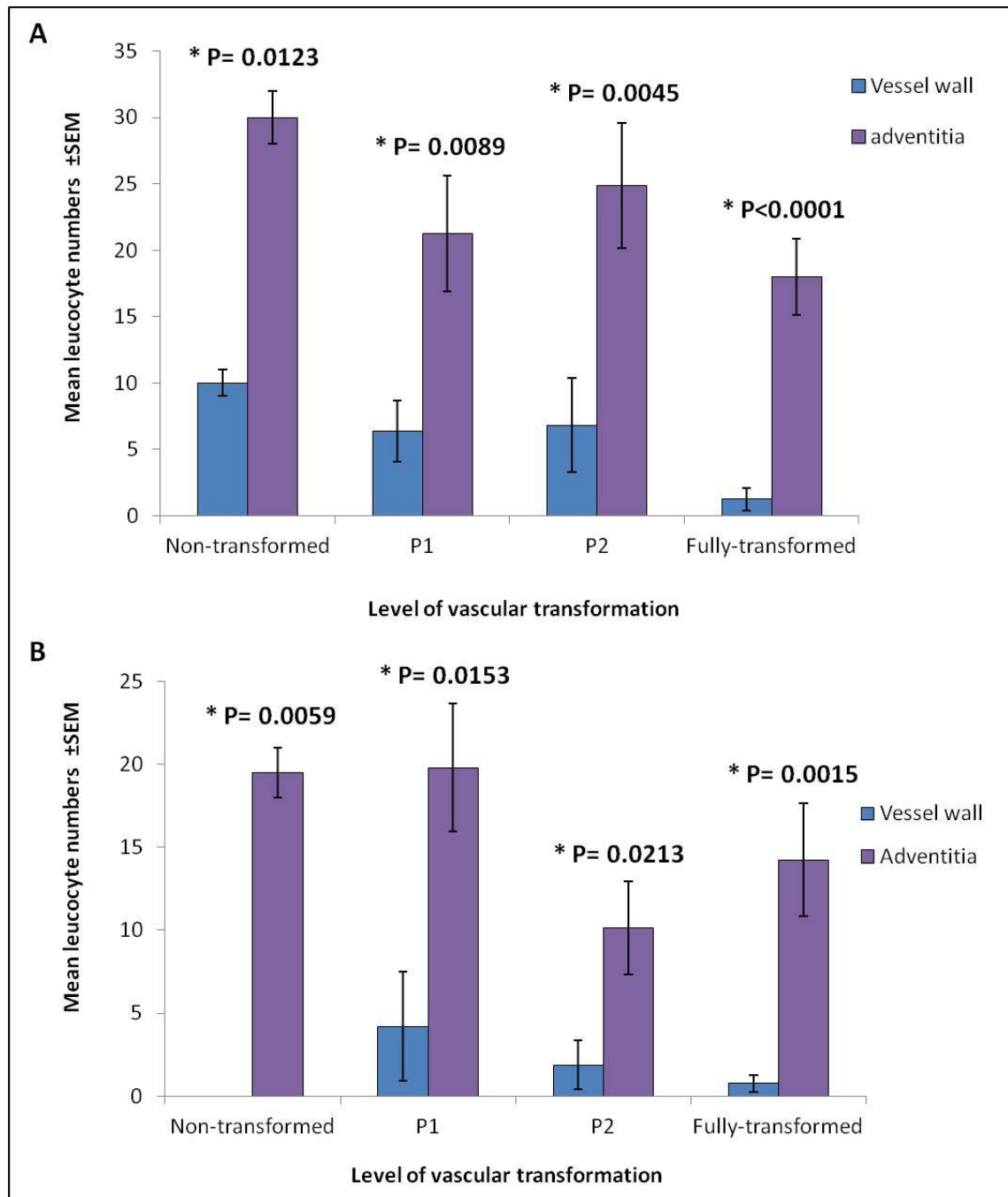
Figure 6.13 Comparison between CD56^{+} cell numbers observed in SpA vessel wall with numbers observed in SpA at both 8-10 weeks and 12-14 weeks gestation
Graphical representation of CD56+ cell numbers in SpA walls compared with numbers observed in adventitia in placental bed biopsies at both 8-10 weeks (A) and 12-14 weeks (B) gestation (8-10 weeks GA: non-transformed n=3, P1 n=8, P2 n=17, fully transformed n=12 vessels and 12-14 weeks GA: non-transformed n=3, P1 n=5, P2 n=8, fully transformed n=8 vessels). Data are expressed as Mean ± SEM. Significance is denoted by P≤0.05.
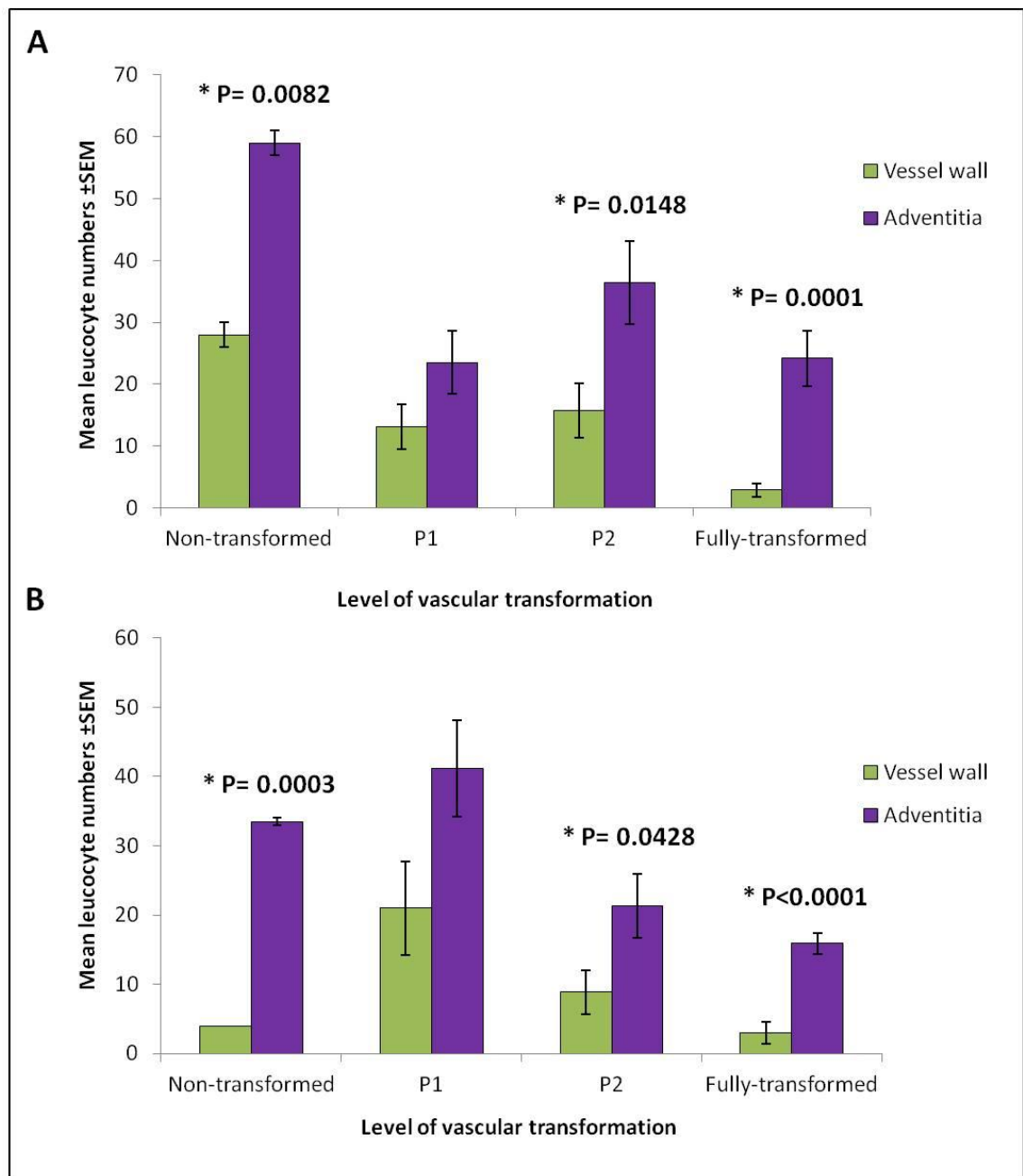
Figure 6.14 Comparison between CD14+ cell numbers observed in SpA vessel wall with numbers observed in SpA at both 8-10 weeks and 12-14 weeks gestation
Graphical representation of CD14+ cell numbers in SpA walls compared with numbers observed in adventitia in placental bed biopsies at both 8-10 weeks (A) and 12-14 weeks (B) gestation (8-10 weeks GA: non-transformed n=3, P1 n=8, P2 n=17, fully transformed n=12 vessels and 12-14 weeks GA: non-transformed n=3, P1 n=5, P2 n=8, fully transformed n=8 vessels). Data are expressed as Mean ± SEM. Significance is denoted by P≤0.05.
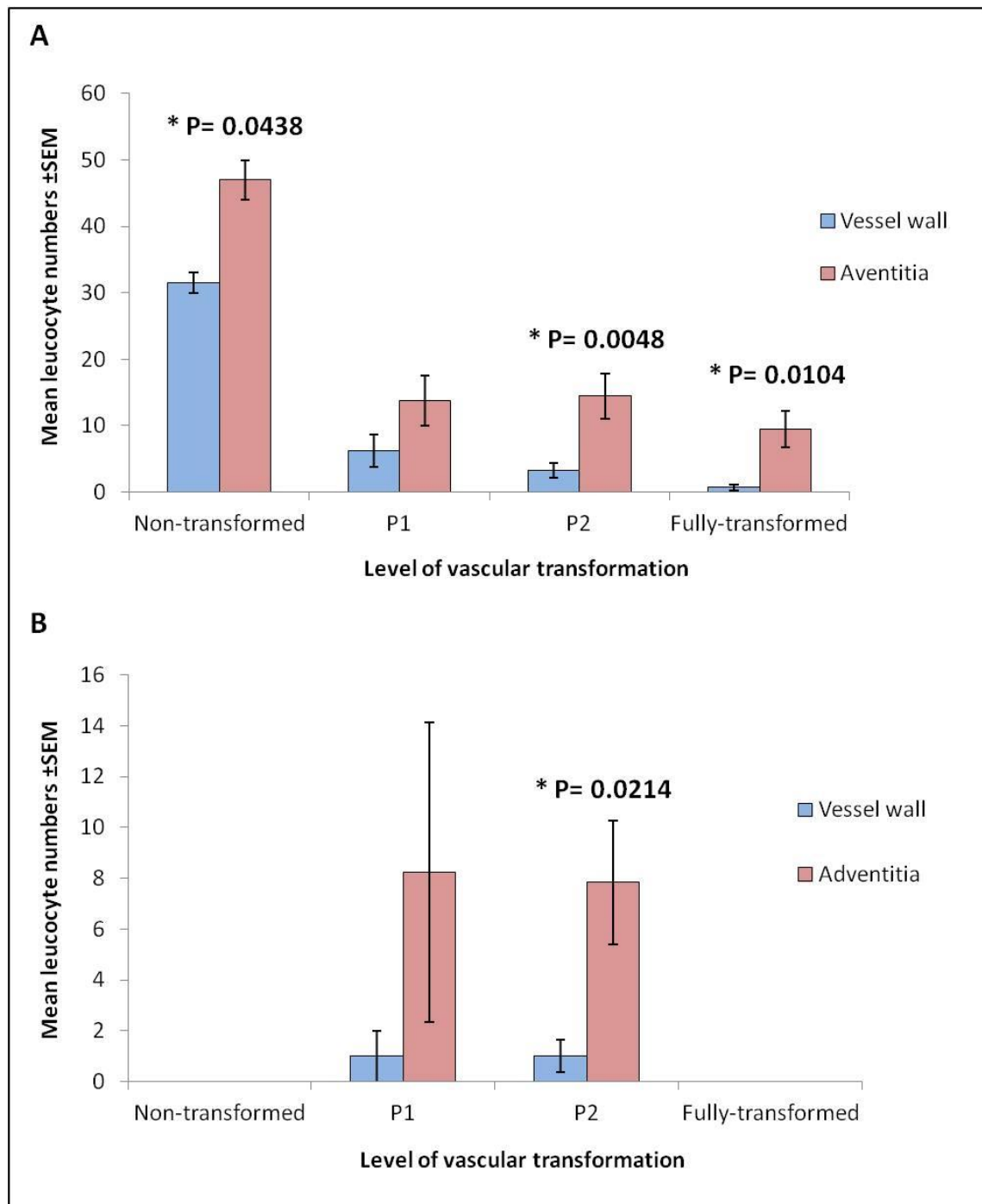
Figure 6.15 Comparison between CD3+ T lymphocyte numbers observed in SpA vessel wall with numbers observed in SpA at both 8-10 weeks and 12-14 weeks gestation
Graphical representation of CD3^{+} cell numbers in SpA walls compared with numbers observed in adventitia in placental bed biopsies at both 8-10 weeks (A) and 12-14 weeks (B) gestation (8-10 weeks GA: non-transformed n=3, P1 n=8, P2 n=17, fully transformed n=12 vessels and 12-14 weeks GA: non-transformed n=3, P1 n=5, P2 n=8, fully transformed n=8 vessels). Data are expressed as Mean ± SEM. Significance is denoted by P≤0.05.

In conclusion there were more leucocytes present in association with spiral arteries at 8-10 weeks gestation compared with 12-14 weeks gestation. In addition, numbers of the three leucocyte populations studied were higher in the adventitia (around the vessel wall) rather than within the vessel wall itself, with CD14$^+$ cells being the most prominent cell population.

6.4.3 Leucocyte numbers in the presence of EVT cells

Leucocyte populations were observed clustered around vessels and in the vessel walls in both gestational age groups in the absence of EVT. However, the numbers of all the leucocyte populations appeared to decline when EVT cells were present.

All of the non-transformed vessels observed in this study did not contain interstitial EVT cells in proximity to the vessels and there were no presence of intramural or endovascular EVT in the wall of the non-transformed SpA either, but the non-transformed SpA were not absent of leucocytes at both GA groups.

At 8-10 weeks gestation 50% of P1 vessels examined had interstitial EVT cells present, 25% of P1 vessels also had intramural EVT cells embedded in the vessel wall but none of the P1 vessels showed endovascular EVT cells. All of the P2 vessels had interstitial EVT cells present, while intramural EVT were observed in 82% and endovascular EVT in 6%. All of the fully transformed vessels observed contained both interstitial and intramural EVT, with 58% of them having endovascular EVT present.

Of all of the P1 vessels assessed at 12-14 weeks gestation, 40% had associated interstitial EVT, while no intramural or endovascular EVT were observed in any of the P1 SpAs. All of the P2 vessels analysed had associated interstitial EVT, 75% contained intramural EVT and 50% had endovascular EVT present in their lumen. All of the fully transformed vessels observed had both interstitial EVT with intramural EVT and 75% endovascular EVT.

Since all non-transformed arteries did not show associated EVT but did show leucocytes, whereas all P2 SpAs and fully transformed spiral arteries had EVT in associated to them, it was therefore decided to focus on the numbers of leucocytes in P1 vessels at both gestations to investigate whether the presence of EVT affected leucocyte cell numbers (Figures 6.16-6.18).

All three leucocyte numbers were slightly higher in both the adventitia and the wall in P1 SpAs when EVT cells were absent (CD56$^+$ Figure 6.16; CD14$^+$ Figure 6.17; CD3$^+$ Figure 6.18). This difference however, was only statistically significant for the numbers of CD3$^+$ leucocytes within the adventitia of P1 SpA, with cell numbers being higher in the absence of EVT cells (Figure 6.18; P=0.0312).
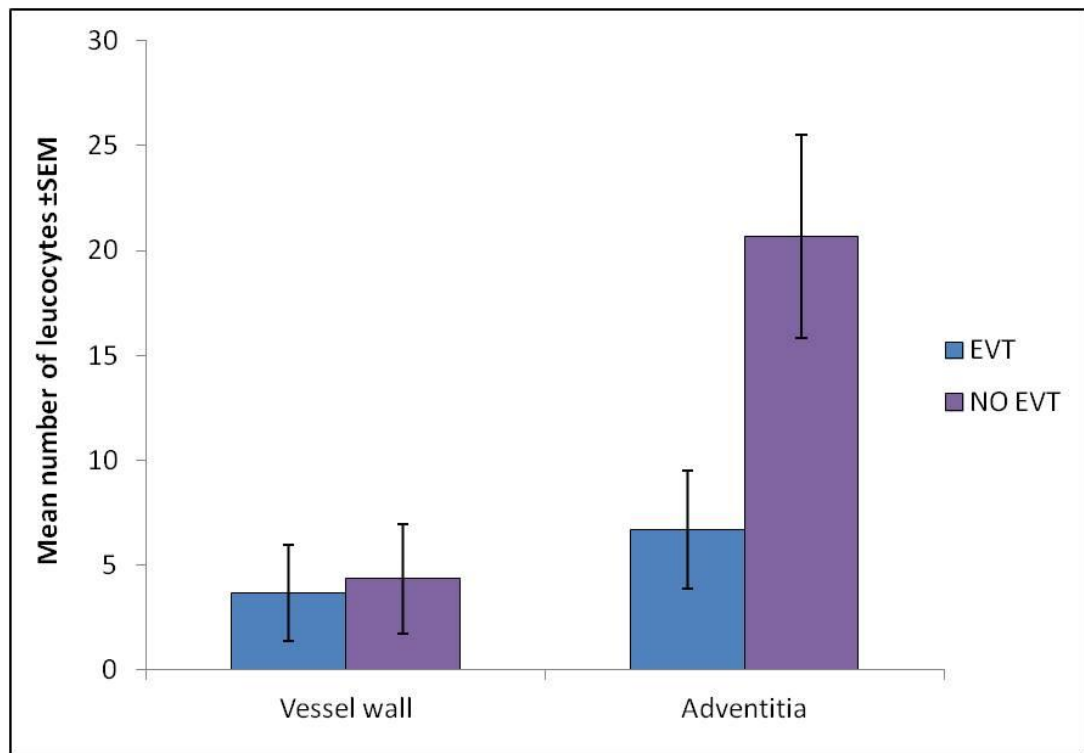
Figure 6.16 Mean CD56$^+$ cell numbers in P1 SpA vessel wall and adventitia in both the presence and absence of EVT at 8-14 weeks gestation
Graph illustrating the average number of CD56$^+$ cells observed in VSMC wall of P1 vessels and in the adventitia of P1 SpA in both the presence and absence of EVT at 8-14 weeks gestation (n=13 vessels assessed). Data expressed as Mean ± SEM.
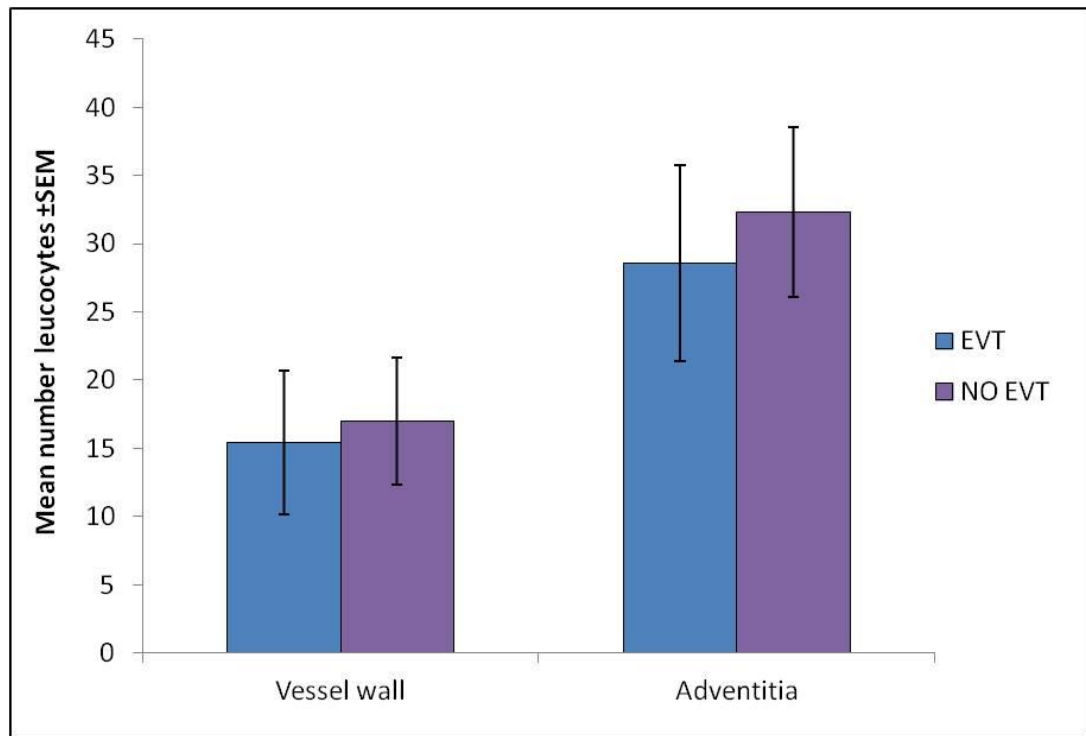
Figure 6.17 Mean CD14$^+$ cell numbers in P1 SpA vessel wall and adventitia in both the presence and absence of EVT at 8-14 weeks gestation
Graph illustrating the average number of CD14$^+$ cells observed in VSMC wall of P1 vessels and in the adventitia of P1 SpA in both the presence and absence of EVT at 8-14 weeks gestation (n=13 vessels assessed). Data expressed as Mean ± SEM.
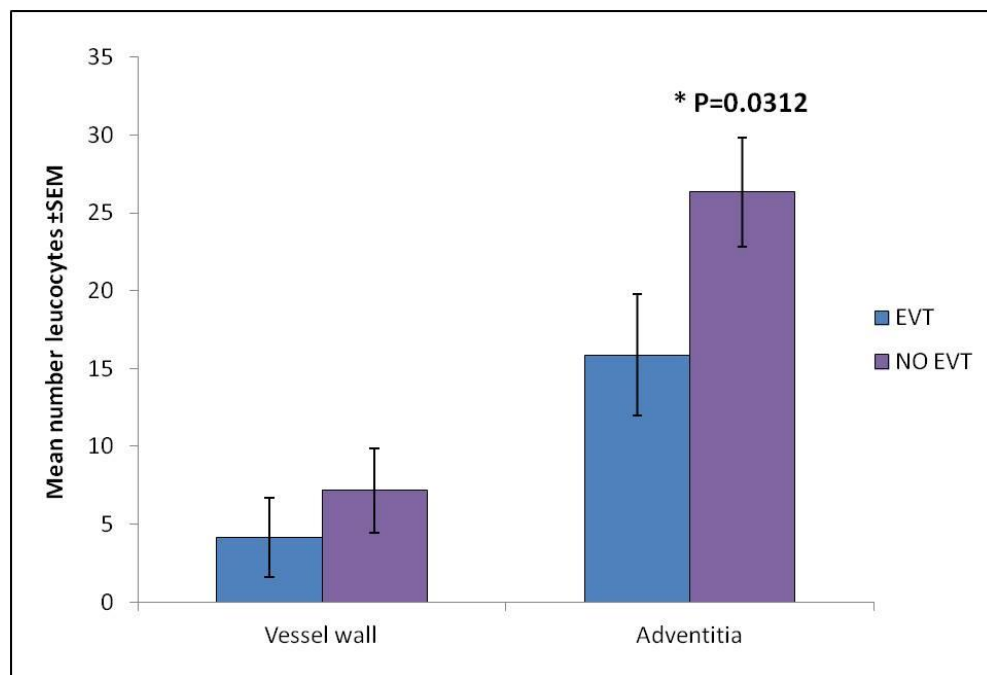
Figure 6.18 Mean CD3$^+$ T lymphocyte numbers in P1 SpA vessel wall and adventitia in both the presence and absence of EVT at 8-14 weeks gestation
Graph illustrating the number of CD3$^+$ cells observed in VSMC wall of P1 vessels and in the adventitia of P1 SpA in both the presence and absence of EVT at 8-14 weeks gestation (n=13 vessels assessed). Data are expressed as Mean ± SEM. Significance is denoted by P≤0.05.

CD14$^+$ cells also appeared to be clustered around interstitial EVT cells in proximity to spiral arteries (Figure 6.19) but this apparent association was not quantified.
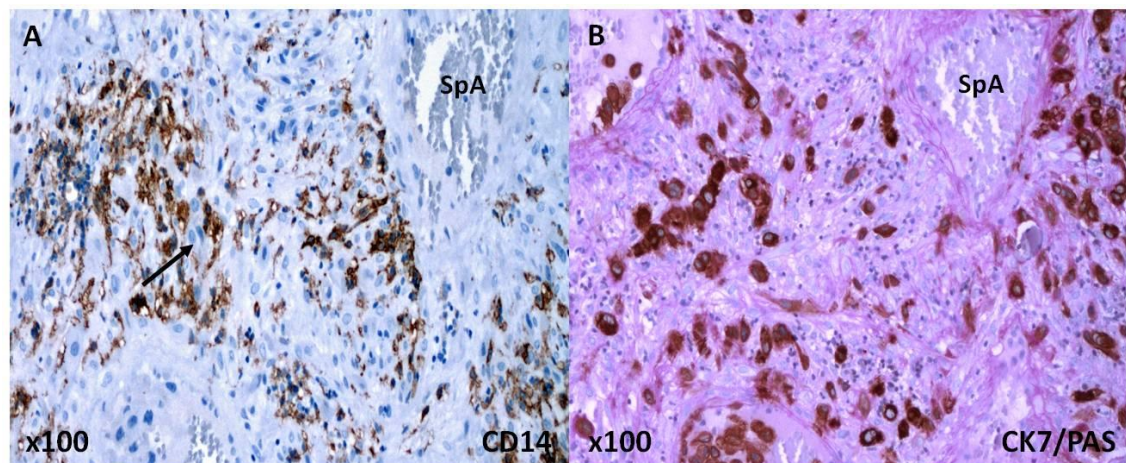
Figure 6.19 Immunohistochemistry image showing the relationship between CD14$^+$ cells and EVT in early pregnant decidua
Representative image showing immunostaining in a placental bed biopsy in early pregnant decidua. A represents CD14 cells and B represents interstitial EVT cells in proximity to one another. Original magnification x100.

6.4.4 Characterisation of pure, viable decidual macrophage population

The isolation of decidual macrophages was achieved in this study but in standard conditions viability decreased after culture (RPMI medium + 10% FCS; section 6.3.4.3). Alternative culture conditions were therefore attempted. The average CD14$^+$ cell viability for all three decidual samples for each 24h culture treatment is outlined in Table 6.3.

Treatment with DMEM media supplemented with 10% ATCC FCS and without GM-CSF addition resulted in 73% of CD14$^+$ cells being viable after24 hours of culture, this treatment was therefore used for further cultures. After optimising culture conditions we managed to isolate and culture a viable, enriched source of decidual macrophage cells. Cell smears prepared at T=0 illustrate key expression markers characteristic of macrophages (Figure 6.20). In the samples examined 99% of the cells were immunopositive for CD14 protein following immediate isolation. All of the CD14$^+$ cells isolated were immunopositive for HLA-DR protein; 90% of the CD14$^+$ cells were immunopositive for CD86 protein; 60% of the CD14$^+$ cells examined were immunopositive for CD83 protein; 30% of these cells were immunopositive for CD11b;

20% of the enriched cells were immunopositive for CD209, whereas only 10% of the enriched CD14$^+$ cells were positively stained for Alpha1-Antitrypsin (A1AT).

Table 6.3 CD14$^+$ cell viability with differing culture conditions
Cell viability in different media conditions used to culture isolated viable decidual CD14$^+$ cells (% of viable cells after 24h in culture; n=3).

Media	FCS	± GM-CSF	% viability
RPMI	10% sigma FCS	NO	35%
RPMI	10% sigma FCS	YES	32%
RPMI	10% ATCC FCS	NO	71%
RPMI	10% ATCC FCS	YES	69%
RPMI	10% Dendritic FCS	NO	50%
RPMI	10% Dendritic FCS	YES	50%
DMEM	10% sigma FCS	NO	40%
DMEM	10% sigma FCS	YES	38%
DMEM	10% ATCC FCS	NO	73%
DMEM	10% ATCC FCS	YES	71%
DMEM	10% Dendritic FCS	NO	50%
DMEM	10% Dendritic FCS	YES	50%

Cells which were cultured for 24hrs following isolation were also labelled for CD14, CD209, CD83 and CD86 and the percentage of positively stained cells compared with the T=0 smears. 100% of the cells were CD14 positive, 90% of the cells stained strongly positive for CD86, 70-80% of the cells showed positive internalised staining for CD83 and 20% of cells showed dispersed staining for CD209, suggesting that these cells maintain phenotype in 24h culture.
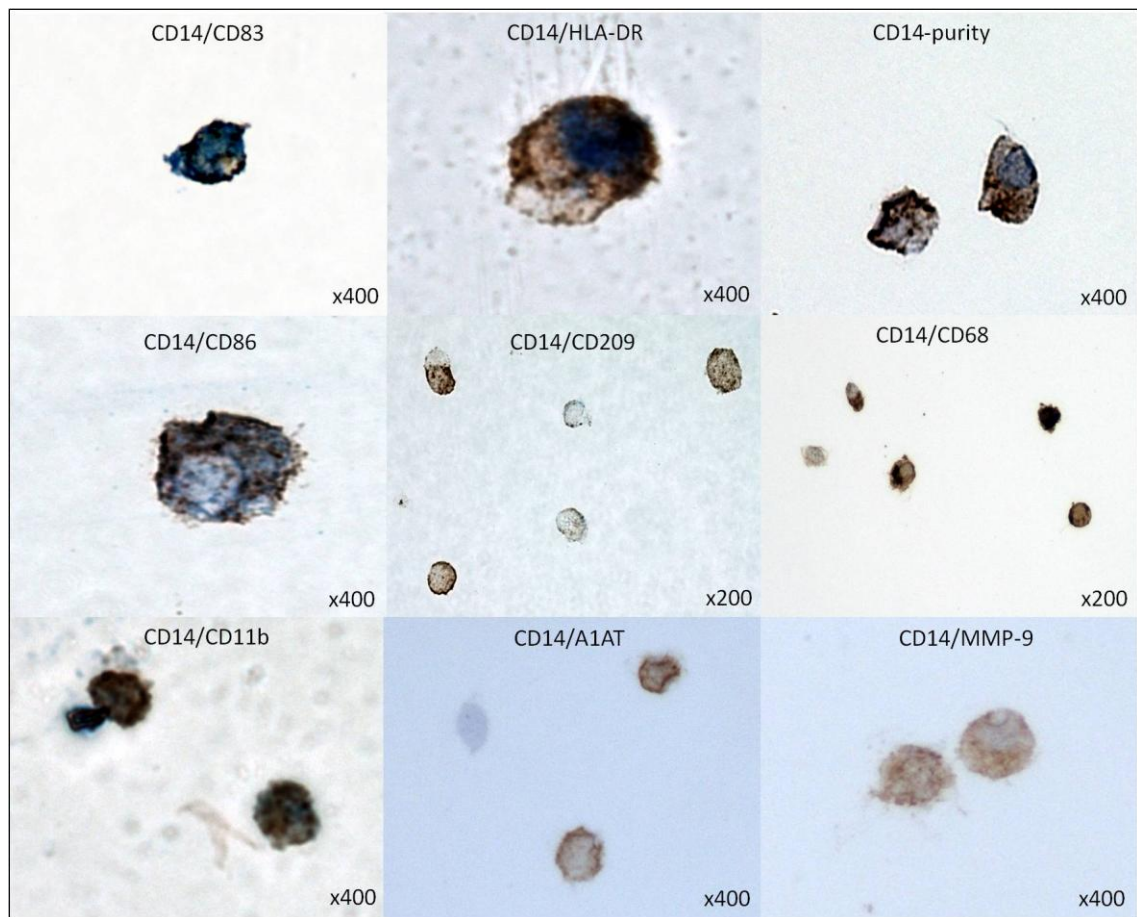
Figure 6.20 Double labelled CD14+ cells with varying macrophage markers
Representative images of decidual CD14$^+$ cell smears following Midimacs cell isolation (T=0).
CD14 positively labelled cells (brown) were double labelled with various macrophage markers
(blue) n=3.

To further confirm the expression of the protein markers and to prove that the positive
immunoreactivity was not a result of the isolation procedure frozen tissue sections of
first trimester decidua were immunostained. *In situ* immunostaining (Figure 6.21)
revealed strong positively stained CD14 cells with strong staining for HLA-DR and CD68.
Cells in the decidua were positively stained for CD86 but we cannot conclude that
these cells are CD14$^+$ cells. Immunostaining for MMP-9 and CD83 was confined to the
walls of spiral arteries. Only a few cells stained positively for CD11b and A1AT was
undetectable in the decidua.
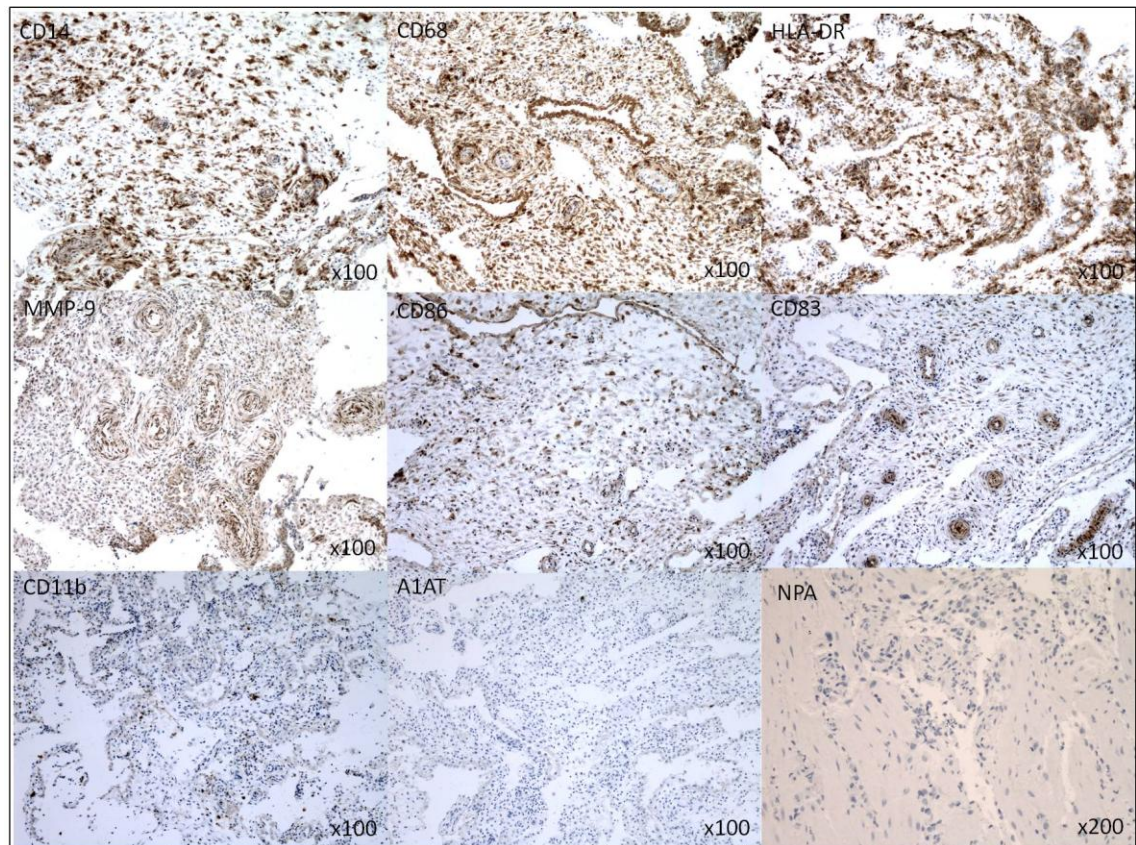
Figure 6.21 Immunostaining of decidual tissue with different macrophage markers
Immunohistochemistry illustrating immunostaining of decidual tissue with different macrophage markers (from left to right: CD14, CD68, HLA-DR, MMP-9, CD86, CD83, CD11b and A1AT all x100 magnification).

6.4.5 Growth factor protein production by isolated CD14$^+$ cell supernatants from 8-10 and 12-14 weeks gestation

The data presented from this study provide evidence that CD14$^+$ cells are the most prominent leucocyte cell population that are associated with VSMCs within the wall of spiral arteries in early pregnancy decidua. Therefore, having isolated an enriched population of CD14$^+$ cells from early pregnancy decidua (section 6.3) and optimised culture conditions, the cells were cultured for 24h for supernatant collection. Secretion of an array of growth factors and cytokines was measured using ELISA and FASTQuant and protein levels were compared between the gestational age groups to investigate whether decidual macrophages produce growth factors and cytokines which could potentially be involved in the remodelling of the spiral arteries in early pregnancy.

6.4.5.1 Human Th1/2 growth factors

Using the Th1/2 FASTQuant multiplex array, it was shown that decidual CD14^{+} cells secreted large quantities of IL-6 (~13000pgml[-1]), IL-1β (ranging from ~11000-70000pgml[-1]) (Figure 6.22A) and TNF-α (ranging from ~1200-5000pgml[-1]) along with substantial quantities of IL-10 (~1000pgml[-1] both GA), IL-4 (~1200pgml[-1]) and IL-13 (~500pgml[-1]). However, IL-5 and IL-2 were only detectable at very low levels (Figure 6.22B).
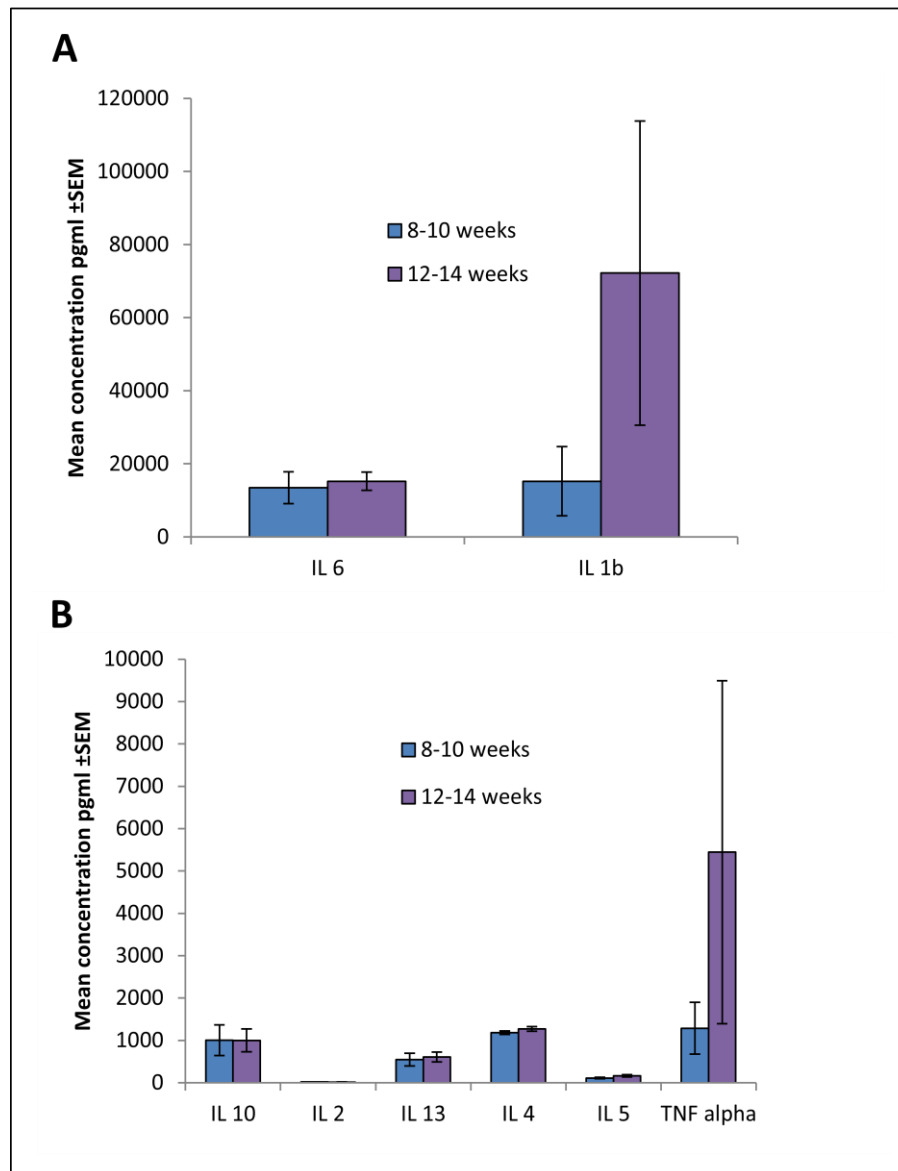
Figure 6.22 Secretion of Th1/Th2 growth factor proteins secreted by CD14^{+} cells at both 8-10 weeks and 12-14 weeks gestation
Graphical representation demonstrating the Th1/Th2 growth factor proteins secreted by isolated decidual CD14^{+} cells at 8-10 weeks GA (n=7) and at 12-14 weeks GA (n=5). A: represents IL-6 and IL-1β protein secretion; B: represents IL-10, IL-2, IL-13, IL-4, IL-5 and TNF-alpha secretion. Data are expressed as Mean ± SEM.

6.4.5.2 Human angiogenic growth factors

From the proteins measured using the angiogenesis FASTQuant multiplex array (8-10 weeks GA n=7; 12-14 weeks GA n=5) and Ang-1 ELISA (8-10 weeks GA n=11; 12-14 weeks GA n=8; duoset, R&D Systems) it was shown that decidual CD14$^+$ cells secreted large quantities of Ang-1 and Ang-2 and FGF-basic protein. Angiogenin, VEGF-A and KGF were secreted at low levels while PDGF-BB was undetectable (Figure 6.23).

The concentrations of TGF-β1, PLGF and VEGF-C in CD14$^+$ cell suspension were all measured using a Duoset sandwich ELISA (R&D Systems) but these proteins were not detected.
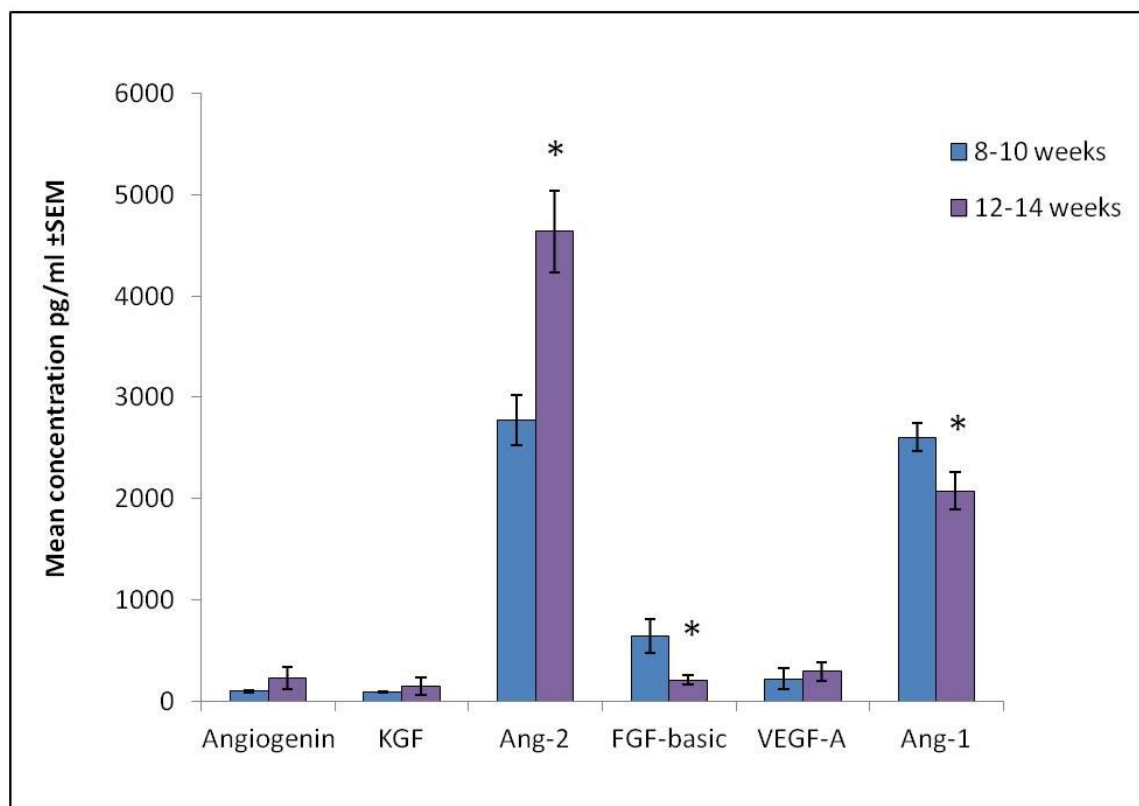
Figure 6.23 Secretion of angiogenic growth factor proteins secreted by CD14$^+$ cells at both 8-10 weeks and 12-14 weeks gestation
Graphical representation demonstrating angiogenic growth factor proteins secreted by isolated decidual CD14$^+$ cells (Angiogenin, KGF, Ang-2, FGF, VEGF-A: 8-10 weeks GA (n=7); 12-14 weeks GA (n=5) and Ang-1 ELISA: 8-10 weeks GA (n=11); 12-14 weeks GA (n=8)). Data are expressed as Mean ± SEM.

6.4.5.3 Differences between gestational ages

There are no significant gestational differences of Th1/2 protein levels, although there is a noticeable increase of IL-1β and TNF-α at 12-14 weeks gestation compared with 8-10 weeks gestation (Figure 6.22). Statistical significance may have not have been achieved due to large variation in levels of these cytokines between samples (8-10 weeks n=7; 12-14 weeks n=5).

Ang-1, Ang-2 and FGF-basic protein levels differed significantly between the gestational age groups (8-10 and 12-14 weeks; Figure 6.23). Ang-2 secretion by CD14$^+$ cells was significantly increased at 12-14 weeks compared with levels at 8-10 weeks gestation (P=0.0019). In contrast, secretion of Ang-1 and FGF-basic protein by CD14$^+$ cells was significantly decreased at 12-14 weeks gestation compared with levels at 8-10 weeks gestation (P=0.0311; P=0.0477 respectively).

6.5 Discussion

6.5.1 Leucocyte involvement in spiral artery remodelling

The remodelling of spiral arteries is a complex series of events that ensures an adequate supply of blood and nutrients to the developing fetus to allow successful pregnancy. However there is still much debate on the mechanisms that control such events due to the difficulty of obtaining appropriate research material from first and second trimester pregnancies. Immunohistochemical evidence has shown that EVT cells invade both the wall and lumen of spiral arteries, indicating that they have relevance in controlling the remodelling events (Pijnenborg et al., 1983). However, VSMC swelling, migration and endothelial vacuolation are apparent prior to the invasion of trophoblast cells (Smith et al., 2009).

This study used an immunohistochemical approach to investigate immune cells of the decidua and their association and potential involvement in SpA remodelling. The release of soluble products of uNK cells and decidual T cells have been reported previously (Lash et al., 2006c, Scaife et al., 2006) but in this study decidual macrophages were closely related to spiral arteries, the soluble products of decidual

CD14$^+$ cells were also analysed to determine whether they may contribute to the remodelling events prior to EVT invasion.

The three leucocyte populations investigated in this study were all observed to be associated with decidual SpAs at 8-10 weeks and 12-14 weeks gestational age using an immunohistochemical approach, indicating a potential role in the events of SpA remodelling. Data from this study also presents a method for characterising stages of vascular remodelling using specialised staining techniques and measurements of immunostained SpA VSMC walls.

There was a large accumulation of leucocytes in proximity to decidual SpAs within early placental bed. The highest numbers of leucocytes were observed in vessels that had not yet begun the process of remodelling and leucocytes were clustered around the vessels. This could be indicative of the leucocytes priming themselves ready for the remodelling events or by simply sending signals to EVT cells. Leucocyte numbers did decline in early transforming vessels (P1) where they were still present within the spiral artery muscular walls and adventitia of the remodelling vessels prior to EVT invasion, suggesting an involvement in the separation and/ or loss of VSMC by apoptosis or aiding cellular migration or VSMC de-differentiation. P1 vessels observed in this study did not have many EVT cells in the vessel wall also suggesting a likely role for leucocytes in early stages of remodelling. These events may be independent or may be mediated by invading EVT, by immune cells of the maternal local environment or by both mechanisms.

The infiltration of leucocytes around and into SpA in the early stages of remodelling demonstrates that initial transformation of the decidua occurs before EVT involvement and provides circumstantial evidence that disruption of SpA VSMC are initially driven by factors released by these cells and the process is not initiated by EVT further concluding the controversial theory that extravillous trophoblast cells are not responsible for early stages of transformation (Pijnenborg et al., 2006b, Smith et al., 2009). Smith et al. (2009) recently demonstrated that uNK cells and macrophages produce MMP-7 and 9 along with reports of production of MMP-2 and -9 by uNK cells (Naruse et al., 2009) providing evidence that these cells have the ability to degrade vascular extracellular matrix (ECM) and therefore contribute to disruptions in vessel

integrity. However, the mechanisms by which the leucocytes may control early remodelling events remains poorly understood. The main focus has been on uNK cells with production of Ang 1 and vessel destability (Robson et al., 2012) but decidual macrophages are equally abundant and therefore have a potential role to play.

The differences of vessel transformation and leucocyte numbers associated with the transforming vessels within the placental bed have not yet been previously investigated across gestational age. In this study the number of leucocytes in VSMC wall of SpA decreased from 8-10 weeks to 12-14 weeks GA, further suggesting a possible role in early vessel transformation before invasion of EVT. Although, leucocyte numbers declined in SpAs they were still prevalent in the VSMC wall of SpAs at 12-14 weeks gestation suggesting a partnership with EVT to achieve full transformation into large, conduit vessels lacking medial muscle and elastic ensuring an adequate blood supply for successful pregnancy. Leucocyte numbers significantly decreased as vessel transformation progressed further indicating a role for EVT in later remodelling stages. The localisation of leucocytes in early stages of remodelling suggests a role for the immune cells in VSMC cell loss, increased vessel permeability and VSMC in SpA undergoing migration, thereby facilitating the infiltration of interstitial EVT into the vessel wall and endovascular EVT invasion into the lumen.

6.5.2 EVT remodelling

It has been documented in this study (Chapter 4) along with numerous previous reports that EVT invasion is controlled by a number of factors including, chemokines, cytokines, MMPs and varying oxygen concentrations (Lash et al., 2006a, Naruse et al., 2010, Dimitriadis et al., 2010). Factors produced by maternal immune cells (including uNK cells, macrophages and T cells) are likely to have an influence on the regulation and behaviour of invading EVT. They may achieve this level of control either directly through molecular interactions with the trophoblast cells or indirectly by the secretion of soluble factors. Immunohistochemical evidence show changes in EVT expression cell surface markers, particularly adhesion markers, as they near spiral arteries preparing the cells for interaction with the VSMCs (Zhou et al., 2003). The interactions with maternal leucocytes or their secretory products may affect EVT phenotype to achieve this. Alternatively co-culture of EVT cells and uNK cells has been shown to reduce

secretion of IL-6, IL-8 and TGF-β1 (Lash et al., 2011); whether inhibiting effects on uNK cell secretion or EVT secretion or both is not known but co-culture of these two cell types leads to inhibition of secretory products.

6.5.3 Mechanisms of leucocyte remodelling

The focus of this study was to investigate the effect of the most abundant leucocyte populations and their relationship with SpAs and their contribution to remodelling events. Maternal leucocytes have been proposed to play a role in SpA remodelling but the mechanisms remain unclear. A role for uNK cells in spiral artery remodelling has been hypothesised (Croy et al., 2003, Lash et al., 2006c, Robson et al., 2012) and has been linked with remodelling in mouse (Ashkar and Croy, 2001, Croy et al., 2003) but has never been established in humans. The factors produced by uNK cells strongly suggest a possible involvement in SpA remodelling, especially a role in initiating the remodelling process. Reports demonstrate that uNK cells are major producer of angiogenic growth factors (Li et al., 2001, Lash et al., 2006c, Hanna et al., 2006); they have been observed in the walls of remodelling vessels in situ before the invasion of EVT (Smith et al., 2009) and uNK cell secretory factors alter vascular integrity of chorionic plate arteries (Robson et al., 2012).

The role of macrophages in SpA remodelling has been proposed due to high numbers of macrophages observed in preeclampsia (Reister et al., 1999, Renaud and Graham, 2008) but this could also be likely of an immune response. CD3^{+} T cells and their role in remodelling has been sparingly investigated, although decreased numbers of CD3^{+} T cells in placental bed biopsies of women suffering preeclampsia has been observed compared with numbers in third trimester decidua controls (Williams et al., 2009b), indicating an importance for this cell type in pregnancy, perhaps in regulating spiral remodelling. The evidence from this study demonstrates a reduction in CD3^{+} T cells as SpA remodelling progresses, suggesting an early involvement prior to trophoblast invasion.

There is increasing evidence, including evidence from this study, that maternal leucocytes, in particular uNK cells and macrophages play an important role in spiral artery remodelling but the mechanisms remain unclear. Early SpA transformation which occurs at 8-10 weeks gestation involves a number of steps including vessel

dilation, swollen endothelial cells and the separation of VSMC layers. As gestational age increases, the VSMCs begin to de-differentiate and EVT cells displace the muscle and elastic arterial media with fibrinoid material which involves the complete loss of VSMC and transient loss of endothelium (Pijnenborg et al., 2006b). In this study we have observed a reduction in maternal leucocytes as gestational age increases, irrespective of the stage of remodelling, indicating that the cells are more than likely to play an important role in the early stages of SpA remodelling. This would also be in keeping with evidence that uNK cells secrete more Ang-1, Ang-2 and VEGF-C at 8-10 weeks compared to levels produced at 12 -14 weeks gestation (Lash et al., 2006c); these angiogenic growth factors are important in ECM matrix degradation and disruption of tight junction proteins between VSMC layers, therefore increasing separation and de-differentiation of VSMCs before the infiltration of EVT.

Macrophages are the second largest leucocyte population in the decidua in early pregnancy and have been detected in close contact with invading EVT and express receptors that are able to interact with ligands found on trophoblast cells (Bulmer and Johnson, 1984, Smith and Kelly, 1988, Reister et al., 2001, Apps et al., 2007). Despite their prevalence and close relationship with EVT little attention has been given to the role of decidual macrophages in normal pregnancy. Although high numbers of macrophages have been observed in early pregnancy their potential involvement in SpA remodelling has only been previously described by Smith et al. (2009). Potential roles for decidual macrophages in pregnancy have been speculated due to their roles in other tissues. Robertson et al. (2003) proposed that during the Th2 cytokine shift that monocytes are activated but there is a decrease in the synthesis of pro-inflammatory cytokines, indicating that the alternatively activated macrophages are likely to play an immunosuppressive role in response to fetal antigens and induction of tolerance. However, activated macrophages are also adept in tissue remodelling observed in other tissues by secretion of proteases, cytokines, growth factors which allow tissue remodelling and angiogenesis. In *in vivo* studies in mouse, activated macrophages have been associated with extensive vascularisation and tumour-bearing mice with depleted monocytes showed a decrease in tumour vascularisation (Evans, 1978). In relation to pregnancy, macrophages could control trophoblast invasion by secretion of TNF-α (Renaud and Graham, 2008). Therefore, providing circumstantial

evidence that macrophages have a possible involvement in SpA remodelling in human pregnancy.

The production of MMPs by uNK cells and macrophages infiltrating remodelling SpAs demonstrated by Smith et al. (2009) suggest that these leucocoytes contribute to vascular remodelling by influencing VSMC migration although evidence for migrating VSMC has not been observed in any other vascular system.

In the present study CD14$^+$ cell numbers were highest in proximity to the remodelling decidual spiral arteries compared to CD56$^+$ and CD3$^+$ T cells at both gestational ages. This further led to the hypothesis that these cells may have a strong involvement in coordinating the events of SpA transformation and that they may achieve this by producing proteins that may play a role in either regulating trophoblast dependent mechanisms or by the cells themselves producing factors that regulate angiogenesis.

The mechanisms that underlie VSMC loss during SpA remodelling events are up for much debate. Harris et al. (2006) and Keogh et al. (2007) have demonstrated initiation of VSMC apoptosis by trophoblast cells via FAS/FASL and TRAIL pathways *in vitro*. Furthermore, Smith et al. (2009) detected VSMC apoptosis using TUNEL staining in decidual SpA undergoing remodelling (*in vivo*) before infiltration of endovascular EVT. However, the degree of VSMC apoptosis was not quantified and TUNEL staining alone is not the most reliable marker of apoptosis. The use of an animal based model also reported evidence of VSMC apoptosis induced by trophoblast cells (Red-Horse et al., 2006). In contrast, (Bulmer et al., 2012) did not detect significant VSMC apoptosis in partially remodelled SpA with only one apoptosed VSMC detected after assessment of 294 myometrial and decidual SpAs regardless of EVT influence.

An alternative explanation proposed for VSMC loss in SpA remodelling is that SpAs undergo de-differentiation. Reports in guinea pig have demonstrated that SpA VSMC de-differentiate into a non-contractile form which remained associated with the vessel until re-muscularising after delivery (Nanaev et al., 2000). However, in humans the VSMCs in the vessel wall are completely displaced with fibrinoid material and intramural EVT. Recent theories are also emerging that SpA VSMCs detach from the vessel and migrate away from the vessel lumen (Whitley and Cartwright, 2010). Bulmer et al. (2012) observed *in situ* migration of VSMCs away from SpAs which were

associated with interstitial, intramural or endovascular EVT; VSMC migration was more apparent in the presence of EVT. Overall, this is accumulating evidence that indicates a role for EVT in the later stages of vascular remodelling and highlights the importance of EVT in finalising the remodelling events, whilst maternal leucocytes may play a role in initiating the primary stages of SpA remodelling. In this study SpA associated leucocytes reduced in number in the later stages of SpA remodelling in the presence of intramural and endovascular EVT. Leucocyte products may play a role in VSMC cellular migration by the production of angiogenic growth factors such as VEGF-A, -C, Ang-1 and -2 which are produced by uNK cells and are also produced by decidual CD14$^+$ cells shown in this study.

6.5.4 Role of macrophages in SpA remodelling

Macrophages are highly versatile cells that adapt to their environment and are capable of performing a variety of functions.

The overwhelming presence of CD14$^+$ cells in association with the SpA walls observed in this study raises the possibility of a role in VSMC loss; this could be by phagocytosis or cellular migration through the production of Ang-1 and -2; production of TNF-α and IL-1β also may suggest roles in differentiation and apoptosis of VSMC. The differences in the secretion of angiogpoeitins between the gestational ages suggest a possible role for macrophages in SpA remodelling. Ang-1 protein secretion by CD14$^+$ cells reduced significantly as gestation increased while Ang-2 secretion significantly increased with gestation. Ang-2 acts as a competitive inhibitor of Ang-1; Ang-2 favouring vessel destabilisation allowing angiogenesis and counteracts vessel maturation, whereas Ang-1 stimulates the formation of new blood vessels. A reduction in the ratio of Ang-1 to Ang-2 would therefore favour vessel destabilisation. Thus in early gestation CD14$^+$ cells may contribute to vessel stability and then as remodelling is initiated the action of macrophage derived Ang-2 facilitates VSMC separation and endothelial cell disruption through its anti-angiogenic effects.

The TNF-α that is secreted by macrophages may also contribute to EVT apoptosis; it has been demonstrated that TNF along with IFN-γ increase the expression of pro-apoptotic factor XAF1 in trophoblast cells initiating the production of caspase-3 inducing cell death (Abrahams et al., 2004b, Straszewski-Chavez et al., 2004). In

complicated pregnancies such as in preeclampsia and spontaneous abortion, trophoblast cells undergo abnormal and extensive apoptosis (DiFederico et al., 1999, Minas et al., 2007). In addition, Reister et al. (2001) have reported that TNF secreted by activated macrophages reduces endovascular EVT invasion by inducing apoptosis observed in the uterine wall of preeclamptic women.

It was also observed in this study that there was a close relationship between EVT and CD14$^+$ cells *in situ*. This interaction is likely to occur through cell surface receptor expression; the homodimer HLA-G expressed uniquely by trophoblast cells is recognised by LILR (leucocyte immunoglobulin-like receptor) which is expressed by macrophages and dendritic cells and LILRB1 and B2 inhibitory receptors have high affinity for HLA-G (Shiroishi et al., 2003) indicating a potential role for trophoblast cells in controlling the maternal immune response in early pregnancy. It has been suggested that HLA-G dimers may act as a pregnancy-specific signal to regulate the maternal immune response and interacts with all maternal leucocytes at the maternal-fetal interface (Apps et al., 2007). With the marrying receptor repertoire of macrophages and EVT through HLA-G/LILRB1 binding this could translate into the release of many growth factors and cytokines by both cell populations and therefore, affect the processes involved in early pregnancy like SpA remodelling and trophoblast invasion by modulating numerous functions. One potential mechanism arising from this interaction is for CD14$^+$ cells to phagocytose interstitial EVT cells as it is evident that EVT cell numbers decline as gestational age increases and as remodelling progresses which is observed in this study. However, further investigation into this hypothesis using phagocytosis assays may conclude this role for macrophages.

6.5.5 Overall summary

This study has provided circumstantial evidence for a role for maternal leucocytes in the early stages of SpA remodelling by their localisation to SpA undergoing early remodelling. For the first time an enriched macrophage population was isolated from human decidual tissue which remained viable after 24h in culture and produced angiogenic growth factors which may be important mediators in the early stages of SpA remodelling independent of trophoblast cells.

The studies developed along the years have given insight into the regulation of SpA remodelling. Further research will finally elucidate the exact roles of EVT dependent and independent pathways in SpA remodelling and may give an understanding of the mechanisms involved in pathological pregnancies which then can be translated into clinical treatment. The interactions between EVT and leucocytes through HLA-G/LILR and KIR recepetors (expressed by uNK cells), SpA VSMCs and leucocytes and SpA VSMCs with EVT are all basis for future studies. The focus has been on uNK cells but macrophages merit further study.

Chapter 7
Discussion and Future Studies

7. Discussion and Future Studies

7.1 Discussion

7.1.1 Purpose of study

Inflammatory cytokines have been implicated in pregnancy function for some time, although exact functional roles for these cytokines remain uncertain, due to the complexity of the cytokine milieu within the uterine decidua.

The angiogenic, migratory function of IL-8 and the invasive capacities of IL-6 in diseases such as cancer have led to interest in their potential roles in pregnancy, particularly on the effects that these cytokines may have on the development of early pregnancy. This has led to investigations of the involvement of cytokines in pregnancy complications such as pre-eclampsia and sporadic miscarriage, with alterations of these cytokines being linked to the pregnancy pathology.

7.1.2 Cytokine production and localisation

This is the first study to date that has documented the exact sites of cytokine location within the decidual tissues of the first half of human pregnancy and has demonstrated receptor expression by specific cells within the decidua, with gestational age comparisons. Many studies in the past have focused on maternal serum cytokine levels or levels within the amniotic fluid (reviewed in Chapter 1). Levels of intrauterine IL-6 and IL-8 secretion were quantified by decidual cell populations abundant during pregnancy development such as uNK cells, macrophages, T-lymphocytes and decidual stromal cells. A gestational age difference in cytokine levels was observed, suggesting a functional role either in initiating the remodelling events that occur in the spiral arteries in normal pregnancy or in regulating EVT invasion. By gaining a clear understanding of the role of these cytokines in normal healthy pregnancy, it will be possible to unravel the mechanisms whereby altered levels contribute to pregnancy complications. This may in turn lead to targeting therapies and in turn have clinical relevance.

7.1.3 Functional role of intrauterine IL-6 and IL-8 in EVT invasion

Uterine natural killer cells and decidual macrophages are sources of uterine IL-6 and IL-8 in the first half of human pregnancy. This provides evidence that these cytokines are involved in important roles in normal pregnancy, although exact *in vivo* roles still need to be clarified. A suggestive role for IL-8 is in the stimulation of EVT invasion; if maternal leucocytes such as uNK cells and decidual macrophages fail to secrete optimum levels for appropriate regulation of trophoblast invasion into the maternal uterine tissues and spiral arteries, aberrant trophoblast invasion could result which may be a factor in the aetiology of SM. We have previously demonstrated that uNK cell derived IL-8 can stimulate EVT invasion (De Oliveira et al., 2010). However, in the current study we did not demonstrate a similar action for IL-6.

There have been many studies of the regulation of EVT invasion but the *in vivo* control mechanisms still remain to be elucidated due to the complexity of the factors involved and the potential interactions between EVT cells and the wide array of cells in the decidua. For a number of years it was suggested that cytokines play important roles in regulating EVT invasion either by inhibitory or stimulating mechanisms, with data providing evidence of TGF-β, IFN-γ, TNF-α, IL-11 and IL-8 involvement, amongst many others (Dimitriadis et al., 2005, Hanna et al., 2006, Lash et al., 2006b, Paiva et al., 2009, Otun et al., 2011). The effect of IL-6 on EVT invasion has been investigated in a number of studies but the use of different immortal trophoblast cell lines, including choriocarcinoma cell lines, has produced contradictory results (reviewed in Chapter 4). Findings in this study provide further evidence that immortalised EVT-like cell lines are an imperfect model for EVT. The present study investigated the effect of IL-6 on EVT invasion using placental explants from early human pregnancy and this has yet to be investigated by others. The use of primary EVT from early pregnancy placentas would have been more desirable to use in this study and would have provided a more pure population but after successful isolation the primary cells showed a restrictive invasive nature, a feature that has been observed by others previously (personal communication Martin Knofler). Although, in the present study IL-6 had no effect on EVT invasion from placental explants, IL-6 did significantly alter phosphorylation of various EVT signalling proteins in primary EVT cells. The cell signalling pathways are

complicated, involving extensive cross talk and it is therefore difficult to determine what effect this would have on EVT *in vivo.* There is some evidence suggesting a role for IL-6 in trophoblast differentiation, or perhaps a secondary affect which may in turn affect the waves of EVT invasion by other cytokines and growth factors. Since this area of research is so complex various different *in vitro* approaches are emerging; co-culture models of EVT and decidual cells or placental tissue co-cultured with decidual tissue from matched patients have been reported (Hazan et al., 2010, Robson et al., 2012) and could more closely mimic the environment of the maternal fetal interface, providing a clearer insight into how these cells behave by protein and genetic analysis.

7.1.4 Functional role of intrauterine IL-6 and IL-8 in SpA remodelling

In this study both IL-6 and IL-8 altered the morphology of VSMCs in CpAs (used as a model of spiral artery remodelling) indicating a possible mechanism in the regulation of SpA remodelling. Uterine NK cells and decidual macrophages have a very close association with SpAs in early pregnancy, providing circumstantial evidence that uterine IL-6 and IL-8 could play a role in SpA remodelling events, especially in the early events that occur prior to EVT invasion. The findings of expression of receptors for the cytokines; IL-6Rα, gp130 and CXCR2, on SpA VSMC in the present study provides further evidence for a role for these cytokines in SpA remodelling. These cytokines could work together synergistically at sites of spiral artery remodelling along with other soluble factors that are also produced by uNK cells and decidual macrophages such as VEGF-C, ANG2 and IFN-γ.

The present study provided circumstantial evidence for a role for uNK and decidual macrophages in the early stages of SpA remodelling prior to trophoblast remodelling. Previous studies have observed a close relationship between maternal leucocytes and decidual spiral arteries undergoing transformation in early pregnancy (Smith et al., 2009, Harris, 2011). In a more recent study factors secreted by first trimester uNK cells facilitate VSMC disruption using *ex vivo* vessel models (Robson et al., 2012). Imunohistochemistry data from the present study provide additional evidence to support this hypothesis, since uNK cells and decidual macrophages surround SpA *in situ* in the early stages of remodelling in early pregnancy. This is the first study to characterise different stages of SpA remodelling *in situ* by analysis of VSMC wall

thickness, although Smith et al. (2009) investigated leucocyte numbers at different remodelling stages, the stages weren't characterised by muscle thickness. This study analysed and compared leucocyte cell numbers associated with transforming SpA at each stage of remodelling in the presence and absence of EVT with gestational age comparisons.

The findings from this study and data from previous studies undoubtedly suggest that uNK cells play a role in initiating early SpA remodelling events, but less attention has been paid to the decidual macrophages. This cell population was dominant in spiral arteries *in situ,* far exceeding the numbers of uNK cells surrounding SpAs. Furthermore the present study provides evidence of secretion of high levels of angiogenic factors, such as Ang-1 and Ang-2, by decidual macrophages with gestational age differences. These results suggest a pivotal role for decidual macrophages in initiating early SpA remodelling events; these cells are equipped to induce vascular destabilisation prior to EVT invasion, priming the vessels for trophoblast mediated remodelling. Decidual macrophages, uNK cells and CD3$^+$T lymphocyte cell numbers in and around SpAs all dramatically reduced as the stage of SpA remodelling progressed with fewer cells present at 12-14 weeks GA compared with cell numbers at 8-10 weeks GA; demonstrating convincing evidence for a potential role in initiating the early SpA remodelling events prior to trophoblast invasion of the vessels. Maternal leucocytes may achieve this by cross talk with one another by coordinating themselves spatially.

Secretion of IL-8 and IL-6 in the decidua may provide the signals for the migration of decidual leucocytes to sites of SpA remodelling, or even contribute to the remodelling events themselves. Leucocyte secretion of IL-8 and IL-6 may also provide signals for invading extravillous trophoblast, so that they locate themselves around and within SpA to complete remodelling.

The mechanisms whereby maternal leucocytes cause VSMC disruption remain to be clarified. It has been suggested that macrophages secrete apoptotic factors that kill VSMCs in order to destabilise the vessel wall and also to phagocytose the dead cells and cellular debris by invading trophoblast (Smith et al., 2009). However, *in situ* studies of placental bed biopsies have found no evidence of VSMC apoptosis but have observed VSMC migration away from the SpA lumen (Bulmer et al., 2012); this is

another potential mechanism whereby secretion factors produced by maternal leucocytes could cause early SpA remodelling changes and could be an explanation for their high cell numbers surrounding early transforming arteries. This has recently been observed by Robson et al. (2012) where Ang-1, Ang-2 and IFN-gamma (factors secreted by both uNK cells and decidual macrophages) all contribute to vascular changes in both SpA and CpA.

7.1.5 Altered cytokine levels in sporadic miscarriage

Levels of both cytokines derived from uNK cells and macrophages were dramatically reduced in SM compared with healthy pregnancy. The reduction in IL-6 and IL-8 in SM may be due to fewer viable leucocyte numbers in SM due to increased apoptosis, although cell viability was checked at the end of the cell culture period, so this is possible but not likely in this study. Alternatively reduction in cytokine levels could be a result of impaired signalling within the uterine environment resulting in down-regulation of cytokine synthesis at either pre transcriptional stages or post translational stages.

A decrease in IL-6 and IL-8 receptors was also observed in SM indicating that cells in the decidua associated with SM have decreased capacity to interact with these cytokines; this may be a response to reduced cytokine production. However, as for all studies of miscarriage samples altered levels of these cytokines may be an effect of the condition and not the cause, although none of the SM samples in this study showed evidence of acute infection, therefore the results observed were not likely to be cause of infection. Although this study did not elucidate specific mechanisms of IL-6 and IL-8 in SM, the reduction in their receptor expression and secretion levels by uterine cells in SM strongly implies that IL-6 and IL-8 are important in early pregnancy. Their functional role may be regulating early events of spiral artery remodelling and this is a potential area for further study that may have future clinical relevance.

Investigation of normal and pathological early pregnancy development in humans is difficult since it is impossible to investigate the role of specific cells/factors *in vivo*. Isolating and culturing primary cells and cell lines in a monolayer destroys three dimensional interactions and the association of the extracellular matrix with neighbouring cells, potentially affecting cell behaviour. However, the use of placental

explants and Matrigel™ in the present study has partly overcome such problems, and the data are supported with *in situ* immunohistochemistry data in placental bed biopsies. Many *in vitro, ex vivo* and *in situ* models have provided great insights into the processes of pregnancy development and advances such as uterine artery Doppler ultrasound screening of spiral arteries is a useful tool for measuring artery resistance which can be used as a predictive measure of pregnancy outcome. A problem that cannot be overcome in using human early pregnancy tissue is that it is not known whether the pregnancy would have continued to term and whether those pregnancies would have developed clinical complications in later pregnancy including SM tissue where some samples collected may have had underlying causes and may lead to recurrent miscarriages.

7.1.6 Conclusion

The present study provides convincing evidence for the importance of maternal leucocytes in early pregnancy and that their secretory factors, including IL-8 and IL-6, have major roles in pregnancy processes with altered levels potentially contributing to pregnancy failure. The functional mechanisms, however, remain unclear; this is due at least in part to cytokine redundancy, pleiotrophy and the complexity of their interactions with one another as they may promote or inhibit actions of other factors. The large cytokine milieu at the maternal fetal interface suggests a large network of cross-talk between factors and that cytokines do not work alone in regulating the uterine environment.

7.2 Future Studies

7.2.1 Further investigation into functional roles of intrauterine IL-6 and IL-8

Although this study has defined some functions of IL-6 and IL-8 such as involvement in VSMC disruption and stimulation of EVT invasion, the effects observed in this study have raised more questions. The localisation and production of IL-6 and IL-8 by four decidual cell populations has been demonstrated in the first half of pregnancy, the total cohort of decidual cells produced large amounts of each cytokine, exceeding the

amounts by the individual cell populations investigated, suggesting that other cell types that were not studied are main producers of IL-6 and IL-8, which was not expected as the initial focus of this study was on uNK cells, but this demonstrates that other cell types are equally or not more important. Therefore, in further studies it would be important to isolate more decidual cell populations such as glandular epithelial cells to analyse their secretion of these cytokines. Immunohistochemistry data revealed intense staining for both IL-6 and IL-8 along with strong staining for gp130 on uterine glands, suggesting that this cell population are producers of these cytokines. Recent evidence has suggested that EVT invade uterine glands in early pregnancy (Moser et al., 2010) and this could raise the possibility of roles for these cytokines in regulating endoglandular EVT invasion.

Further investigation is required to determine the effect of IL-6 on trophoblast function as the present study demonstrated that IL-6 stimulated many proteins involved in the EVT signalling pathways and reduced EVT secretion of RANTES, although no effect on EVT invasiveness was observed. Furture investigation could gain insight to the alternative effects IL-6 has on EVT migration and EVT hCG secretion; the differentiation of trophoblast cells from cyototrophoblasts into invading extravillous trophoblasts or invading EVT into non invasive giant cells. It would be interesting to see whether decidual CD14$^+$ supernatants, cultured with isolated primary EVT from the same patient, had a similar effect and whether using neutralising antibodies against IL-6 would abrogate the effect. Although previous studies from our group (unpublished data) observed no evidence of IL-6 treatment on EVT apoptosis using M30 protein detection by Western blotting, this still could be an area for further clarification using immunocytochemistry, immunofluorescence and Western blot analysis for different apoptotic markers. EVT migration assays would be a further area to research also, given that production of RANTES by EVT was reduced after IL-6 treatment. In addition the effects of IL-8 on EVT signalling and secretion should be investigated to elucidate the mechanisms whereby IL-8 stimulates EVT invasion. Regulatory proteins may then be targeted for therapies in disorders where excessive EVT invasion is apparent; this could potentially be a cause of placenta accreta, although the decidua is absent in placenta accreta making it difficult to assess the extent of EVT invasion. The exact

mechanism for pathogenesis of the disease is currently unknown and is a major pregnancy complication, recently reviewed by Garmi and Salim (2012).

Observations of disruptions of CpA VSMC after cytokine treatment indicated a potential role for these cytokines in SpA remodelling. The effect of these cytokines on VSMCs can be further studied by treating primary vascular smooth muscle cells lines such as primary human aorta cells and measuring the protease activity and secretion of angiogenic proteins. Non-pregnant myometrial arteries are another possible source of a more directly relevant tissue and it would be good to investigate the effect of IL-6 and IL-8 on this vasculature and compare this with the effect observed with CpAs. It is accepted that CpAs are not a perfect model for SpAs as they differ in arterial composition compared with myometrial arteries, but endometrial arteries may differ in composition to myometrial arteries and the process of SpA remodelling may be very different in the decidua and myometrium. Chorionic plate artery vessels are, however, readily accessible and easy to dissect compared with myometrial and decidual SpAs. Recently it has been reported that both first trimester EVT and uNK cell conditioned medium disrupted VSMC morphology and disorganisation in both CpAs and myometrial arteries dissected from non-pregnant hysterectomy specimens (Robson et al., 2012). Potential soluble mediators of this effect were studies including Ang-1, Ang-2, IFN-γ and VEGF-C. However, other uNK and EVT cell derived factors including IL-8 and IL-6 may contribute to VSMC disruption; evidence from a recent study has demonstrated that both uNK and EVT cells produce a variety of other cytokines and angiogenic growth factors that are potentially involved in SpA remodelling (Lash et al., 2010a). This could be achieved by culturing CpAs and myometrial arteries with uNK cell supernatants as well as CD14$^+$ supernatants with neutralising antibodies against IL-6 and IL-8. Comparison of VSMC disruption in CpAs and non-pregnant myometrial arteries cultured with uNK cell and CD14$^+$ cell supernatants isolated from early SM decidua compared with supernatants from healthy pregnant decidua would be an interesting area to research; the hypothesis would be that VSMC disruption would be reduced in SM due to less IL-6 and IL-8 being produced by uNK and CD14$^+$ cells compared with these cells in normal pregnancy. This could also be coupled with Doppler measurements; levels of IL-6 and IL-8 could be measured in decidual supernatants in pregnancies which have had Doppler measurements taken. Abnormal

Doppler versus normal Doppler can be compared with decidual cytokine levels; blood vessel changes observed may be related to differences in decidual IL-6 and IL-8 levels, an area which has yet to be studied.

7.2.2 Further investigation into the role of maternal leucocytes

Further studies into association of maternal leucocytes and remodelling of spiral arteries in the myometrium are required to assess whether they play a role in initiating remodelling events deeper in the placental bed. Uterine NK cells are only present in decidua (Bulmer and Lash, 2005), and so it would be interesting to investigate macrophage and T lymphocyte cell numbers in the myometrium and their involvement in remodelling myometrial SpA. These cells could potentially provide signals to recruit invading EVT from the decidua or eEVT in the second wave of deeper EVT invasion to modify the SpAs deeper in the uterine tissues. Spiral artery invasion in the myometrium has been observed where uNK cells are absent, therefore other leucocytes may have role; this has been originally hypothesised but never proven (Pijnenborg et al., 2006b).

7.2.3 Limitations of tissue collection

Normal early pregnancy tissue was readily obtainable; a limitation of the present study was the difficulty in obtaining SM tissue, due to restricted availability, especially in obtaining late (≥13 weeks gestational age) SM tissue. Due to the difficulties encountered only early (≤ 12^{+6} weeks gestational age) SM decidua was collected. Many early miscarriages are now managed medically with misoprostol treatment and this limited the number of miscarriage cases that were available as hormone treatment could potentially alter cytokine levels. This study would have benefited from studies on late miscarriage tissue but late miscarriage samples are less common and may be associated with abnormal SpA remodelling (Ball et al., 2006a). Emotional issues of collecting samples from women suffering miscarriage may have also contributed to the limited availability of samples as many women were too upset to approach for consent. Therefore if there was more time to collect the tissue then cytokine levels from later SM decidua would have been compared to cytokine levels with those in early SM decidua and also levels from chromosomally normal (euploid) and abnormal

(aneuploid) miscarriages would have been investigated. Although immunohistochemistry data in this study observed no differences in staining patterns which is confirmed by reports demonstrating that decidual defects are similar in both euploid and aneuploid miscarriages (Brosens et al., 1978, Ball et al., 2006a, Ball et al., 2006b) and leucocyte numbers do not significantly differ between them (Williams et al., 2009b) but it would be worthwhile to compare cytokine secretion levels in both miscarriages as this has yet to investigated.

7.2.4 Overall summary

In summary, in the present study IL-6, IL-8 and their receptors were localised in the decidua of early human pregnancy and it was shown that cells such as uNK cells, uterine macrophages, T-lymphocytes and stromal cells are producers of both cytokines at the fetal maternal interface. The present study also demonstrated gestational age differences in cytokine production by particular decidual cell populations, suggesting different roles and importance at different stages of early pregnancy. From the cells studied, uterine macrophages are the most abundant source of IL-6 and IL-8 although they are the second most abundant leucocyte in the decidua in early pregnancy. A possible role for this cell population is to mediate VSMC and endothelial cell changes in early stages of SpA remodelling may be through the localised production of both IL-6 and IL-8. Further evidence for this is demonstration that receptors for both IL-6 and IL-8 on SpA endothelium and VSMCs have altered expression in SM. Both cytokines play roles in regulating trophoblast biology suggesting further involvement in pregnancy processes.

The importance of IL-6 and IL-8 in early human pregnancy is apparent from the evidence in the present study and is further supported by reduced cytokine expression in SM; this warrants further investigations in order to clarify specific roles for both cytokines in these crucial processes. Further studies of the mechanisms that underlie the functional effects of these cytokines are required. Functional studies with a cohort of decidual cytokines should be also investigated due to the large and complex cytokine milieu at the maternal fetal interface which may alter with gestational age, potentially even from one week to the next; there is a wide array of cytokines and growth factors which may have synergistic or antagonising effects on each other and

therefore affect the outcome of such processes *in vivo*. Mouse cytokine gene knockout studies would be one approach to achieve this by investigating how this impacts upon mouse reproduction reviewed by Ingman and Jones (2008). Better understanding of the roles of these cytokines in healthy pregnancy thereby creating a clearer picture *in vivo* will allow better understanding of their roles in pathology pregnancy and may eventually lead to targeted therapies of clinical importance.

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Appendix

Appendix

Materials and solutions

All chemicals were obtained from Sigma Chemical Company Ltd., Poole, Dorset, UK except where otherwise stated.

Cell culture reagents:

Complete medium (RPMI 1640)

RPMI 1640 medium

10% fetal calf serum (FCS; heat inactivated)

0.4mM/ml L-glutamine

5U/ml Penicillin

0.05mg/ml Streptomycin

All components mixed together at room temperature and stored at 4°C

Complete medium (DMEM-F12)

Dulbecco's modified eagle medium nutrient mixture:F-12 HAM [1:1 ratio]

10% FCS (heat inactivated)

0.4mM/ml L-glutamine

5U/ml Penicillin

0.05mg/ml Streptomycin

All components mixed together at room temperature and stored at 4°C

Incomplete medium (RPMI 1640)

RPMI 1640 medium

0.4mM/ml L-glutamine

5U/ml Penicillin

0.05mg/ml Streptomycin

All components mixed together at room temperature and stored at 4°C

Charcoal stripped medium (0.5, 5 or 10%)

RPMI 1640 medium

0.5, 5 or 10% charcoal stripped FCS

0.4mM/ml L-glutamine

5U/ml Penicillin

0.05mg/ml Streptomycin

All components mixed together at room temperature and stored at 4°C

Complete medium for CD14+ cells

Dulbecco's modified eagle medium

10% FCS (ATCC; Manassas, VA, USA)

0.4mM/ml L-glutamine

5U/ml Penicillin

0.05mg/ml Streptomycin

All components mixed together at room temperature and stored at 4°C

MidiMACS buffer

0.19g EDTA

0.5g bovine serum albumin (BSA)

100ml PBS

Adjusted to pH7.4

Filtered through a sterile 0.2µm syringe filter (Nalgene) and stored at 4°C.

General laboratory reagents and buffers:

Sterile phosphate buffered saline (PBS)

1 phosphate buffered saline tablet dissolved in 200ml distilled H_2O, to obtain 0.01M phosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride adjusted to pH7.4. The mixture was filtered through a sterile 0.2µm

syringe filter (Nalgene Thermo Scientific; Germany) then autoclaved and stored at 4°C.

0.1M Tris buffered saline (TBS)

10L distilled H_2O

80g sodium chloride

6.05g Tris (hydroxymethyl) methyl amine

38ml 1N hydrochloric acid

Sodium chloride and Tris were dissolved overnight and adjusted to pH7.6 before use.

Citrate buffer

2.1g citric acid monohydrate

13ml 2M NaOH

900ml distilled water

Dissolved overnight and adjusted to pH6.0 before use.

Ethylenediaminetetraacetic acid (EDTA) buffer

2.26g EDTA

5L distilled water

Adjusted to pH8.0

Trypsin working solution

Trypsin solution (0.5%)

0.05g trypsin

10ml distilled water

Dissolved and stored at -20°C

Calcium chloride solution (1%)

0.1g calcium chloride

10ml distilled water

Dissolved and stored at -20°C

1ml of trypsin solution and 1ml calcium chloride solution added to 8ml of water, adjusted to pH 7.8 with 1N NaOH.

Sections were immersed in warmed trypsin working solution at 37°C waterbath and incubated for 20 minutes and then placed in distilled water. Slides were then added to TBS (room temperature).

Tris acetate TEA buffer (50x stock solution)

242g Tris base dissolved in 250ml deionised water

57.1ml acetic acid

100ml EDTA (pH 8.0)

Adjusted to make the final volume of 1L pH 8.5

The working solution of 1x TAE buffer is made by diluting the stock solution by 50x in deionised water.

Dimethyl sulfoxide (DMSO) freezing mix

20% DMSO

80% FCS

Aliquoted into 1ml aliquots in cryovials and stored at -20°C until required.

Histochemical Stains and solutions:

Mayer's haematoxylin

2g haematoxylin (Raymond A Lamb, London, UK)

100g potassium alum (BDH, Poole, UK)

0.4g sodium iodate

2L distilled water

Dissolved at room temperature overnight. Then added:-

100g chloral hydrate (BDH)

2g citric acid

All components were brought to the boil and left overnight. Filtered before use.

Eosin

11.25g eosin (Raymond A Lamb)

5.0g erythrosin (Raymond A Lamb)

2.0g phloxine (Raymond A Lamb)

200ml 95% ethanol

800ml tap water

All components were dissolved together at room temperature.

Scott's tap water substitute

17.5g sodium bicarbonate (BDH)

100g magnesium sulphate (BDH)

5L distilled water

A few crystals of thymol

All components were dissolved together at room temperature.

Preparation of APES coated slides

4ml APES solution (3-aminopropyltriethoxysilane)

1L acetone

1L 99% industrial methylated spirits

Washed glass slides in a dilute detergent and rinsed in water several times. The slides were then immersed into distilled water followed by washes in 99% industrial methylated spirits. The slides were air dried at room temperature before coating with the APES solution. The slides were then immersed into acetone, acetone and APES solution (196ml acetone and 4ml APES solution), acetone only and distilled water. Once again, the slides were air dried at room temperature overnight and stored at 4°C until required.

Elastic Van Gieson staining

This stain is used to visualise elastin and mast cell granules; elastic tissue-appears blue/black. The Van Gieson counterstain is used to visualise connective tissue within the slides; collagen stains red.

Miller's elastin

2g victoria blue

2g new fuchsin

2g crsyal violet

Dissolved the dyes in 400ml distilled water then added:-

8g resorcin

2g dextrin

100ml 30% aqueous ferric nitrate

The mixture was boiled for 5 minutes and filtered. The precipitate was re-dissolved in 400ml of 95% alcohol and boiled for 20 minutes. The mixtures was cooled and filtered before topping up with 95% alcohol to make 400ml. 4ml of concentrated hydrochloric acid was then added.

Van Gieson's counterstain

15ml 1% aqueous acid fuchsin

50ml saturated picric acid

50ml distilled water

The sections were immersed in running tap water and sections were then oxidised in potassium permanganate solution for 5 minutes. The slides were rinsed in water before being decolourised with oxalic acid (approximately 1 minute) rinsed in water and then rinsed in 95% alcohol. The slides were then placed in a coplin jar containing Miller's elastin stain and incubated for 2 hours at room temperature. The slides were then washed in 95% alcohol to remove excess stain and differentiated microscopically using alcohol until elastic fibres appeared blue/black. Once differentiated Van Gieson's counterstain was applied to the stained sections for 3 minutes followed by a rinse in 95% alcohol

and dehydration in absolute alcohol. The slides were then cleared in xylene and mounted using DPX synthetic resin.

Periodic Acid Schiff (PAS) staining

50ml 50% Periodic acid solution

2450ml Distilled water

(To make 1% periodic acid solution)

Schiffs reagent obtained from Sigma (S-5133) store at 4°C

Following immunohistochemistry the sections were rinsed thoroughly in tap water. Each section was then incubated for 5 minutes in 1% periodic acid and rinsed with distilled water. Sections were then incubated for 8 minutes in Schiffs reagent. The slides were then immersed in running tap water for 12 minutes to further develop the PAS stain. The slides were then counterstained in Mayer's haematoxylin, dehydrated in ascending concentrations of alcohol, cleared in xylene and mounted with DPX synthetic resin.

Orcein staining (Shikata technique)

1g Shikata orcein

100ml 70% alcohol

1ml concentrated hydrochloric acid

Sections were rinsed in tap water and treated with acidified potassium permanganate solution for 3 minutes. Slides were washed in water then decolourised with 1% oxalic acid, washed in water then immersed into the Shikata stain for 90 minutes at room temperature. Following staining the slides were rinsed in water, dehydrated in alcohol, cleared in xylene and mounted with DPX synthetic resin.

Immunohistochemistry staining kits:

Vectastain Elite mouse IgG ABC kit *(Vector Laboratories Ltd, Peterborough, UK); avidin-biotin peroxidase complex method*

Blocking serum: 15μl Vectastain normal horse serum diluted in 1ml 0.1M Tris buffered saline (TBS)

Secondary antibody: 15μl normal horse serum and 5μl Vectastain biotinylated anti-mouse IgG antibody in 1ml TBS

Tertiary antibody: 20μl Vectastain A and 20μl Vectastain B, diluted in 1ml TBS

Vectastain Elite rabbit IgG ABC kit (Vector Laboratories Ltd.); avidin-biotin peroxidase complex method

Blocking serum: 15μl Vectastain normal goat serum diluted in 1ml TBS

Secondary antibody: 15μl normal goat serum and 5μl Vectastain biotinylated anti-rabbit IgG antibody in 1ml TBS

Tertiary antibody: 20μl Vectastain A and 20μl Vectastain B, diluted in 1ml TBS

Vectastain Elite goat IgG ABC kit (Vector Laboratories Ltd.); avidin-biotin peroxidase complex method

Blocking serum: 15μl Vectastain normal rabbit serum diluted in 1ml TBS

Secondary antibody: 15μl normal rabbit serum and 5μl Vectastain biotinylated anti-goat IgG antibody in 1ml TBS

Tertiary antibody: 20μl Vectastain A and 20μl Vectastain B, diluted in 1ml TBS

ImPRESS kit mouse/rabbit Ig (MP-7500) (Vector Laboratories Ltd, California, USA); polymerised reporter enzyme staining system

Blocking serum: normal horse serum (2.5%)

Universal peroxidase: anti-mouse/rabbit Ig

APAAP double labelling reagents

Blocking serum: normal rabbit serum (from Vectastain Elite goat IgG ABC kit)

Secondary antibody: Rabbit anti-mouse IgG (Z259; Dako UK Ltd, Cambride, UK) 1:100 TBS pH7.6

Tertiary antibody: Alkaline phosphatase anti-phosphatase (APAAP) complex antibody (D651; Dako) 1:50 in TBS pH7.6

Chromogens used for development of immunostaining:

3,3'- Diaminobenzidine tetrachloride (DAB)

One DAB tablet (Sigma) was added to 0.2ml 1% aqueous H_2O_2 (Sigma) in 10mls TBS pH7.6 and left to dissolve. This solution was then filtered and applied to the sections for approximately 2-3 minutes. The colour reaction was ended by placing slides in running tap water for 5 minutes.

Vector NovaRed Substrate kit (SK-4800; Vector laboratories)

To 5ml distilled water add:-

3 drops of reagent 1, mix well

2 drops of reagent 2, mix well

2 drops of reagent 3, mix well

2 drops of hydrogen peroxide, mix well

This mix was applied to the slides for approximately 5 minutes and the reaction was ended by placing the slides in running tap water.

Alkaline phosphatase substrate kit III (SK-5300; Vector laboratories)

To 5ml of TBS pH8.2 add:-

2 drops reagent 1, mix well

2 drops reagent 2, mix well

2 drops reagent 3, mix well

The mixture was immediately applied to the slides and developed in the dark for between 5-20 minutes. Following this the slides were washed in running tap water to end the reaction.

Western Blotting solutions:

HEPES Protein Extraction Buffer

20mM HEPES buffer, pH7.4

150mM NaCl

2mM CaCl$_2$

10mM CHAPS

1% protease inhibitor cocktail (to be added just before homogenising/lysing)

The buffer was kept ice cold prior to homogenisation/lysis. Tissue was homogenised or cells were lysed in the buffer and the lysates were microfuged for 10 minutes at full speed (14.1rcf (relative centrifugal force)).

2x Separating buffer

0.75mM Tris

0.2% SDS

100ml deionised H$_2$O

pH 8.8

2x Stacking buffer

0.25mM Tris

0.2% SDS

100ml ddH$_2$O

pH 6.8

Separating gel

Acrylamide (40%)

5ml 2x separating buffer

ddH$_2$O

20µl of temed*

100µl of 10% APS (ammonium persulfate; 50mg in 500µl of ddH2O)*

*Temed and APS were added just before gel casting. The gel was loaded onto the glass plate apparatus, topping the gel with isopropanol to prevent air bubbles. The gel was allowed to polymerise for 45 minutes.

The size of the separating gel could be adjusted using different volumes of acrylamide to water:

7.5% gel= 2ml acrylamide: 3ml water

10% gel= 2.7ml acrylamide: 3ml water

12.5% gel= 3.3ml acrylamide: 1.7ml water

Stacking gel

1ml acrylamide

2.5ml 2x stacking buffer

1.5ml ddH$_2$O

10µl temed*

50µl APS*

*Temed and APS were added just before gel casting. The isopropanol layer was poured off and gel was added to set the separating gel. Combs were placed in the stacking gel before casting. The gel was allowed to polymerise for 45 minutes and the combs were then removed.

5x electrode buffer

Tris-base 15g/L or Tris-HCL 19.5g/L

Glycine 72g/L

SDS 5g/L

1L ddH2O

pH 8.3

To make 1x electrode buffer 200mls of 5x buffer was added to 800ml deionised H$_2$O.

Transfer buffer

Tris-HCL 3.93g/L

Glycine 14.26g/L

Methanol 200ml/L

pH8.3

The buffer was stored at 4°C prior to transblotting.

Protein loading buffer (2x Laemmli buffer) - reducing conditions

Reducing conditions = disrupts the tertiary structure of the proteins by breaking the disulphide bridges (S-S) into sulphydryl (SH-SH) groups allowing the proteins to separate by molecular weight (kDa).

4% Sodium dodecyl sulphate (SDS)

10% 2-mercaptoethanol

20% glycerol

0.004% bromophenol blue

0.125M Tris-HCL

pH 6.8

The samples were boiled for 5 minutes at 95-100°C for denaturing of protein. If detecting epiptope on native protein SDS was omitted and the sample was not boiled. If detecting oxidised proteins (non-reducing conditions) 2-mercaptoethanol was omitted. 5μl of dye was added to each sample.

Blocking buffer (10%)

5g of non-fat dried powdered milk

50ml 1xTBS/PBS-T (TBS/PBS + 0.05% Tween20)

Milk was dissolved in TBS/PBS-T and stored at 4°C

FastQuant Array:

Human Th1/Th2 kit

Detects IL-6, IL-1β, IL-12, IL-13, IL-4, IFN-γ, IL-5 and TNF-α proteins.

Human II kit

Detects IL-8, GM-CSF, IL-10, IL-1β, IL-12p70, IL-2, MCP-1, IL-4, RANTES and IL-6 proteins.

Human Angiogenesis kit

Detects Angiogenin, Angopoietin-2, KGF, PDGF-BB, FGF-basic, TIMP-1, ICAM-1, and VEGF growth factors.

Each kit contains 4 slides, each with 16 3D nitrocellulose arrays with anti cytokine monoclonal antibodies arrayed in triplicate.

Solutions

Standard dileunt: 0.1% BSA, 0.05% Tween 20 in PBS pH7.4

1x blocking buffer (6mls provided)

10x wash solution (125mls provided) - for 1x wash buffer added 50ml of 10x washing solution to 450mls dH$_2$0.

600µl biotinylated antibody solution in 0.5mgml^{-1} BSA, 1X TBS – diluted 500µl of antibody cocktail solution with 4500µl of 1x wash buffer.

Stock SA-Cy5 solution (GE healthcare): 1mg of lyophilised SA-Cy5 fluorescent reporter – added 1ml of dH$_2$0 and dispensed 5µl aliquots and stored at -20°C until required.

Working SA-Cy5 solution: added 1µl of SA-Cy5 stock solution to 7999µl of 1x wash buffer and protected from light.

Three 25µl vials of recombinant antigen cocktail in 1mgml^{-1} BSA, PBS (20µl of recombinant antigen cocktail was added to 280µl of diluents to make standard A, then an 8 point standard curve was prepared).

Publications

1. ***Effects of interleukin-6 on extravillous trophoblast invasion in early human pregnancy*** H Champion Molecular Human Reproduction 2012 (Champion et al., 2012)